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Title: IMMUNIZATION WITH A COCKTAIL OF ANTIGENS FUSED WITH OprI REDUCES NEOSPORA CANINUM VERTICAL TRANSMISSION AND POSTNATAL MORTALITY IN MICE

Article Type: Original article

Keywords: Neosporosis; mouse model; pregnancy; vaccine; dendritic cells; immunomodulation; rhoptries; protein disulfide isomerase

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Abstract: OprI is an outer membrane lipoprotein from Pseudomonas aeruginosa, and when fused to a recombinant antigen, will exert adjuvant properties by engaging Toll-like receptor 2, leading to dendritic cell activation. Previous studies have shown that the Neospora caninum (Nc) antigens NcPDI, NcROP2 and NcROP40 are implicated in host cell interactions and are promising vaccine candidates. In two independent experiments, the efficacy of a polyvalent vaccine formulation composed of OprI-NcPDI, OprI-NcROP2 and OprI-NcROP40 (collectively named O-Ags) was assessed in non-pregnant and pregnant Balb/c mouse models challenged with tachyzoites of the high-virulence isolate Nc-Spain7. Parameters that were investigated were clinical signs, fertility, parasite burden in adult mice, humoral and cellular immune responses at different time-points prior to and after challenge infection, vertical transmission and post-natal survival of offspring mice, all to explore potential correlations with efficacy. Vaccination of mice with O-Ags induced a mixed Th1/Th2 immune response in adult mice and led to significantly increased protection against cerebral infection. Vaccination with O-Ags also resulted in reduced vertical transmission, and postnatal disease in offspring was significantly inhibited at a rate not observed in mice infected with a high-virulence isolate to date. However, O-Ags mixed with TLR ligands targeting TLR3 and TLR7, which are known to induce clear Th1-biased responses, or vaccination with OprI fused to the non-N. caninum antigen ovalbumin (OprI-OVA) did not confer protection.

Cover Letter

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Prof. Daniel Altmann
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Bern, 15.11.2018

Dear Prof. Altmann

I am hereby submitting our revised manuscript **JVAC-D-18-01227** paper for publication *Vaccine.*

Thank you for providing us the opportunity to submit a revision.
We have followed the reviewer's comments and altered the manuscript accordingly. In addition we gone through the paper and eliminated also a number of typing errors that we found. We also include a compare file that makes it easier to follow the changes.

We hope that pour manuscript is now up to the standards of the journal. If other changes are required, please let me know.

Yours sincerely

Andrew Hemphill

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***Response to Reviewers**

Author's responses to Reviewers' comments:

Reviewer #2: JVAC-D-18-01227
The study reports immunization experiments in mice with OprI-fused antigens to prevent Neospora caninum induced vertical transmission and disease. The experiments are well planned and the manuscript nicely written and I have only a few comments.

Line 140-175, Figure 2: The description of experiments is a bit confusing; it is not that clear what applies for experiment 1 or experiment 2 or for both experiments. You referred to Figure 1 but I think it would be better to refer to Figure 2.

Response
Thank you for this comment. We have slightly altered the text and made it much clearer, which investigations are part of experiment 1, experiment 2, or both. In addition, there was actually a mistake in the figure numbers, which has now been corrected. Fig. 1 now refers to the schematic presentation of the experimental design, Fig. 2 shows the recombinant antigens

Figure 2 is not optimal, yet. It is difficult to understand the three arrows. It took me a while to understand that the first arrow is for time of experiment, the second is for time of pregnancy and the third is for time post challenge. Why is the third line with a step? Why have the second and the third lines different arrow-heads as compared to the first/top line?

Response
We have modified Figure 1 (Schematic presentation of experiments 1 and 2) making it much clearer to read and easier to understand.

Line 288: "EPT" was already explained in Line 214.

Response
This was actually on line 227, and is now corrected

Line 266 and entire manuscript: Always mention statistical test, once reporting OR or P-values . What are the 95% confidence intervals for OR?

Response
We have now mentioned statistical test every time. OR confidence intervals were added

Line 333, 334: "Mann-Whitney U test" would be better. There are several ways to mention this tests in the manuscript, actually.

Response
This was corrected along the entire manuscript

Line 364: What is the reference "0" ?

Response
This is now deleted

Line 481: One of the authors of this reference is almost not visible in this citation.

Response
Corrected

Reviewer #3: Some points are addressed.
OprI-fused N. caninum's antigens was investsifgated as Neosporesis vaccine. However, the Oprl was not explained in this MS.
Why the OprI was fused and what effects are expected are explained for readers' understanding.

Response

We have rephrased the Introduction (Line 81-91) and added some more information into the discussion (lines 432-437), to explain the rational for exploiting OprI and its adjuvant properties.

Suggested reviewers

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Vaccination of mice with OprI-linked recombinant antigens results in mixed Th1/Th2 immune response.

Vaccination confers protection against *Nesopora caninum* infection in non-pregnant mice and dams.

Vaccination results in significant reduction in offspring mortality and vertical transmission.

Addition of adjuvants inducing a Th1-biased response abolish the protective efficacy.

***Abstract**

OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa*, and when fused to a recombinant antigen, will exert adjuvant properties by engaging Toll-like receptor 2, leading to dendritic cell activation. Previous studies have shown that the *Neospora caninum* (Nc) antigens NcPDI, NcROP2 and NcROP40 are implicated in host cell interactions and are promising vaccine candidates. In two independent experiments, the efficacy of a polyvalent vaccine formulation composed of OprI-NcPDI, OprI-NcROP2 and OprI-NcROP40 (collectively named O-Ags) was assessed in non-pregnant and pregnant Balb/c mouse models challenged with tachyzoites of the high-virulence isolate Nc-Spain7. Parameters that were investigated were clinical signs, fertility, parasite burden in adult mice, humoral and cellular immune responses at different time-points prior to and after challenge infection, vertical transmission and post-natal survival of offspring mice, all to explore potential correlations with efficacy. Vaccination of mice with O-Ags induced a mixed Th1/Th2 immune response in adult mice and led to significantly increased protection against cerebral infection. Vaccination with O-Ags also resulted in reduced vertical transmission, and postnatal disease in offspring was significantly inhibited at a rate not observed in mice infected with a high-virulence isolate to date. However, O-Ags mixed with TLR ligands targeting TLR3 and TLR7, which are known to induce clear Th1-biased responses, or vaccination with OprI fused to the non-*N. caninum* antigen ovalbumin (OprI-OVA) did not confer protection.

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Note: Supplementary data associated with this article

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Abstract

OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa*, and when fused to a recombinant antigen, will exert adjuvant properties by engaging Toll-like receptor 2, leading to dendritic cell activation. Previous studies have shown that the *Neospora caninum* (Nc) antigens NcPDI, NcROP2 and NcROP40 are implicated in host cell interactions and are promising vaccine candidates. In two independent experiments, the efficacy of a polyvalent vaccine formulation composed of OprI-NcPDI, OprI-NcROP2 and OprI-NcROP40 (collectively named O-Ags) was assessed in non-pregnant and pregnant Balb/c mouse models challenged with tachyzoites of the high-virulence isolate Nc-Spain7. Parameters that were investigated were clinical signs, fertility, parasite burden in adult mice, humoral and cellular immune responses at different time-points prior to and after challenge infection, vertical transmission and post-natal survival of offspring mice, all to explore potential correlations with efficacy. Vaccination of mice with O-Ags induced a mixed Th1/Th2 immune response in adult mice and led to significantly increased protection against cerebral infection. Vaccination with O-Ags also resulted in reduced vertical transmission, and postnatal disease in offspring was significantly inhibited at a rate not observed in mice infected with a high-virulence isolate to date. However, O-Ags mixed with TLR ligands targeting TLR3 and TLR7, which are known to induce clear Th1-biased responses, or vaccination with OprI fused to the non-*N. caninum* antigen ovalbumin (OprI-OVA) did not confer protection.

Keywords: Neosporosis; mouse model; pregnancy; vaccine; dendritic cells; immunomodulation; rhoptries; protein disulfide isomerase

1. Introduction

Neospora caninum is a cyst-forming coccidian closely related to *Toxoplasma gondii*, and is one of major causes of infective abortion in cattle [1]. In addition, *N. caninum* infection in cattle often results in birth of weak calves or persistently infected offspring, which then transmits the parasite to the next generation. Vaccination has been considered to be the most cost-effective strategy to control bovine neosporosis [2,3], but no commercial vaccines are available [4].

Live vaccines based on attenuated *N. caninum* isolates partially protected mice and cattle against fetal death upon experimental challenge infection during pregnancy [5,6,7]. However, several disadvantages of live vaccines related to costs of parasite *in vitro* culture, storage, limited shelf life of the final product and logistics of its distribution, as well as the potential risks of virulence reversion and recombination between attenuated and circulating strains, render live vaccines a rather unattractive option [4]. Vaccines based on total antigenic extracts or different antigenic fractions of the parasite have shown variable results with overall lower success [7]. Subunit vaccines composed of recombinant antigens would represent an interesting option and would solve many of the inherent logistic and financial disadvantages of live vaccines [4]. Admittedly, recombinant vaccine formulations have so far not shown very promising effects in pregnant neosporosis animal models. However, the versatile potential of recombinant vaccines should be much more exhaustively exploited by applying antigen combinations with adjuvants targeting different components of the immune system [4].

There is “proof-of-concept” that a reduction of vertical transmission and postnatal mortality due to *N. caninum* infection can be achieved by vaccination not only with live vaccines [8,9], but also with recombinant subunit vaccines in mice [10,11]. Bacterially expressed recNcPDI, recNcROP2 and recNcROP40 represent promising vaccine candidates. Immunization

of mice with recNcROP2 formulated in saponin resulted in a significantly increased survival rate in adult mice [12] and in offspring born to mice infected during pregnancy with the *N. caninum* Nc-1 isolate [10]. NcROP40 is another rhoptry protein that is abundantly expressed in virulent *N. caninum* isolates [13]. Immunization of mice with both recNcROP40 and recNcROP2 recombinant proteins lead to reduction of vertical transmission and postnatal mortality of 16% in offspring born to mice infected with the highly virulent isolate Nc-Spain7 [11]. RecNcPDI (*N. caninum* protein disulfide isomerase) had conferred protection against cerebral neosporosis when applied intranasally [14], but failed to protect dams and their offspring [2].

The TLR2-ligand OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa*. It has been used to confer adjuvant properties when expressed in fusion with different antigens. [15]. Different immune functions have been attributed to TLR2 signaling including the promotion of a mixed and balanced Th1-, Th2- and Treg cell response [16] and a T-cell tropism to mucosal tissues even when inoculated via a non-mucosal route [17]. In previous immunization studies, OprI had been fused to the *N. caninum* chimeric antigen Mic3-1-R, and vaccinated mice were challenged during pregnancy. This did not result in protection, neither in dams nor in offspring mice, but we nevertheless detected that fusion of OprI to MIC3-1-R induced a mixed Th1/Th2 profile response [18], while the immune response in mice immunized with MIC3-1-R emulsified in saponin was largely Th2-biased, thus confirming the immunomodulatory properties of OprI-fusions. This well-adjusted Th1/Th2 balance is required to protect *N. caninum* vaccinated mice during pregnancy when a strong Th1 response could be detrimental [30].

In this study, we performed two independent experiments to assess the efficacy of a polyvalent vaccine formulation composed of OprI-NcPDI, OprI-NcROP2 and OprI-NcROP40 (collectively named O-Ags) in non-pregnant and pregnant Balb/c mouse models challenged with *N. caninum* tachyzoites. In one of the experiments, the same O-Ags formulation was mixed with

two additional TLR ligands targeting TLR3 and TLR7, which are known to induce clear Th1-biased responses [19] and likewise effects in dams and offspring were assessed. In the other experiment, a group vaccinated with OprI fused to ovalbumin (OprI-OVA) was included to provide an irrelevant antigen control.

2. Materials and methods

2.1. Neospora caninum *culture, inoculum and crude extract preparation*

The *N. caninum* Nc-Spain7 isolate [20] was grown *in vitro* by continuous passages in Vero cell cultures [18]. The parasite inoculum was prepared as described [21]. *N. caninum* crude extract for lymphocyte re-stimulation assay and Western blot, and soluble antigen fractions for ELISA, were prepared as described earlier [18].

2.2. Production of recombinant antigens fused to OprI

The coding sequence of NcPDI (aa 25-446) [22], a fragment of NcROP2 (aa 238-594) [12] and the complete coding sequence of NcROP40 [11] were cloned in the pOLP plasmid in frame with OprI [15]. A previously described plasmid, containing the ovalbumin (OVA) partial sequence (aa 203-386) in frame with OprI [16] was also used to obtain the unrelated antigen OprI-OVA. Proteins were all expressed in Rosetta(DE3)pLysS *Escherichia coli* strain (Novagen). They were isolated from the bacterial outer membranes as previously described [18]. Purified OprI-NcPDI was dialyzed against PBS over-night at 4°C and stored at -80°C. OprI-NcROP2 and OprI-NcROP40, expressed less efficiently and insoluble in aqueous solution, were dialyzed over-night against 1g/L ammonium bicarbonate at 4°C and lyophilized. They were resuspended in

PBS in one-tenth of the original volume, vortexed, and were sonicated at 30°C in a water bath until a homogeneous suspension was obtained. All purified OprI-antigens were finally stored at -80°C. Non-OprI-fused NcPDI, NcROP2 and NcROP40 [11,22,22], were expressed and purified as described [18]. The purity and integrity of all proteins were analyzed by SDS-PAGE and the protein concentration was determined (BCA Protein Assay Kit, Thermo Scientific Pierce). Endotoxin levels were quantified by the Limulus-Amebocyte-Lysate-Kinetic-QCL (Lonza). The final LPS content was below 0.1EU/µg.

2.3. Dendritic cell (DC) stimulation

OprI-fused and non-fused recombinant proteins were assessed for DC stimulation [18]. Briefly, DCs were obtained *in vitro* from bone marrow cells obtained from a naïve Balb/c mouse. DCs ($0.5x10^6$ cells/ml) were cultured in the presence of each protein (1 or 5µg/ml), LPS (0.1µg/ml) or medium during 24hours, and TNF-α was measured in the culture supernatants by ELISA [18].

2.4. Immunization schedule, clinical monitoring and sample collection

All animal protocols were approved by the Bernese Animal Welfare Committee (license BE115/14.) Two experiments were carried out. Schedules of immunization, mating, blood sample collection and euthanasia for both studies are depicted in Fig.1. Six weeks-old BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained as described [18].

In Experiment 1, 16 to 20 female mice per group were immunized three times, at two-week intervals. The O-Ags group was subcutaneously injected with all three OprI-antigens, 5µg

each; the O-Ags+TLR group was immunized with the same antigens mixed with poly I:C (TLR3 ligand) (Invivogen) and R848 (TLR7 ligand) (Invivogen), 25µg each. Two control groups, one of which remained non-infected throughout the experiment (C-), the other one that was infected (C+), received PBS only. Eight days after the second injection, just prior to the oestrus synchronization, a blood sample was obtained from 4 mice per group (see Fig.1; PrCh-1) and serum recovered for IgG analysis.

In experiment 2, experimental groups of 20 female mice were immunized subcutaneously three times, at two-week intervals. The O-Ags group received the three antigens as above, the O-OVA group was injected with 15µg of OprI-OVA, the two additional groups (C- and C+ groups, see above) received PBS. A blood sample from the tail was obtained from all mice 4 days after the third immunization (at 4-7 days post-mating) just prior to challenge (Fig.1; PrCh-2).

Eight days post- second immunization, mice in both experiment 1 and experiment 2 were submitted to oestrus synchronization [23] and 2 females and 1 male per cage were mated during 72h. The third immunization was applied after separation of males and females, 5 days prior to challenge infection. At 5-8 days of pregnancy, mice from the O-Ags, O-Ags+TLR, O-OVA and C+ groups were subcutaneously infected with 10^5 Nc-Spain7 tachyzoites [24]. Mice of the C- groups received only PBS. At day 9 post-infection, a blood sample was obtained from the tail of 3 pregnant mice of each group from experiment 1 and of all mice from experiment 2 (Fig.1; PstCh-pm). Two days before birth, pregnant and non-pregnant mice were separated. Dams were allowed to give birth and rear their offspring in individual cages. Non-pregnant mice were kept in groups of 4-5 mice per cage. Following infection, all mice were daily monitored for clinical signs using a standardized score sheet: coat (ruffled coat=score1; stary stiff coat=score2); weight loss (10% loss=score1, 15-20% loss=score 4); behavior (hunched appearance, walking in circles, head tilt, apathy and ataxia, all=score 1). Animal were euthanized when the score exceeded 3 points.

At 30 days post-infection (30 dpi), all surviving non-pregnant mice in both experiments were euthanized (Fig.1; PstCh-NonPreg). Dams and their pups were maintained together until day 30 post-partum (pp) (=41-44 dpi), subsequently all animals were euthanized (Fig.1; PstCh-D).

Data on clinical signs, fertility (percentage of pregnant mice), litter size (average of number of pups born per dam), neonatal mortality of pups (stillborn pups or pups dying within the first 2 days post-partum (dpp) and postnatal mortality (pups dying between day 3 and 30pp) were recorded. Upon euthanasia, blood was extracted by cardiac puncture and sera stored at -80 °C. Brains from non-pregnant mice, dams and surviving pups were also collected and immediately frozen at -20°C. Spleens from 5 to 6 non-pregnant mice and dams per group were aseptically recovered and processed for splenocyte re-stimulation *in vitro*.

2.5.Analysis of the cerebral parasite burden

Brains from non-pregnant mice, dams and surviving pups were analyzed by *N. caninum*-specific real-time PCR [25]. DNA extraction was performed using the Nucleospin Kit (Macherey-Nagel, Oensingen, Switzerland). The DNA concentration in all samples was determined using the QuantiFluor dsDNA System (Promega, Madison, Wi.) and was adjusted to 5ng/µl with sterile DNase free water. Quantitative real-time PCR was performed using the Rotor-Gene 6000 real-time PCR machine. The parasite load was calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 *N. caninum* tachyzoites included in each run.

194 2.6. *Assessment of cytokine expression levels in serum and splenic lymphocytes stimulated* in
195 vitro
196
197 Levels of mouse IFN-γ, IL-10, IL-2 and IL-5 were assessed at two different time-points in
198 experiment 2: (i) at 9dpi, coinciding with days 14-17 of pregnancy (PstCh-pm, see Fig.1) in
199 blood, and (ii) at the late post-challenge phase in non-pregnant mice (30 dpi) and dams (43-45
200 dpi) by splenocyte re-stimulation *in vitro* as described [18]. For cytokine assessment in blood, the
201 serum samples were analysed by bead-based multiplex assay for the Luminex® platform
202 (Hertogenbosch, The Netherlands) . Microtiter filter plates were run on Luminex instruments
203 (Bio-Plex^{TM}200 system). Calibration curves were calculated with Bio-Plex Manager software
204 using a five-parametric logistic curve fitting [18]. For cytokine analysis upon splenocyte recall
205 responses, spleens were disaggregated and splenocyte cell suspensions were prepared as
206 described [18], The resulting cell suspensions were seeded in 48-well plates and re-stimulated
207 with either concanavalin A (ConA; 5µg/ml; Sigma), recNc-PDI, NcROP2, NcROP40 (20µg/ml),
208 whole *N. caninum* crude extract (10µg/ml) or remained non-stimulated. Supernatants were
209 collected after 72h and stored at -80°C until cytokines were assessed by ELISA (BD
210 OptEIA™ Mouse ELISA Set, LifeSpan Biosciences Inc., Seattle, WA, USA).
211
212 *2.7. Analysis of serum immunoglobulins*
213
214 Immunoglobulins were analyzed at different time-points: (i) during the pre-challenge
215 phase prior to mating in experiment 1 (PrCh-1); (ii) during pre-challenge phase after mating in
216 experiment 2 (PrCh-2); (iii) during the early post-challenge phase in experiments 1 and 2 (PstCh-
217 pm); (iv) at the late post-challenge phase just prior to euthanasia in non-pregnant mice (PstCh-
218 NonPreg) and in dams (PstCh-D) (see Fig.1). Serum levels of *N. caninum*, NcPDI-, NcROP2- or

NcROP40-specific IgG1 and IgG2a were measured by ELISA [26]. Four 4-fold serial dilutions were analyzed for each sample and results were expressed as end-point titer (EPT) calculated as the inverse value of the dilution giving an OD≥cut-off [27]. The cut-off was independently positioned in each plate in the lower linear part of the dilution curve of the same positive control which allowed the normalization between plates.

PrCh-1 and PstCh-pm serum samples from experiment 1 were also analyzed in pools by immunoblotting for NcROP2- and NcROP40-specific IgG. Non-OprI NcROP2 and NcROP40 (10µg each) were submitted to Western blot analysis following standard procedures [26]. Pooled sera were incubated at 1:50, 1:200 and 1:800 dilutions and an anti-mouse IgG antibody conjugated to alkaline phosphatase (eBioscience) at 1:5000 dilution was employed.

2.8.Statistical analysis

Cytokines levels, clinical signs scores and cerebral parasite burdens were compared between groups by the non-parametric Kruskal-Wallis test; upon detection of statistical differences, a Dunn's multiple comparison test was subsequently applied to compare pair-by-pair. End point titers of IgG1 and IgG2a or ratios IgG1:IgG2a were compared between groups by Kruskal-Wallis test followed by Dunn's multiple comparison post-test or by Mann-Whitney U test when only two groups were compared at pre-challenge and pregnancy phases or between dams and non-pregnant mice in each group. Repeated measures of PDI antibodies along time were compared by Wilcoxon matched pairs test. Correlations between parasite burden or vertical transmission and any immune parameters were analyzed by Pearson correlation coefficient. To compare the mortality of pups along time, survival proportions at each time-point were plotted in Kaplan-Meier graphs and survival curves were compared by Log-rank test. The percentages of

survivors at the end of the experiment or percentages of *N. caninum* PCR positive samples were analyzed by Chi-square test with Yates' continuity correction in a contingency table.

Regarding the parameters measured in both experiments and compared between groups (C-, O-Ags and C+), a unique statistical analysis was done with all data from both experiments. A mixed-effects log-linear model (Venables and Ripley, 2002) was used to analyze pregnancy and immunization with the O-Ags formulation as fixed effects on parasite burden, having experiment as random effect. Effect sizes were estimated with a penalized quasi-likelihood method (glmmPQL from MASS library). A logistic mixed model with Laplace parameter estimation via Lapplace approximation (glmer of lme4 package) was used to evaluate clinical signs presence and vertical transmission. The odds-ratio (OR) relative to the O-Ags group was calculated with the respective 95% confidence interval (CI 95%). Survival of pups was compared with a Cox model with fixed (immunization) and random (experiment) effects (coxme library). Statistical significance was established at $P<0.05$.

3. Results

3.1. Increased dendritic cell stimulation by OprI-fused recombinant antigens

The purified OprI-fused recombinant proteins shown in Fig.2A were used for stimulation of bone marrow-derived DCs, which were cultured in the presence of 1 or 5 µg/ml of each protein, LPS (0.1µg/ml) or medium during 24 hours, and the production of TNF-α was measured in the culture supernatants by ELISA (Fig.2B). In contrast to the non-fused recombinant antigens, the OprI-proteins induced strong and specific DC stimulation (Fig.2B).

3.2. Immunization with OprI-fused Neospora *antigens reduced vertical transmission and increased postnatal survival*

Table 1 shows the outcome of *N. caninum* infection in dams and pups in both experiments. Comparing the O-Ags groups and the C+ infection control groups in both experiments, using the experiment as random effect in the mixed model, there was a significant increase in survival of offspring of O-Ags groups $P < 0.001$) and a significant reduction in vertical transmission (OR=4.52, CI 95% [2.06;10.63], $P<0.001$).

Analyzing experiment 1 separately, offspring from O-Ags group also showed a significant higher survival compared to C+ (χ^2 and Log-rank test, $P < 0.05$). In the O-Ags+TLR group in experiment 1 (Fig.3A), the pup survival curve was also significantly different from the O-Ags group (Log-rank test, $P<0.01$), showing that supplementation of the O-Ags formulation by TLR3 plus TLR7 ligands abolished the protective effect of O-Ags immunization. In fact, the O-Ags+TLR group the survival of pups was not significantly different from C+ (χ^2 and Log-rank test, $P > 0.05$) (Table 1; Fig. 3A).

In experiment 2 (Fig. 3B), comparing the C+ group with the O-Ags group, the percentage of pups survival and the pup survival curves from O-Ags were also significantly higher (χ^2 and Log-rank test, $P < 0.01$) and the vertical transmission significantly lower than C+ (χ^2 and Log-rank test, $P < 0.01$). Here, a group of mice was immunized with OprI fused to ovalbumin (O-OVA group). In this latter group immunized with O-OVA, the percentage of surviving pups, the pup survival curve and the vertical transmission were identical to C+ (O-OVA *vs* C+; χ^2 and Log-rank test, $P > 0.05$) (Table 1; Fig.3B).

3.3. Immunization with OprI-fused Neospora *antigens reduced cerebral neosporosis*

Taking into account both experiments in the mixed-effects logistic model there was a significant effect of O-Ags vaccination on the likelihood of animals developing clinical signs, with C+ animals at higher risk (OR=12.6, CI 95% [1.76; 260.66], $P<0.05$) independent of pregnancy. A significant effect of both O-Ags immunization ($P<0.001$) and pregnancy ($P<0.01$) on the cerebral parasite burden, without a significant interaction between these two variables ($P>0.05$), was observed with the mixed effects log linear model (Fig.4A,B). Compared to non-immunized mice (C+), O-Ags vaccinated dams showed lower parasite burdens.

In experiment 1 and 2, dams immunized with O-Ags+TLR or O-OVA did not show reduced cerebral parasite burden compared to C+ (Kruskal-Wallis, $P>0.05$) (Fig.4A,B). Regarding non-pregnant mice, immunization with O-Ags also reduced significantly the cerebral parasite burden and clinical signs (Fig.4C,D). This could only be seen in experiment 2 (O-Ags vs C+; Kruskal-Wallis, $P<0.05$), since in experiment 1 only one mouse from O-Ags was not pregnant. No protective effect was observed in mice immunized with O-Ags+TLR (data not shown) or O-OVA (Kruskal-Wallis, $P>0.05$) (Fig.4C).

3.4. Humoral immune responses against recombinant antigens

Specific antibody responses against each of the recombinant antigens used for immunization were studied by ELISA in both experiments at different time-points in the pre-challenge phase, either prior to mating in experiment 1 (PrCh-1) or 5 days post-mating in experiment 2 (PrCh-2), and at 9dpi (14-16 days post-mating; PstCh-pm.). Specific anti-ROP2 and anti-ROP40 antibody levels were very low at both pre-challenge time-points and several animals remained ELISA-seronegative when assessed even at 9dpi in both experiments. Thus, pools of sera collected at the pre- and post-challenge time-points in experiment 1 were analyzed by

Western blotting (Fig.5A). Anti-ROP2 antibodies remained virtually undetectable in sera taken during the pre-challenge phase in the groups immunized with O-Ags and O-Ags+TLR. However, IgG levels in O-Ags and O-Ags+TLR were higher in sera taken during pregnancy at 9 dpi (PstCh-pm), especially in the group immunized with O-Ags. Anti-ROP40 IgG levels were also low in both groups prior to challenge but were dramatically elevated in the group immunized with O-Ags+TLR in post-challenge sera taken from pregnant mice (PstCh-pm) (Fig.5A). Sera obtained from the O-OVA and the C+ group remained negative for ROP2 and ROP40 at both PrCh-1 and PstCh-pm.

PDI-specific antibodies were detected by ELISA during the pre-challenge phase. In experiment 1, levels in mice vaccinated with O-Ags+TLR were higher than those in mice vaccinated with O-Ags, and in spite of the low number of samples the difference reached statistical significance for IgG2a at the pre-challenge phase in experiment 1 (PrCh-1; Mann-Whitney U test, $P<0.05$) (Fig.5B). Moreover, the IgG1:IgG2a ratio was markedly lower in the O-Ags+TLR group compared to the O-Ags group, and was statistically significant during the pre-challenge phase (Mann-Whitney U test, $P< 0.05$) (Fig.5C). Surprisingly, PDI antibody levels were lower at 9dpi (PstCh-pm) than before mating in both vaccinated groups (Fig.5B). Since the values at 9dpi corresponded to just three pregnant animals and no identification of mice was carried out, we could not deduce the individual evolution of antibody levels in experiment 1. Thus, in experiment 2 mice were individually tracked. With the exception of one, those O-Ags-vaccinated mice with confirmed pregnancy exhibited very little changes or decreased anti-PDI antibody levels during the timespan between pre-challenge to 9dpi (Wilcoxon matched pairs test; $P>0.05$), whereas those mice that did not become pregnant exhibited a clear boost of anti-PDI antibody levels (Wilcoxon matched pairs test, $P<0.01$) (suppl. Fig.1), confirming the results from experiment 1. The same tendency was observed with IgG2a levels (data not shown). Only one

pregnant mouse exhibited a strong increase of antibody levels, and this animal had a very high parasite burden, elevated clinical signs score, and vertical transmission of *N. caninum* tachyzoites to its offspring (suppl Fig.1).

3.5.Antibody responses against soluble N. caninum antigens

No significant differences in IgG1 or IgG2a levels between groups were detected (Kruskal-Wallis, $P>0.05$) (Fig.6). In sera from mice immunized with O-Ags, and O-OVA, and from infected non-immunized C+ mice, similar IgG2a levels were noted in dams and non-pregnant mice (Mann-Whitney U test, $P>0.05$), whereas IgG1 levels were significantly lower in non-pregnant mice compared to dams (Mann-Whitney U test, $P<0.05$) pointing towards an IgG2a-biased response in non-pregnant mice compared to dams. In contrast, in sera of mice vaccinated with O-Ags+TLR ligands, no such differences between dams and non-pregnant were detected (Mann-Whitney U test, $P>0.05$), and the humoral immune response was IgG2a-biased in both cases (Fig. 6).

3.6.Cytokine responses

Cytokines were measured in experiment 2 at 9dpi in peripheral blood and at the end of the experiment upon *in vitro* stimulation of splenocytes and analysis of culture supernatants. At 9dpi, the levels of IFNγ, IL-10 and IL-5 in peripheral blood were significantly elevated in all infected animals when compared to group C- (Fig.7). However, IFNγ-levels in pregnant as well as non-pregnant mice vaccinated with O-Ags were significantly lower than in the C+ group (Kruskal-Wallis, $P<0.05$). IL-10 levels were also significantly lower in non-pregnant mice vaccinated with

O-Ags compared to non-pregnant mice in the C+ group. No significant levels of IL-2 were detected in peripheral blood samples of any group (data not shown).

At the end of the experiment mice were sacrificed, splenocytes were cultured and cytokine recall responses were measured in medium supernatants. In non-pregnant mice, only splenocytes from O-Ags and O-OVA vaccinated mice displayed a consistent increase of IFNγ and IL-10 secretion upon stimulation with *N. caninum* crude extract, compared to splenocytes from C- mice (Kruskal-Wallis, $P<0.05$) (Fig.8). Splenocytes derived from the C+ group showed no changes or very little increase of cytokine production. In the case of vaccinated dams, splenocyte cytokine levels were more heterogeneously distributed, with high individual variations, with the exception of highly elevated IL-2 responses in splenocytes isolated from O-Ags vaccinated dams, (Fig.8).

4. Discussion

The development of sub-unit vaccines against parasitic diseases is a major challenge due to the complexity of the biology of parasites [28]. This is especially true in the case of congenital neosporosis, for which so far only live-attenuated vaccines have achieved reasonable levels of protection against vertical transmission [29]. In the present work, an immunogenic formulation composed of three recombinant *N. caninum* antigens fused to OprI was assessed in a standardized pregnant mouse model of neosporosis based on the highly virulent *N. caninum* isolate Nc-Spain7. The vaccine formulation was composed of bacterially expressed and purified recNcPDI, NcROP2 and NcROP40, all three N-terminally fused to the OprI, a tri-acylated bacterial outer membrane protein. OprI targets TLR2, and thus stimulates mixed Th1, Th2 and Treg responses, favoring cross-presentation by APCs [16] Moreover, OprI was able to modulate the cellular immune

response against *N. caninum* towards a mixed Th1/Th2 response in mice vaccinated with the *N. caninum* chimeric antigen Mic3-1-R [18]. The Nc*ROP40* gene was found to be identical in three *N. caninum* isolates displaying different virulence, which renders this a promising vaccine candidate [31,32]. RecNcROP2 had been demonstrated to confer significant protection in non-pregnant and pregnant neosporosis mouse models based on the *N. caninum* Nc1 isolate [10,12]. RecNcPDI had conferred excellent protection in non-pregnant mice when applied intranasally emulsified in cholera toxin, but failed to prevent congenital neosporosis in pregnant mice [2, 14]. Nevertheless, since TLR2-ligands have shown to induce mucosal immunity by imprinting lymphocyte tropism to mucosae, even though they are applied by non-mucosal route [17], and NcPDI had conferred mucosal immunity [14], we incorporated this protein into the polyvalent O-Ags formulation.

We show here in two independent experiments that immunization with O-Ags confers significant protection in offspring born to dams that were challenged with *N. caninum* tachyzoite infection on day 7-9 of pregnancy. Postnatal mortality was reduced by 25.0 and 26.9% of pups in experiment 1 and 2, respectively, the mean survival time was extended and, overall, there was a clear difference in the survival curves. This rate of protection against congenital neosporosis has not been seen with recombinant antigens before (11). Vertical transmission (including dead pups and PCR positive survivors) was reduced in 17.2 and 24.4% of pups, respectively, showing a significant effect of immunization. In addition, vaccinated dams and non-pregnant mice exhibited significantly less parasite burden, compared to the corresponding control groups, and the likelihood of developing clinical signs was strongly reduced, with non-vaccinated animals at higher risk.

In contrast to vaccination protocols applied earlier [2,10,11], the third immunization was applied just after males and females were separated, 5 days prior to challenge infection. This

410 immunization protocol was safe and did not impair pregnancy. Applying the final immunization
411 during, rather than prior to, pregnancy could favor an immune response that is adequate for
412 pregnancy maintenance without losing efficacy against *N. caninum* infection, as shown for
413 several human vaccines [33].

414 The protection induced by immunization of mice with O-Ags was abrogated by adding
415 the TLR-3 ligand Poly I:C and the TLR7-ligand R848 as additional adjuvants. Thus, the
416 protection observed was associated with the particular OprI-adjuvant effect. TLR3 and TLR7 are
417 potent inducers of a Th1-type response [34]. In experiment 1, the O-Ags+TLR group exhibited
418 hallmarks of a strongly Th1-biased immunity. The more balanced Th1/Th2 immunity elicited
419 through O-Ags was more favorable for successful pregnancy. In addition, the complete absence
420 of protection in mice immunized with OprI-fused ovalbumin, and our previous studies employing
421 a chimeric *N. caninum* antigen (NcMIC1-3-R) [18], showed that the protection achieved with the
422 polyvalent O-Ags formulation employed here was clearly antigen-dependent.

423 OprI-PDI elicited the strongest humoral immune response. PDI-specific IgG levels were
424 elevated already prior to challenge, before and after mating. However, further investigation of
425 anti-PDI antibody levels at 9dpi in pregnant mice then demonstrated a decrease (or a lack of
426 boost) of antibody levels. This was observed in experiments 1 and 2, but mice could be
427 individually tracked only in experiment 2. In contrast, sera of only few mice had detectable
428 antibodies directed against ROP2 and ROP40 when assessed by ELISA prior to challenge and
429 this low response was confirmed by Western blotting using sera from experiment 1. Nevertheless,
430 immunoblotting confirmed specific recNcROP2 and recNcROP40 antibody responses, since a
431 signal against recNcROP2 was detected at 9dpi in both O-Ags and O-Ags+TLR vaccinated
432 groups, with no signal in the C+ group. RecNcROP40 was also detected slightly by both
433 vaccinated groups at pre-challenge phase, boosting at 9dpi in the O-Ags+TLR group.

The decrease, or lack of boost, of anti-PDI antibody levels after the third immunization in the vaccinated mice was only observed in pregnant animals. This indicates immune modulation due to the pregnancy, but it is unclear how this affects the overall protectivity of the vaccine formulation. An inverse correlation between anti-PDI-antibody levels and protection against cerebral infection could be observed in dams. Those pregnant mice with a slightly increased anti-PDI antibody levels between the pre-challenge and 9 dpi were those exhibiting high parasite burden and clinical signs score afterwards. Conversely, those dams showing the strongest drop of antibody levels between these two time-points were those with lowest parasite burden afterwards. However, in the pups, no correlation with vertical transmission was detected. Whether the impairment in anti-PDI antibodies boost was a consequence of an early control of parasite replication or a requisite for improved protection is not clear. A study on *N. caninum* profilin vaccinated mice [32] also reported on a drop of profilin-specific antibody titers shortly after challenge, and levels recovered at 21dpi. It was suggested that antibodies were being consumed by playing an active role against the infection [35]. However, only non-pregnant animals were assessed, and in our study non-pregnant mice actually showed a strong boost of antibody levels between pre-challenge and 9 dpi, and we did not note a correlation between antibody levels at 9dpi and cerebral parasite burden. Thus, the differential antibody kinetics observed here is likely to be a consequence of a pregnancy-associated phenomenon. Antibody levels are also altered during the course of *N. caninum* infection in cattle. In naturally infected heifers, antibody fluctuations occurring between 90 and 240 days of gestation were shown to be associated with a higher probability of vertical transmission [36, 37]. Cows that aborted also showed more pronounced fluctuations and overall higher antibody levels, especially between months 3 and 8 of gestation, compared to non-aborting cows [38]. Whether the vaccine-associated drop in antibody levels in dams compared to non-pregnant mice reflects a downregulation of total blood

458 immunoglobulins during pregnancy needs to be further investigated. In humans, an overall
459 reduction of total IgG and IgM was observed in healthy pregnant women compared to healthy
460 non-pregnant women [39].

461 In experiment 2, cytokine levels were studied at 9dpi in peripheral blood samples, and at
462 30dpi for non-pregnant mice and 43dpi for dams by analyzing splenocyte recall responses after
463 stimulation with crude *N. caninum* extracts. Moreover, all mice were individually tracked. At
464 9dpi, IFNγ blood levels were lower in O-Ags vaccinated mice compared to the C+ group, in both
465 pregnant and non-pregnant mice. However, at chronic phase, namely at 43dpi, when IFNγ levels
466 were measured in culture supernatants of stimulated splenocytes, this effect was not observed.
467 Splenocytes from vaccinated dams with lower vertical transmission rates tended to produce more
468 IFNγ, thus the correlation between IFNγ levels and vertical transmission was inverted. When
469 splenocytes from non-pregnant mice were stimulated and supernatants assessed for IFNγ, higher
470 levels were recorded in the O-Ags vaccinated group compared to C+ group. It was shown earlier
471 that in *N. caninum* infected mice IFNγ levels reach a peak around 10dpi [40], which is in
472 accordance with the highest IFNγ levels observed in our C+ group at 9 dpi. We suggest that the
473 down-regulation of IFNγ levels at 9dpi in O-Ags mice may have contributed to the reduction of
474 cerebral infection in non-pregnant mice and of vertical transmission in dams. A similar
475 interesting pattern of IFNγ responses was already described [41]. Dams primo-infected during
476 pregnancy (corresponding to our C+ group) showed increased production of IFNγ compared to
477 non-pregnant mice peaking at 11dpi. However, those dams which were protected against vertical
478 transmission by being infected before breeding showed a decreasing kinetic of IFNγ levels along
479 the pregnancy and increasing at delivery [41]. Further investigations should be carried out to
480 elucidate the role of IFNγ induced by protective vaccine formulations and how this IFNγ
481 response is affected during pregnancy.

In summary, these vaccination studies in a neosporosis mouse model have shown that promising efficacy against congenital and cerebral neosporosis can be achieved by immunization with a polyvalent combination vaccine composed of recombinant NcPDI, NcROP2 and NcROP40, all three fused to the TLR2-ligand OprI. This vaccine induced a balanced Th1/Th2 immune response in adults, which reduced vertical transmission, but was not highly efficacious in preventing cerebral infection in dams. To the best of our knowledge, this is a major advance in terms of efficacy achieved with a recombinant vaccine formulation. Further studies should aim to optimize the dosage and the timing of immunizations, and it will be highly interesting to elucidate the immunological mechanisms that are responsible for the protective effects against vertical transmission.

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631 **Figure Legends**
632 **Fig. 1.** Schematic presentation of experiments 1 and 2. Female mice were immunized in two-
633 week intervals, twice prior to mating (Imm. I; Imm. II), and a third time (Imm. III) shortly after
634 mating. All mice were challenged by subcutaneous inoculation of 10^5 *N. caninum* tachyzoites.
635 Mice were closely monitored for clinical signs and mortality. Non-pregnant mice were
636 euthanized on day 30 post-infection, dams and pups were euthanized on days 43-45 post-
637 infection. Immunoglobulins (Igs) and/or cytokine responses were analyzed at different time-
638 points for experiment 1 and 2: PrCh-1= Pre-challenge time-point experiment 1 (only Igs
639 analyzed); PrCh-2 = Pre-challenge time-point experiment 2 (only Igs analyzed); PstCh-pm =
640 Post-challenge “post-mating” time-point (Igs analyzed for experiment 1, both Igs and cytokines
641 for experiment 2); PstCh-NonPreg = Post-challenge “non-pregnant mice” time-point (Igs
642 analyzed for experiment 1, both Igs and cytokines for experiment 2); PstCh-D = Post-challenge

643 "dams" time-point (Igs analyzed for experiment 1, both Igs and cytokines for experiment 2).W =
644 time-point of oestrus synchronization (Whitten effect); M = mating
645
646 **Fig. 2.** SDS-PAGE and dendritic cell stimulation assays. (A) SDS-PAGE and Comassie staining
647 of OprI-PDI, OprI-ROP2, OprI-ROP40 and OprI-OVA, all expressed in *E. coli* and purified as
648 described in materials and methods. For each protein, 3, 1 and 0.3 µg (from left to right) was
649 loaded. M = molecular weight marker. (B) TNF-alpha levels in medium supernatants following
650 stimulation of mouse bone marrow-derived dendritic cells with 1 or 5 µg of OprI-PDI, OprI-
651 ROP2 or OprI-ROP40, compared with TNF-alpha-levels in supernatants of DC cultures
652 stimulated with the corrersponding non-OprI-antigens. Each assay was done in triplicates, and the
653 error bar indicates the standard error of the mean (SEM).
654
655 **Fig. 3.** Kaplan Meier survival curves of pups from experiment 1 (A) and experiment 2 (B).
656
657 **Fig. 4.** Cerebral parasite burden measured by real-time PCR in dams from experiment 1 (A) and
658 2 (B) at 40-44 dpi, and from non-pregnant mice from experiment 2 at 30 dpi (C). Experiment 1 is
659 not shown since only one mouse from the O-Ags group remained non-pregnant. (D) shows the
660 clinical signs score of non-pregnant mice in experiment 2.
661
662 **Fig. 5.** Humoral immune responses against recombinant antigens. A) Western blot analysis of
663 pooled sera obtained from experiment 1. Recombinant ROP2 (rROP2) (MW = 41 kDa) and
664 rROP40 (MW = 49 kDa) were separated by SDS-PAGE, blotted onto nitrocellulose, and rROP2-
665 and rROP40-specific IgG were detected by immunoblotting in mouse sera obtained at the pre-
666 challenge phase prior to mating (PrCh-1) and at 9 dpi coinciding with days 14-16 of pregnancy

(PstCh-pm.). "+" depicts a positive control serum from a mouse that was chronically infected with *N. caninum*. 1, 2, and 3 strips represent dilutions 1:50, 1:200 and 1:800 of pooled sera. Arrowheads indicate the location of recombinant proteins. (B) ELISA-based detection and quantification of PDI-specific IgG1 and IgG2a levels in experiment 1. PDI-specific antibodies in mice vaccinated with O-Ags and O-Ags+TLR ligands collected at the pre-challenge phase prior to mating (PrCh-1) and at 9 dpi coinciding with days 14-16 post mating (PstCh-pm). (C) Ratio of IgG1:IgG2a. Dots represent individual values of 4 randomly chosen mice euthanized at PrCh-1 and 3 pregnant mice randomly chosen to be euthanized at PstCh-pm. Horizontal lanes represent the median in each group.

Fig. 6. IgG1 and IgG2a antibodies against soluble *N. caninum* extract in sera from mice from experiment 2. Sera were from vaccinated groups (O-Ags, O-Ags+TLR, O-Ova) and the non-vaccinated and infected group (C+) collected at the end of the experiment (30 dpi for non-pregnant mice and 40 dpi for dams). (*) indicates statistically significant differences (Mann-Whitney U test, $P<0.05$).

Fig. 7. Cytokine levels measured in blood samples obtained at 9 dpi coinciding with days 14-16 post-mating (PstCh-pm.) in experiment 2. Boxes represent median, 25^{th} and 75^{th} percentiles; whiskers and individual points represent extreme values by Tukey method. (*) indicates statistically significant differences, Kruskal-Wallis, $P<0.05$.

Fig. 8. Cytokine responses after splenocyte restimulation *in vitro* with *N. caninum* crude extract in dams (40 dpi) and non-pregnant (30 dpi) mice at the end of experiment 2. Boxes represent median, 25^{th} and 75^{th} percentiles; whiskers and individual points represent extreme values by

691 Tukey method. (*) and (**) show Kruskal-Wallis, $P < 0.05$ and $P < 0.01$ statistically significant

692 differences, respectively, compared to C- group.

693

694

Table 1. Outcome of *Neospora caninum* infection in dams and pups in experiments 1 and 2.

Group	Experiment	Fertility[a]	No. dams[b]	Litter size[c]	Neonatal mortality[d]	Postnatal mortality[e]	Postnatal survival[f]	Median survival time[g]	Vertical transmission[h]
O-Ags	Exp. 1	14/15 (93.3%)	11	5.2	8/57 (14.0%)	32/49 (65.3%)	17/49 (34.7%)	18	39/49 (79.6%)
O-Ags	Exp 2.	9/20 (45%)	9	6.6	5/59 (8.5%)	31/54 (57.4%)	23/54 (42.6%)	21.5	35/54 (64.8%)
O-Ags+TLR	Exp. 1	9/16 (56.3%)	6	5.7	3/34 (8.8%)	26/31 (83.9%)	5/31 (16.1%)	16	28/31 (90.3%)
O-OVA	Exp. 2	9/20 (45%)	9	6.3	3/57 (5.3%)	47/54 (87.0%)	7/54 (12.9%)	16.5	48/54 (88.9%)
C+	Exp. 1	11/20 (55%)	8	5.6	14/45 (31.1%)	28/31 (90.3%)	3/31 (9.7%)	15	30/31 (96.8%)
C+	Exp. 2	14/20 (70%)	14	6.4	6/89 (6.7%)	70/83 (84.3%)	13/83 (15.7%)	16	74/83 (89.2)
C-	Exp. 1	11/16 (68.8%)	8	5.9	14/47 (29.8%)	0/33 (0%)	33/33 (100%)	Undef.	0/33 (0%)
C-	Exp. 2	10/20 (50%)	10	6.2	2/62 (3.2%)	0/60 (0%)	60/60 (100%)	Undef.	0/60 (0%)

[a] Proportion of pregnant mice per group (%)
[b] In experiment 1, three pregnant mice were excluded because they were euthanized before birth for blood sampling.
[c] Number of delivered pups per dam
[d] Proportion of pups born dead or that died within the first 2 days post-partum (%)
[e] Proportion of pups died from day 3 to 30 post-partum (%)
[f] Proportion of survival pups at day 30 post-partum (%)
[g] Day post-partum at which 50% of pups were dead
[h] Proportion of *Neospora caninum*-PCR positive surviving pups plus those which died from day 3 post-partum (dead pups from day 3 post-partum are considered *N. caninum*-PCR positive as previously shown (Dellarupe et al., 2014)).
Undef.: undefined, no pup mortality.

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IMMUNIZATION WITH A COCKTAIL OF ANTIGENS FUSED WITH OprI REDUCES *NEOSPORA CANINUM* VERTICAL TRANSMISSION AND POSTNATAL MORTALITY IN MICE

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Note: Supplementary data associated with this article

Abstract

OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa*, and when fused to a recombinant antigen, will exert adjuvant properties by engaging Toll-like receptor 2, leading to dendritic cell activation. Previous studies have shown that the *Neospora caninum* (Nc) antigens NcPDI, NcROP2 and NcROP40 are implicated in host cell interactions and are promising vaccine candidates. In two independent experiments, the efficacy of a polyvalent vaccine formulation composed of OprI-NcPDI, OprI-NcROP2 and OprI-NcROP40 (collectively named O-Ags) was assessed in non-pregnant and pregnant Balb/c mouse models challenged with tachyzoites of the high-virulence isolate Nc-Spain7. Parameters that were investigated were clinical signs, fertility, ~~efficacy~~parasite burden in adult mice, humoral and cellular immune responses at different time-points prior to and after challenge infection, vertical transmission and post-natal survival of offspring mice, all to explore potential correlations with efficacy. Vaccination of mice with O-Ags induced a mixed Th1/Th2 immune response in adult mice and led to significantly increased protection against cerebral infection. Vaccination with O-Ags also resulted in reduced vertical transmission, and postnatal disease in offspring was significantly inhibited at a rate not observed in mice infected with a high-virulence isolate to date. However, O-Ags mixed with TLR ligands targeting TLR3 and TLR7, which are known to induce clear Th1-biased responses, or vaccination with OprI fused to the non-*N. caninum* antigen ovalbumin (OprI-OVA) did not confer protection.

Keywords: Neosporosis; mouse model; pregnancy; vaccine; dendritic cells; immunomodulation; rhoptries; protein disulfide isomerase

1. Introduction

Neospora caninum is a cyst-forming coccidian closely related to *Toxoplasma gondii*, and is one of major causes of infective abortion in cattle [1]. In addition, *N. caninum* infection in cattle often results in birth of weak calves or persistently infected offspring, which then transmits the parasite to the next generation. Vaccination has been considered to be the most cost-effective strategy to control bovine neosporosis [2,3], but no commercial vaccines are available [4].

Live vaccines based on attenuated *N. caninum* isolates partially protected mice and cattle against fetal death upon experimental challenge infection during pregnancy [5,6,7]. However, several disadvantages of live vaccines related to costs of parasite *in vitro* culture, storage, limited shelf life of the final product and logistics of its distribution, as well as the potential risks of virulence reversion and recombination between attenuated and circulating strains, render live vaccines a rather unattractive option [4]. Vaccines based on total antigenic extracts or different antigenic fractions of the parasite have shown variable results with overall lower success [7]. Subunit vaccines composed of recombinant antigens would represent an interesting option and would solve many of the inherent logistic and financial disadvantages of live vaccines [4]. Admittedly, recombinant vaccine formulations have so far not shown very promising effects in pregnant neosporosis animal models. However, the versatile potential of recombinant vaccines should be much more exhaustively exploited by applying antigen combinations with adjuvants targeting different components of the immune system [4].

There is “proof-of-concept” that a reduction of vertical transmission and postnatal mortality due to *N. caninum* infection can be achieved by vaccination not only with live vaccines [8,9], but also with recombinant subunit vaccines in mice [10,11]. Bacterially expressed recNcPDI, recNcROP2 and recNcROP40 represent promising vaccine candidates. Immunization

of mice with recNcROP2 formulated in saponin resulted in a significantly increased survival rate in adult mice [12] and in offspring born to mice infected during pregnancy with the *N. caninum* Nc-1 isolate [10]. NcROP40 is another rhoptry protein that is abundantly expressed in virulent *N. caninum* isolates [13]. Immunization of mice with both recNcROP40 and recNcROP2 recombinant proteins lead to reduction of vertical transmission and postnatal mortality of 16% in offspring born to mice infected with the highly virulent isolate Nc-Spain7 [11]. RecNcPDI (*N. caninum* protein disulfide isomerase) had conferred protection against cerebral neosporosis when applied intranasally [14], but failed to protect dams and their offspring [2].

The TLR2-ligand OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa* ~~[15].~~ It has been used to confer adjuvant properties when expressed in fusion with different antigens. [15]. Different immune functions have been attributed to TLR2 signaling including the promotion of a mixed and balanced Th1-, Th2- and ~~Th2~~ Treg cell response [16] and a T-cell tropism to mucosal tissues even when inoculated via a non-mucosal route [17]. In previous immunization studies, OprI had been fused to the *N. caninum* chimeric antigen Mic3-1-R, and vaccinated mice were challenged during pregnancy. This did not result in protection, neither in dams nor in offspring mice, but we nevertheless detected that fusion of OprI to MIC3-1-R induced a mixed Th1/Th2 profile response [18], while the immune response in mice immunized with MIC3-1-R emulsified in saponin was largely Th2-biased, thus confirming the immunomodulatory properties of OprI-fusions. This well-adjusted Th1/Th2 balance is required to protect *N. caninum* vaccinated mice during pregnancy when a strong Th1 response could be detrimental [30].

In this study, we performed two independent experiments to assess the efficacy of a polyvalent vaccine formulation composed of OprI-NcPDI, OprI-NcROP2 and OprI-NcROP40 (collectively named O-Ags) in non-pregnant and pregnant Balb/c mouse models challenged with

N. caninum tachyzoites. In one of the experiments, the same O-Ags formulation was mixed with two additional TLR ligands targeting TLR3 and TLR7, which are known to induce clear Th1-biased responses [19] and likewise effects in dams and offspring were assessed. In the other experiment, a group vaccinated with OprI fused to ovalbumin (OprI-OVA) was included to provide an irrelevant antigen control.

2. Materials and methods

2.1. Neospora caninum *culture, inoculum and crude extract preparation*

The *N. caninum* Nc-Spain7 isolate [20] was grown *in vitro* by continuous passages in Vero cell cultures [18]. The parasite inoculum was prepared as described [21]. *N. caninum* crude extract for lymphocyte re-stimulation assay and Western blot, and soluble antigen fractions for ELISA, were prepared as described earlier [18].

2.2. Production of recombinant antigens fused to OprI

The coding sequence of NcPDI (aa 25-446) [22], a fragment of NcROP2 (aa 238-594) [12] and the complete coding sequence of NcROP40 [11] were cloned in the pOLP plasmid in frame ~~wiuth~~with OprI [15]. A previously described plasmid, containing the ovalbumin (OVA) partial sequence (aa 203-386) in frame with OprI [16] was also used to obtain the unrelated antigen OprI-OVA. Proteins were all expressed in Rosetta(DE3)pLysS *Escherichia coli* strain (Novagen). They were isolated from the bacterial outer membranes as previously described [18]. Purified OprI-NcPDI was dialyzed against PBS over-night at 4°C and stored at -80°C. OprI-NcROP2 and OprI-NcROP40, expressed less efficiently and insoluble in aqueous solution, were

dialyzed over-night against 1g/L ~~ammoniumbicarbonate~~ammonium bicarbonate at 4°C and lyophilized. They were resuspended in PBS in one-tenth of the original volume, vortexed, and were sonicated at 30°C in a water bath until a homogeneous suspension was obtained. All purified OprI-antigens were finally stored at -80°C. Non-OprI-fused NcPDI, NcROP2 and NcROP40 [11,22,22], were expressed and purified as described [18]. The purity and integrity of all proteins were analyzed by SDS-PAGE and the protein concentration was determined (BCA Protein Assay Kit, Thermo Scientific Pierce). Endotoxin levels were quantified by the Limulus-Amebocyte-Lysate-Kinetic-QCL (Lonza). The final LPS content was below 0.1EU/µg.

2.3. Dendritic cell (DC) stimulation

OprI-fused and non-fused recombinant proteins were assessed for DC stimulation [18]. Briefly, DCs were obtained *in vitro* from bone marrow cells obtained from a naïve Balb/c mouse. DCs ($0.5x10^6$ cells/ml~~),~~) were cultured in the presence of each protein (1 or 5µg/ml), LPS (0.1µg/ml) or medium during 24hours, and TNF-α was measured in the culture supernatants by ELISA [18].

2.4. Immunization schedule, clinical monitoring and sample collection

All animal protocols were approved by the Bernese Animal Welfare Committee (license BE115/14.) Two experiments were carried out. Schedules of immunization, mating, blood sample collection and euthanasia for both studies are depicted in Fig.1. Six weeks-old BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained as described [18].

In Experiment 1, 16 to 20 female mice per group were immunized three times, at two-week intervals. The O-Ags group was subcutaneously injected with all three OprI-antigens, 5µg each; the O-Ags+TLR group was immunized with the same antigens mixed with poly I:C (TLR3 ligand) (Invivogen) and R848 (TLR7 ligand) (Invivogen), 25µg each. Two control groups, one of which remained non-infected throughout the experiment (C-), the other one that was infected (C+), received PBS only. Eight days after the second injection, just prior to the oestrus synchronization, a blood sample was obtained from 4 mice per group (see Fig.1; PrCh-1) and serum recovered for IgG analysis.

In experiment 2, experimental groups of 20 female mice were immunized subcutaneously three times, at two-week intervals. The O-Ags group received the three antigens as above, the O-OVA group was injected with 15µg of OprI-OVA, the two additional groups (C- and C+ groups, see above) received PBS. A blood sample from the tail was obtained from all mice ~~3~~4 days after the third immunization (at 4-7 days post-mating) just prior to challenge (Fig.1; PrCh-2).

Eight days post- second immunization, ~~all mice~~mice in both experiment 1 and experiment 2 were submitted to oestrus synchronization [23] and 2 females and 1 male per cage were mated during 72h. The third immunization was applied after separation of males and females, 5 days prior to challenge infection. At 5-8 days of pregnancy, mice from the O-Ags, O-Ags+TLR, O-OVA and C+ groups were subcutaneously infected with 10^5 Nc-Spain7 tachyzoites [24]. Mice of the C- ~~group~~groups received only PBS. At day 9 post-~~challenge,~~ infection, a blood sample was obtained from the tail of 3 pregnant mice of each group from experiment 1 ~~were euthanized,~~ and ~~blood was collected by intracardiac puncture, whereas in~~of all mice from experiment 2 ~~a blood sample was obtained from the tail of all mice~~ (Fig.1; PstCh-pm). ~~On day 18 post-mating~~Two days before birth, pregnant and non-pregnant mice were separated. Dams were allowed to give birth and rear their offspring in individual cages. Non-pregnant mice were kept in groups of 4-5 mice

per cage. Following infection, all mice were daily monitored for clinical signs using a standardized score sheet: coat (ruffled coat=score1; stary stiff coat=score2); weight loss (10% loss=score1, 15-20% loss=score 4); behavior (hunched appearance, walking in circles, head tilt, apathy and ataxia, all=score 1). Animal were euthanized when the score exceeded 3 points.

At 30 days post-infection (30 dpi), all surviving non-pregnant mice in both experiments were euthanized (Fig.1; PstCh-NonPreg). Dams and their pups were maintained together until day 30 post-partum (pp) (=41-44 dpi), subsequently all animals were euthanized (Fig.1; PstCh-D).

Data on clinical signs, fertility (percentage of pregnant mice), litter size (average of number of pups born per dam), neonatal mortality of pups (stillborn pups or pups dying within the first 2 days post-partum (dpp) and postnatal mortality (pups dying between day 3 and 30pp) were recorded. Upon euthanasia, blood was extracted by cardiac puncture and sera stored at -80 °C. Brains from non-pregnant mice, dams and surviving pups were also collected and immediately frozen at -20°C. Spleens from 5 to 6 non-pregnant mice and dams per group were aseptically recovered and processed for splenocyte re-stimulation *in vitro*.

2.5.Analysis of the cerebral parasite burden

Brains from non-pregnant mice, dams and surviving pups were analyzed by *N. caninum*-specific real-time PCR [25]. DNA extraction was performed using the Nucleospin Kit (Macherey-Nagel, Oensingen, Switzerland). The DNA concentration in all samples was determined using the QuantiFluor dsDNA System (Promega, Madison, Wi.) and was adjusted to 5ng/µl with sterile DNase free water. Quantitative real-time PCR was performed using the Rotor-Gene 6000 real-time PCR machine. The parasite load was calculated by interpolation from a

194 standard curve with DNA equivalents from 1000, 100 and 10 *N. caninum* tachyzoites included in
195 each run.
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197 2.6. *Assessment of cytokine expression levels in serum and splenic lymphocytes stimulated* in
198 vitro
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200 Levels of mouse IFN-γ, IL-10, IL-2 and IL-5 were assessed at two different time-points in
201 experiment 2: (i) at 9dpi, coinciding with days 14-~~16~~17 of pregnancy (PstCh-pm, see Fig.1) in
202 blood, and (ii) at the late post-challenge phase in non-pregnant mice (30 dpi) and dams (43-45
203 dpi) by splenocyte re-stimulation *in vitro* as described [18]. For cytokine assessment in blood, the
204 serum samples were analysed by bead-based multiplex assay for the Luminex® platform
205 (Hertogenbosch, The Netherlands) . Microtiter filter plates were run on Luminex instruments
206 (Bio-Plex^{TM}200 system). Calibration curves were calculated with Bio-Plex Manager software
207 using a five-parametric logistic curve fitting [18]. For cytokine analysis upon splenocyte recall
208 responses, spleens were disaggregated and splenocyte cell suspensions were prepared as
209 described [18], The resulting cell suspensions were seeded in 48-well plates and re-stimulated
210 with either concanavalin A (ConA; 5µg/ml; Sigma), recNc-PDI, NcROP2, NcROP40 (20µg/ml),
211 whole *N. caninum* crude extract (10µg/ml) or remained non-stimulated. Supernatants were
212 collected after 72h and stored at -80°C until cytokines were assessed by ELISA (BD
213 OptEIA™ Mouse ELISA Set, LifeSpan Biosciences Inc., Seattle, WA, USA).
214
215 *2.7. Analysis of serum immunoglobulins*
216
217 Immunoglobulins were analyzed at different time-points: (i) during the pre-challenge
218 phase prior to mating in experiment 1 (PrCh-1); (ii) during pre-challenge phase after mating in

experiment 2 (PrCh-2); (iii) during the early post-challenge phase in experiments 1 and 2 (PstCh-pm); (iv) at the late post-challenge phase just prior to euthanasia in non-pregnant mice (PstCh-NonPreg) and in dams (PstCh-D) (see Fig.1). Serum levels of *N. caninum*, NcPDI-, NcROP2- or NcROP40-specific IgG1 and IgG2a were measured by ELISA [26]. Four 4-fold serial dilutions were analyzed for each sample and results were expressed as end-point titer (EPT) calculated as the inverse value of the dilution giving an OD≥cut-off [27]. The cut-off was independently positioned in each plate in the lower linear part of the dilution curve of the same positive control which allowed the normalization between plates.

PrCh-1 and PstCh-pm serum samples from experiment 1 were also analyzed in pools by immunoblotting for NcROP2- and NcROP40-specific IgG. Non-OprI- NcROP2 and NcROP40 (10µg each) were submitted to Western blot analysis following standard procedures [26]. Pooled sera were incubated at 1:50, 1:200 and 1:800 dilutions and an anti-mouse IgG antibody conjugated to alkaline phosphatase (eBioscience) at 1:5000 dilution was employed.

2.8.Statistical analysis

Cytokines levels, clinical signs scores and cerebral parasite burdens were compared between groups by the non-parametric Kruskal-Wallis test; upon detection of statistical differences, a Dunn's multiple comparison test was subsequently applied to compare pair-by-pair. End point ~~titer (EPT)~~titers of IgG1 and IgG2a or ratios IgG1:IgG2a were compared between groups by Kruskal-Wallis test followed by Dunn's multiple comparison post-test or by ~~*U*~~ Mann-Whitney U test when only two groups were compared at pre-challenge and pregnancy phases or between dams and non-pregnant mice in each group. Repeated measures of PDI antibodies along time were compared by Wilcoxon matched pairs test. Correlations between parasite burden or vertical transmission and any immune parameters were analyzed by Pearson correlation

coefficient. To compare the mortality of pups along time, survival proportions at each time-point were plotted in Kaplan-Meier graphs and survival curves were compared by Log-rank test. The percentages of survivors at the end of the experiment or percentages of *N. caninum* PCR positive samples were analyzed by Chi-square test with Yates' continuity correction in a contingency table.

Regarding the parameters measured in both experiments and compared between groups (C-, O-Ags and C+), a unique statistical analysis was done with all data from both experiments. A mixed-effects log-linear model (Venables and Ripley, 2002) was used to analyze pregnancy and immunization with the O-Ags formulation as fixed effects on parasite burden, having experiment as random effect. Effect sizes were estimated with a penalized quasi-likelihood method (glmmPQL from MASS library). A logistic mixed model with Laplace parameter estimation via Lapplace approximation (glmer of lme4 package) was used to evaluate clinical signs presence and vertical transmission. The odds-ratio (OR) relative to the O-Ags group was calculated. with the respective 95% confidence interval (CI 95%). Survival of pups was compared with a Cox model with fixed (immunization) and random (experiment) effects (coxme library). Statistical significance was established at $P<0.05$.

3. Results

3.1. Increased dendritic cell stimulation by OprI-fused recombinant antigens

The purified OprI-fused recombinant proteins shown in Fig.2A were used for stimulation of bone marrow-derived DCs, which were cultured in the presence of 1 or 5 µg/ml of each protein, LPS (0.1µg/ml) or medium during 24 hours, and the production of TNF-α was measured

in the culture supernatants by ELISA (Fig.2B). In contrast to the non-fused recombinant antigens, the OprI-proteins induced strong and specific DC stimulation (Fig.2B).

3.2. Immunization with OprI-fused Neospora *antigens reduced vertical transmission and increased postnatal survival*

Table 1 shows the outcome of *N. caninum* infection in dams and pups in both experiments. Comparing the O-Ags groups and the C+ infection control groups in both experiments, using the experiment as random effect in the mixed model, there was a significant ~~difference~~increase in survival of offspring of ~~immunized animals (~~O-Ags groups P <0.001) and a significant reduction in vertical transmission (OR=4.52, CI 95% [2.06;10.63], P<0.001).

Analyzing experiment 1 separately, offspring from O-Ags group also showed a significant higher survival compared to C+ (χ^2 and Log-rank test, P <0.05). In the O-Ags+TLR group in experiment 1 (Fig.3A), the pup survival ~~curves were~~curve was also significantly different from the O-Ags group (Log-rank test, P<0.01), showing that supplementation of the O-Ags formulation by TLR3 plus TLR7 ligands abolished the protective effect of O-Ags immunization. In fact, the O-Ags+TLR group the ~~pup~~ survival ~~curve~~of pups was not significantly different from C+ (~~O-Ags+TLR *vs* C+;~~ χ^2 and Log-rank test, P >0.05) (Table 1; Fig. 3A).

In experiment 2 (Fig. 3B), comparing the C+ group with the O-Ags group, the ~~pup~~percentage of pups survival and the pup survival curves from O-Ags were also significantly ~~different (~~higher (χ^2 and Log-rank test, P <0.01~~).~~) and the vertical transmission significantly lower than C+ (χ^2 and Log-rank test, P <0.01). Here, a group of mice was immunized with OprI fused to ovalbumin (O-OVA group). In ~~the~~this latter group immunized with O-OVA, the

percentage of surviving pups, the pup survival curve ~~was~~and the vertical transmission were identical to C+ (O-OVA *vs* C+; χ^2 and Log-rank test, P >0.05) (Table 1; Fig.3B).

3.3. Immunization with OprI-fused Neospora *antigens reduced cerebral neosporosis*

Taking into account both experiments in the mixed-effects ~~log linear~~logistic model there was a significant effect of O-Ags vaccination on the likelihood of animals developing clinical signs, with C+ animals at higher risk (OR=12.6, CI 95% [1.76; 260.66], $P<0.05$) independent of pregnancy. A significant effect of both O-Ags immunization ($P<0.001$) and pregnancy ($P<0.01$) on the cerebral parasite burden, without a significant interaction between these two variables~~,~~ ($P>0.05$), was observed with the mixed effects log linear model (Fig.4A,B~~)~~). Compared to non-immunized mice~~,~~ (C+), O-Ags vaccinated dams showed lower parasite burdens ~~(Fig.4A,B). In~~, In experiment 1 and 2, dams immunized with O-Ags+TLR or O-OVA~~, no reduction in~~ did not show reduced cerebral parasite burden ~~was detected~~compared to C+ (Kruskal-Wallis, $P>0.05$) (Fig.4A,B). ~~Immunization~~Regarding non-pregnant mice, immunization with O-Ags also ~~impacted on~~ reduced significantly the cerebral parasite burden and clinical signs ~~in non pregnant immunized animals~~ (Fig.4C,D). This could only be seen in experiment 2 (O-Ags vs C+; Kruskal-Wallis, $P<0.05$), since in experiment 1 only one mouse from O-Ags was not pregnant. No protective effect was observed in ~~the one non pregnant mouse~~ mice immunized with O-Ags+TLR (data not shown) or ~~in the~~ O-OVA ~~immunized mice~~ (Kruskal-Wallis, $P>0.05$) (Fig.4C).

3.4. Humoral immune responses against recombinant antigens

Specific antibody responses against each of the recombinant antigens used for immunization were studied by ELISA in both experiments at different time-points in the pre-

challenge phase, either prior to mating in experiment 1 (PrCh-1) or 5 days post-mating in experiment 2 (PrCh-2), and at 9dpi (14-16 days post-mating; PstCh-pm.). Specific anti-ROP2 and anti-ROP40 antibody levels were very low at both pre-challenge time-points and several animals remained ELISA-seronegative when assessed even at 9dpi~~)~~ in both experiments. Thus, pools of sera collected at the pre- and post-challenge time-points in experiment 1 were analyzed by Western blotting (Fig.5A). Anti-ROP2 antibodies remained virtually undetectable in sera taken during the pre-challenge phase in the groups immunized with O-Ags and O-Ags+TLR~~, but.~~ However, IgG levels in O-Ags and O-Ags+TLR were higher in sera taken during pregnancy at 9 dpi~~,~~ (PstCh-pm), especially in the group immunized with O-Ags. Anti-ROP40 IgG levels were also low in both groups prior to challenge but were dramatically elevated in the group immunized with O-Ags+TLR in post-challenge sera taken from pregnant mice (PstCh-pm) (Fig.5A). Sera obtained from the O-OVA and the C+ group remained negative for ROP2 and ROP40 at both PrCh-1 and PstCh-pm.

PDI-specific antibodies were detected by ELISA during the pre-challenge phase. In experiment 1, levels in mice vaccinated with O-Ags+TLR were higher than those in mice vaccinated with O-Ags, and in spite of the low number of samples the difference reached statistical significance for IgG2a at the pre-challenge phase in experiment 1 (PrCh-1; ~~*U*Mann~~Mann-Whitney U test, $P<0.05$) (Fig.5B). Moreover, the IgG1:IgG2a ratio was markedly lower in the O-Ags+TLR group compared to the O-Ags group, and was statistically significant during the pre-challenge phase (~~*U*~~ Mann-Whitney U test, $P< 0.05$) (Fig.~~5B~~5C). Surprisingly, PDI antibody levels were lower at 9dpi (PstCh-pm) than before mating in both vaccinated groups (Fig.5B). Since the values at 9dpi corresponded to just three pregnant animals and no identification of mice was carried out, we could not deduce the individual evolution of antibody levels in experiment 1. Thus, in experiment 2 mice were individually tracked. With the exception

of one, those O-Ags-vaccinated mice with confirmed pregnancy exhibited very little changes or decreased anti-PDI antibody levels during the timespan between pre-challenge to 9dpi (Wilcoxon matched pairs test; $P>0.05$), whereas those mice that did not become pregnant exhibited a clear boost of anti-PDI antibody levels (Wilcoxon matched pairs test, $P<0.01$) (suppl. Fig.1), confirming the results from experiment 1. The same tendency was observed with IgG2a levels (data not shown). Only one pregnant mouse exhibited a strong increase of antibody levels, and this animal had a very high parasite burden, elevated clinical signs score, and vertical transmission of *N. caninum* tachyzoites to its offspring (suppl Fig.1).

3.5.Antibody responses against soluble N. caninum antigens

No significant differences in IgG1 or IgG2a levels between groups were detected (~~two way ANOVA~~Kruskal-Wallis, $P>0.05$) (Fig.6). In sera from mice immunized with O-Ags, and O-OVA, and from infected non-immunized C+ mice, similar IgG2a levels were noted in dams and non-pregnant mice (~~*U* mann~~Mann-Whitney U test, $P>0.05$), whereas IgG1 levels were significantly lower in non-pregnant mice compared to dams (~~*U*mann~~Mann-Whitney U test, $P<0.05$) pointing towards an IgG2a-biased response in non-pregnant mice compared to dams. In contrast, in sera of mice vaccinated with O-Ags+TLR ligands, no such differences between dams and non-pregnant were detected (~~*U*mann~~Mann-Whitney U test, $P>0.05$), and the humoral immune response was IgG2a-biased in both cases~~.~~ (Fig. 6).

3.6.Cytokine responses

Cytokines were measured in experiment 2 at 9dpi in peripheral blood and at the end of the experiment upon *in vitro* stimulation of splenocytes and analysis of culture supernatants. At 9dpi, the levels of IFNγ, IL-10 and IL-5 in peripheral blood were significantly elevated in all infected animals when compared to group C- (Fig.7). However, IFNγ-levels in pregnant as well as non-pregnant mice vaccinated with O-Ags were significantly lower than in the C+ group (Kruskal-Wallis, $P<0.05$). IL-10 levels were also significantly lower in non-pregnant mice vaccinated with O-Ags compared to non-pregnant mice in the C+ group. No significant levels of IL-2 were detected in peripheral blood samples of any group. (data not shown).

At the end of the experiment mice were sacrificed, splenocytes were cultured and cytokine recall responses were measured in medium supernatants. In non-pregnant mice, only splenocytes from O-Ags and O-OVA vaccinated mice displayed a consistent increase of IFNγ and IL-10 secretion upon stimulation with *N. caninum* crude extract, compared to splenocytes from C- mice (Kruskal-Wallis, $P<0.05$) (Fig.8). Splenocytes derived from the C+ group showed no changes or very little increase of cytokine production. In the case of vaccinated dams, splenocyte cytokine levels were more heterogeneously distributed, with high individual variations, with the exception of highly elevated IL-2 responses in splenocytes isolated from O-Ags vaccinated dams, (Fig.8).

4. Discussion

The development of sub-unit vaccines against parasitic diseases is a major challenge due to the complexity of the biology of parasites [28]. This is especially true in the case of congenital neosporosis, for which so far only live-attenuated vaccines have achieved reasonable levels of protection against vertical transmission [29, 0]. In the present work, an immunogenic formulation

composed of three recombinant *N. caninum* antigens fused to OprI was assessed in a standardized pregnant mouse model of neosporosis based on the highly virulent *N. caninum* isolate Nc-Spain7. The vaccine formulation was composed of bacterially expressed and purified recNcPDI, NcROP2 and NcROP40, all three N-terminally fused to the OprI ~~lipoprotein.~~, a tri-acylated bacterial outer membrane protein. OprI targets TLR2, and thus stimulates mixed Th1, Th2 and Treg responses, favoring cross-presentation by APCs [16] Moreover, OprI was able to modulate the cellular immune response against *N. caninum* towards a mixed Th1/Th2 response in mice vaccinated with the *N. caninum* chimeric antigen Mic3-1-R [18]. The Nc*ROP40* gene was found to be identical in three *N. caninum* isolates displaying different virulence, which renders this a promising vaccine candidate [31,32]. RecNcROP2 had been demonstrated to confer significant protection in non-pregnant and pregnant neosporosis mouse models based on the ~~-~~*N. caninum* Nc1 isolate [10,12]. RecNcPDI had conferred excellent protection in non-pregnant mice when applied intranasally emulsified in cholera toxin, but failed to prevent congenital neosporosis in pregnant mice [2, 14]. Nevertheless, since TLR2-ligands have shown to induce mucosal immunity by imprinting lymphocyte tropism to mucosae, even though they are applied by non-mucosal route [17], and NcPDI had conferred mucosal immunity [14], we incorporated this protein into the polyvalent O-Ags formulation.

We show here in two independent experiments that immunization with O-Ags confers significant protection in offspring born to dams that were challenged with *N. caninum* tachyzoite infection on day 7-9 of pregnancy. Postnatal mortality was reduced by 25.0 and 26.9% of pups in experiment 1 and 2, respectively, the mean survival time was extended and, overall, there was a clear difference in the survival curves. This rate of protection against congenital neosporosis has not been seen with recombinant antigens before (11). Vertical transmission (including dead pups and PCR positive survivors) was reduced in 17.2 and 24.4% of pups, respectively, showing a

significant effect of immunization. In addition, vaccinated dams and non-pregnant mice exhibited significantly less parasite burden, compared to the corresponding control groups, and the likelihood of developing clinical signs was strongly reduced, with non-vaccinated animals at higher risk.

In contrast to vaccination protocols applied earlier [2,10,11], the third immunization was applied just after males and females were separated, 5 days prior to challenge infection. This immunization protocol was safe and did not impair pregnancy. Applying the final immunization during, rather than prior to, pregnancy could favor an immune response that is adequate for pregnancy maintenance without losing efficacy against *N. caninum* infection, as shown for several human vaccines [33].

The protection induced by immunization of mice with O-Ags was abrogated by adding the TLR-3 ligand Poly I:C and the TLR7-ligand R848 as additional adjuvants. Thus, the protection observed was associated with the particular OprI-adjuvant effect. TLR3 and TLR7 are potent inducers of a Th1-type response [34]. In experiment 1, the O-Ags+TLR group exhibited hallmarks of a strongly Th1-biased immunity. The more balanced Th1/Th2 immunity elicited through O-Ags was more favorable for successful pregnancy. In addition, the complete absence of protection in mice immunized with OprI-fused ovalbumin, and our previous studies employing a chimeric *N. caninum* antigen (NcMIC1-3-R;) [18], showed that the protection achieved with the polyvalent O-Ags formulation employed here was clearly antigen-dependent.

OprI-PDI elicited the strongest humoral immune response. PDI-specific IgG levels were elevated already prior to challenge, before and after mating. However, further investigation of anti-PDI antibody levels at 9dpi in pregnant mice then demonstrated a decrease (or a lack of boost) of antibody levels. This was observed in experiments 1 and 2, but mice could be individually tracked only in experiment 2. In contrast, sera of only few mice had detectable

antibodies directed against ROP2 and ROP40 when assessed by ELISA prior to challenge and ~~at 9dpi., and~~ this low response was confirmed by Western blotting using sera from experiment 1. Nevertheless, immunoblotting confirmed specific recNcROP2 and recNcROP40 antibody responses, since a signal against recNcROP2 was detected at 9dpi in both O-Ags and O-Ags+TLR vaccinated groups, with no signal in the C+ group. RecNcROP40 was also detected slightly by both vaccinated groups at pre-challenge phase, boosting at 9dpi in the O-Ags+TLR group.

The decrease, or lack of boost, of anti-PDI antibody levels ~~was observed only in pregnant animals. The lack of PDI-specific antibody boost~~ after the third immunization in the vaccinated ~~pregnant~~ mice was only observed in pregnant animals. This indicates immune modulation due to the pregnancy, but it is unclear how this affects the overall protectivity of the vaccine formulation. An inverse correlation between anti-PDI-antibody levels and protection against cerebral infection could be observed in dams. Those pregnant mice with a slightly increased anti-PDI antibody ~~level~~levels between the pre-challenge and 9 dpi were those exhibiting high parasite burden and clinical signs score afterwards. Conversely, those dams showing the strongest drop of antibody levels between these two time-points were those with lowest parasite burden afterwards. However, in the pups, no correlation with vertical transmission was detected. Whether the impairment in anti-PDI antibodies boost was a consequence of an early control of parasite replication or a requisite for improved protection is not clear. A study on *N. caninum* profilin vaccinated mice [32] also reported on a drop of profilin-specific antibody titers shortly after challenge, and levels recovered at 21dpi. It was suggested that antibodies were being consumed by playing an active role against the infection [35]. However, only non-pregnant animals were assessed, and in our study non-pregnant mice actually showed a strong boost of antibody levels between pre-challenge and 9 dpi, and we did not note a correlation between antibody levels at

9dpi and cerebral parasite burden. Thus, the differential antibody kinetics observed here is likely to be a consequence of a pregnancy-associated phenomenon. Antibody levels are also altered during the course of *N. caninum* infection in cattle. In naturally infected heifers, antibody fluctuations occurring between 90 and 240 days of gestation were shown to be associated with a higher probability of vertical transmission [36, 37]. Cows that aborted also showed more pronounced fluctuations and overall higher antibody levels, especially between months 3 and 8 of gestation, compared to non-aborting cows [38]. Whether the vaccine-associated drop in antibody levels in dams compared to non-pregnant mice ~~is a reflection of~~reflects a downregulation of total blood immunoglobulins during pregnancy needs to be further investigated. In humans, an overall reduction of total IgG and IgM was observed in healthy pregnant women compared to healthy non-pregnant women [39].

In experiment 2, cytokine levels were studied at 9dpi in peripheral blood samples, and at 30dpi for non-pregnant mice and 43dpi for dams by analyzing splenocyte recall responses after stimulation with crude *N. caninum* extracts. Moreover, all mice were individually tracked. At 9dpi, IFNγ blood levels were lower in O-Ags vaccinated mice compared to the C+ group, in both pregnant and non-pregnant mice. However, at chronic phase, namely at 43dpi, when IFNγ levels were measured in culture supernatants of stimulated splenocytes, this effect was not observed. Splenocytes from vaccinated dams with lower vertical transmission rates tended to produce more IFNγ, thus the correlation between IFNγ levels and vertical transmission was inverted. When splenocytes from non-pregnant mice were stimulated and supernatants assessed for IFNγ, higher levels were recorded in the O-Ags vaccinated group compared to C+ group. It was shown earlier that in *N. caninum* infected mice IFNγ levels reach a peak around 10dpi [40], which is in accordance with the highest IFNγ levels observed in our C+ group at 9 dpi. We suggest that the down-regulation of IFNγ levels at 9dpi in O-Ags mice may have contributed to the reduction of

cerebral infection in non-pregnant mice and of vertical transmission in dams. A similar interesting pattern of IFNγ responses was already described [41]. Dams primo-infected during pregnancy (corresponding to our C+ group) showed increased production of IFNγ compared to non-pregnant mice peaking at 11dpi. However, those dams which were protected against vertical transmission by being infected before breeding showed a decreasing kinetic of IFNγ levels along the pregnancy and increasing at delivery [41]. Further investigations should be carried out to elucidate the role of IFNγ induced by protective vaccine formulations and how this IFNγ response is affected during pregnancy.

In summary, these vaccination studies in a neosporosis mouse model have shown that promising efficacy against congenital and cerebral neosporosis can be achieved by immunization with a polyvalent combination vaccine composed of recombinant NcPDI, NcROP2 and NcROP40, all three fused to the TLR2-ligand OprI. This vaccine induced a balanced Th1/Th2 immune response in adults, which reduced vertical transmission, but was not highly efficacious in preventing cerebral infection in dams. To the best of our knowledge, this is a major advance in terms of efficacy achieved with a recombinant vaccine formulation. Further studies should aim to optimize the dosage and the timing of immunizations, and it will be highly interesting to elucidate the immunological mechanisms that are responsible for the protective effects against vertical transmission.

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Figure Legends

~~**Fig. 1**~~**Fig. 1.** Schematic presentation of experiments 1 and 2. Female mice were immunized in two-week intervals, twice prior to mating (Imm. I; Imm. II), and a third time (Imm. III) shortly after mating. All mice were challenged by subcutaneous inoculation of 10^5 *N. caninum* tachyzoites. Mice were closely monitored for clinical signs and mortality. Non-pregnant mice were euthanized on day 30 post-infection, dams and pups were euthanized on days 43-45 post-infection. Immunoglobulins (Igs) and/or cytokine responses were analyzed at different time-points for experiment 1 and 2: PrCh-1= Pre-challenge time-point experiment 1 (only Igs

analyzed); PrCh-2 = Pre-challenge time-point experiment 2 (only Igs analyzed); PstCh-pm = Post-challenge "post-mating" time-point (Igs analyzed for experiment 1, both Igs and cytokines for experiment 2); PstCh-NonPreg = Post-challenge "non-pregnant mice" time-point (Igs analyzed for experiment 1, both Igs and cytokines for experiment 2); PstCh-D = Post-challenge "dams" time-point (Igs analyzed for experiment 1, both Igs and cytokines for experiment 2).W = time-point of oestrus synchronization (Whitten effect); M = mating

Fig. 2. SDS-PAGE and dendritic cell stimulation assays. (A) SDS-PAGE and Comassie staining of OprI-PDI, OprI-ROP2, OprI-ROP40 and OprI-OVA, all expressed in *E. coli* and purified as described in materials and methods. For each protein, 3, 1 and 0.3 µg (from left to right) was loaded. M = molecular weight marker. (B) TNF-alpha levels in medium supernatants following stimulation of mouse bone marrow-derived dendritic cells with 1 or 5 µg of OprI-PDI, OprI-ROP2 or OprI-ROP40, compared with TNF-alpha-levels in supernatants of DC cultures stimulated with the corrersponding non-OprI-antigens. Each assay was done in triplicates, and the error bar indicates the standard error of the mean (SEM).

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~~**Fig. 2.** Timeline of experiments 1 and 2. Female mice were immunized in two week intervals, twice prior to mating (Imm. I; Imm. II), and a third time (Imm. III) after mating. All mice were challenged by subcutaneous inoculation of 10^5 *N. caninum* tachyzoites. Mice were closely monitored for clinical signs and mortality. Non pregnant mice were euthanized on 30 days post infection, dams and pups were euthanized on 43-45 days post infection. W = time point of oestrus synchronization (Whitten effect); M = mating; PrCh 1= Pre-challenge time point experiment 1; PrCh-2 = Pre-challenge time-point experiment 2; PstCh-pm = Post-challenge "post-mating" time-point; PstCh-NonPreg = Post-challenge "non-pregnant mice" time-point;~~

~~PstCh-D = Post-challenge "dams" time-point; Igs = immunoglobulins; Bold arrows indicate time-points of immune response analysis.~~

Fig. 3. Kaplan Meier survival curves of pups from experiment 1 (A) and experiment 2 (B).

Fig. 4. Cerebral parasite burden measured by real-time PCR in dams from experiment 1 (A) and 2 (B) at 40-44 dpi, and from non-pregnant mice from experiment 2 at 30 dpi (C). Experiment 1 is not shown since only one mouse from the O-Ags group remained non-pregnant. (D) shows the clinical signs score of non-pregnant mice in experiment 2.

Fig. 5. Humoral immune responses against recombinant antigens. A) Western blot analysis of pooled sera obtained from experiment 1. Recombinant ROP2 (rROP2) (MW = 41 kDa) and rROP40 (MW = 49 kDa) were separated by SDS-PAGE, blotted onto nitrocellulose, and rROP2- and rROP40-specific IgG were detected by immunoblotting in mouse sera obtained at the pre-challenge phase prior to mating (PrCh-1) and at 9 dpi coinciding with days 14-16 of pregnancy (PstCh-pm.). "+" depicts a positive control serum from a mouse that was chronically infected with *N. caninum*. 1, 2, and 3 strips represent dilutions 1:50, 1:200 and 1:800 of pooled sera. Arrowheads indicate the location of recombinant proteins. (B) ELISA-based detection and quantification of PDI-specific IgG1 and IgG2a levels in experiment 1. PDI-specific antibodies in mice vaccinated with O-Ags and O-Ags+TLR ligands collected at the pre-challenge phase prior to mating (PrCh-1) and at 9 dpi coinciding with days 14-16 post mating (PstCh-pm). (C) Ratio of IgG1:IgG2a. Dots represent individual values of 4 randomly chosen mice euthanized at PrCh-1 and 3 pregnant mice randomly chosen to be euthanized at PstCh-pm. Horizontal lanes represent the median in each group.

Fig. 6. IgG1 and IgG2a antibodies against soluble *N. caninum* extract in sera from mice from experiment 2. Sera were from vaccinated groups (O-Ags, O-Ags+TLR, O-Ova) and the non-vaccinated and infected group (C+) collected at the end of the experiment (30 dpi for non-pregnant mice and 40 dpi for dams). (*) indicates statistically significant differences (~~*U*~~ Mann-Whitney U test, P<0.05).

Fig. 7. Cytokine levels measured in blood samples obtained at 9 dpi coinciding with days 14-16 post-mating (PstCh-pm.) in experiment 2. Boxes represent median, 25^{th} and 75^{th} percentiles; whiskers and individual points represent extreme values by Tukey method. (*) indicates statistically significant differences, Kruskal-Wallis, P <0.05.

Fig. 8. Cytokine responses after splenocyte restimulation *in vitro* with *N. caninum* crude extract in dams (40 dpi) and non-pregnant (30 dpi) mice at the end of experiment 2. Boxes represent median, 25^{th} and 75^{th} percentiles; whiskers and individual points represent extreme values by Tukey method. (*) and (**) show Kruskal-Wallis, $P < 0.05$ and $P < 0.01$ statistically significant differences, respectively, compared to C- group.

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713

714 **Table 1. Outcome of *Neospora caninum* infection in dams and pups in experiments 1 and 2.**

Group	Experiment	Fertility[a]	No. dams[b]	Litter size[c]	Neonatal mortality[d]	Postnatal mortality[e]	Postnatal survival[f]	Median survival time[g]	Vertical transmission[h]
O-Ags	Exp. 1	14/15 (93.3%)	11	5.2	8/57 (14.0%)	32/49 (65.3%)	17/49 (34.7%)	18	39/49 (79.6%)
O-Ags	Exp 2.	9/20 (45%)	9	6.6	5/59 (8.5%)	31/54 (57.4%)	23/54 (42.6%)	21.5	35/54 (64.8%)
O-Ags+TLR	Exp. 1	9/16 (56.3%)	6	5.7	3/34 (8.8%)	26/31 (83.9%)	5/31 (16.1%)	16	28/31 (90.3%)
O-OVA	Exp. 2	9/20 (45%)	9	6.3	3/57 (5.3%)	47/54 (87.0%)	7/54 (12.9%)	16.5	48/54 (88.9%)
C+	Exp. 1	11/20 (55%)	8	5.6	14/45 (31.1%)	28/31 (90.3%)	3/31 (9.7%)	15	30/31 (96.8%)
C+	Exp. 2	14/20 (70%)	14	6.4	6/89 (6.7%)	70/83 (84.3%)	13/83 (15.7%)	16	74/83 (89.2)
C-	Exp. 1	11/16 (68.8%)	8	5.9	14/47 (29.8%)	0/33 (0%)	33/33 (100%)	Undef.	0/33 (0%)
C-	Exp. 2	10/20 (50%)	10	6.2	2/62 (3.2%)	0/60 (0%)	60/60 (100%)	Undef.	0/60 (0%)

715 [a] Proportion of pregnant mice per group (%)
716 [b] In experiment 1, three pregnant mice were excluded because they were euthanized before birth for blood
717 sampling.
718 [c] Number of delivered pups per dam
719 [d] Proportion of pups born dead or that died within the first 2 days post-partum (%)
720 [e] Proportion of pups died from day 3 to 30 post-partum (%)
721 [f] Proportion of survival pups at day 30 post-partum (%)
722 [g] Day post-partum at which 50% of pups were dead
723 [h] Proportion of *Neospora caninum*-PCR positive surviving pups plus those which died from day 3 post-partum
724 (dead pups from day 3 post-partum are considered *N. caninum*-PCR positive as previously shown (Dellarupe et
725 al., 2014)).
726 Undef.: undefined, no pup mortality.
727
728

Table 1

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[a] Proportion of pregnant mice per group (%)
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[g] Day post-partum at which 50% of pups were dead
[h] Proportion of *Neospora caninum*-PCR positive surviving pups plus those which died from day 3 post-partum (dead pups from day 3 post-partum are considered *N. caninum*-PCR positive as previously shown (Dellarupe et al., 2014)).
Undef.: undefined, no pup mortality.

Figure1

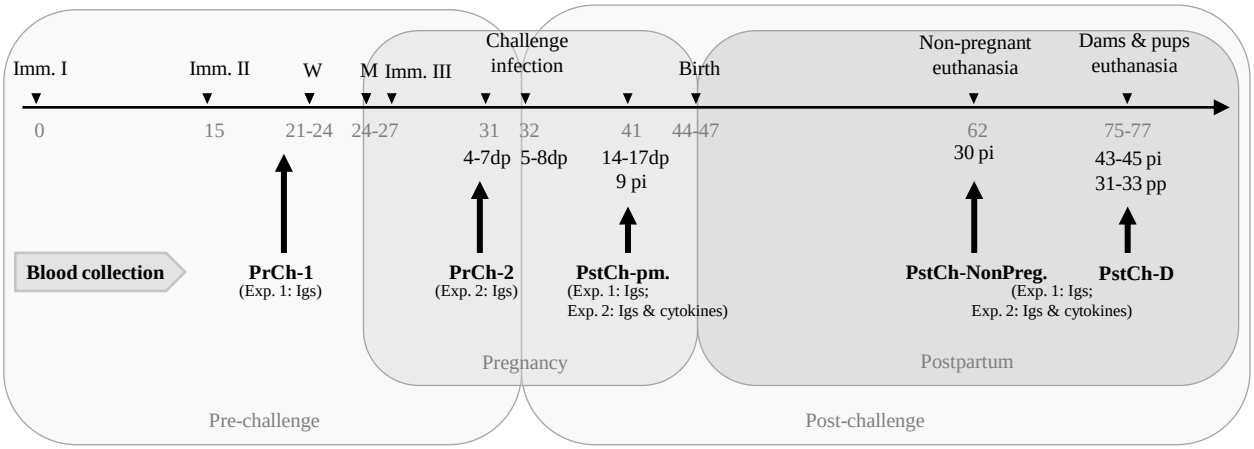

Fig. 1

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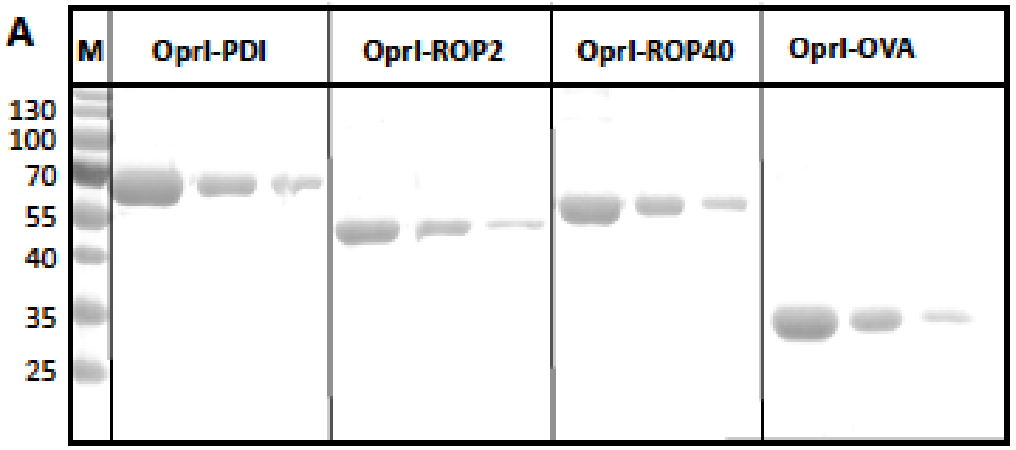

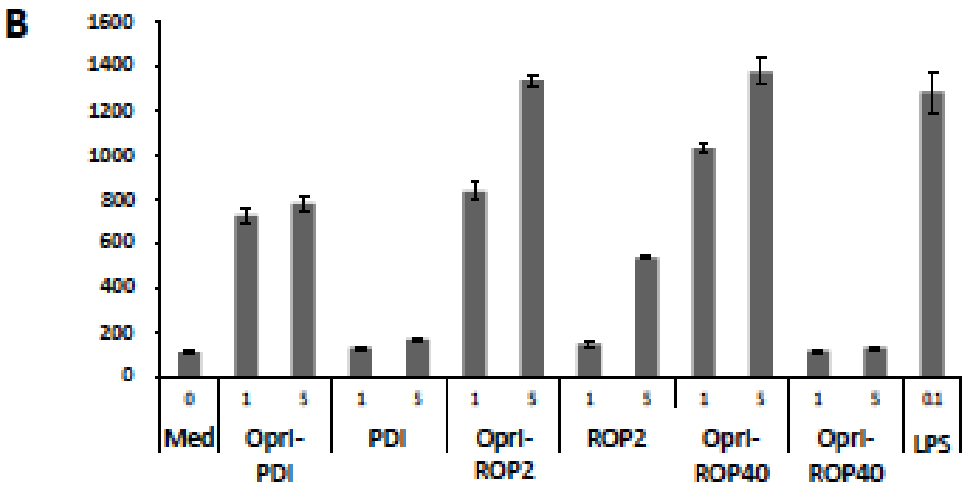

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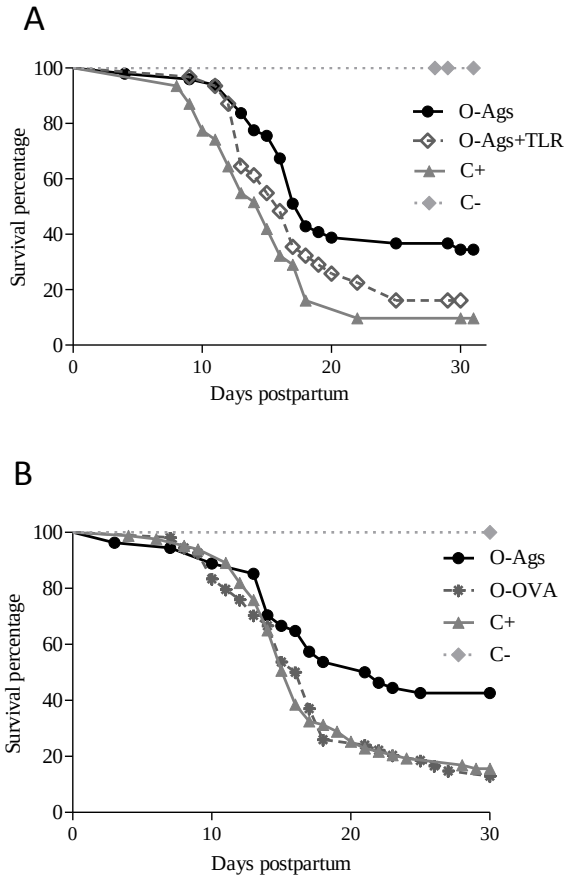

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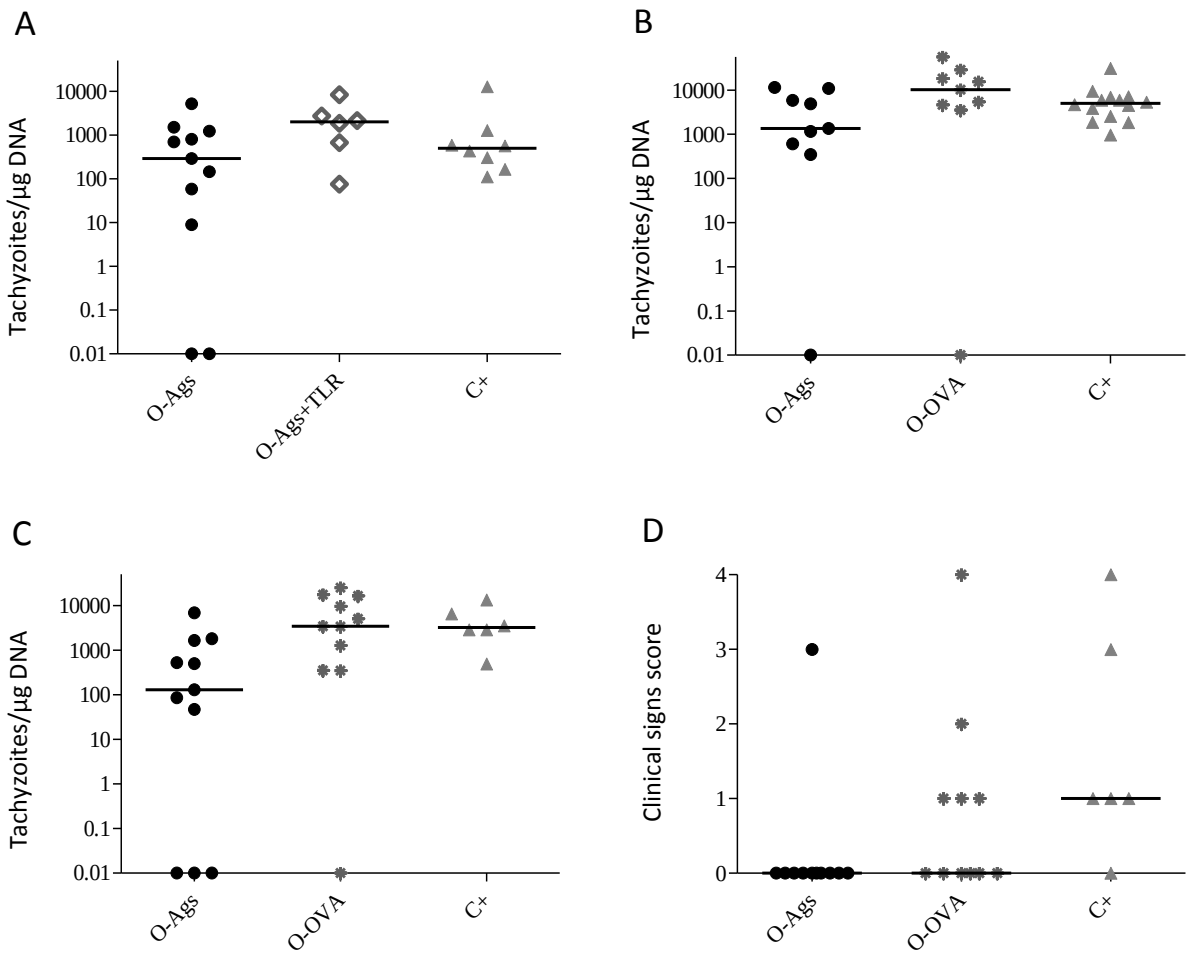

Fig. 4

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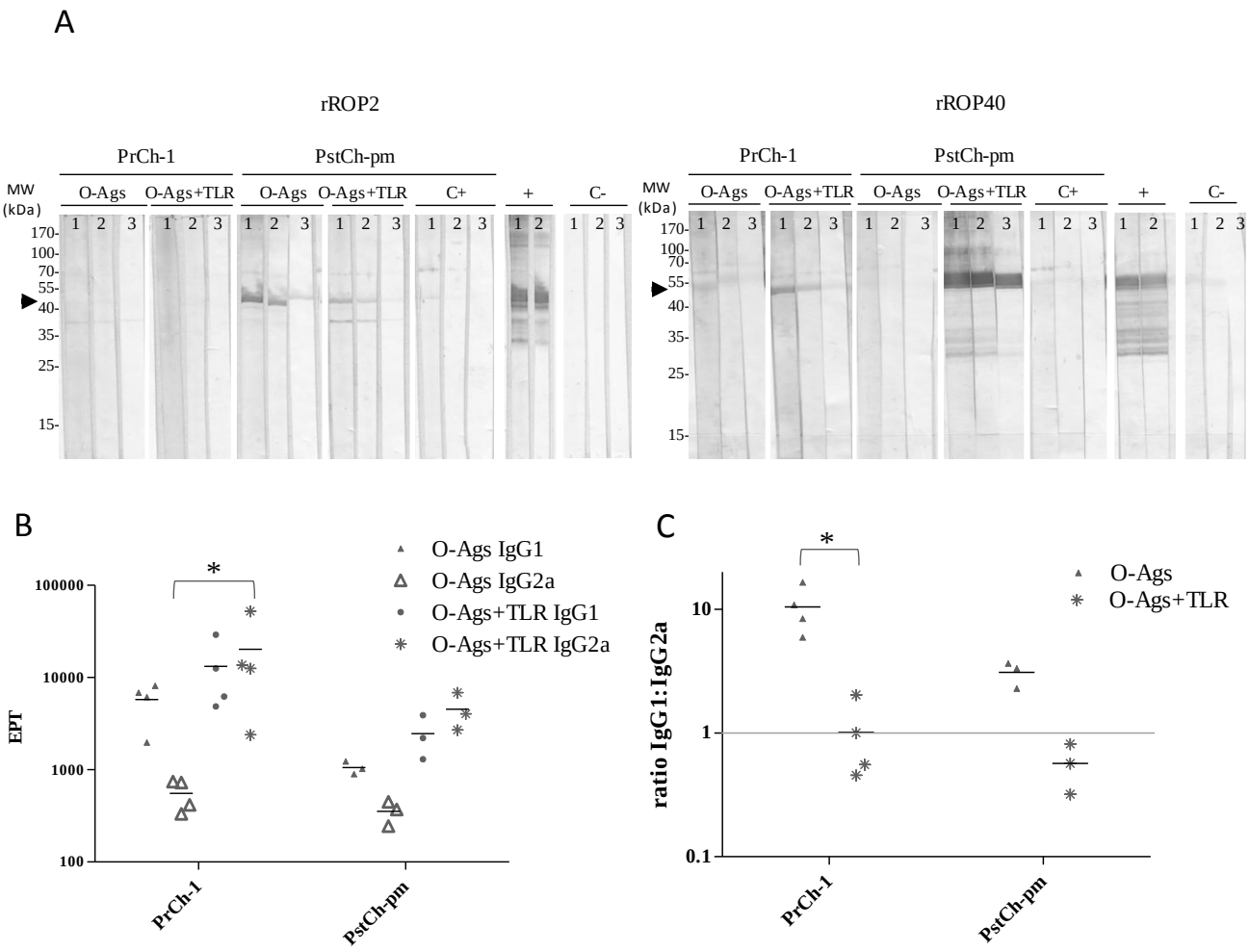

Fig. 5

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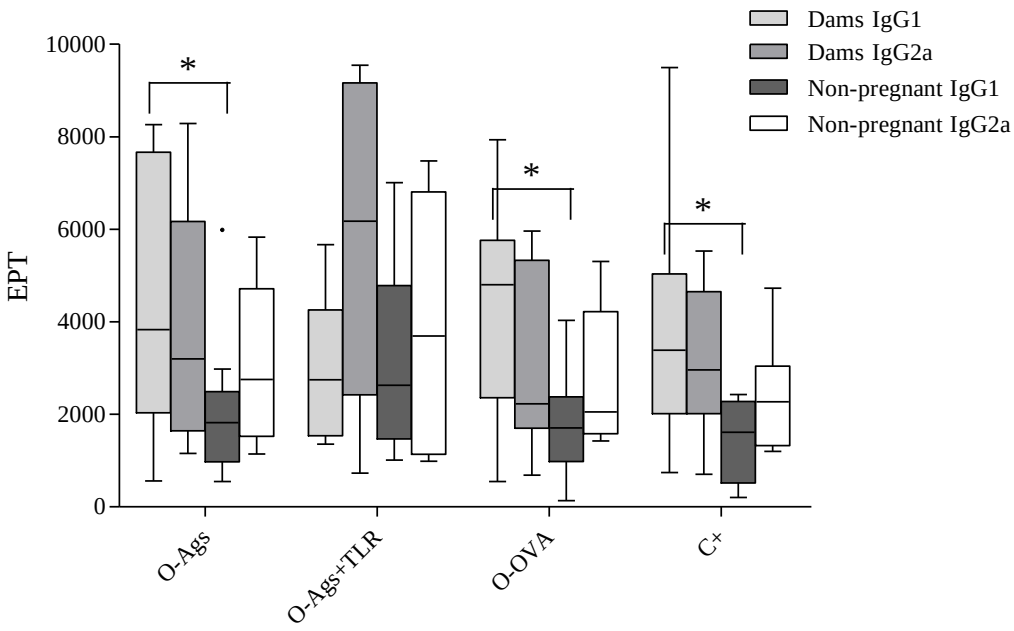

Fig. 6

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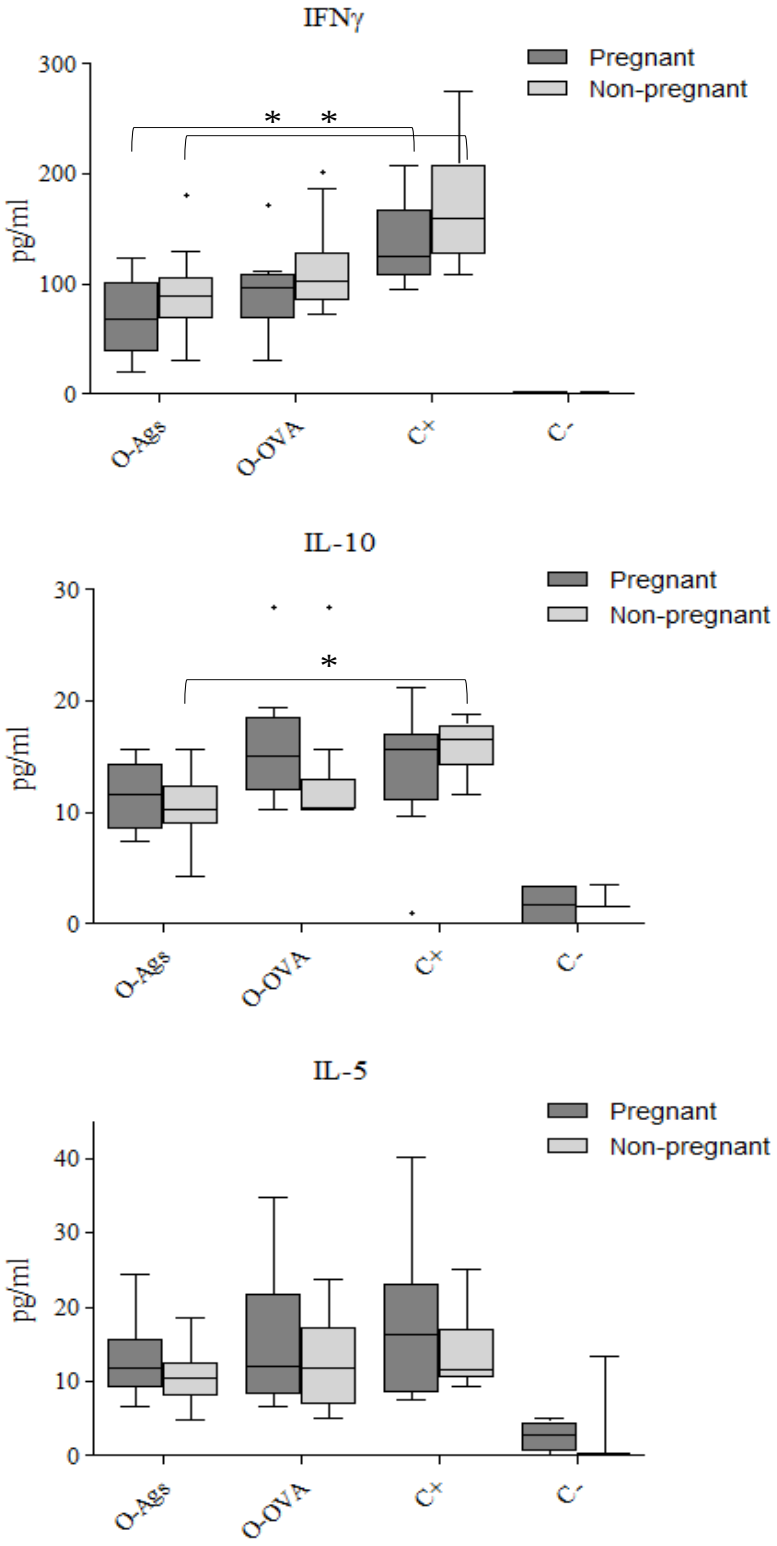

Fig. 7

Figure8

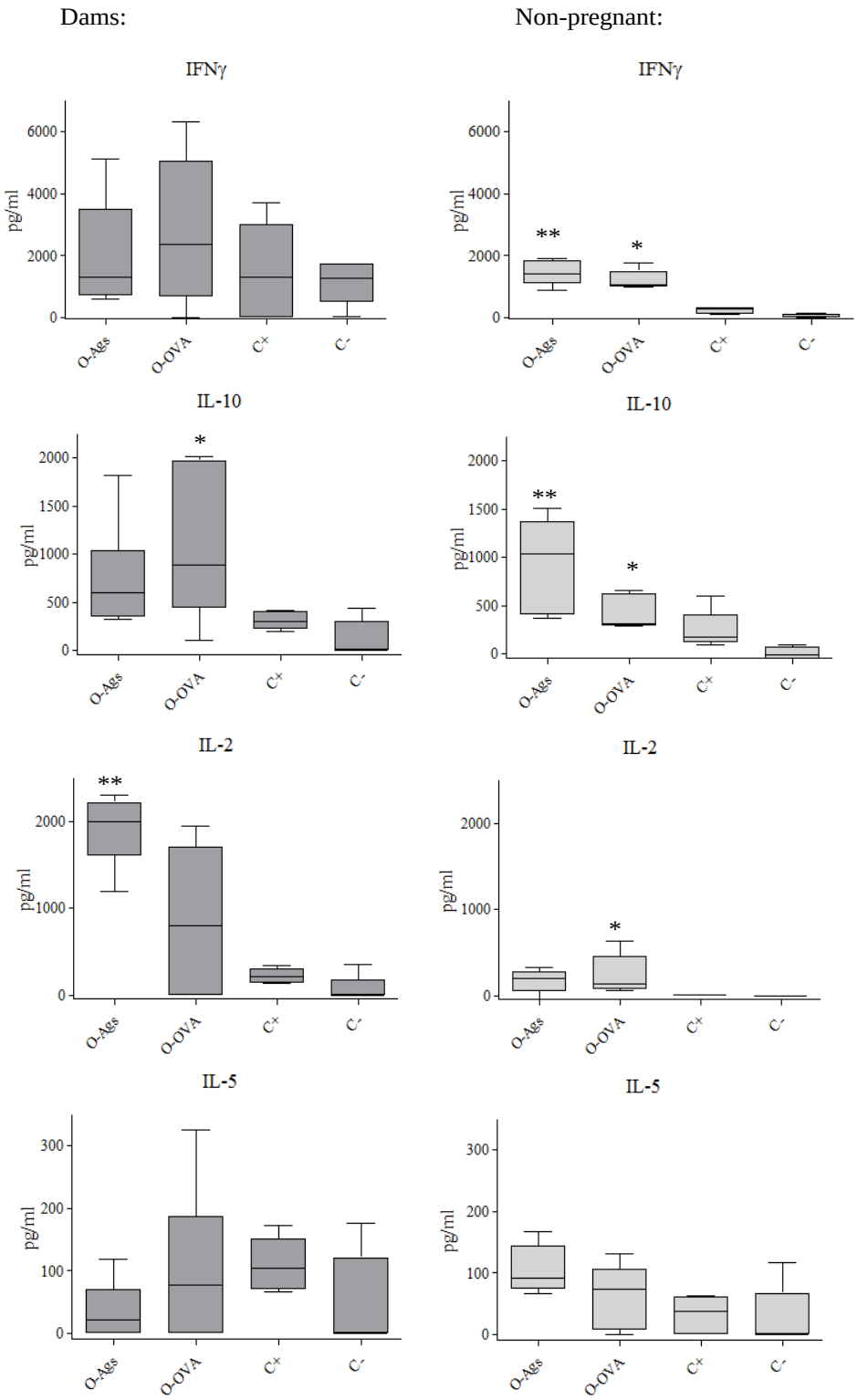

Fig. 8

Supplemental Files

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