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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.

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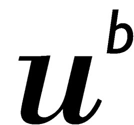

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Editor
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Bern, 15.11.2018

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

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*Response to Reviewers

1 Author's responses to Reviewers' comments:
2
3
4 Reviewer #2: JVAC-D-18-01227
5 The study reports immunization experiments in mice with OprI-fused antigens to prevent Neospora
6 caninum induced vertical transmission and disease. The experiments are well planned and the
7 manuscript nicely written and I have only a few comments.
8
9 Line 140-175, Figure 2: The description of experiments is a bit confusing; it is not that clear what
10 applies for experiment 1 or experiment 2 or for both experiments. You referred to Figure 1 but I think
11 it would be better to refer to Figure 2.
12
13 Response
14 Thank you for this comment. We have slightly altered the text and made it much clearer, which
15 investigations are part of experiment 1, experiment 2, or both. In addition, there was actually a
16 mistake in the figure numbers, which has now been corrected. Fig. 1 now refers to the schematic
17 presentation of the experimental design, Fig. 2 shows the recombinant antigens
18
19 Figure 2 is not optimal, yet. It is difficult to understand the three arrows. It took me a while to
20 understand that the first arrow is for time of experiment, the second is for time of pregnancy and the
21 third is for time post challenge. Why is the third line with a step? Why have the second and the third
22 lines different arrow-heads as compared to the first/top line?
23
24 Response
25 We have modified Figure 1 (Schematic presentation of experiments 1 and 2) making it much clearer
26 to read and easier to understand.
27
28 Line 288: "EPT" was already explained in Line 214.
29
30 Response
31 This was actually on line 227, and is now corrected
32
33 Line 266 and entire manuscript: Always mention statistical test, once reporting OR or P-values . What
34 are the 95% confidence intervals for OR?
35
36 Response
37 We have now mentioned statistical test every time. OR confidence intervals were added
38
39
40 Line 333, 334: "Mann-Whitney U test" would be better. There are several ways to mention this tests
41 in the manuscript, actually.
42
43 Response
44 This was corrected along the entire manuscript
45
46 Line 364: What is the reference "0" ?
47
48 Response
49 This is now deleted
50
51 Line 481: One of the authors of this reference is almost not visible in this citation.
52
53 Response
54 Corrected
55
56 Reviewer #3: Some points are addressed.
57 OprI-fused N. caninum's antigens was investsifgated as Neosporesis vaccine. However, the Oprl was
58 not explained in this MS.
59 Why the OprI was fused and what effects are expected are explained for readers' understanding.

60
61 Response
62 We have rephrased the Introduction (Line 81-91) and added some more information into the
63 discussion (lines 432-437), to explain the rational for exploiting OprI and its adjuvant properties.

**Suggested Reviewers*

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Highlights (for review)

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

1 OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa,* and when fused to
2 a recombinant antigen, will exert adjuvant properties by engaging Toll-like receptor 2, leading
3 to dendritic cell activation. Previous studies have shown that the *Neospora caninum* (Nc)
4 antigens NcPDI, NcROP2 and NcROP40 are implicated in host cell interactions and are
5 promising vaccine candidates. In two independent experiments, the efficacy of a polyvalent
6 vaccine formulation composed of OprI-NcPDI, OprI-NcROP2 and OprI-NcROP40
7 (collectively named O-Ags) was assessed in non-pregnant and pregnant Balb/c mouse models
8 challenged with tachyzoites of the high-virulence isolate Nc-Spain7. Parameters that were
9 investigated were clinical signs, fertility, parasite burden in adult mice, humoral and cellular
10 immune responses at different time-points prior to and after challenge infection, vertical
11 transmission and post-natal survival of offspring mice, all to explore potential correlations
12 with efficacy. Vaccination of mice with O-Ags induced a mixed Th1/Th2 immune response in
13 adult mice and led to significantly increased protection against cerebral infection. Vaccination
14 with O-Ags also resulted in reduced vertical transmission, and postnatal disease in offspring
15 was significantly inhibited at a rate not observed in mice infected with a high-virulence isolate
16 to date. However, O-Ags mixed with TLR ligands targeting TLR3 and TLR7, which are
17 known to induce clear Th1-biased responses, or vaccination with OprI fused to the non-*N.*
18 *caninum* antigen ovalbumin (OprI-OVA) did not confer protection.
19

*Title Page

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2 **REDUCES *NEOSPORA CANINUM* VERTICAL TRANSMISSION AND POSTNATAL**
3 **MORTALITY IN MICE**

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24 Note: Supplementary data associated with this article
25

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3 **IN MICE**

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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

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

81 The TLR2-ligand OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa.*
82 It has been used to confer adjuvant properties when expressed in fusion with different antigens.
83 [15]. Different immune functions have been attributed to TLR2 signaling including the promotion
84 of a mixed and balanced Th1-, Th2- and Treg cell response [16] and a T-cell tropism to mucosal
85 tissues even when inoculated via a non-mucosal route [17]. In previous immunization studies,
86 OprI had been fused to the *N. caninum* chimeric antigen Mic3-1-R, and vaccinated mice were
87 challenged during pregnancy. This did not result in protection, neither in dams nor in offspring
88 mice, but we nevertheless detected that fusion of OprI to MIC3-1-R induced a mixed Th1/Th2
89 profile response [18], while the immune response in mice immunized with MIC3-1-R emulsified
90 in saponin was largely Th2-biased, thus confirming the immunomodulatory properties of OprI-
91 fusions. This well-adjusted Th1/Th2 balance is required to protect *N. caninum* vaccinated mice
92 during pregnancy when a strong Th1 response could be detrimental [30].

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

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

101

102 **2. Materials and methods**

103

104 *2.1.* Neospora caninum *culture, inoculum and crude extract preparation*

105

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

110

111 *2.2. Production of recombinant antigens fused to OprI*

112

113 The coding sequence of NcPDI (aa 25-446) [22], a fragment of NcROP2 (aa 238-594)
114 [12] and the complete coding sequence of NcROP40 [11] were cloned in the pOLP plasmid in
115 frame with OprI [15]. A previously described plasmid, containing the ovalbumin (OVA) partial
116 sequence (aa 203-386) in frame with OprI [16] was also used to obtain the unrelated antigen
117 OprI-OVA. Proteins were all expressed in Rosetta(DE3)pLysS *Escherichia coli* strain (Novagen).
118 They were isolated from the bacterial outer membranes as previously described [18]. Purified
119 OprI-NcPDI was dialyzed against PBS over-night at 4°C and stored at -80°C. OprI-NcROP2 and
120 OprI-NcROP40, expressed less efficiently and insoluble in aqueous solution, were dialyzed over-
121 night against 1g/L ammonium bicarbonate at 4°C and lyophilized. They were resuspended in

122 PBS in one-tenth of the original volume, vortexed, and were sonicated at 30°C in a water bath
123 until a homogeneous suspension was obtained. All purified OprI-antigens were finally stored at -
124 80°C. Non-OprI-fused NcPDI, NcROP2 and NcROP40 [11,22,22], were expressed and purified
125 as described [18]. The purity and integrity of all proteins were analyzed by SDS-PAGE and the
126 protein concentration was determined (BCA Protein Assay Kit, Thermo Scientific Pierce).
127 Endotoxin levels were quantified by the Limulus-Amebocyte-Lysate-Kinetic-QCL (Lonza). The
128 final LPS content was below 0.1EU/µg.

129

130 *2.3. Dendritic cell (DC) stimulation*

131
132 OprI-fused and non-fused recombinant proteins were assessed for DC stimulation [18].
133 Briefly, DCs were obtained *in vitro* from bone marrow cells obtained from a naïve Balb/c mouse.
134 DCs (0.5x10^6 cells/ml) were cultured in the presence of each protein (1 or 5µg/ml), LPS
135 (0.1µg/ml) or medium during 24hours, and TNF-α was measured in the culture supernatants by
136 ELISA [18].

137

138 *2.4. Immunization schedule, clinical monitoring and sample collection*

139
140 All animal protocols were approved by the Bernese Animal Welfare Committee (license
141 BE115/14.) Two experiments were carried out. Schedules of immunization, mating, blood sample
142 collection and euthanasia for both studies are depicted in Fig.1. Six weeks-old BALB/c mice
143 were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained as
144 described [18].
145 In Experiment 1, 16 to 20 female mice per group were immunized three times, at two-
146 week intervals. The O-Ags group was subcutaneously injected with all three OprI-antigens, 5µg

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

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

158 Eight days post- second immunization, mice in both experiment 1 and experiment 2 were
159 submitted to oestrus synchronization [23] and 2 females and 1 male per cage were mated during
160 72h. The third immunization was applied after separation of males and females, 5 days prior to
161 challenge infection. At 5-8 days of pregnancy, mice from the O-Ags, O-Ags+TLR, O-OVA and
162 C+ groups were subcutaneously infected with 10^5 Nc-Spain7 tachyzoites [24]. Mice of the C-
163 groups received only PBS. At day 9 post-infection, a blood sample was obtained from the tail of
164 3 pregnant mice of each group from experiment 1 and of all mice from experiment 2 (Fig.1;
165 PstCh-pm). Two days before birth, pregnant and non-pregnant mice were separated. Dams were
166 allowed to give birth and rear their offspring in individual cages. Non-pregnant mice were kept in
167 groups of 4-5 mice per cage. Following infection, all mice were daily monitored for clinical signs
168 using a standardized score sheet: coat (ruffled coat=score1; stary stiff coat=score2); weight loss
169 (10% loss=score1, 15-20% loss=score 4); behavior (hunched appearance, walking in circles, head
170 tilt, apathy and ataxia, all=score 1). Animal were euthanized when the score exceeded 3 points.

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

175 Data on clinical signs, fertility (percentage of pregnant mice), litter size (average of
176 number of pups born per dam), neonatal mortality of pups (stillborn pups or pups dying within
177 the first 2 days post-partum (dpp) and postnatal mortality (pups dying between day 3 and 30pp)
178 were recorded. Upon euthanasia, blood was extracted by cardiac puncture and sera stored at -80
179 °C. Brains from non-pregnant mice, dams and surviving pups were also collected and
180 immediately frozen at -20°C. Spleens from 5 to 6 non-pregnant mice and dams per group were
181 aseptically recovered and processed for splenocyte re-stimulation *in vitro*.

182

183 *2.5.Analysis of the cerebral parasite burden*

184

185 Brains from non-pregnant mice, dams and surviving pups were analyzed by *N. caninum*-
186 specific real-time PCR [25]. DNA extraction was performed using the Nucleospin Kit
187 (Macherey-Nagel, Oensingen, Switzerland). The DNA concentration in all samples was
188 determined using the QuantiFluor dsDNA System (Promega, Madison, Wi.) and was adjusted to
189 5ng/µl with sterile DNase free water. Quantitative real-time PCR was performed using the Rotor-
190 Gene 6000 real-time PCR machine. The parasite load was calculated by interpolation from a
191 standard curve with DNA equivalents from 1000, 100 and 10 *N. caninum* tachyzoites included in
192 each run.

193

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™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

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

224 PrCh-1 and PstCh-pm serum samples from experiment 1 were also analyzed in pools by
225 immunoblotting for NcROP2- and NcROP40-specific IgG. Non-OprI NcROP2 and NcROP40
226 (10µg each) were submitted to Western blot analysis following standard procedures [26]. Pooled
227 sera were incubated at 1:50, 1:200 and 1:800 dilutions and an anti-mouse IgG antibody
228 conjugated to alkaline phosphatase (eBioscience) at 1:5000 dilution was employed.

229

230 *2.8. Statistical analysis*

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

242 survivors at the end of the experiment or percentages of *N. caninum* PCR positive samples were
243 analyzed by Chi-square test with Yates' continuity correction in a contingency table.
244 Regarding the parameters measured in both experiments and compared between groups
245 (C-, O-Ags and C+), a unique statistical analysis was done with all data from both experiments.
246 A mixed-effects log-linear model (Venables and Ripley, 2002) was used to analyze pregnancy
247 and immunization with the O-Ags formulation as fixed effects on parasite burden, having
248 experiment as random effect. Effect sizes were estimated with a penalized quasi-likelihood
249 method (glmmPQL from MASS library). A logistic mixed model with Laplace parameter
250 estimation via Lapplace approximation (glmer of lme4 package) was used to evaluate clinical
251 signs presence and vertical transmission. The odds-ratio (OR) relative to the O-Ags group was
252 calculated with the respective 95% confidence interval (CI 95%). Survival of pups was compared
253 with a Cox model with fixed (immunization) and random (experiment) effects (coxme library).
254 Statistical significance was established at $P<0.05$.
255
256 **3. Results**
257
258 *3.1. Increased dendritic cell stimulation by OprI-fused recombinant antigens*
259
260 The purified OprI-fused recombinant proteins shown in Fig.2A were used for stimulation
261 of bone marrow-derived DCs, which were cultured in the presence of 1 or 5 μg/ml of each
262 protein, LPS (0.1μg/ml) or medium during 24 hours, and the production of TNF-α was measured
263 in the culture supernatants by ELISA (Fig.2B). In contrast to the non-fused recombinant antigens,
264 the OprI-proteins induced strong and specific DC stimulation (Fig.2B).
265

266 *3.2. Immunization with OprI-fused* Neospora *antigens reduced vertical transmission and*
267 *increased postnatal survival*

268

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

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

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

288

289 *3.3. Immunization with OprI-fused* Neospora *antigens reduced cerebral neosporosis*

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

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

304

305 *3.4. Humoral immune responses against recombinant antigens*

306

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

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

322 PDI-specific antibodies were detected by ELISA during the pre-challenge phase. In
323 experiment 1, levels in mice vaccinated with O-Ags+TLR were higher than those in mice
324 vaccinated with O-Ags, and in spite of the low number of samples the difference reached
325 statistical significance for IgG2a at the pre-challenge phase in experiment 1 (PrCh-1; Mann-
326 Whitney U test, $P<0.05$) (Fig.5B). Moreover, the IgG1:IgG2a ratio was markedly lower in the O-
327 Ags+TLR group compared to the O-Ags group, and was statistically significant during the pre-
328 challenge phase (Mann-Whitney U test, $P< 0.05$) (Fig.5C). Surprisingly, PDI antibody levels
329 were lower at 9dpi (PstCh-pm) than before mating in both vaccinated groups (Fig.5B). Since the
330 values at 9dpi corresponded to just three pregnant animals and no identification of mice was
331 carried out, we could not deduce the individual evolution of antibody levels in experiment 1.
332 Thus, in experiment 2 mice were individually tracked. With the exception of one, those O-Ags-
333 vaccinated mice with confirmed pregnancy exhibited very little changes or decreased anti-PDI
334 antibody levels during the timespan between pre-challenge to 9dpi (Wilcoxon matched pairs test;
335 $P>0.05$), whereas those mice that did not become pregnant exhibited a clear boost of anti-PDI
336 antibody levels (Wilcoxon matched pairs test, $P<0.01$) (suppl. Fig.1), confirming the results from
337 experiment 1. The same tendency was observed with IgG2a levels (data not shown). Only one

338 pregnant mouse exhibited a strong increase of antibody levels, and this animal had a very high
339 parasite burden, elevated clinical signs score, and vertical transmission of *N. caninum* tachyzoites
340 to its offspring (suppl Fig.1).
341
342 *3.5.Antibody responses against soluble N. caninum antigens*
343
344 No significant differences in IgG1 or IgG2a levels between groups were detected (Kruskal-
345 Wallis, $P>0.05$) (Fig.6). In sera from mice immunized with O-Ags, and O-OVA, and from
346 infected non-immunized C+ mice, similar IgG2a levels were noted in dams and non-pregnant
347 mice (Mann-Whitney U test, $P>0.05$), whereas IgG1 levels were significantly lower in non-
348 pregnant mice compared to dams (Mann-Whitney U test, $P<0.05$) pointing towards an IgG2a-
349 biased response in non-pregnant mice compared to dams. In contrast, in sera of mice vaccinated
350 with O-Ags+TLR ligands, no such differences between dams and non-pregnant were detected
351 (Mann-Whitney U test, $P>0.05$), and the humoral immune response was IgG2a-biased in both
352 cases (Fig. 6).
353
354 *3.6.Cytokine responses*
355
356 Cytokines were measured in experiment 2 at 9dpi in peripheral blood and at the end of the
357 experiment upon *in vitro* stimulation of splenocytes and analysis of culture supernatants. At 9dpi,
358 the levels of IFNγ, IL-10 and IL-5 in peripheral blood were significantly elevated in all infected
359 animals when compared to group C- (Fig.7). However, IFNγ-levels in pregnant as well as non-
360 pregnant mice vaccinated with O-Ags were significantly lower than in the C+ group (Kruskal-
361 Wallis, $P<0.05$). IL-10 levels were also significantly lower in non-pregnant mice vaccinated with

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

364 At the end of the experiment mice were sacrificed, splenocytes were cultured and cytokine
365 recall responses were measured in medium supernatants. In non-pregnant mice, only splenocytes
366 from O-Ags and O-OVA vaccinated mice displayed a consistent increase of IFNγ and IL-10
367 secretion upon stimulation with *N. caninum* crude extract, compared to splenocytes from C- mice
368 (Kruskal-Wallis, $P<0.05$) (Fig.8). Splenocytes derived from the C+ group showed no changes or
369 very little increase of cytokine production. In the case of vaccinated dams, splenocyte cytokine
370 levels were more heterogeneously distributed, with high individual variations, with the exception
371 of highly elevated IL-2 responses in splenocytes isolated from O-Ags vaccinated dams, (Fig.8).
372
373

374 **4. Discussion**

375

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

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

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

408 In contrast to vaccination protocols applied earlier [2,10,11], the third immunization was
409 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.

434 The decrease, or lack of boost, of anti-PDI antibody levels after the third immunization in
435 the vaccinated mice was only observed in pregnant animals. This indicates immune modulation
436 due to the pregnancy, but it is unclear how this affects the overall protectivity of the vaccine
437 formulation. An inverse correlation between anti-PDI-antibody levels and protection against
438 cerebral infection could be observed in dams. Those pregnant mice with a slightly increased anti-
439 PDI antibody levels between the pre-challenge and 9 dpi were those exhibiting high parasite
440 burden and clinical signs score afterwards. Conversely, those dams showing the strongest drop of
441 antibody levels between these two time-points were those with lowest parasite burden afterwards.
442 However, in the pups, no correlation with vertical transmission was detected. Whether the
443 impairment in anti-PDI antibodies boost was a consequence of an early control of parasite
444 replication or a requisite for improved protection is not clear. A study on *N. caninum* profilin
445 vaccinated mice [32] also reported on a drop of profilin-specific antibody titers shortly after
446 challenge, and levels recovered at 21dpi. It was suggested that antibodies were being consumed
447 by playing an active role against the infection [35]. However, only non-pregnant animals were
448 assessed, and in our study non-pregnant mice actually showed a strong boost of antibody levels
449 between pre-challenge and 9 dpi, and we did not note a correlation between antibody levels at
450 9dpi and cerebral parasite burden. Thus, the differential antibody kinetics observed here is likely
451 to be a consequence of a pregnancy-associated phenomenon. Antibody levels are also altered
452 during the course of *N. caninum* infection in cattle. In naturally infected heifers, antibody
453 fluctuations occurring between 90 and 240 days of gestation were shown to be associated with a
454 higher probability of vertical transmission [36, 37]. Cows that aborted also showed more
455 pronounced fluctuations and overall higher antibody levels, especially between months 3 and 8 of
456 gestation, compared to non-aborting cows [38]. Whether the vaccine-associated drop in antibody
457 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.

482 In summary, these vaccination studies in a neosporosis mouse model have shown that
483 promising efficacy against congenital and cerebral neosporosis can be achieved by immunization
484 with a polyvalent combination vaccine composed of recombinant NcPDI, NcROP2 and
485 NcROP40, all three fused to the TLR2-ligand OprI. This vaccine induced a balanced Th1/Th2
486 immune response in adults, which reduced vertical transmission, but was not highly efficacious in
487 preventing cerebral infection in dams. To the best of our knowledge, this is a major advance in
488 terms of efficacy achieved with a recombinant vaccine formulation. Further studies should aim to
489 optimize the dosage and the timing of immunizations, and it will be highly interesting to
490 elucidate the immunological mechanisms that are responsible for the protective effects against
491 vertical transmission.
492
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497
498
499 **References**
500 [1] Dubey , J.P. Hemphill, A; Calero-Bernal, R.; Schares, G., 2017. Neosporosis in Animals.
501 CRC Press, Florida, United States.
502 [2] Debache, K., Hemphill, A., 2013. Differential effects of intranasal vaccination with
503 recombinant NcPDI in different mouse models of *Neospora caninum* infection. Parasite Immunol
504 35, 11-20.

505 [3] Reichel, M.P., Alejandra Ayanegui-Alcerreca, M., Gondim, L.F., Ellis, J.T., 2013. What is the
506 global economic impact of *Neospora caninum* in cattle - the billion dollar question. Int J Parasitol
507 43, 133-142.
508 [4] Reichel, M.P., Moore, D.P., Hemphill, A., Ortega-Mora, L.M., Dubey, J.P., Ellis, J.T., 2015.
509 A live vaccine against *Neospora caninum* abortions in cattle. Vaccine 33, 1299-1301.
510 [5] Rojo-Montejo, S., Collantes-Fernandez, E., Perez-Zaballos, F., Rodriguez-Marcos, S.,
511 Blanco-Murcia, J., Rodriguez-Bertos, A., Prenafeta, A., Ortega-Mora, L.M., 2013. Effect of
512 vaccination of cattle with the low virulence Nc-Spain 1H isolate of *Neospora caninum* against a
513 heterologous challenge in early and mid-gestation. Vet Res 44, 106.
514 [6] Weber, F.H., Jackson, J.A., Sobecki, B., Choromanski, L., Olsen, M., Meinert, T., Frank, R.,
515 Reichel, M.P., Ellis, J.T., 2013. On the efficacy and safety of vaccination with live tachyzoites of
516 *Neospora caninum* for prevention of neospora-associated fetal loss in cattle. Clin Vaccine
517 Immunol 20, 99-105.
518 [7] Horcajo, P., Regidor-Cerrillo, J., Aguado-Martinez, A., Hemphill, A., Ortega-Mora, L.M.,
519 2016. Vaccines for bovine neosporosis: current status and key aspects for development. Parasite
520 Immunol 38, 709-723.
521 [8] Rojo-Montejo, S., Collantes-Fernandez, E., Lopez-Perez, I., Risco-Castillo, V., Prenafeta, A.,
522 Ortega-Mora, L.M., 2012. Evaluation of the protection conferred by a naturally attenuated
523 *Neospora caninum* isolate against congenital and cerebral neosporosis in mice. Vet Res 43, 62.
524 [9] Miller, C., Quinn, H., Ryce, C., Reichel, M.P., Ellis, J.T., 2005. Reduction in transplacental
525 transmission of *Neospora caninum* in outbred mice by vaccination. Int J Parasitol 35, 821-828.
526 [10] Debache, K., Alaeddine, F., Guionaud, C., Monney, T., Müller, J., Strohbusch, M., Leib,
527 S.L., Grandgirard, D., Hemphill, A., 2009. Vaccination with recombinant NcROP2 combined

528 with recombinant NcMIC1 and NcMIC3 reduces cerebral infection and vertical transmission in
529 mice experimentally infected with *Neospora caninum* tachyzoites. Int J Parasitol 39, 1373-1384.
530 [11] Pastor-Fernandez, I., Arranz-Solis, D., Regidor-Cerrillo, J., Alvarez-Garcia, G., Hemphill,
531 A., Garcia-Culebras, A., Cuevas-Martin, C., Ortega-Mora, L.M., 2015. A vaccine formulation
532 combining rhoptry proteins NcROP40 and NcROP2 improves pup survival in a pregnant mouse
533 model of neosporosis. Vet Parasitol 207, 203-215.
534 [12] Debache, K., Guionaud, C., Alaeddine, F., Mevissen, M., Hemphill, A., 2008. Vaccination
535 of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice
536 infected with *Neospora caninum* tachyzoites. Int J Parasitol 38, 1455-1463.
537 [13] Regidor-Cerrillo, J., Alvarez-Garcia, G., Pastor-Fernandez, I., Marugan-Hernandez, V.,
538 Gomez-Bautista, M., Ortega-Mora, L.M., 2012. Proteome expression changes among virulent
539 and attenuated *Neospora caninum* isolates. J Proteomics 75, 2306-2318.
540 [14] Debache, K., Guionaud, C., Alaeddine, F., Hemphill, A., 2010. Intraperitoneal and intra-
541 nasal vaccination of mice with three distinct recombinant *Neospora caninum* antigens results in
542 differential effects with regard to protection against experimental challenge with *Neospora*
543 *caninum* tachyzoites. Parasitology 137, 229-240.
544 [15] Basto, A.P., Piedade, J., Ramalho, R., Alves, S., Soares, H., Cornelis, P., Martins, C.,
545 Leitao, A., 2012. A new cloning system based on the OprI lipoprotein for the production of
546 recombinant bacterial cell wall-derived immunogenic formulations. J Biotechnol 157, 50-63.
547 [16] Basto, A.P., Leitao, A., 2014. Targeting TLR2 for vaccine development. J Immunol Res
548 2014, 619410.
549 [17] Wang, S., Villablanca, E.J., De Calisto, J., Gomes, D.C., Nguyen, D.D., MizoguchI, E.,
550 Kagan, J.C., Reinecker, H.C., Hacohen, N., Nagler, C., Xavier, R.J., Rossi-Bergmann, B., Chen,

551 Y.B., Blomhoff, R., Snapper, S.B., Mora,J.R.,2011. MyD88-dependent TLR1/2 signals educate
552 dendritic cells with gut-specific imprinting properties. J Immunol. 187, 141-150.
553 [18] Aguado-Martinez, A., Basto, A.P., Muller, J., Balmer, V., Manser, V., Leitao, A., Hemphill,
554 A., 2016. N-terminal fusion of a toll-like receptor 2-ligand to a *Neospora caninum* chimeric
555 antigen efficiently modifies the properties of the specific immune response. Parasitology 143,
556 606-616.
557 [19] Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F., Lanzavecchia, A., 2005. Selected Toll-
558 like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in
559 dendritic cells. Nat Immunol 6, 769-776.
560 [20] Regidor-Cerrillo, J., Gomez-Bautista, M., Pereira-Bueno, J., Aduriz, G., Navarro-Lozano,
561 V., Risco-Castillo, V., Fernandez-Garcia, A., Pedraza-Diaz, S., Ortega-Mora, L.M., 2008.
562 Isolation and genetic characterization of *Neospora caninum* from asymptomatic calves in Spain.
563 Parasitology 135, 1651-1659.
564 [21] Arranz-Solis, D., Benavides, J., Regidor-Cerrillo, J., Horcajo, P., Castano, P., del Carmen
565 Ferreras, M., Jimenez-Pelayo, L., Collantes-Fernandez, E., Ferre, I., Hemphill, A., Perez, V.,
566 Ortega-Mora, L.M., 2016. Systemic and local immune responses in sheep after *Neospora*
567 *caninum* experimental infection at early, mid and late gestation. Vet Res 47, 2.
568 [22] Muller, J., Naguleswaran, A., Muller, N., Hemphill, A., 2008. *Neospora caninum*: functional
569 inhibition of protein disulfide isomerase by the broad-spectrum anti-parasitic drug nitazoxanide
570 and other thiazolides. Exp Parasitol 118, 80-88.
571 [23] Whitten, M.K., 1957. Effect of exteroceptive factors on the oestrous cycle of mice. Nature
572 180, 1436.

573 [24] Arranz-Solís, D., Aguado-Martínez, A., Müller, J., Regidor-Cerrillo, J., Ortega-Mora, L. M.,
574 Hemphill, A., 2015. Dose-dependent effects of experimental infection with the virulent *Neospora*
575 *caninum* Nc-Spain7 isolate in a pregnant mouse model. Vet. Parasitol. 211:133-140.
576 [25] Muller, N., Vonlaufen, N., Gianinazzi, C., Leib, S.L., Hemphill, A., 2002. Application of
577 real-time fluorescent PCR for quantitative assessment of *Neospora caninum* infections in
578 organotypic slice cultures of rat central nervous system tissue. J Clin Microbiol 40, 252-255.
579 [26] Monney, T., Rutti, D., Schorer, M., Debache, K., Grandgirard, D., Leib, S.L., Hemphill, A.,
580 2011. RecNcMIC3-1-R is a microneme- and rhoptry-based chimeric antigen that protects against
581 acute neosporosis and limits cerebral parasite load in the mouse model for *Neospora caninum*
582 infection. Vaccine 29, 6967-6975.
583 [27] Jenum, P.A., Stray-Pedersen, B., Gundersen, A.G., 1997. Improved diagnosis of primary
584 *Toxoplasma gondii* infection in early pregnancy by determination of anti-*Toxoplasma*
585 immunoglobulin G avidity. J Clin Microbiol 35, 1972-1977.
586 [28] Morrison, W.I., Tomley, F., 2016. Development of vaccines for parasitic diseases of
587 animals: Challenges and opportunities. Parasite Immunol 38, 707-708.
588 [29] Hemphill, A., Aguado-Martinez, A., Müller, J., 2016. Approaches for the vaccination and
589 treatment of *Neospora caninum* infections in mice and ruminant models. Parasitology 143, 245-
590 259.
591 [30] Aguado-Martínez, A., Basto, A. P., Leitão, A., Hemphill, A. 2017. *Neospora caninum* in
592 non-pregnant and pregnant mouse models: cross-talk between infection and immunity. Int. J.
593 Parasitol. 47:723-735.
594 [31] Pastor-Fernandez, I., Regidor-Cerrillo, J., Jimenez-Ruiz, E., Alvarez-Garcia, G., Marugan-
595 Hernandez, V., Hemphill, A., Ortega-Mora, L.M., 2016. Characterization of the *Neospora*

596 *caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins during the tachyzoite lytic cycle.
597 Parasitology 143, 97-113.
598 [32] MacHugh, N.D., Weir, W., Burrells, A., Lizundia, R., Graham, S.P., Taracha, E.L., Shiels,
599 B.R., Langsley, G., Morrison, W.I., 2011. Extensive polymorphism and evidence of immune
600 selection in a highly dominant antigen recognized by bovine CD8 T cells specific for *Theileria*
601 *annulata*. Infect Immun 79, 2059-2069.
602 [33] Bethancourt, C.N., Wang, T.L., Bocchini, J.A., Jr., 2017. Vaccination during pregnancy:
603 first line of defense for expecting mothers and vulnerable young infants. Curr Opin Pediatr 29,
604 737-743.
605 [34] Papaioannou, A.I., Spathis, A., Kostikas, K., Karakitsos, P., Papiris, S., Rossios, C., 2017.
606 The role of endosomal toll-like receptors in asthma. Eur J Pharmacol 808, 14-20.
607 [35] Mansilla, F.C., Quintana, M.E., Langellotti, C., Wilda, M., Martinez, A., Fonzo, A., Moore,
608 D.P., Cardoso, N., Capozzo, A.V., 2016. Immunization with *Neospora caninum* profilin induces
609 limited protection and a regulatory T-cell response in mice. Exp Parasitol 160, 1-10.
610 [36] Pare, J., Thurmond, M.C., Hietala, S.K., 1997. *Neospora caninum* antibodies in cows during
611 pregnancy as a predictor of congenital infection and abortion. J Parasitol 83, 82-87.
612 [37] Guy, C.S., Williams, D.J.L., Kelly, D.F., McGarry, J.W., Guy, F., Bjorkman, C., Smith,
613 R.F., Trees, A.J., 2001. *Neospora caninum* in persistently infected, pregnant cows: spontaneous
614 transplacental infection is associated with an acute increase in maternal antibody. Vet Rec 149,
615 443-449.
616 [38] Quintanilla-Gozalo, A., Pereira-Bueno, J., Seijas-Carballedo, J., Costas, E., Ortega-Mora,
617 L.M., 2000. Observational studies in *Neospora caninum* infected dairy cattle: relationship
618 infection-abortion and gestational antibody fluctuations. In "A European perspective on
619 *Neospora caninum*". Int J Parasitol 30, 877-924.

620 [39] Amah-Tariah, F.S., Bekinbo, M.T., Dapper, D.V., 2016. Comparative study of Serum
621 Immunoglobulin levels in Healthy Pregnant and Pregnant Subjects with HIV and Malaria
622 Infection in Port Harcourt, Nigeria. Int Res J Med Sci 4, 11-16.
623 [40] Khan, I.A., Schwartzman, J.D., Fonseka, S., Kasper, L.H., 1997. *Neospora caninum*: role
624 for immune cytokines in host immunity. Exp Parasitol 85, 24-34.
625 [41] Rettigner, C., De Meerschman, F., Focant, C., Vanderplasschen, A., Losson, B., 2004. The
626 vertical transmission following the reactivation of a *Neospora caninum* chronic infection does not
627 seem to be due to an alteration of the systemic immune response in pregnant CBA/Ca mice.
628 Parasitology 128, 149-160.
629
630
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

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

676

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

682

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

687

688 **Fig. 8.** Cytokine responses after splenocyte restimulation *in vitro* with *N. caninum* crude extract
689 in dams (40 dpi) and non-pregnant (30 dpi) mice at the end of experiment 2. Boxes represent
690 median, 25th and 75th 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

695

696 **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%)

697 [a] Proportion of pregnant mice per group (%)
698 [b] In experiment 1, three pregnant mice were excluded because they were euthanized before birth for blood
699 sampling.
700 [c] Number of delivered pups per dam
701 [d] Proportion of pups born dead or that died within the first 2 days post-partum (%)
702 [e] Proportion of pups died from day 3 to 30 post-partum (%)
703 [f] Proportion of survival pups at day 30 post-partum (%)
704 [g] Day post-partum at which 50% of pups were dead
705 [h] Proportion of *Neospora caninum*-PCR positive surviving pups plus those which died from day 3 post-partum
706 (dead pups from day 3 post-partum are considered *N. caninum*-PCR positive as previously shown (Dellarupe et
707 al., 2014)).
708 Undef.: undefined, no pup mortality.
709
710

Manuskript compare version
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2 **IMMUNIZATION WITH A COCKTAIL OF ANTIGENS FUSED WITH OprI REDUCES**
3 ***NEOSPORA CANINUM* VERTICAL TRANSMISSION AND POSTNATAL MORTALITY**
4 **IN MICE**

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24 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

49

50 **1. Introduction**

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

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

69 There is "proof-of-concept" that a reduction of vertical transmission and postnatal
70 mortality due to *N. caninum* infection can be achieved by vaccination not only with live vaccines
71 [8,9], but also with recombinant subunit vaccines in mice [10,11]. Bacterially expressed
72 recNcPDI, recNcROP2 and recNcROP40 represent promising vaccine candidates. Immunization

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

81 The TLR2-ligand OprI is an outer membrane lipoprotein from *Pseudomonas aeruginosa*
82 ~~[15].~~. It has been used to confer adjuvant properties when expressed in fusion with different
83 antigens. [15]. Different immune functions have been attributed to TLR2 signaling including the
84 promotion of a mixed and balanced Th1-, Th2- and ~~Th2-~~Treg cell response [16] and a T-cell
85 tropism to mucosal tissues even when inoculated via a non-mucosal route [17]. In previous
86 immunization studies, OprI had been fused to the *N. caninum* chimeric antigen Mic3-1-R, and
87 vaccinated mice were challenged during pregnancy. This did not result in protection, neither in
88 dams nor in offspring mice, but we nevertheless detected that fusion of OprI to MIC3-1-R
89 induced a mixed Th1/Th2 profile response [18], while the immune response in mice immunized
90 with MIC3-1-R emulsified in saponin was largely Th2-biased, thus confirming the
91 immunomodulatory properties of OprI-fusions. This well-adjusted Th1/Th2 balance is required to
92 protect *N. caninum* vaccinated mice during pregnancy when a strong Th1 response could be
93 detrimental [30].

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

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

102

103 **2. Materials and methods**

104

105 *2.1.* Neospora caninum *culture, inoculum and crude extract preparation*

106

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

111

112 *2.2. Production of recombinant antigens fused to OprI*

113

114 The coding sequence of NcPDI (aa 25-446) [22], a fragment of NcROP2 (aa 238-594)
115 [12] and the complete coding sequence of NcROP40 [11] were cloned in the pOLP plasmid in
116 frame ~~wiuth~~with OprI [15]. A previously described plasmid, containing the ovalbumin (OVA)
117 partial sequence (aa 203-386) in frame with OprI [16] was also used to obtain the unrelated
118 antigen OprI-OVA. Proteins were all expressed in Rosetta(DE3)pLysS *Escherichia coli* strain
119 (Novagen). They were isolated from the bacterial outer membranes as previously described [18].
120 Purified OprI-NcPDI was dialyzed against PBS over-night at 4°C and stored at -80°C. OprI-
121 NcROP2 and OprI-NcROP40, expressed less efficiently and insoluble in aqueous solution, were

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

130

131 *2.3. Dendritic cell (DC) stimulation*

132

133 OprI-fused and non-fused recombinant proteins were assessed for DC stimulation [18].
134 Briefly, DCs were obtained *in vitro* from bone marrow cells obtained from a naïve Balb/c mouse.
135 DCs ($0.5x10^6$ cells/ml~~),~~) were cultured in the presence of each protein (1 or 5µg/ml), LPS
136 (0.1µg/ml) or medium during 24hours, and TNF-α was measured in the culture supernatants by
137 ELISA [18].

138

139 *2.4. Immunization schedule, clinical monitoring and sample collection*

140

141 All animal protocols were approved by the Bernese Animal Welfare Committee (license
142 BE115/14.) Two experiments were carried out. Schedules of immunization, mating, blood sample
143 collection and euthanasia for both studies are depicted in Fig.1. Six weeks-old BALB/c mice
144 were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained as
145 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

170 per cage. Following infection, all mice were daily monitored for clinical signs using a
171 standardized score sheet: coat (ruffled coat=score1; stary stiff coat=score2); weight loss (10%
172 loss=score1, 15-20% loss=score 4); behavior (hunched appearance, walking in circles, head tilt,
173 apathy and ataxia, all=score 1). Animal were euthanized when the score exceeded 3 points.
174 At 30 days post-infection (30 dpi), all surviving non-pregnant mice in both experiments
175 were euthanized (Fig.1; PstCh-NonPreg). Dams and their pups were maintained together until
176 day 30 post-partum (pp) (=41-44 dpi), subsequently all animals were euthanized (Fig.1; PstCh-
177 D).
178 Data on clinical signs, fertility (percentage of pregnant mice), litter size (average of
179 number of pups born per dam), neonatal mortality of pups (stillborn pups or pups dying within
180 the first 2 days post-partum (dpp) and postnatal mortality (pups dying between day 3 and 30pp)
181 were recorded. Upon euthanasia, blood was extracted by cardiac puncture and sera stored at -80
182 °C. Brains from non-pregnant mice, dams and surviving pups were also collected and
183 immediately frozen at -20°C. Spleens from 5 to 6 non-pregnant mice and dams per group were
184 aseptically recovered and processed for splenocyte re-stimulation *in vitro*.
185
186 *2.5.Analysis of the cerebral parasite burden*
187
188 Brains from non-pregnant mice, dams and surviving pups were analyzed by *N. caninum*-
189 specific real-time PCR [25]. DNA extraction was performed using the Nucleospin Kit
190 (Macherey-Nagel, Oensingen, Switzerland). The DNA concentration in all samples was
191 determined using the QuantiFluor dsDNA System (Promega, Madison, Wi.) and was adjusted to
192 5ng/µl with sterile DNase free water. Quantitative real-time PCR was performed using the Rotor-
193 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.

2.6. *Assessment of cytokine expression levels in serum and splenic lymphocytes stimulated* in vitro

Levels of mouse IFN-γ, IL-10, IL-2 and IL-5 were assessed at two different time-points in experiment 2: (i) at 9dpi, coinciding with days 14-~~16~~17 of pregnancy (PstCh-pm, see Fig.1) in blood, and (ii) at the late post-challenge phase in non-pregnant mice (30 dpi) and dams (43-45 dpi) by splenocyte re-stimulation *in vitro* as described [18]. For cytokine assessment in blood, the serum samples were analysed by bead-based multiplex assay for the Luminex® platform (Hertogenbosch, The Netherlands) . Microtiter filter plates were run on Luminex instruments (Bio-Plex™200 system). Calibration curves were calculated with Bio-Plex Manager software using a five-parametric logistic curve fitting [18]. For cytokine analysis upon splenocyte recall responses, spleens were disaggregated and splenocyte cell suspensions were prepared as described [18], The resulting cell suspensions were seeded in 48-well plates and re-stimulated with either concanavalin A (ConA; 5µg/ml; Sigma), recNc-PDI, NcROP2, NcROP40 (20µg/ml), whole *N. caninum* crude extract (10µg/ml) or remained non-stimulated. Supernatants were collected after 72h and stored at -80°C until cytokines were assessed by ELISA (BD OptEIA™ Mouse ELISA Set, LifeSpan Biosciences Inc., Seattle, WA, USA).

2.7. Analysis of serum immunoglobulins

Immunoglobulins were analyzed at different time-points: (i) during the pre-challenge 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

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

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

259

260 **3. Results**

261

262 *3.1. Increased dendritic cell stimulation by OprI-fused recombinant antigens*

263

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

267 in the culture supernatants by ELISA (Fig.2B). In contrast to the non-fused recombinant antigens,

268 the OprI-proteins induced strong and specific DC stimulation (Fig.2B).

269

270 *3.2. Immunization with OprI-fused* Neospora *antigens reduced vertical transmission and*

271 *increased postnatal survival*

272

273 Table 1 shows the outcome of *N. caninum* infection in dams and pups in both

274 experiments. Comparing the O-Ags groups and the C+ infection control groups in both

275 experiments, using the experiment as random effect in the mixed model, there was a significant

276 differenceincrease in survival of offspring of immunized animals (O-Ags groups *P* <0.001) and a

277 significant reduction in vertical transmission (OR=4.52, CI 95% [2.06;10.63], *P*<0.001).

278 Analyzing experiment 1 separately, offspring from O-Ags group also showed a significant

279 higher survival compared to C+ (χ^2 and Log-rank test, *P* <0.05). In the O-Ags+TLR group in

280 experiment 1 (Fig.3A), the pup survival curves werecurve was also significantly different from

281 the O-Ags group (Log-rank test, *P*<0.01), showing that supplementation of the O-Ags

282 formulation by TLR3 plus TLR7 ligands abolished the protective effect of O-Ags immunization.

283 In fact, the O-Ags+TLR group the pup survival curveof pups was not significantly different from

284 C+ (O-Ags+TLR *vs* C+; χ^2 and Log-rank test, *P* >0.05) (Table 1; Fig. 3A).

285 In experiment 2 (Fig. 3B), comparing the C+ group with the O-Ags group, the

286 puppercentage of pups survival and the pup survival curves from O-Ags were also significantly

287 different (higher (χ^2 and Log-rank test, *P* <0.01).) and the vertical transmission significantly

288 lower than C+ (χ^2 and Log-rank test, *P* <0.01). Here, a group of mice was immunized with OprI

289 fused to ovalbumin (O-OVA group). In thethis latter group immunized with O-OVA, the

290 percentage of surviving pups, the pup survival curve ~~was~~and the vertical transmission were
291 identical to C+ (O-OVA *vs* C+; χ^2 and Log-rank test, P >0.05) (Table 1; Fig.3B).
292
293 *3.3. Immunization with OprI-fused* Neospora *antigens reduced cerebral neosporosis*

294 Taking into account both experiments in the mixed-effects ~~log-linear~~logistic model there
295 was a significant effect of O-Ags vaccination on the likelihood of animals developing clinical
296 signs, with C+ animals at higher risk (OR=12.6, CI 95% [1.76; 260.66], *P*<0.05) independent of
297 pregnancy. A significant effect of both O-Ags immunization (*P*<0.001) and pregnancy (*P*<0.01)
298 on the cerebral parasite burden, without a significant interaction between these two variables~~,~~
299 (*P*>0.05), was observed with the mixed effects log linear model (Fig.4A,B~~)~~). Compared to non-
300 immunized mice~~,~~ (C+), O-Ags vaccinated dams showed lower parasite burdens~~ (Fig.4A,B). In~~.
301 In experiment 1 and 2, dams immunized with O-Ags+TLR or O-OVA~~, no reduction in~~
302 did not show reduced cerebral parasite burden ~~was detected~~compared to C+ (Kruskal-Wallis,
303 *P*>0.05) (Fig.4A,B). ~~Immunization~~Regarding non-pregnant mice, immunization with O-Ags also
304 ~~impacted on~~ reduced significantly the cerebral parasite burden and clinical signs ~~in non-pregnant~~
305 ~~immunized animals~~ (Fig.4C,D). This could only be seen in experiment 2 (O-Ags vs C+; Kruskal-
306 Wallis, *P* <0.05), since in experiment 1 only one mouse from O-Ags was not pregnant. No
307 protective effect was observed in ~~the one non-pregnant mouse~~ mice immunized with O-Ags+TLR
308 (data not shown) or ~~in the~~ O-OVA ~~immunized mice~~ (Kruskal-Wallis, *P*>0.05) (Fig.4C).
309
310 *3.4. Humoral immune responses against recombinant antigens*
311
312 Specific antibody responses against each of the recombinant antigens used for
313 immunization were studied by ELISA in both experiments at different time-points in the pre-

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

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

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

346

347 *3.5.Antibody responses against soluble N. caninum antigens*

348

349 No significant differences in IgG1 or IgG2a levels between groups were detected (~~two-way~~
350 ~~ANOVA~~Kruskal-Wallis, $P>0.05$) (Fig.6). In sera from mice immunized with O-Ags, and O-
351 OVA, and from infected non-immunized C+ mice, similar IgG2a levels were noted in dams and
352 non-pregnant mice (~~*U*- mann~~Mann-Whitney U test, $P>0.05$), whereas IgG1 levels were
353 significantly lower in non-pregnant mice compared to dams (~~*U*mann~~Mann-Whitney U test,
354 $P<0.05$) pointing towards an IgG2a-biased response in non-pregnant mice compared to dams. In
355 contrast, in sera of mice vaccinated with O-Ags+TLR ligands, no such differences between dams
356 and non-pregnant were detected (~~*U*mann~~Mann-Whitney U test, $P>0.05$), and the humoral
357 immune response was IgG2a-biased in both cases~~.~~ (Fig. 6).

358

359 *3.6.Cytokine responses*

360

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

385 composed of three recombinant *N. caninum* antigens fused to OprI was assessed in a standardized
386 pregnant mouse model of neosporosis based on the highly virulent *N. caninum* isolate Nc-Spain7.
387 The vaccine formulation was composed of bacterially expressed and purified recNcPDI, NcROP2
388 and NcROP40, all three N-terminally fused to the OprI lipoprotein., a tri-acylated bacterial outer
389 membrane protein. OprI targets TLR2, and thus stimulates mixed Th1, Th2 and Treg responses,
390 favoring cross-presentation by APCs [16] Moreover, OprI was able to modulate the cellular
391 immune response against *N. caninum* towards a mixed Th1/Th2 response in mice vaccinated with
392 the *N. caninum* chimeric antigen Mic3-1-R [18]. The Nc*ROP40* gene was found to be identical in
393 three *N. caninum* isolates displaying different virulence, which renders this a promising vaccine
394 candidate [31,32]. RecNcROP2 had been demonstrated to confer significant protection in non-
395 pregnant and pregnant neosporosis mouse models based on the -*N. caninum* Nc1 isolate [10,12].
396 RecNcPDI had conferred excellent protection in non-pregnant mice when applied intranasally
397 emulsified in cholera toxin, but failed to prevent congenital neosporosis in pregnant mice [2, 14].
398 Nevertheless, since TLR2-ligands have shown to induce mucosal immunity by imprinting
399 lymphocyte tropism to mucosae, even though they are applied by non-mucosal route [17], and
400 NcPDI had conferred mucosal immunity [14], we incorporated this protein into the polyvalent O-
401 Ags formulation.
402 We show here in two independent experiments that immunization with O-Ags confers
403 significant protection in offspring born to dams that were challenged with *N. caninum* tachyzoite
404 infection on day 7-9 of pregnancy. Postnatal mortality was reduced by 25.0 and 26.9% of pups in
405 experiment 1 and 2, respectively, the mean survival time was extended and, overall, there was a
406 clear difference in the survival curves. This rate of protection against congenital neosporosis has
407 not been seen with recombinant antigens before (11). Vertical transmission (including dead pups
408 and PCR positive survivors) was reduced in 17.2 and 24.4% of pups, respectively, showing a

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

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

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

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

433 antibodies directed against ROP2 and ROP40 when assessed by ELISA prior to challenge and ~~at~~
434 ~~9dpi., and~~ this low response was confirmed by Western blotting using sera from experiment 1.
435 Nevertheless, immunoblotting confirmed specific recNcROP2 and recNcROP40 antibody
436 responses, since a signal against recNcROP2 was detected at 9dpi in both O-Ags and O-
437 Ags+TLR vaccinated groups, with no signal in the C+ group. RecNcROP40 was also detected
438 slightly by both vaccinated groups at pre-challenge phase, boosting at 9dpi in the O-Ags+TLR
439 group.

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

457 9dpi and cerebral parasite burden. Thus, the differential antibody kinetics observed here is likely
458 to be a consequence of a pregnancy-associated phenomenon. Antibody levels are also altered
459 during the course of *N. caninum* infection in cattle. In naturally infected heifers, antibody
460 fluctuations occurring between 90 and 240 days of gestation were shown to be associated with a
461 higher probability of vertical transmission [36, 37]. Cows that aborted also showed more
462 pronounced fluctuations and overall higher antibody levels, especially between months 3 and 8 of
463 gestation, compared to non-aborting cows [38]. Whether the vaccine-associated drop in antibody
464 levels in dams compared to non-pregnant mice ~~is a reflection of~~reflects a downregulation of total
465 blood immunoglobulins during pregnancy needs to be further investigated. In humans, an overall
466 reduction of total IgG and IgM was observed in healthy pregnant women compared to healthy
467 non-pregnant women [39].

468 In experiment 2, cytokine levels were studied at 9dpi in peripheral blood samples, and at
469 30dpi for non-pregnant mice and 43dpi for dams by analyzing splenocyte recall responses after
470 stimulation with crude *N. caninum* extracts. Moreover, all mice were individually tracked. At
471 9dpi, IFNγ blood levels were lower in O-Ags vaccinated mice compared to the C+ group, in both
472 pregnant and non-pregnant mice. However, at chronic phase, namely at 43dpi, when IFNγ levels
473 were measured in culture supernatants of stimulated splenocytes, this effect was not observed.
474 Splenocytes from vaccinated dams with lower vertical transmission rates tended to produce more
475 IFNγ, thus the correlation between IFNγ levels and vertical transmission was inverted. When
476 splenocytes from non-pregnant mice were stimulated and supernatants assessed for IFNγ, higher
477 levels were recorded in the O-Ags vaccinated group compared to C+ group. It was shown earlier
478 that in *N. caninum* infected mice IFNγ levels reach a peak around 10dpi [40], which is in
479 accordance with the highest IFNγ levels observed in our C+ group at 9 dpi. We suggest that the
480 down-regulation of IFNγ levels at 9dpi in O-Ags mice may have contributed to the reduction of

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

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

499

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504

References

[1] Dubey , J.P.H., Hemphill, A; Calero-Bernal, R.; Schares, G., 2017. Neosporosis in Animals. CRC Press, Florida, United States.

[2] Debache, K., Hemphill, A., 2013. Differential effects of intranasal vaccination with recombinant NcPDI in different mouse models of *Neospora caninum* infection. Parasite Immunol 35, 11-20.

[3] Reichel, M.P., Alejandra Ayanegui-Alcerreca, M., Gondim, L.F., Ellis, J.T., 2013. What is the global economic impact of *Neospora caninum* in cattle - the billion dollar question. Int J Parasitol 43, 133-142.

[4] Reichel, M.P., Moore, D.P., Hemphill, A., Ortega-Mora, L.M., Dubey, J.P., Ellis, J.T., 2015. A live vaccine against *Neospora caninum* abortions in cattle. Vaccine 33, 1299-1301.

[5] Rojo-Montejo, S., Collantes-Fernandez, E., Perez-Zaballos, F., Rodriguez-Marcos, S., Blanco-Murcia, J., Rodriguez-Bertos, A., Prenafeta, A., Ortega-Mora, L.M., 2013. Effect of vaccination of cattle with the low virulence Nc-Spain 1H isolate of *Neospora caninum* against a heterologous challenge in early and mid-gestation. Vet Res 44, 106.

[6] Weber, F.H., Jackson, J.A., Sobecki, B., Choromanski, L., Olsen, M., Meinert, T., Frank, R., Reichel, M.P., Ellis, J.T., 2013. On the efficacy and safety of vaccination with live tachyzoites of *Neospora caninum* for prevention of neospora-associated fetal loss in cattle. Clin Vaccine Immunol 20, 99-105.

[7] Horcajo, P., Regidor-Cerrillo, J., Aguado-Martinez, A., Hemphill, A., Ortega-Mora, L.M., 2016. Vaccines for bovine neosporosis: current status and key aspects for development. Parasite Immunol 38, 709-723.

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[8] Rojo-Montejo, S., Collantes-Fernandez, E., Lopez-Perez, I., Risco-Castillo, V., Prenafeta, A., Ortega-Mora, L.M., 2012. Evaluation of the protection conferred by a naturally attenuated *Neospora caninum* isolate against congenital and cerebral neosporosis in mice. Vet Res 43, 62.

[9] Miller, C., Quinn, H., Ryce, C., Reichel, M.P., Ellis, J.T., 2005. Reduction in transplacental transmission of *Neospora caninum* in outbred mice by vaccination. Int J Parasitol 35, 821-828.

[10] Debache, K., Alaeddine, F., Guionaud, C., Monney, T., Müller, J., Strohbusch, M., Leib, S.L., Grandgirard, D., Hemphill, A., 2009. Vaccination with recombinant NcROP2 combined with recombinant NcMIC1 and NcMIC3 reduces cerebral infection and vertical transmission in mice experimentally infected with *Neospora caninum* tachyzoites. Int J Parasitol 39, 1373-1384.

[11] Pastor-Fernandez, I., Arranz-Solis, D., Regidor-Cerrillo, J., Alvarez-Garcia, G., Hemphill, A., Garcia-Culebras, A., Cuevas-Martin, C., Ortega-Mora, L.M., 2015. A vaccine formulation combining rhoptry proteins NcROP40 and NcROP2 improves pup survival in a pregnant mouse model of neosporosis. Vet Parasitol 207, 203-215.

[12] Debache, K., Guionaud, C., Alaeddine, F., Mevissen, M., Hemphill, A., 2008. Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with *Neospora caninum* tachyzoites. Int J Parasitol 38, 1455-1463.

[13] Regidor-Cerrillo, J., Alvarez-Garcia, G., Pastor-Fernandez, I., Marugan-Hernandez, V., Gomez-Bautista, M., Ortega-Mora, L.M., 2012. Proteome expression changes among virulent and attenuated *Neospora caninum* isolates. J Proteomics 75, 2306-2318.

[14] Debache, K., Guionaud, C., Alaeddine, F., Hemphill, A., 2010. Intraperitoneal and intra-nasal vaccination of mice with three distinct recombinant *Neospora caninum* antigens results in differential effects with regard to protection against experimental challenge with *Neospora caninum* tachyzoites. Parasitology 137, 229-240.

Formatted: French (Switzerland)

551 [15] Basto, A.P., Piedade, J., Ramalho, R., Alves, S., Soares, H., Cornelis, P., Martins, C.,
552 Leitao, A., 2012. A new cloning system based on the OprI lipoprotein for the production of
553 recombinant bacterial cell wall-derived immunogenic formulations. J Biotechnol 157, 50-63.
554 [16] Basto, A.P., Leitao, A., 2014. Targeting TLR2 for vaccine development. J Immunol Res
555 2014, 619410.
556 [17] Wang, S., Villablanca, E.J., De Calisto, J., Gomes, D.C., Nguyen, D.D., MizoguchI, E.,
557 Kagan, J.C., Reinecker, H.C., Hacohen, N., Nagler, C., Xavier, R.J., Rossi-Bergmann, B., Chen,
558 Y.B., Blomhoff, R., Snapper, S.B., Mora,J.R.,2011. MyD88-dependent TLR1/2 signals educate
559 dendritic cells with gut-specific imprinting properties. J Immunol. 187, 141-150.
560 [18] Aguado-Martinez, A., Basto, A.P., Muller, J., Balmer, V., Manser, V., Leitao, A., Hemphill,
561 A., 2016. N-terminal fusion of a toll-like receptor 2-ligand to a *Neospora caninum* chimeric
562 antigen efficiently modifies the properties of the specific immune response. Parasitology 143,
563 606-616.
564 [19] Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F., Lanzavecchia, A., 2005. Selected Toll-
565 like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in
566 dendritic cells. Nat Immunol 6, 769-776.
567 [20] Regidor-Cerrillo, J., Gomez-Bautista, M., Pereira-Bueno, J., Aduriz, G., Navarro-Lozano,
568 V., Risco-Castillo, V., Fernandez-Garcia, A., Pedraza-Diaz, S., Ortega-Mora, L.M., 2008.
569 Isolation and genetic characterization of *Neospora caninum* from asymptomatic calves in Spain.
570 Parasitology 135, 1651-1659.
571 [21] Arranz-Solis, D., Benavides, J., Regidor-Cerrillo, J., Horcajo, P., Castano, P., del Carmen
572 Ferreras, M., Jimenez-Pelayo, L., Collantes-Fernandez, E., Ferre, I., Hemphill, A., Perez, V.,
573 Ortega-Mora, L.M., 2016. Systemic and local immune responses in sheep after *Neospora*
574 *caninum* experimental infection at early, mid and late gestation. Vet Res 47, 2.

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575 [22] Muller, J., Naguleswaran, A., Muller, N., Hemphill, A., 2008. *Neospora caninum*: functional
576 inhibition of protein disulfide isomerase by the broad-spectrum anti-parasitic drug nitazoxanide
577 and other thiazolides. Exp Parasitol 118, 80-88.
578 [23] Whitten, M.K., 1957. Effect of exteroceptive factors on the oestrous cycle of mice. Nature
579 180, 1436.
580 [24] Arranz-Solís, D., Aguado-Martínez, A., Müller, J., Regidor-Cerrillo, J., Ortega-Mora, L. M.,
581 Hemphill, A., 2015. Dose-dependent effects of experimental infection with the virulent *Neospora*
582 *caninum* Nc-Spain7 isolate in a pregnant mouse model. Vet. Parasitol. 211:133-140.
583 [25] Muller, N., Vonlaufen, N., Gianinazzi, C., Leib, S.L., Hemphill, A., 2002. Application of
584 real-time fluorescent PCR for quantitative assessment of *Neospora caninum* infections in
585 organotypic slice cultures of rat central nervous system tissue. J Clin Microbiol 40, 252-255.
586 [26] Monney, T., Rutti, D., Schorer, M., Debache, K., Grandgirard, D., Leib, S.L., Hemphill, A.,
587 2011. RecNcMIC3-1-R is a microneme- and rhoptry-based chimeric antigen that protects against
588 acute neosporosis and limits cerebral parasite load in the mouse model for *Neospora caninum*
589 infection. Vaccine 29, 6967-6975.
590 [27] Jenum, P.A., Stray-Pedersen, B., Gundersen, A.G., 1997. Improved diagnosis of primary
591 *Toxoplasma gondii* infection in early pregnancy by determination of anti-*Toxoplasma*
592 immunoglobulin G avidity. J Clin Microbiol 35, 1972-1977.
593 [28] Morrison, W.I., Tomley, F., 2016. Development of vaccines for parasitic diseases of
594 animals: Challenges and opportunities. Parasite Immunol 38, 707-708.
595 [29] Hemphill, A., Aguado-Martinez, A., Müller, J., 2016. Approaches for the vaccination and
596 treatment of *Neospora caninum* infections in mice and ruminant models. Parasitology 143, 245-
597 259.
598 [30] Aguado-Martínez, A., Basto, A. P., Leitão, A., Hemphill, A. 2017. *Neospora caninum* in

599 non-pregnant and pregnant mouse models: cross-talk between infection and immunity. Int. J.
600 Parasitol. 47:723-735.
601 [31] Pastor-Fernandez, I., Regidor-Cerrillo, J., Jimenez-Ruiz, E., Alvarez-Garcia, G., Marugan-
602 Hernandez, V., Hemphill, A., Ortega-Mora, L.M., 2016. Characterization of the *Neospora*
603 *caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins during the tachyzoite lytic cycle.
604 Parasitology 143, 97-113.
605 [32] MacHugh, N.D., Weir, W., Burrells, A., Lizundia, R., Graham, S.P., Taracha, E.L., Shiels,
606 B.R., Langsley, G., Morrison, W.I., 2011. Extensive polymorphism and evidence of immune
607 selection in a highly dominant antigen recognized by bovine CD8 T cells specific for *Theileria*
608 *annulata*. Infect Immun 79, 2059-2069.
609 [33] Bethancourt, C.N., Wang, T.L., Bocchini, J.A., Jr., 2017. Vaccination during pregnancy:
610 first line of defense for expecting mothers and vulnerable young infants. Curr Opin Pediatr 29,
611 737-743.
612 [34] Papaioannou, A.I., Spathis, A., Kostikas, K., Karakitsos, P., Papiris, S., Rossios, C., 2017.
613 The role of endosomal toll-like receptors in asthma. Eur J Pharmacol 808, 14-20.
614 [35] Mansilla, F.C., Quintana, M.E., Langellotti, C., Wilda, M., Martinez, A., Fonzo, A., Moore,
615 D.P., Cardoso, N., Capozzo, A.V., 2016. Immunization with *Neospora caninum* profilin induces
616 limited protection and a regulatory T-cell response in mice. Exp Parasitol 160, 1-10.
617 [36] Pare, J., Thurmond, M.C., Hietala, S.K., 1997. *Neospora caninum* antibodies in cows during
618 pregnancy as a predictor of congenital infection and abortion. J Parasitol 83, 82-87.
619 [37] Guy, C.S., Williams, D.J.L., Kelly, D.F., McGarry, J.W., Guy, F., Bjorkman, C., Smith,
620 R.F., Trees, A.J., 2001. *Neospora caninum* in persistently infected, pregnant cows: spontaneous
621 transplacental infection is associated with an acute increase in maternal antibody. Vet Rec 149,
622 443-449.

623 [38] Quintanilla-Gozalo, A., Pereira-Bueno, J., Seijas-Carballedo, J., Costas, E., Ortega-Mora, L.M., 2000. Observational studies in *Neospora caninum* infected dairy cattle: relationship infection-abortion and gestational antibody fluctuations. In "A European perspective on *Neospora caninum*". Int J Parasitol 30, 877-924.

627 [39] Amah-Tariah, F.S., Bekinbo, M.T., Dapper, D.V., 2016. Comparative study of Serum Immunoglobulin levels in Healthy Pregnant and Pregnant Subjects with HIV and Malaria Infection in Port Harcourt, Nigeria. Int Res J Med Sci 4, 11-16.

630 [40] Khan, I.A., Schwartzman, J.D., Fonseka, S., Kasper, L.H., 1997. *Neospora caninum*: role for immune cytokines in host immunity. Exp Parasitol 85, 24-34.

632 [41] Rettigner, C., De Meerschman, F., Focant, C., Vanderplasschen, A., Losson, B., 2004. The vertical transmission following the reactivation of a *Neospora caninum* chronic infection does not seem to be due to an alteration of the systemic immune response in pregnant CBA/Ca mice. Parasitology 128, 149-160.

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637

638 **Figure Legends**

639

640 ~~**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 correrspondig 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;

670 ~~PstCh-D = Post-challenge "dams" time-point; Igs = immunoglobulins; Bold arrows indicate time-~~

671 ~~points of immune response analysis.~~

672

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

674

675 **Fig. 4.** Cerebral parasite burden measured by real-time PCR in dams from experiment 1 (A) and

676 2 (B) at 40-44 dpi, and from non-pregnant mice from experiment 2 at 30 dpi (C). Experiment 1 is

677 not shown since only one mouse from the O-Ags group remained non-pregnant. (D) shows the

678 clinical signs score of non-pregnant mice in experiment 2.

679

680 **Fig. 5.** Humoral immune responses against recombinant antigens. A) Western blot analysis of

681 pooled sera obtained from experiment 1. Recombinant ROP2 (rROP2) (MW = 41 kDa) and

682 rROP40 (MW = 49 kDa) were separated by SDS-PAGE, blotted onto nitrocellulose, and rROP2-

683 and rROP40-specific IgG were detected by immunoblotting in mouse sera obtained at the pre-

684 challenge phase prior to mating (PrCh-1) and at 9 dpi coinciding with days 14-16 of pregnancy

685 (PstCh-pm.). "+" depicts a positive control serum from a mouse that was chronically infected

686 with *N. caninum*. 1, 2, and 3 strips represent dilutions 1:50, 1:200 and 1:800 of pooled sera.

687 Arrowheads indicate the location of recombinant proteins. (B) ELISA-based detection and

688 quantification of PDI-specific IgG1 and IgG2a levels in experiment 1. PDI-specific antibodies in

689 mice vaccinated with O-Ags and O-Ags+TLR ligands collected at the pre-challenge phase prior

690 to mating (PrCh-1) and at 9 dpi coinciding with days 14-16 post mating (PstCh-pm). (C) Ratio of

691 IgG1:IgG2a. Dots represent individual values of 4 randomly chosen mice euthanized at PrCh-1

692 and 3 pregnant mice randomly chosen to be euthanized at PstCh-pm. Horizontal lanes represent

693 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).

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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, 25th and 75th 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, 25th and 75th 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.

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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[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.

Figure1

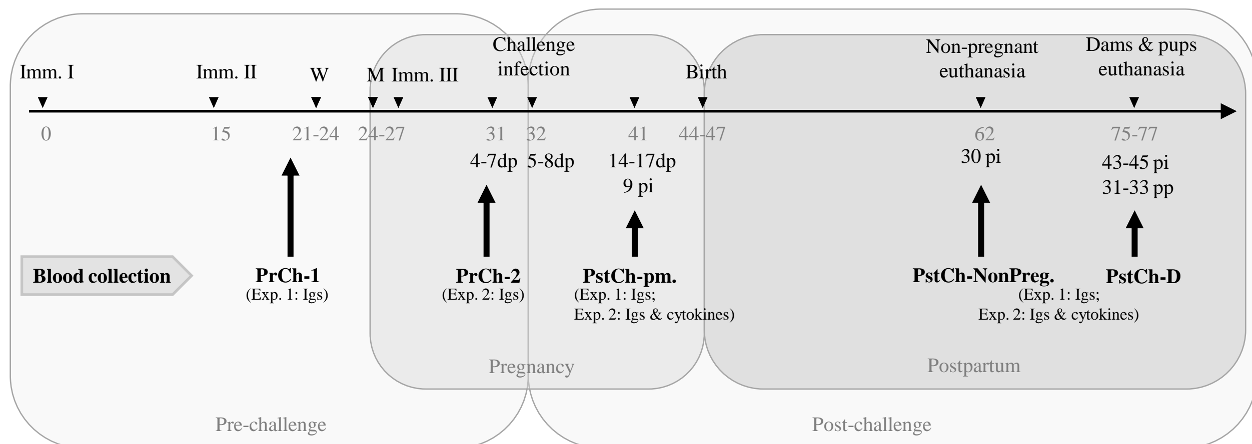
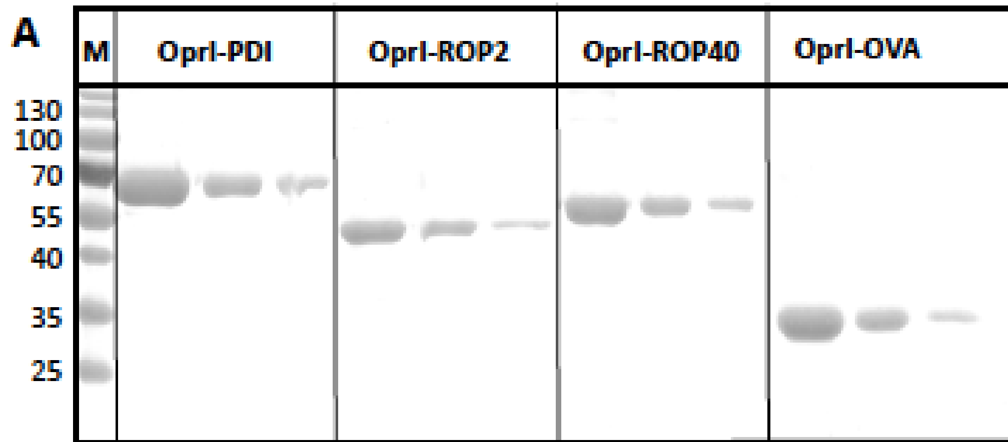


Fig. 1

Figure2



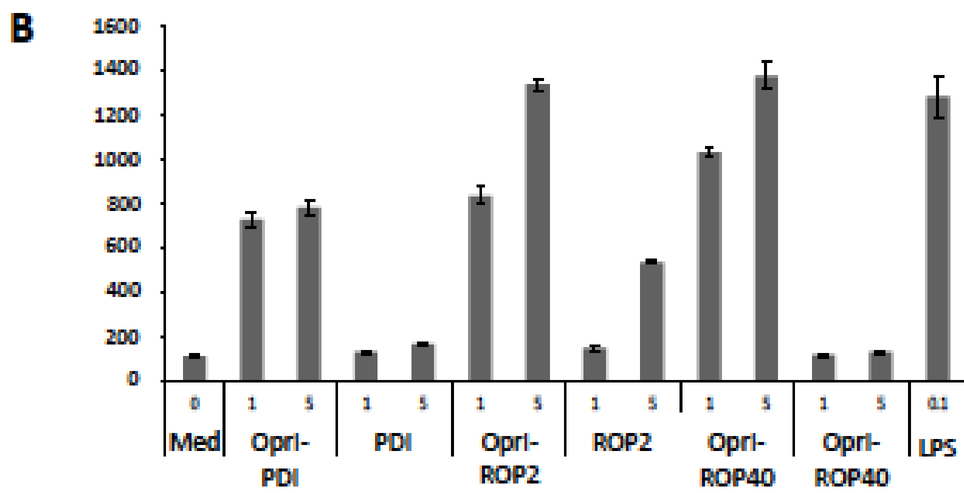


Fig. 2

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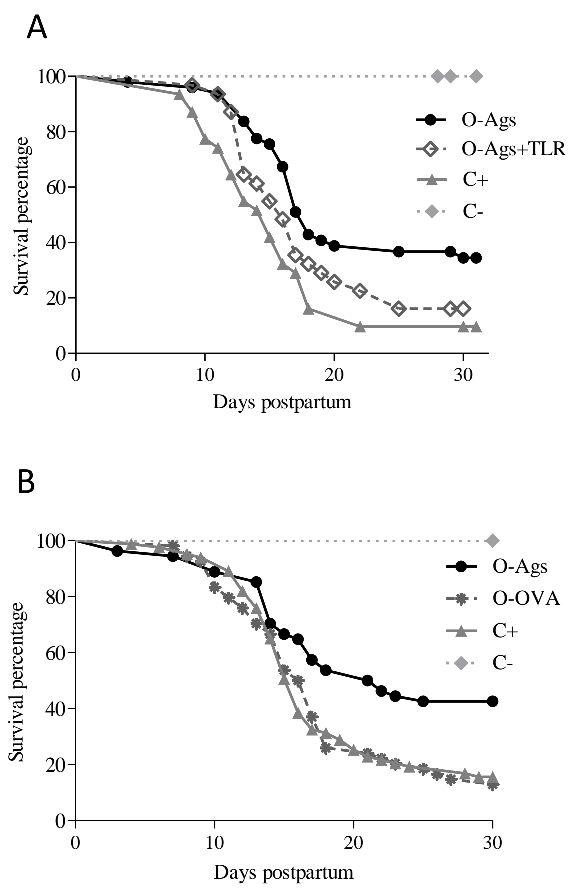

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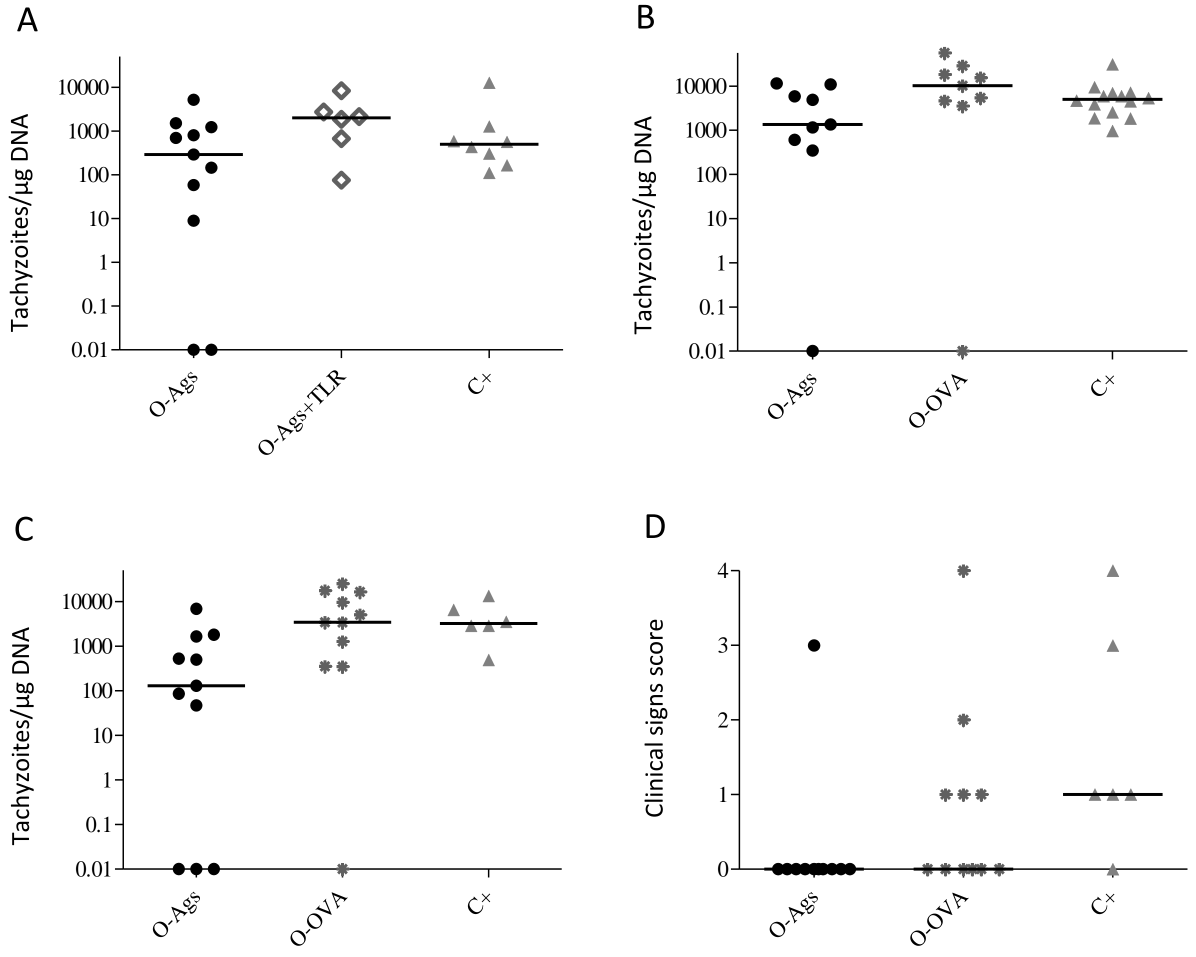

Fig. 4

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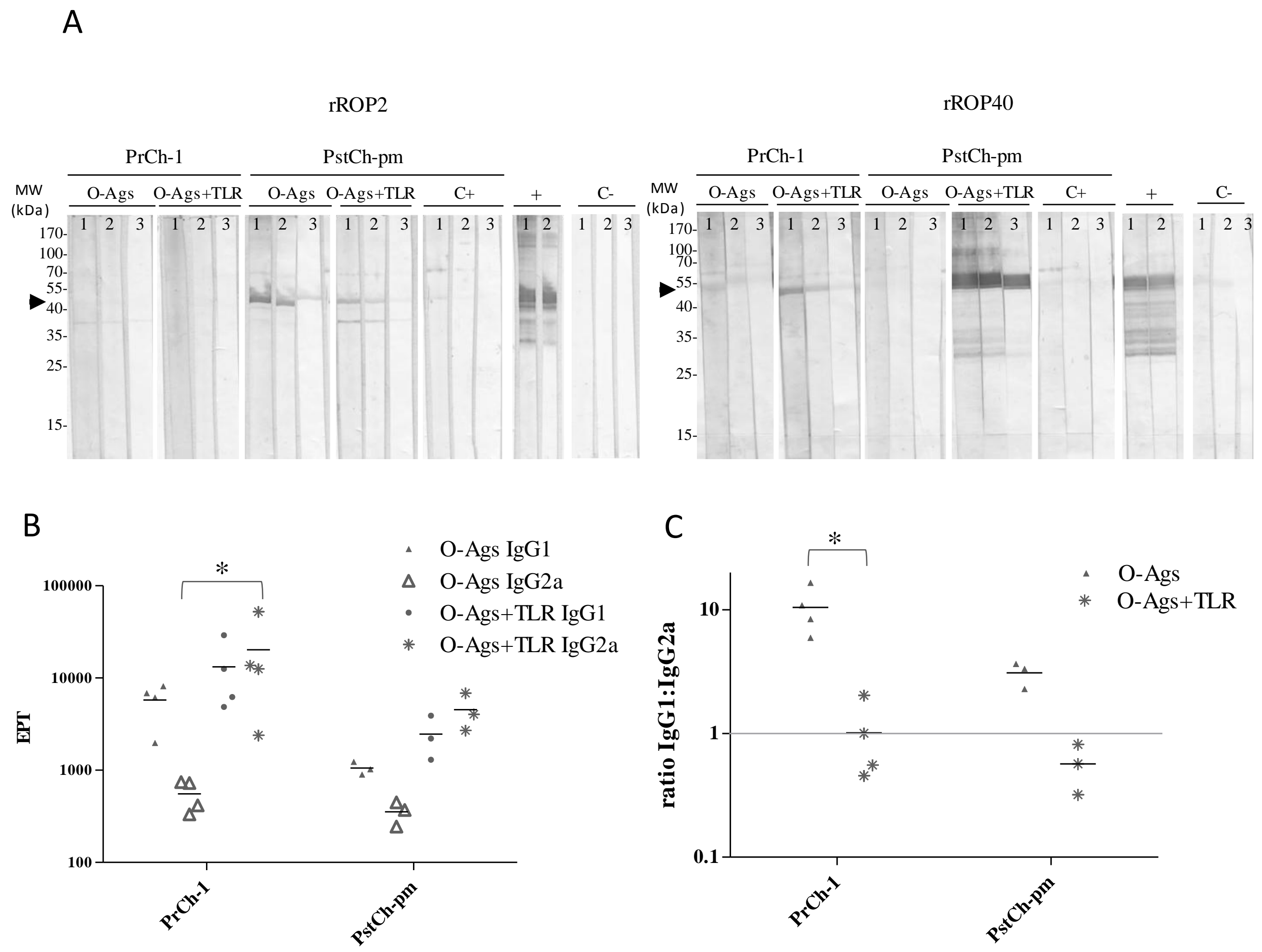
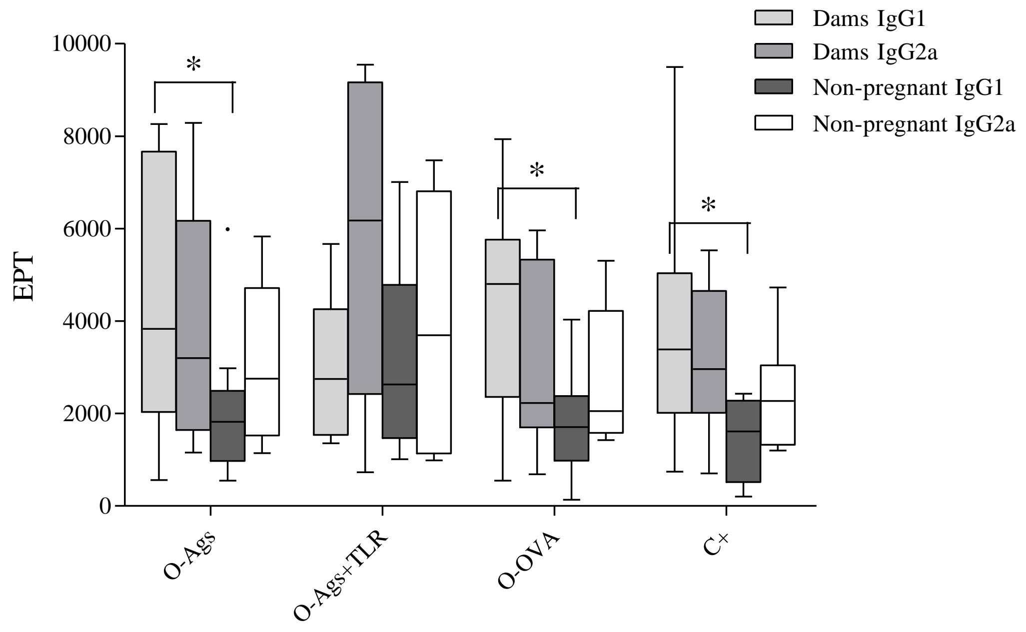


Fig. 5

Figure6



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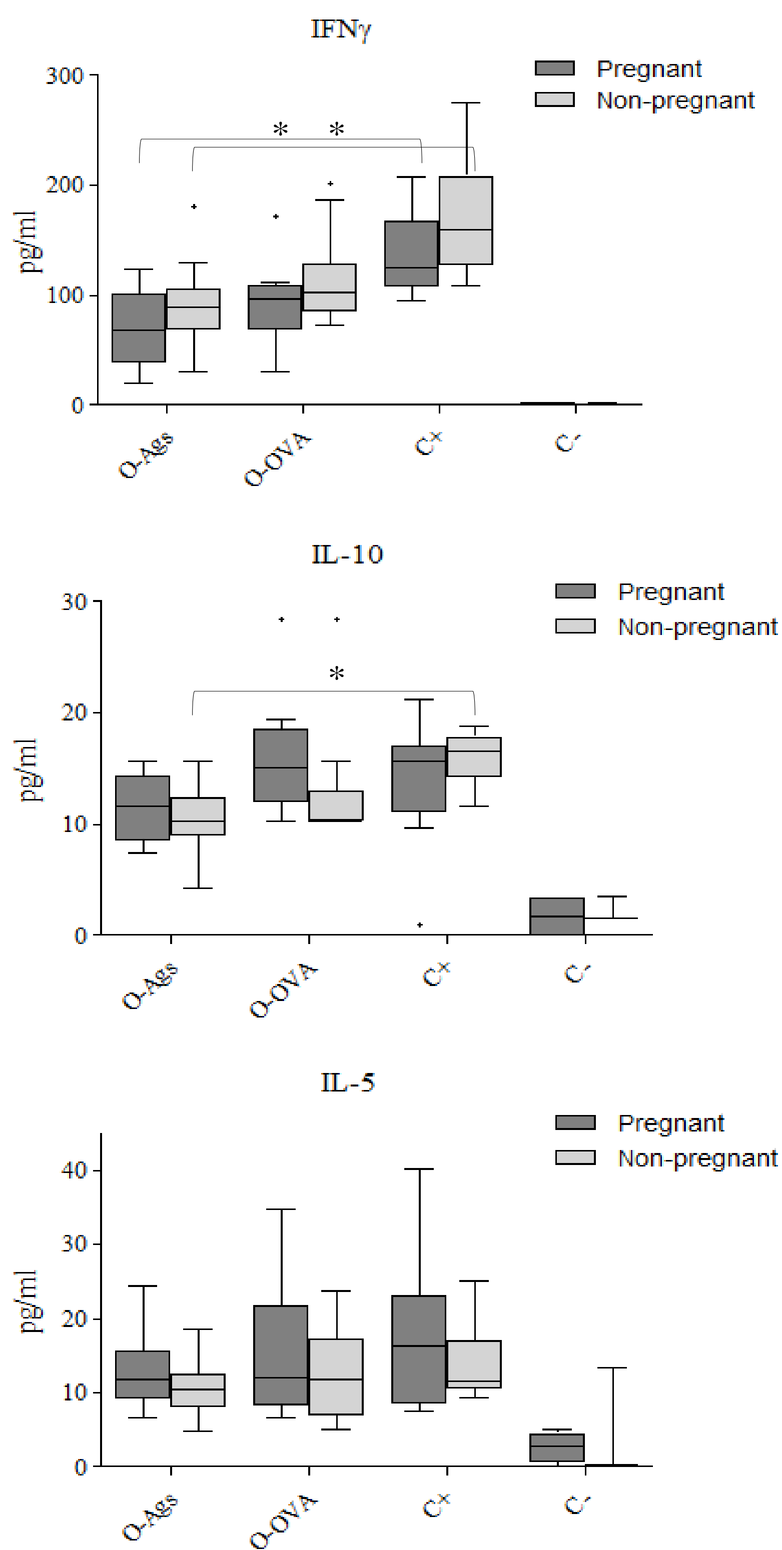

Fig. 7

Figure8

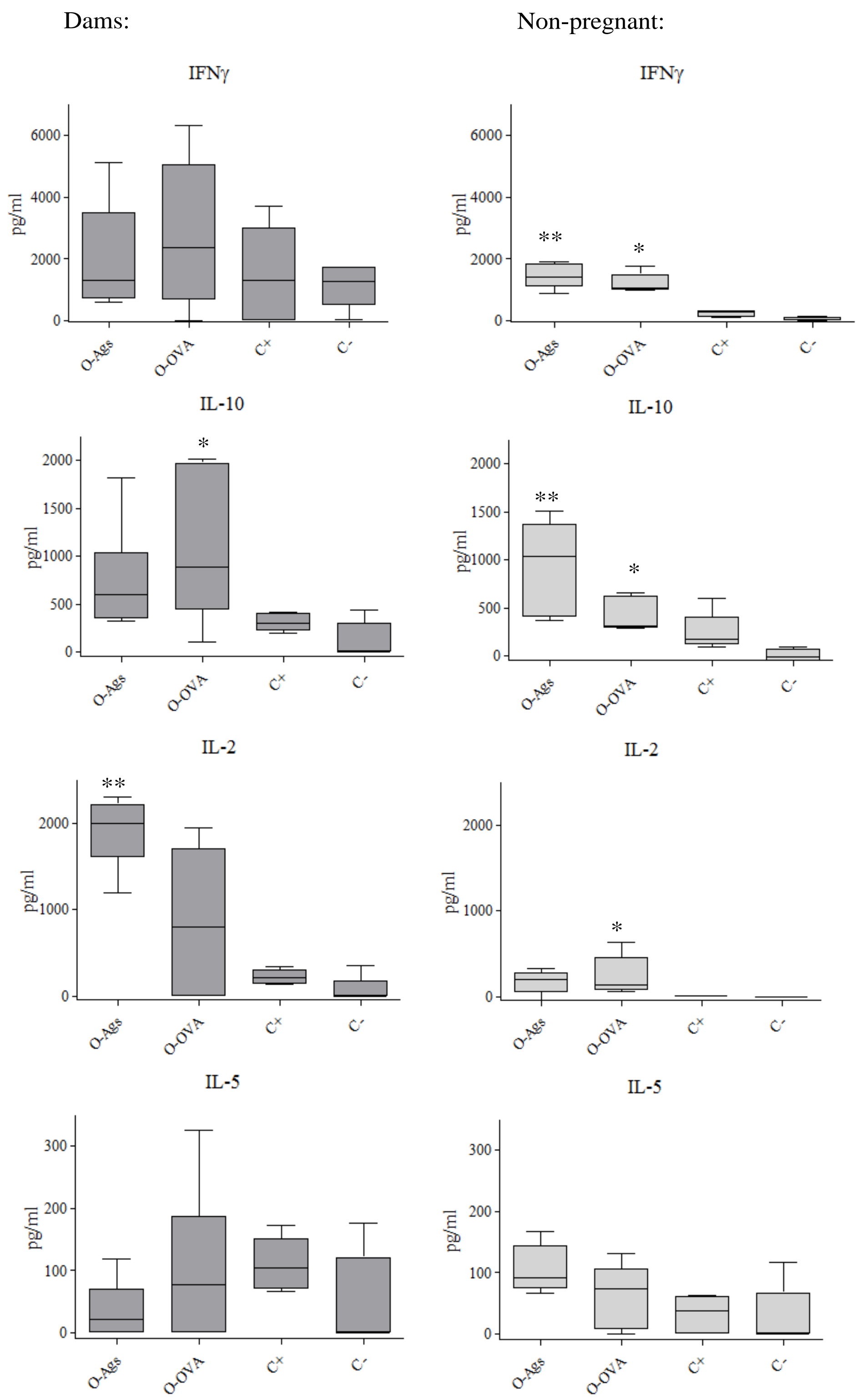

Fig. 8

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