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Critical role of TLR2 in triggering protective immunity with cyclophilin entrapped in oligomannose-coated liposomes against *Neospora caninum* infection in mice

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Abbreviations: BCA, bicinchoninic acid assay; CCK-8, cell counting kit-8; ConA, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; DPI, days post-infection; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HRP, horse radish peroxidase; IFN-γ, interferon γ; IgG, immunoglobulin G; IL-4, interleukin 4; PBS, phosphate buffered saline; Nc-1, a strain of *Neospora caninum*; NcCyp, *N. caninum* cyclophilin antigen;

NF-κB, nuclear factor of kappa B pathway; OML, oligomannose-coated-liposomes; RPMI-1640 medium, Roswell Park Memorial Institute 1640 medium; TLR2, Toll-like receptor 2.

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54 **1. Introduction**

55 *Neospora caninum* is an intracellular protozoan parasite closely related to *Toxoplasma*
56 *gondii,* infecting dogs as the definitive host as well as a wide range of warm-blooded animals,
57 as intermediate hosts [1]. To date, there is no effective treatment or potent vaccine against *N.*
58 *caninum* [2], [3].

59 A balance between T-helper 1 (Th)1 and Th2 mediated immunity is critical for the
60 control of infection and successful development of vaccines against *N. caninum* [4], [5].
61 Immune responses to *N. caninum* consist of humoral and cellular immunity. However, the
62 most effective protection is via cellular immunity, especially the activation of Th1 cells and
63 the production of cytokines including interleukin (IL)-12 and gamma-interferon (IFN-γ) [6].

64 Professional antigen presenting cells (APCs) such as dendritic cells and macrophages
65 also play a vital role in triggering immune responses against invading pathogens by
66 presenting antigens to major histocompatibility complex molecules (MHC) class I and II,
67 which activates lymphocytes, leading to antigen-specific acquired immunity along with
68 immunologic memory [7]. This event is mediated by pattern recognition receptors including
69 Toll-like receptors (TLRs), which are highly expressed by APCs. TLRs recognize many
70 pathogens by matching conserved pathogen-associated molecular patterns [8]. Previous
71 reports revealed that mice lacking TLR2 exhibited a higher parasite burden than wild-type
72 (WT) mice against *N. caninum* and *T. gondii* infections [9], [10]. Moreover, the TLR2 is
73 involved in innate and adaptive immunity against *N. caninum* in murine cells [11]. The
74 activation of TLRs is strongly correlated to activation of the signal transduction of nuclear
75 factor kappa-B, the major integral component of immune responses and protection against
76 microbial infections [12], [13]. Moreover, the chemokine receptor, CCR5, was reported to be
77 associated with the induction of protective immunity in mice infected with *N. caninum*. Mice
78 lacking the CCR5 gene were more vulnerable to *N. caninum* infection [14], possibly because

79 interactions of CCR5 with parasite-derived cyclophilin elicits the migration and activation of
80 innate immune cells [5], [15]. Oligomannose-coated-liposomes (OML) are used as a delivery
81 system and potential adjuvant in vaccine development against several pathogens of protozoan
82 origin [5].

83 Only one study has reported the usefulness of NcCyp as a vaccine candidate when
84 formulated with ImmuMax and CpG as an adjuvant [16]. In the current study, the vaccination
85 of mice with NcCyp with or without entrapment in OML was investigated in different mouse
86 strains including TLR2-deficient mice. TLR2 was specifically targeted because of its
87 protective role in *N. caninum* infection via the induction of innate and adaptive immunity. In
88 addition, recent studies reported the indispensable role of TLR2 in vaccine development
89 against infectious agents. After challenge infection, TLR2 is upregulated on antigen-
90 presenting cells leading to the increased expressions of MHC II and co-stimulatory molecules
91 (CD80, CD83, CD86), which are required for the priming of Th1 and Th2-mediated
92 immunity [17]. We investigated the induction of cellular and humoral immunity as key
93 factors for protective immunity. Excellent prophylactic potential was conferred in mice using
94 NcCyp-OML against lethal infection with *N. caninum*.

95

96 **2. Materials and Methods**

97 *2.1. Ethical statement*

98 We followed the guidelines and recommendations of the Guide for the Care and Use
99 of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and
100 Technology, Japan. The procedures were approved by the Committee on the Ethics of
101 Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine
102 (numbers: 29-58, 29-61). Experiments that are painful for mice such as the collection of
103 cardiac blood, injection with parasites, recombinant proteins or thioglycolate medium, and
104 surgical operations to collect the brain and spleen were performed under general anesthesia
105 with isoflurane. Cervical dislocation was performed for the euthanization of mice.

106

107 *2.2. Animals*

108 Six-to-seven-week-old female BALB/c mice were purchased from Clea Japan
109 (Tokyo, Japan) and used for vaccination experiments. Male and female C57BL/6 mice at 7-
110 weeks of age were purchased from Clea Japan and used for the vaccination study. The
111 macrophage assay was conducted using cells isolated from male mice. C57BL/6-background
112 TLR2-knockout ($TLR2^{-/-}$) mice were a kind gift from Dr. Satoshi Uematsu and Dr. Shizuo
113 Akira (Osaka University, Osaka, Japan) [18].

114

115 *2.3. RAW 264.7 cell lines and NF-κB analysis*

116 The NF-κB secreting alkaline phosphatase reporter cell line generated from RAW
117 264.7 cells (NF-κB/SEAP cells) was obtained from Novus Biological Inc. (Littleton, CO,
118 USA). The cells were seeded in 96-well plates (2×10^5 in 100 μL/well) and incubated at 37°C
119 for 4 h to allow adherence of the cells to the bottom of the plate. The NcCyp, NcCyp-OML,
120 and GST-OML proteins, and LPS as a positive control and medium alone as a negative

121 control, were added to the cells, and then the plates were incubated at 37°C for 24 h. The
122 procedures for expression and purification of recombinant proteins and OML were described
123 in Supplemental Information file. The culture supernatant from NF-κB/SEAP cells was
124 collected and the levels of secreted alkaline phosphatase were measured using a SEAP
125 reporter assay kit (Novus) according to the manufacturer's instructions. Further details of the
126 culture of NF-κB/SEAP cells, expression and purification of recombinant proteins, and
127 preparation of OML can be found in the Supplemental Methods in the Supplemental
128 Information.

129

130 *2.4. Macrophages and IL-12p40 production*

131 The macrophage suspension was added to 96-well tissue culture microplates at 3×10^5
132 cells/well, incubated at 37°C for 4 h, washed thoroughly to remove nonadherent cells, and
133 further incubated after addition of the indicated stimulants, including positive and negative
134 controls, at 37°C in a 5% CO_2 incubator. The culture supernatant was collected and the levels
135 of IL-12p40 were measured using a commercial ELISA (Pierce Biotechnology Inc.,
136 Rockford, IL, USA), according to the manufacturer's recommendations. Further details of
137 monolayer cultures of mouse peritoneal macrophages can be found in the Supplemental
138 Methods in the Supplemental Information.

139

140 *2.5. Immunization and infection*

141 Different mouse strains (BALB/c and C57BL/6), genders (male and female), and genetic
142 backgrounds (wild-type and $TLR2^{-/-}$) were used in this study to investigate the
143 immunoprophylactic properties of NcCyp. Mice were injected subcutaneously with
144 recombinant NcCyp, NcCyp-OML, and GST-OML, or with PBS alone (each 100 μL) three
145 times at 14-day intervals. To inoculate the same molecular number of the antigens, the

146 inoculum was calculated by molar number based on the molecular weight of each antigen
147 (NcCyp; 20.5 kDa, GST; 27 kDa). Each mouse was immunized with 25 pmol from each
148 antigen (NcCyp-OML; 0.51 μg, NcCyp; 0.51 μg, GST-OML; 0.68 μg). In the BALB/c
149 mouse model, only female mice were used (12 mice per group from 2 independent trials). In
150 the C57BL/6 mouse model, immunizations were administered to male and female wild type
151 or $TLR2^{-/-}$ mice (males: 14 mice per group except for the NcCyp-immunized $TLR2^{-/-}$ group
152 (13 mice) from 2 independent trials; females: 7 per group from one trial). Two weeks after
153 the third immunization, mice were infected with 1×10^6 tachyzoites of the *N. caninum* Nc-1
154 strain via the intraperitoneal route. The mouse survival rates, body weight, and clinical scores
155 were monitored for 32 and 70 days after challenge for BALB/c and C57BL/6 mice,
156 respectively. Serum (20 μL) was collected from mice via the tail veins at −2, 7, 21, and 35
157 days after the first immunization, and at 7 dpi to investigate the dynamics of specific
158 antibodies generated against NcCyp, using an indirect enzyme-linked immunosorbent assay
159 (ELISA). At the indicated times, serum and brain samples were collected from all surviving
160 mice after they were euthanized. Further details of parasite preparation, clinical score
161 observation, and ELISAs can be found in the Supplemental Methods in the Supplemental
162 Information.

163

164 *2.6. Splenocyte isolation and stimulation for the measurement of proliferation and cytokine*
165 *production*

166 Fourteen days after the third immunization, spleens were removed from immunized
167 and PBS-inoculated mice (*n* = 4/group) under aseptic conditions. The spleen cells were
168 placed into 96-well plates (3×10^5/100 μl/well) and stimulated with recombinant NcCyp,
169 concanavalin A (ConA; Sigma-Aldrich, St Louis, MO) as the positive control, or with
170 stimulant-free medium as the negative control. The cells were incubated for 48 h at 37°C in

171 5% CO_2. A 100-μl aliquot of culture supernatant was collected and assayed for cytokines (IL-
172 4, IL-10 and IFN-γ). At the same time, the proliferation of spleen cells was measured by a
173 Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Further details of the
174 collection of splenocytes and stimulation indices can be found in the Supplemental Methods
175 in the Supplemental Information.
176
177 *2.7. Statistical analyses*
178 Statistical analyses were performed using a one- or two-way analysis of variance
179 (ANOVA) followed by the Tukey–Kramer test for group comparisons. The significance of
180 differences in mouse survival was analyzed by the χ^2 test. All statistical analyses were
181 performed with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). *P*
182 values < 0.05 were considered statistically significant.
183
184 **3. Results**
185 *3.1. Immune stimulating activity of NcCyp*
186 Responses in the NF-κB signaling pathway in RAW 264.7 cells secreting NF-κB
187 dependent SEAP, and IL-12p40 production from naturally isolated peritoneal macrophage of
188 mice against stimulation with NcCyp were investigated (Fig. 1). Regarding the NF-κB
189 pathway, a statistically significant difference against mock treatment (negative control) was
190 only detected for high concentrations of NcCyp-OML (100 nM) and LPS (positive control)
191 (Fig. 1A). In the peritoneal mouse macrophages, Il-12p40 production was observed in the
192 treatment with LPS (10 ng/mL) and NcCyp-OML at concentrations of 10 nM and 100 nM,
193 but not naked NcCyp- or GST-OML-treated cells (Fig. 1B). These results suggest NcCyp-
194 OML has immune-stimulating activity.
195

196 3.2. *Immunoprotective efficacy of NcCyp in female BALB/c mice*

197 Anti-NcCyp IgG1 was produced 1 week after the third immunization with NcCyp-
198 OML or NcCyp alone, and reached a peak after 1 week of challenge with *N. caninum* (Fig.
199 2A). Of note, the IgG1 level in NcCyp-OML-immunized mice was significantly higher than
200 in those immunized with NcCyp alone. IgG2a was only reported in mice receiving NcCyp-
201 OML followed by naked NcCyp after 1 week of infection, which indicates a boosting effect
202 in these groups (Fig. 2B). These results suggest that immunization with NcCyp induces both
203 Th1 and Th2 immune responses against *N. caninum* in BALB/c mice.

204 To investigate the potential of NcCyp to stimulate cellular immunity, spleen cell
205 proliferation and cytokine production was evaluated in immunized mice 2 weeks after the
206 third immunization. Both splenocyte proliferation and IFN-γ production were increased in
207 NcCyp-OML and NcCyp-immunized mice when stimulated with NcCyp at 10 and 50 μg/ml
208 (Fig. 3A, 3B). Higher levels of IFN-γ production were recorded in the NcCyp-OML group
209 compared with the NcCyp group alone at a concentration of 50 μg/ml of rNcCyp. IL-4 and
210 IL-10 productions in spleen cells from mice immunized with NcCyp with or without OML,
211 and control mice inoculated with PBS or GST-OML, were not significantly enhanced by
212 stimulation with immunized antigens (Fig. 3C, 3D). Collectively, these results imply that
213 immunization with NcCyp-OML followed by NcCyp triggered antigen-specific cell-mediated
214 immune responses in BALB/c mice.

215 The immunization of mice with NcCyp-OML increased their survival rate (83.3%),
216 and attenuated the severity of infection as confirmed by lower changes in clinical scores and
217 body weight compared with all other groups, especially the control groups (survival rate;
218 16.7% in PBS-injected mice, 25.0% in GST-OML-injected mice) (Fig. 4). When compared
219 with control groups, protective efficacy was also observed in mice immunized by NcCyp
220 alone determined by their higher survival rate (66.7%) and lower clinical score. Furthermore,

221 parasite burden in brain was lower in NcCyp-OML-immunized group than all other groups,
222 although it was statistically significant against the control PBS-inoculated mice (Fig. S3A).
223
224 *3.3. Protective efficacy of NcCyp in C57BL/6 mice and the role of TLR2*
225 The ability of NcCyp-OML and NcCyp to generate specific antibodies or to induce
226 cellular immunity was investigated in the C57BL/6 mouse model to reveal the mechanism of
227 protection. In contrast to the results observed in BALB/c mice, no detectable levels of anti-
228 NcCyp antibodies (IgG1 or IgG2c) were observed in C57BL/6 mice (Figs. 5 and S1). Spleen
229 cell proliferation was only observed in NcCyp-OML-immunized wild-type mice but not in
230 TLR2$^{-/-}$ mice when stimulated with rNcCyp at 50 μg/ml (Fig. 6A). Additionally, increased
231 IFN-γ production was predominant in NcCyp-OML-immunized wild-type mice when
232 stimulated with rNcCyp at 50 μg/ml (Fig. 6B). Spleen cell proliferation and IFN-γ production
233 was determined in all groups with ConA stimulation as a positive control. These results
234 suggest NcCyp-OML induces cellular immunity in male C57BL/6 mice, and might be
235 partially dependent on TLR2.
236 Next, we investigated the protective efficacy of NcCyp with and without OML using
237 male and female C57BL/6 mice. TLR2$^{-/-}$ mice were used to evaluate the role of TLR2 in the
238 induction of immunity and in conferring protection (Figs. 7, S2 and S3). Using male mice,
239 NcCyp-OML protected wild-type mice (survival rate: 85.7%) against infection with *N.*
240 *caninum* compared with the PBS treated wild-type mice (survival rate: 28.6%) (Fig. 7A).
241 This effect was markedly abrogated when the same antigen was inoculated in TLR2$^{-/-}$ mice
242 (survival rate: 35.7%) (Fig. 7A). There were minimal changes in clinical score and body
243 weight in the wild-type mice immunized with NcCyp-OML (Fig. 7B, C). Similar tendencies
244 regarding the protective efficacy of NcCyp with and without OML were observed in female
245 mice (Fig. S2). No tangible differences in cerebral parasite burden among all groups of male

246 or female wild-type and TLR2KO mice were observed because of low number of survived

247 mice of control group (Fig. S3B, C).

248

Discussion

Previously, we demonstrated that OML is an efficacious adjuvant in many vaccine studies of *N. caninum*. In a mouse model, a formulation using OML improved the protective properties of apical membrane antigen 1 (AMA1) [19], and dense granular protein 7 [20]. NcGRA7-OML also demonstrated protective efficacy in cattle [21]. Similar to these studies, the current study revealed that immunization with NcCyp-OML triggered protective immunity against *N. caninum* infection. Herein, different genders and strains of mice were used to provide inclusive results on the characterization of NcCyp-OML as potential vaccine candidate. Differences in mouse sex and strain cause variations in susceptibility and also vaccine development against *N. caninum* infection [22]. Noteworthy, the immune response in BALB/c mice is predominantly Th2-biased, whereas C57BL/6 mice possess higher Th1-immunity, which may variously affect the kind of protective immunity of vaccine antigens [23], [24]. Moreover, the extents and key molecules of different immune responses are varied between male and female mice; numbers of macrophages and B and T lymphocytes are greatly higher in females than males [25]. In BALB/c mice, substantial levels of IgG1 specific antibodies (a marker for humoral immunity) were produced in NcCyp-OML-immunized mice. In addition, levels of IFN-γ higher than IL-4 or IL-10 were observed in the supernatants of spleen cells from mice immunized with NcCyp-OML, and to lower extent with NcCyp alone. These results indicate that immunization with NcCyp-OML triggers antigen-specific humoral and cellular immunity in BALB/c mice. In NcCyp-OML-immunized C57BL/6 mice, the induction of antigen-specific antibody production was not detected although antigen-specific spleen cell responses were confirmed. The protective efficacy of NcCyp-OML was observed in BALB/c and C57BL/6 mice, indicating that antigen-specific cellular immune responses are critical for the protection induced by NcCyp-

273 OML immunization. The importance of cellular immunity for resistance against *N*. *caninum*
274 infection was previously reported [5].

275 A recent study suggested a role for TLR2 in vaccine development against *N*. *caninum*
276 was targeted, although no tangible progress has been achieved. The TLR2 ligand OprI (a
277 bacterial lipoprotein) exerts strong adjuvant activity when combined with the *N*. *caninum*
278 chimeric protein Mic3-1-R, but no protection was reported in mice [26]. Activation of TLR2
279 by vaccine inoculation via any route including mucosal tissues in the digestive tract, the main
280 entrance for infection by *N*. *caninum* cysts, induces generalized protection. This effect is
281 triggered by the homing of T cells and dendritic cells to the gut mucosa via MyD88-
282 dependent TLR2 signals [27]. Such an approach has achieved variable success in vaccine
283 development for other pathogens such as *Leishmania major* and *Brugia malayi* [28],
284 *Mycobacterium tuberculosis* [29], and influenza virus [30]. Nevertheless, our study has
285 provided the first direct evidence for the contribution of TLR2 in protective immunity against
286 *N*. *caninum* infection. The immunization of C57BL/6 mice with NcCyp-OML conferred
287 substantial protection against challenge with *N*. *caninum*. Strikingly, this protection was
288 dramatically decreased in $TLR2^{-/-}$ mice, indicating the contribution of TLR2 to the induction
289 of protective immunity. This protection was associated with Th1 or cell-mediated immunity
290 because cell proliferation and IFN-γ production of spleen cells is dependent on TLR2.

291 Based on the high immune stimulatory effect of NcCyp-OML compared with NcCyp
292 alone or other control stimulants, we believe NcCyp-OML has the potential to protect host
293 animals from infection with *N*. *caninum*. Notably, treatment of macrophages with NcCyp-
294 OML induced IL-12p40 production via the activation of NF-κB signals, indicating the
295 immune-stimulating activity of NcCyp-OML. Several studies have reported a role for the
296 TLR2/NF-κB-signaling axis in eliciting immune responses triggered primarily via cytokine
297 production [31], [32]. Because macrophages are powerful effector immune cells against *N*.

298 *caninum* infection [33], the immune regulatory effects of NcCyp-OML will be an important
299 factor in controlling infection.

300 Few reports have revealed the efficacy of OML via the manipulation of cell receptors
301 and signaling pathways. The mannose-binding C-type lectin receptor, SIGNR1, was reported
302 to be a physiological receptor for OML during its uptake by macrophages. The activation of
303 SIGNR1 was associated with IL-12 production via NF-κB signaling [34]. Moreover, OMLs
304 were reported to induce extracellular signal-regulated kinase activation in a murine
305 macrophage cell line via the phosphorylation of Src family kinases and enhancement of the
306 PI3K/Akt pathway [35]. In a more recent study, IFN-γ production by spleen cells from mice
307 immunized with OML was highly dependent on TLR4 but not TLR2, using knockout mice
308 for each receptor. However, neither the removal of TLR2 nor TLR4 affected the production
309 of IL-12 after mouse macrophages were treated with OML [36]. Our results were similar to
310 those obtained in the aforementioned study regarding TLR2-independent IL-12 production
311 from macrophages (Fig. S4).

312 Interactions between TLR2 and NcCyp-OML are important for the induction of
313 protection against infection in mice by immunization with NcCyp-OML. When NcCyp-OML
314 is administered, it is preferentially taken up by macrophages and/or other APCs [37]. The
315 delivered antigen is then processed and its peptides are presented to T-cells by MHC class I
316 and class II molecules, leading to the activation of T-cell subsets responsible for antigen-
317 specific immune responses. IL-12 production was observed in $TLR2^{-/-}$ macrophages treated
318 with NcCyp-OML, indicating that TLR2 was not involved in APC activation. Instead, TLR2
319 may contribute to the induction of acquired immunity triggered by immunization with
320 NcCyp-OML. The synergistic effect between OML and NcCyp is crucial for the potent
321 immunogenicity and protective efficacy of NcCyp-OML. In conclusion, our data suggest that
322 TLR2 has a crucial role in NcCyp-OML-conferred protection against *N. caninum* infection of

323 mice. Proficiency in triggering immune response and protective effect for NcCyp, in
324 particular those formulated in OML, is a promising aspect for further evaluation against the
325 vertical transmission of *N*. *caninum*. In addition, role of TLR2 should be investigated to
326 reveal its effect in pregnant mouse model.

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332

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336

337 **Conflict of interest**

338 The authors declare that they have no financial or competing interests concerning this study.

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341 **References**

342

343 [1] Dubey JP, Schares G. Neosporosis in animals – the last five years. Vet Parasitol
344 2011;180:90–108.

345

346 [2] VanLeeuwen JA, Greenwood S, Clark F, Acorn A, Markham F, McCarron J, et al.
347 Monensin use against *Neospora caninum* challenge in dairy cattle. Vet Parasitol
348 2011;175:372–6.

349

350 [3] Weber FH, Jackson JA, Sobecki B, Choromanski L, Olsen M, Meinert T, et al. On the
351 efficacy and safety of vaccination with live tachyzoites of *Neospora caninum* for prevention
352 of *Neospora*-associated fetal loss in cattle. Clin Vac Immunol 2013;20:99–105.

353

354 [3] Nishikawa Y, Inoue N, Makala L, Nagasawa H. A role for balance of interferon-gamma
355 and interleukin-4 production in protective immunity against *Neospora caninum* infection. Vet
356 Parasitol. 2003;116:175–84.

357

358 [5] Nishikawa Y. Towards a preventive strategy for neosporosis: challenges and future
359 perspectives for vaccine development against infection with *Neospora caninum*. J Vet Med
360 Sci 2017;79:1374–80.

361

362 [6] Innes EA, Buxton D, Maley S, Wright S, Marks J, Esteban I, et al. Neosporosis: Aspects
363 of epidemiology and host immune response. Ann N Y Acad Sci 2000;916:93–101.

364

365 [7] O'Garra A, Murphy KM. From IL-10 to IL-12: how pathogens and their products
366 stimulate APCs to induce T(H)1 development. Nat Immunol 2009;10:929–32.

367

368 [8] Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY, et al. MyD88 is
369 required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12
370 production by dendritic cells. J Immunol 2002;168:5997–6001.

371

372 [9] Mineo TW, Oliveira CJ, Gutierrez FR, Silva JS. Recognition by Toll-like receptor 2
373 induces antigen presenting cell activation and Th1 programming during infection by
374 *Neospora caninum*. Immunol Cell Biol 2010;88:825–33.

375

376 [10] Mun HS, Aosai F, Norose K, Chen M, Piao LX, Takeuchi O, et al. TLR2 as an essential
377 molecule for protective immunity against *Toxoplasma gondii* infection. Int
378 Immunol 2003;15:1081–7.

379

380 [11] Gibson J, Gow N, Wong SYC. Expression and functions of innate pattern recognition
381 receptors in T and B cells. Immunol Endocr & Metab Agents in Med Chem 2010;10:11–20.

382

383 [12] Rahman MM, McFadden G. Modulation of NF-κB signalling by microbial pathogens.
384 Nat Rev Microbiol 2011;9:291–306.

385

386 [13] Liu T, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther
387 2017;2:17023.

388

389 [14] Abe C, Tanaka S, Nishimura M, Ihara F, Xuan X, Nishikawa Y. Role of the chemokine
390 receptor CCR5-dependent host defense system n *Neospora caninum* infections. Parasit
391 Vectors 2015;8:5.
392
393 [15] Kameyama K, Nishimura M, Punsantsogvoo M, Ibrahim HM, Xuan X, Furuoka H, et al.
394 Immunological characterization of *Neospora caninum* cyclophilin. Parasitol 2012;139:294–
395 301.
396
397 [16] Tuo W, Zhao Y, Zhu D, Jenkins MC. Immunization of female BALB/c mice
398 with *Neospora* cyclophilin and/or NcSRS2 elicits specific antibody response and prevents
399 against challenge infection by *Neospora caninum*. Vaccine 2011;29:2392–9.
400
401 [17] Basto AP, Leitão A. Targeting TLR2 for vaccine development. J Immunol Res
402 2014;2014: 619410.
403
404 [18] Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficint
405 mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol 2000;165:5392–6
406
407 [19] Zhang H, Nishikawa Y, Yamagishi J, Zhou J, Kojima N, Yokoyama N, et al. *Neospora*
408 *caninum:* application of apical membrane antigen 1 encapsulated in the oligomannose-coated
409 liposomes for reduction of offsprings mortality from infection in BALB/c mice. Exp Parasitol
410 2010;125:130–6.
411

412 [20] Nishikawa Y, Zhang H, Ikehara Y, Kojima N, Xuan X, Yokoyama N. Immunization
413 with oligomannose-coated liposome-entrapped dense granule protein 7 protects dams and
414 offspring from *Neospora caninum* infection in mice. Clin Vaccine Immunol 2009;16:792–7.
415
416 [21] Nishimura M, Kohara J, Kuroda Y, Hiasa J, Tanaka S, Muroi Y, et al. Oligomannose-
417 coated liposome-entrapped dense granule protein 7 induces protective immune response to
418 *Neospora caninum* in cattle. Vaccine 2013;31:3528–35.
419
420 [22] Aguado-Martínez A, Basto AP, Leitão A, Hemphill A. *Neospora caninum* in non-
421 pregnant and pregnant mouse models: cross-talk between infection and immunity. Int J
422 Parasitol 2017;47:723–35.
423
424 [23] Chen X, Oppenheim JJ, Howard OM. BALB/c mice have more CD4+CD25+ T
425 regulatory cells and show greater susceptibility to suppression of their CD4+CD25-
426 responder T cells than C57BL/6 mice. J Leukoc Biol 2005;78:114–21.
427
428 [24] Ferreira BL, Ferreira ÉR, de Brito MV, Salu BR, Oliva MLV, Mortara RA, et al.
429 BALB/c and C57BL/6 mice cytokine responses to *Trypanosoma cruzi* infection are
430 independent of parasite strain infectivity. Front Microbiol 2018;9:553.
431
432 [25] Scotland RS, Stables MJ, Madalli S, Watson P, Gilroy DW. Sex differences in resident
433 immune cell phenotype underlie more efficient acute inflammatory responses in female mice.
434 Blood 2011;118:5918–27.

435

436 [26] Aguado-Martínez A, Basto AP, Müller J, Balmer V, Manser V, Leitão A, et al. N-
437 terminal fusion of a toll-like receptor 2-ligand to a *Neospora caninum* chimeric antigen
438 efficiently modifies the properties of the specific immune response. Parasitol 2016;143:606–
439 16.

440

441 [27] Wang S, Villablanca EJ, De Calisto J, Gomes DC, Nguyen DD, MizoguchI E, et al.
442 MyD88-dependent TLR1/2 signals educate dendritic cells with gut-specific imprinting
443 properties. J Immunol 2011;187:141–50.

444

445 [28] Halliday A, Turner JD, Guimarães A, Bates PA, Taylor MJ. The TLR2/6 ligand
446 PAM2CSK4 is a Th2 polarizing adjuvant in *Leishmania major* and *Brugia malayi*
447 murine vaccine models. Parasit Vectors 2016;9:96.

448

449 [29] Kim WS, Kim JS, Cha SB, Kim H, Kwon KW, Kim SJ, et al. *Mycobacterium*
450 *tuberculosis* Rv3628 drives Th1-type T cell immunity via TLR2-mediated activation of
451 dendritic cells and displays vaccine potential against the hyper-virulent Beijing K strain.
452 Oncotarget 2016;7:24962–82.

453

454 [30] Shepardson KM, Schwarz B, Larson K, Morton RV, Avera J, McCoy K, et al. Induction
455 of antiviral immune response through recognition of the repeating subunit pattern of viral
456 capsids is toll-like receptor 2 dependent. MBio 2017;8:e01356-17.

457

458 [31] Hawiger J. Innate immunity and inflammation: a transcriptional paradigm. Immunol Res
459 2001; 23: 99–109.

460

461 [32] Zhang G, Ghosh S. Toll-like receptor-mediated NF-B activation: a phylogenetically
462 conserved paradigm in innate immunity. J Clin Invest 2001;107:13–19.

463

464 [33] Abe C, Tanaka S, Ihara F, Nishikawa Y. Macrophage depletion prior to *Neospora*
465 *caninum* infection results in severe neosporosis in mice. Clin Vaccine Immunol
466 2014;21:1185–8.

467

468 [34] Kato C, Kojima N. SIGNR1 ligation on murine peritoneal macrophages induces IL-
469 12 production through NFkappaB activation. Glycoconj J 2010;27:525–31.

470

471 [35] Kato C, Kajiwara T, Numazaki M, Takagi H, Kojima N. Oligomannose-coated
472 liposomes activate ERK via Src kinases and PI3K/Akt in J774A.1 cells. Biochem Biophys
473 Res Commun 2008;372:898–901.

474

475 [36] Matsuoka Y, Takagi H, Yamatani M, Kuroda Y, Sato K, Kojima N. Requirement of
476 TLR4 signaling for the induction of a Th1 immune response elicited by oligomannose-coated
477 liposomes. Immunol Lett 2016;178:61–7.

478

479 [37] Shimizu Y, Takagi H, Nakayama T, Yamakami K, Tadakuma T, Yokoyama N, et al.
480 Intraperitoneal immunization with oligomannose-coated liposome-entrapped soluble
481 leishmanial antigen induces antigen-specific T-helper type immune response in BALB/c mice
482 through uptake by peritoneal macrophages. Parasit Immunol 2007;29:229–39.

483

484 **Figure legends**

485 **Fig. 1. NF-κB activation and cytokine production.** (A) NF-κB/SEAP cells were treated
486 with LPS (10 and 50 ng/mL), medium only (mock), and recombinant proteins of NcCyp and
487 GST (10 and 100 nM) with or without OML. Stimulated cells were incubated for 24 h to
488 measure secreted alkaline phosphatase levels. Each value represents the mean ± standard
489 deviation of quadruple samples. Asterisks (*) above the bars in graphs indicate statistically
490 significant differences among the test groups and the mock group (one-way ANOVA plus
491 Tukey ± Kramer *post hoc* analysis, $P < 0.05$). (B) Production of IL-12p40 from murine
492 peritoneal macrophages. Macrophages were treated with LPS (10 ng/mL), medium only
493 (mock), and recombinant NcCyp, NcCyp-OML, and GST-OML (10 and 100 nM) for 20 h.
494 The IL-12p40 value represents the mean ± standard deviation of triplicate samples. The
495 results are representative of two repeated experiments with similar results. The different
496 letters above the bars in the graphs indicate statistically significant differences among all
497 other groups (one-way ANOVA plus Tukey–Kramer *post hoc* analysis, $P < 0.05$). NcCyp; 10
498 nM (0.205 μg/mL), 100 nM (2.05 μg/mL), GST; 10 nM (0.27 μg/mL), 100 nM (2.7 μg/mL).

499

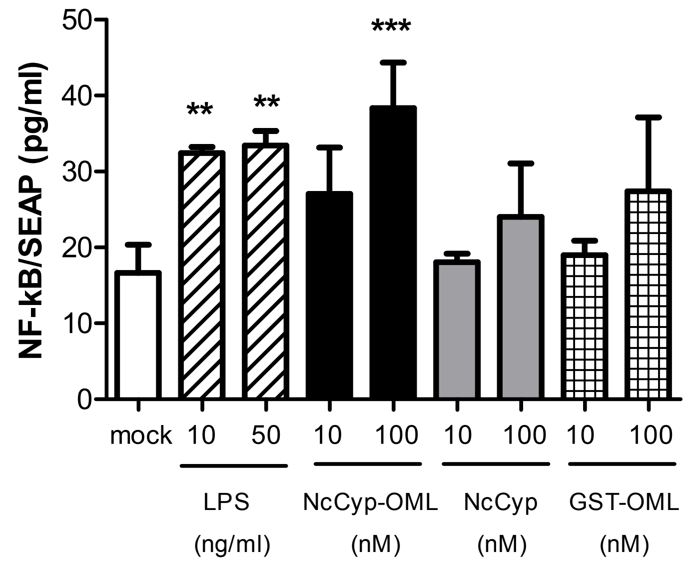
500 **Fig. 2. Production of specific antibodies against NcCyp in female BALB/c mice.** Mice
501 were immunized with 25 pmol of NcCyp-OML, NcCyp, GST-OML, or PBS alone via the
502 subcutaneous route and then challenged with a lethal dose (1×10^6) of *N. caninum* tachyzoites
503 via an intraperitoneal route. Sera were collected from all mouse groups 2 days before
504 immunization and tested for NcCyp-specific IgG1 and IgG2a antibodies using indirect
505 ELISAs. The mean optical density (OD) was determined at a wavelength of 415 nm. Each
506 bar represents the mean ± standard deviation for mice in each group ($n = 6$). The results are
507 representative of one trial from two repeated experiments with similar results. The different
508 letters above the bars in the graphs indicate statistically significant differences among the

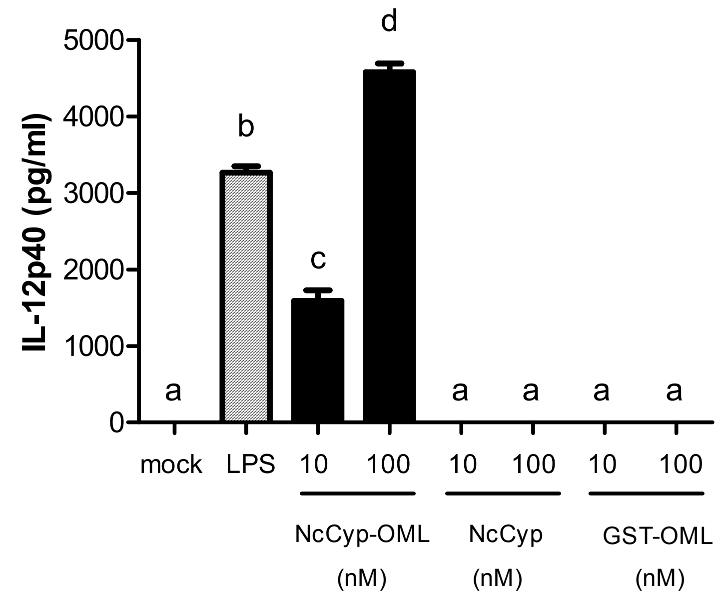
509 same immunization group by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P <$
510 0.05). *, statistically significant differences were observed compared with day 0 after the first
511 immunization in the same immunized group by two-way ANOVA and Tukey–Kramer *post*
512 *hoc* analysis ($P < 0.05$).
513
514 **Fig. 3. Splenocyte proliferation and cytokine production in female BALB/c mice.**
515 Spleens were dissected aseptically from immunized mice 2 weeks after the third
516 immunization with PBS, NcCyp-OML, NcCyp alone, or GST-OML. Cells were stimulated
517 with Concanavalin A (ConA, 5 μg/mL), NcCyp, GST, or without any stimulator (Medium)
518 for 48 h. (A) Cell proliferation, (B) IFN-γ, (C) IL-4, and (D) IL-10 production. Each bar
519 represents the mean ± standard deviation ($n = 4$ for all groups). The different letters above the
520 bars in the graphs indicate statistically significant differences among groups with the same
521 stimulation by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$). #,
522 Statistically significant differences were observed compared with medium only in the same
523 immunized group by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$).
524
525 **Fig. 4. Clinical parameters and protective indices in immunized female BALB/c mice.**
526 After the third immunization, all mice (6 per group) were intraperitoneally challenged with a
527 lethal dose (1×10^6) of *N. caninum* tachyzoites. (A) The survival rates (surviving mice/total
528 mice) were calculated from two pooled independent experiments: PBS; 2/12 (16.7%), GST-
529 OML; 3/12 (25%), NcCyp-OML; 10/12 (83.3%) and NcCyp; 8/12 (66.7%). *, the differences
530 were significant between the PBS- and GST-OML-injected groups and those of NcCyp-OML
531 or NcCyp-immunized groups by $\chi2$ test ($P < 0.05$). Changes in the clinical score (B) and
532 body weight (C) were calculated as the means ± standard deviation of clinical score and body
533 weight values of all mice in a group from −2 to +32 dpi. The significance in change in body

534 weight or clinical score was determined by two-way ANOVA plus Tukey–Kramer *post hoc*
535 analysis ($P < 0.05$). Asterisk (*) refers to a significant difference in the test groups (NcCyp-
536 OML and NcCyp alone) compared with either control group (PBS and GST-OML). #,
537 Significant difference among the test groups.
538
539 **Fig. 5. Specific antibody response against NcCyp in male C57BL/6 and TLR2$^{-/-}$ mice.**
540 Sera were collected from all mice in each group (NcCyp-OML, NcCyp, GST-OML, or PBS
541 alone) 2 days before immunization and 1 week after the third immunization to test for
542 NcCyp-specific IgG1 (A) and IgG2c antibodies (B) using indirect ELISA. In addition, sera
543 were collected from these mice 1 week after challenge with a lethal dose (1×10^6) of *N.*
544 *caninum* tachyzoites to estimate the boosting effect of immunization. The mean optical
545 density (OD) was determined at a wavelength of 415 nm. Each bar represents the mean ±
546 standard deviation for mice in each group ($n = 6$). Data are representative of one trial from
547 two repeated experiments with similar results. No statistically significant differences were
548 observed among the immunization groups as determined by one-way ANOVA plus Tukey–
549 Kramer *post hoc* analysis ($P < 0.05$).
550
551 **Fig. 6. Splenocyte responses in male C57BL/6 and TLR2$^{-/-}$ mice.** At 2 weeks after the last
552 immunization, spleens were dissected from wild-type and TLR2$^{-/-}$ male mice immunized with
553 PBS, NcCyp-OML, or NcCyp alone. Cells were seeded in plates and stimulated with
554 Concanavalin A (Con A, 1 μg/mL), NcCyp, or without any stimulator (Medium) for 48 h.
555 For cell proliferation (A) and IFN-γ production (B), each bar represents the mean ± standard
556 deviation ($n = 4$ for all groups). The different letters above the bars in the graphs indicate
557 statistically significant differences among groups with the same stimulation by two-way
558 ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$). #, Statistically significant

559 differences were observed compared with medium alone in the same immunized group with
560 two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$).
561
562 **Fig. 7. Survival rate and clinical parameters in male C57BL/6 and TLR2$^{-/-}$ mice.** (A) To
563 determine the survival rate, wild type (WT) or TLR2$^{-/-}$ (KO) C57BL/6 male mice were
564 immunized (14 mice per group except for the NcCyp-TLR2$^{-/-}$ group (13 mice) from 2
565 independent trials). After the third immunization, all mice were intraperitoneally challenged
566 with a lethal dose (1×10^6) of *N. caninum* tachyzoites. The survival rates were calculated for
567 two pooled independent trials: PBS-WT; 4/14 (28.6%), PBS-KO; 2/14 (14.3%), NcCyp-
568 OML-WT; 12/14 (85.7%), NcCyp-OML-KO; 5/14 (35.7%), NcCyp-WT; 6/14 (42.9%) and
569 NcCyp-KO; 7/13 (53.8%). *, Indicates significant differences compared with the PBS group
570 of the same mouse type and #, indicates a significant difference among wild-type and TLR2$^{-/-}$
571 mice of the same vaccine antigen as calculated by the χ^2 test ($P < 0.05$). Regarding clinical
572 findings, data were calculated as the means ± standard deviation of clinical score (B) and
573 body weight (C) values of all mice in a group ($n = 6$) from −2 until +70 dpi. The significance
574 in clinical score was determined by two-way ANOVA plus Tukey–Kramer *post hoc* analysis
575 ($P < 0.05$). *, Significant differences in the groups (NcCyp-OML, NcCyp alone, or GST-
576 OML) compared with the PBS control group. #, Significant differences among C57BL/6 and
577 TLR2$^{-/-}$ mice in the same immunization group. The results are representative of one trial from
578 two repeated experiments with similar results using male mice.
579

Fig. 1. Fereig et al.





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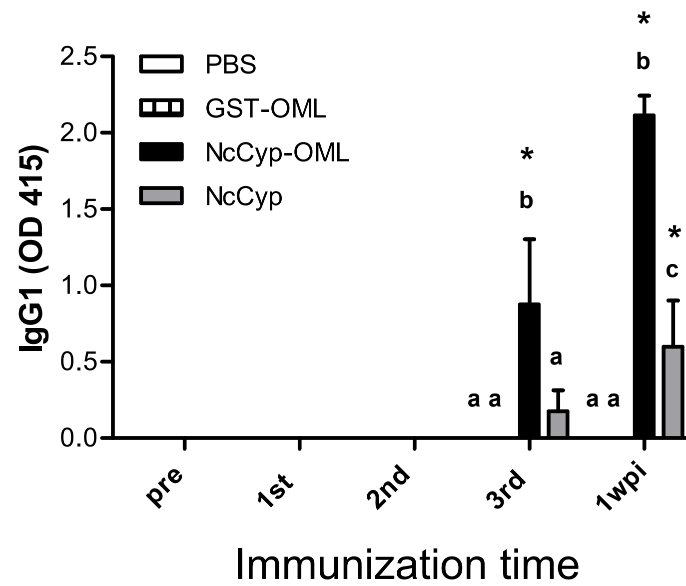

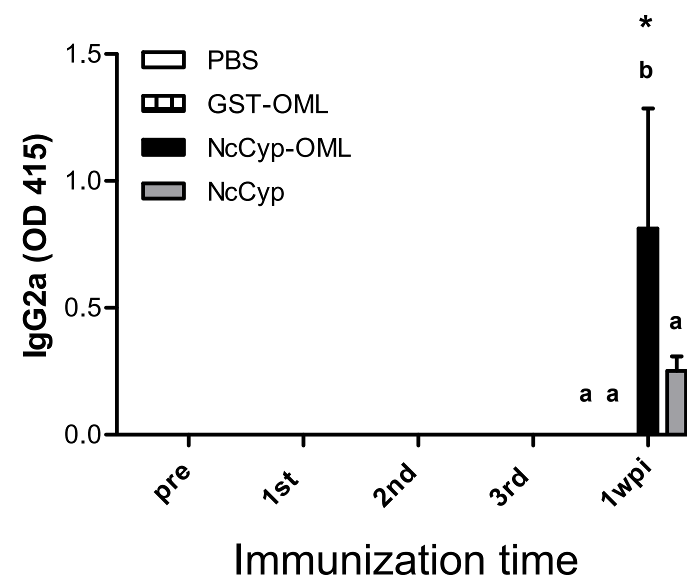

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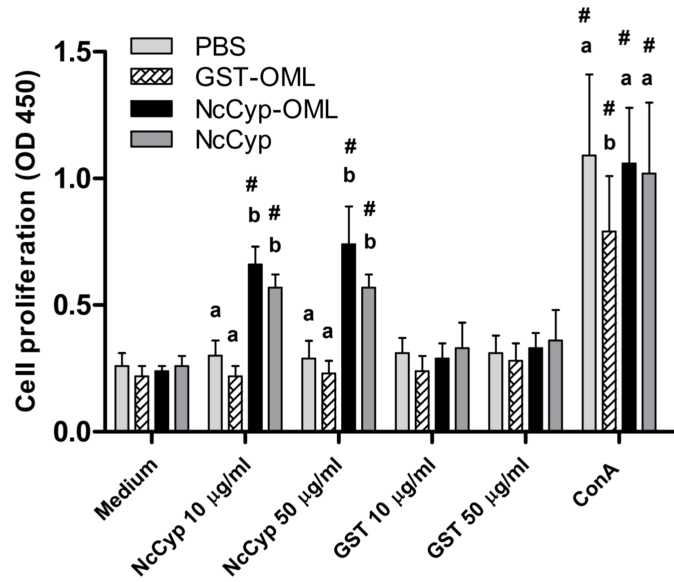

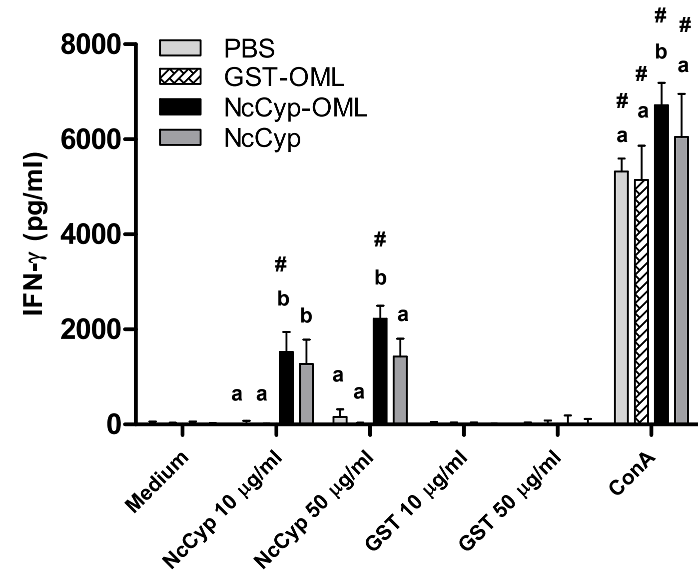

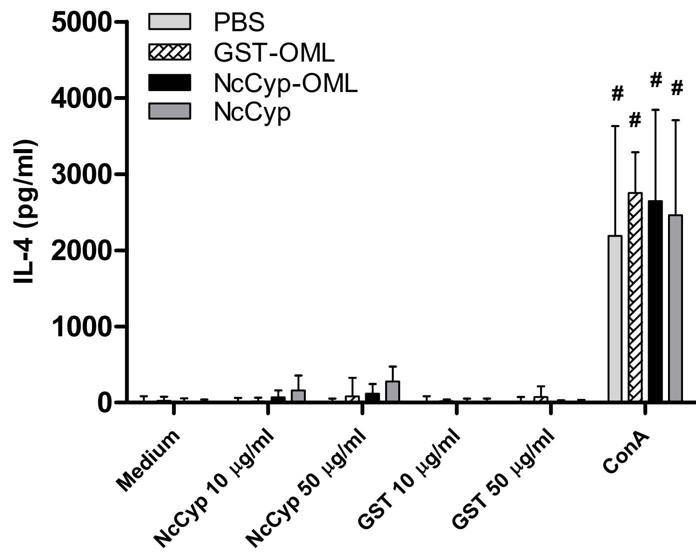

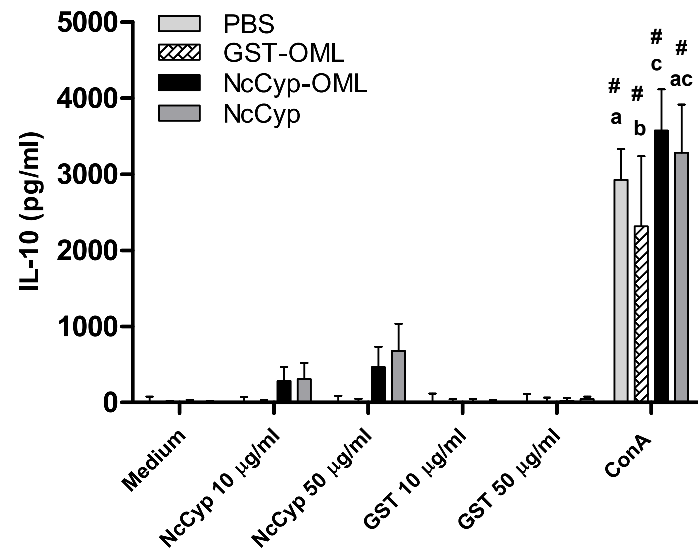

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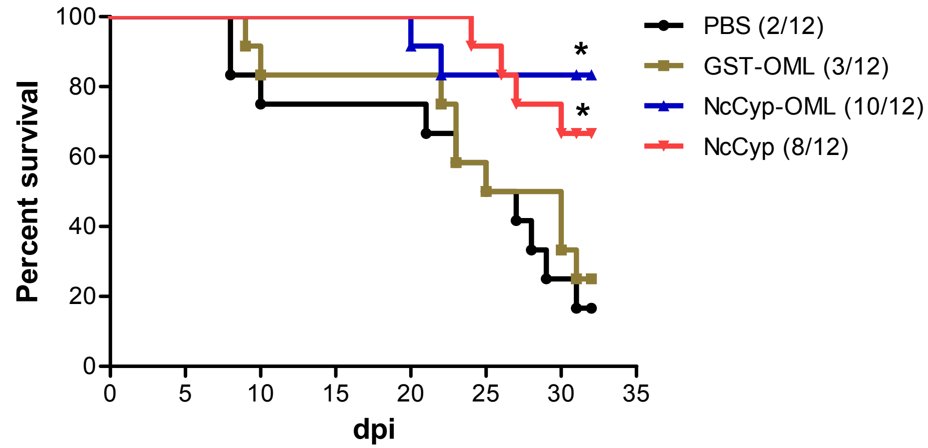
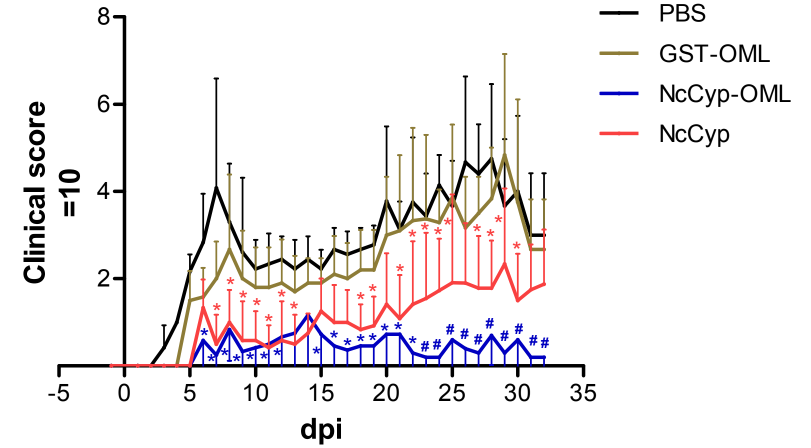




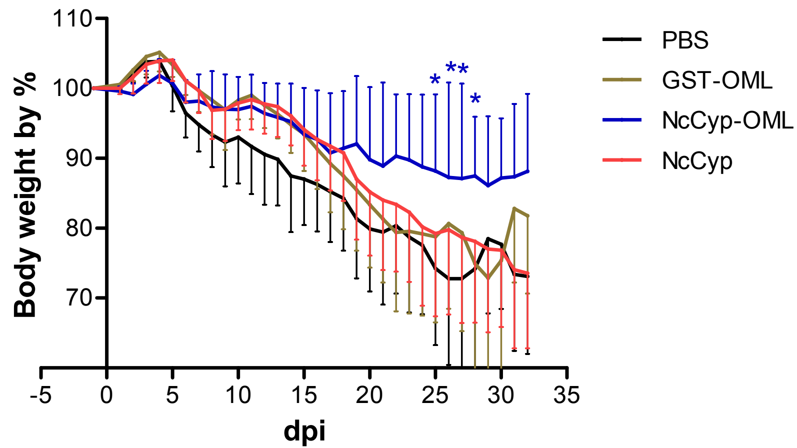
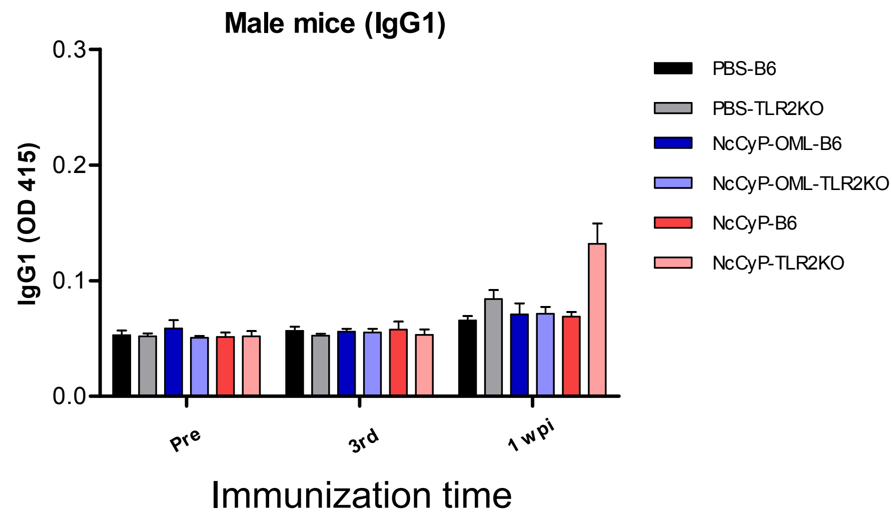


Fig. 5. Fereig et al.

A



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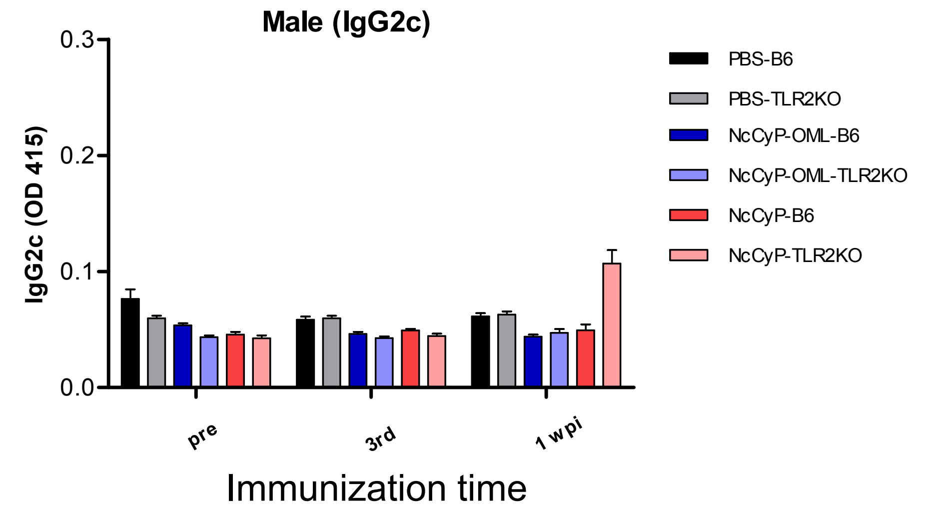

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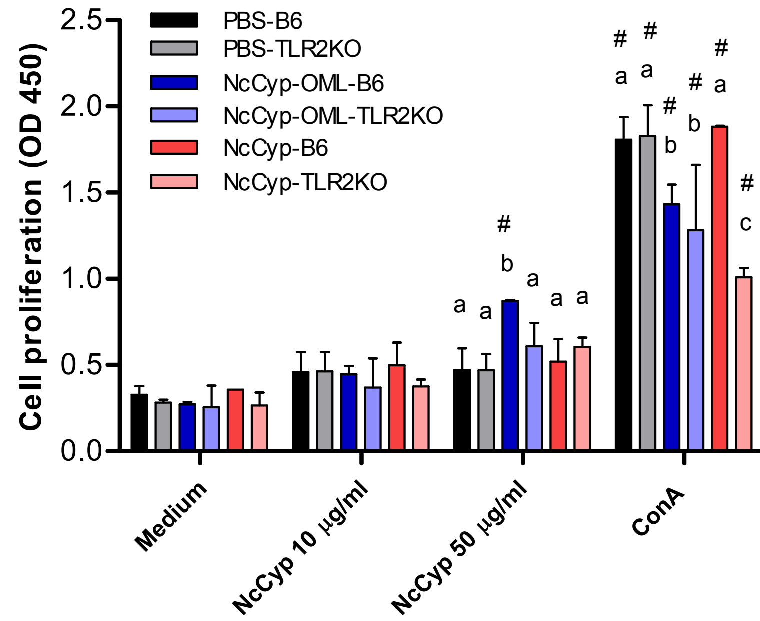
A



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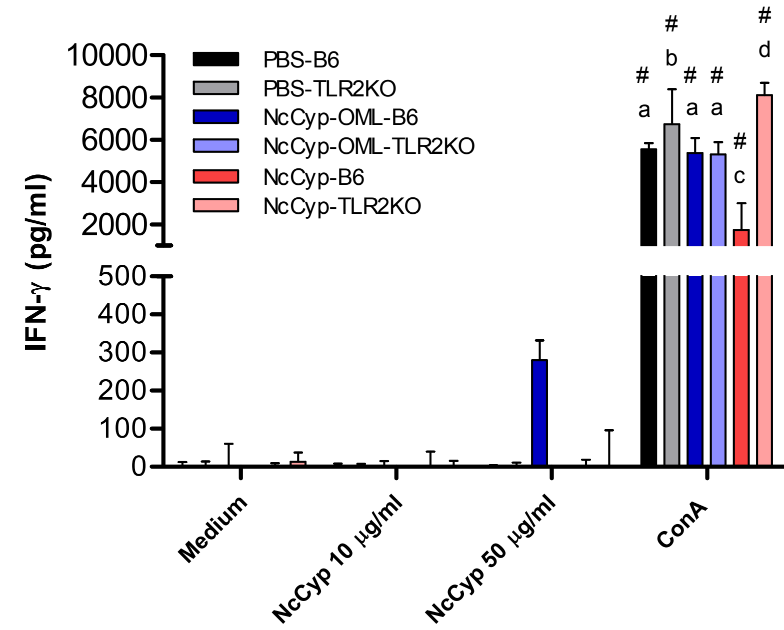

Fig. 7. Fereig et al.