Supplemental information

Supplemental Methods

Parasites and cell cultures

For parasite sustainability, *N. caninum* (strain Nc-1) was maintained in Vero cells (African green monkey kidney epithelial cells) cultured in Eagle's minimum essential medium (EMEM; Sigma, St. Louis, MO, USA) containing 8% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma). For the purification of tachyzoites, the parasites and host-cell debris were washed in cold phosphate-buffered saline (PBS), and the infected cell monolayer was scraped with a cell scraper (BD Bioscience, San Jose, CA, USA), harvested in medium and centrifuged at 800 ×*g* for 5 minutes at 20°C. The cell pellet was resuspended in RPMI-1640 medium (Sigma) containing 10% FBS and then passed through a 27-gauge needle and a filter with a pore size of 5.0 μm (Millipore, Bedford, MA, USA). The NF-κB-secreting alkaline phosphatase reporter cell line generated from RAW 264.7 (NF-κB/SEAP) cells was obtained from Novus biological Inc. (Littleton, CO, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (*v/v*) fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Darmstadt). For the drug based-selection of NF-κB/SEAP cells, 0.5

19 mg/mL G418 (Geneticin, Roche, Mannheim, Germany) was added and then cells were

20 cultivated in a 5% CO_2 incubator at 37°C.

21

22 *Expression and purification of recombinant proteins*

23 Recombinant NcCyp18 (NcCyp) was expressed and purified as described previously [1],

24 with slight modifications. In brief, it was expressed as a GST fusion protein in

25 *Escherichia coli* DH5α cells (Takara, Bio, Inc.). Removal of the GST tags from rNcCyp

26 was performed by thrombin protease (GE Healthcare) according to the manufacturer's

27 instructions. Protein concentrations (μg/ml) were measured using the bicinchoninic acid

28 (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Either the

29 naked or entrapped recombinant GST in OML was used as a negative control protein in

30 some experiments. For use in cell culture, proteins were filtered with a 0.45-μm low-

31 protein binding Supor® membrane, and endotoxin was removed using Acrodisc® Units

32 with Mustang® E Membrane (Pall Life Sciences, Ann Arbor, MI, USA). Before use, the

33 level of endotoxin was estimated with Limulus Amebocyte Lysate reagents (Seikagaku

34 Inc., Tokyo, Japan), and no endotoxin was detected in the tested protein lots.

35

36 *Preparation of oligomannose-coated-liposomes (OML)*

37 Liposomes were prepared as previously described [2]. In brief, a chloroform/methanol
38 (2:1, *v/v*) solution containing 1.5 μmol of dipalmitoylphosphatidylcholine (DPPC), 1.5
39 μmol of cholesterol, and 0.15 μmol of mannopentose and
40 dipalmitoylphosphatidylethanolamine (M3–DPPE) was added to a conical flask and
41 rotary evaporated to prepare a lipid film containing neoglycolipids. For the protein-
42 entrapping of OMLs, 200 μl of NcCyp (500 μg/ml) was added to the dried lipid film, and
43 multilamellar vesicles were prepared by intense vortex dispersion. The multilamellar
44 vesicles were extruded 5 times through a 1-μm-pore polycarbonate membrane (Whatman,
45 USA). Liposome-encapsulated NcCyp was separated from recombinant-nonentrapped
46 NcCyp proteins by three successive cycles of washing in PBS with centrifugation (20,000
47 ×*g*, 30 min, 4°C). The concentrations of the entrapped proteins were measured using a
48 modified Lowry protein assay kit (Thermo Scientific, USA) in the presence of 0.3% (*w/v*)
49 SDS using BSA to prepare the standard.
50
51 *Monolayer cultures of mouse peritoneal macrophages.*
52 Natural macrophages were collected from the peritoneal cavity of male C57BL/6 and
53 TLR2$^{-/-}$ mice via the intraperitoneal injection of 1 ml of 4.05% brewer modified BBL
54 thioglycolate medium (Becton Dickinson, Sparks, MD). Four days later, cells were

55 harvested by peritoneal lavage with 5 ml of cold PBS. After harvesting, the cells were

56 centrifuged at 800 ×*g* for 10 min at 4°C, and red blood cells were removed with lysis

57 buffer (0.83% NH_4Cl, 0.01 M Tris-HCl [pH 7.2]) and then suspended again in DMEM

58 containing 10% FBS. The macrophage suspension was then added to 96-well tissue

59 culture microplates at 3×10^5 cells/well. The suspensions were incubated at 37°C for 4 h,

60 washed thoroughly to remove nonadherent cells, and further incubated after the addition

61 of the indicated stimulants, including positive and negative controls, at 37°C in a 5% CO_2

62 incubator.

63

64 *Clinical scores and body weights*

65 The clinical score was determined by recording the clinical signs exhibited by each mouse

66 in each group from -2 to +32 or +70 dpi, in BALB/c or C57BL/6 mice, respectively,

67 following *N. caninum* infection as described in our previous study [3]. In brief, each

68 recorded clinical sign was categorized by a certain score ranging from 0 (no signs) to 10

69 (all signs). The mild clinical finding manifested by mice was the backwards curvature of

70 the spine, a rough and shaggy appearance of hair, and hiding beneath the bedding (scores:

71 1–3). As the infection progresses, mice stop feeding because their eyes are closed, and

72 pain results in emaciation and sluggish movements (scores: 4–7). In the severe stages, the

73 mouse shows ataxia if forced to move followed by lying on its stomach, and the loss of

74 reflexes, which usually results in coma and death (scores: 8–10). Alterations in the body

75 weight of each mouse in each group were recorded daily at the abovementioned indicated

76 times after infection with *N. caninum*, and were compared with the weights recorded on

77 the first day of measurement.

78

79 *Real time-PCR for measuring parasite burdens*

80 After the mice were euthanized, the brains were aseptically dissected and procedures of

81 the parasite DNA extraction and measurement were applied as described previously [4].

82 The brain tissues were digested with extraction buffer (0.1 M Tris-HCl, pH 9.9; 1% SDS,

83 0.1 M NaCl, 1 mM EDTA, 1 mg/mL proteinase K) at 55°C. The parasite DNA was

84 obtained and purified by phenol–chloroform extraction and ethanol precipitation. Parasite

85 DNA was measured and analyzed by real-time PCR using specific primers for the Nc5

86 gene, the specific gene for *N. caninum* quantification y [5], (forward primer: nucleotides

87 248–257, 5′-ACT GGA GGC ACG CTG AAC AC-3′; reverse primer: nucleotides 303–

88 323, 5′-AAC AAT GCT TCG CAA GAG GAA-3′. The PCR was done on genomic DNA

89 and guided by the SYBR green detection method (Applied Biosystems, Carlsbad, CA,

90 USA). ABI Prism 7900HT Sequence Detection System (Applied Biosystems) was used

91 for amplifications, data processing and analysis. A known *N. caninum* DNA extracted

92 from 1 × 10^5 parasites was used for preparation of standard curve using. The data were

93 processed and analyzed using Dissociation Curves version 1.0 F (AB Applied

94 Biosystems).

95

96 *Indirect ELISA to detect NcCyp-specific antibodies*

97 Mouse sera were tested against recombinant NcCyp as a coating antigen. Purified antigen

98 was diluted in a coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) to a final

99 concentration of 0.1 μM. The ELISA plates (Nunc, Roskilde, Denmark) were coated with

100 the antigen at 4°C overnight and blocked with 3% (*w/v*) skim milk prepared in PBS

101 solution for 1 h at 37°C. The plates were incubated with 50 μL 1:100 diluted serum

102 samples from immunized and control mice after washing once with PBST (PBS-

103 containing 0.05% Tween 20). The plates were washed 6 times with PBST, and incubated

104 with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG1 and IgG2a or IgG2c

105 (1:4000) for 1 h at 37°C. After a further 6 washes, the enzyme reaction was developed

106 with 2, 2-azinobis (3-ethylbenzthiazolinesulfonic acid) (ABTS) (Sigma). The optical

107 density (OD) was measured at a wavelength of 415 nm.

108

109 *Collection of splenocytes and determination of stimulation indices*

110 Fourteen days after the third immunization, the spleens were aseptically collected from
111 the vaccinated and PBS-inoculated mice (n = 4/group). Single cell suspensions were
112 prepared from purified spleen cells as described previously [6], with slight modifications.
113 The spleen cells were placed into 96-well plates (3×10^5/100 µl/well) and stimulated with
114 100 µL of NcCyp (10 and 50 µg/ml) or concanavalin A (ConA; Sigma-Aldrich, St Louis,
115 MO) (5 µg/ml in female BALB/c mice or 1 µg/ml in C57BL/6 and TLR2$^{-/-}$ mice) as the
116 positive control, or with stimulant-free medium as the negative control. Cells with
117 different stimulants were incubated for 48 h at 37°C in 5% CO_2. A 100-µl aliquot of
118 culture supernatant was collected and assayed for cytokines (IL-4, IL-10 and IFN-γ). At
119 the same time, 10 µl of Cell Counting Kit-8 reagent (CCK-8, Dojindo Laboratories,
120 Kumamoto, Japan) was added to the previously stimulated cell wells in the dark to
121 estimate splenocyte proliferation. The plates were incubated at 37°C in 5% CO_2 for 2 h
122 and then the optical density of each well was measured using an ELISA reader at 450 nm.

123

124 **References**

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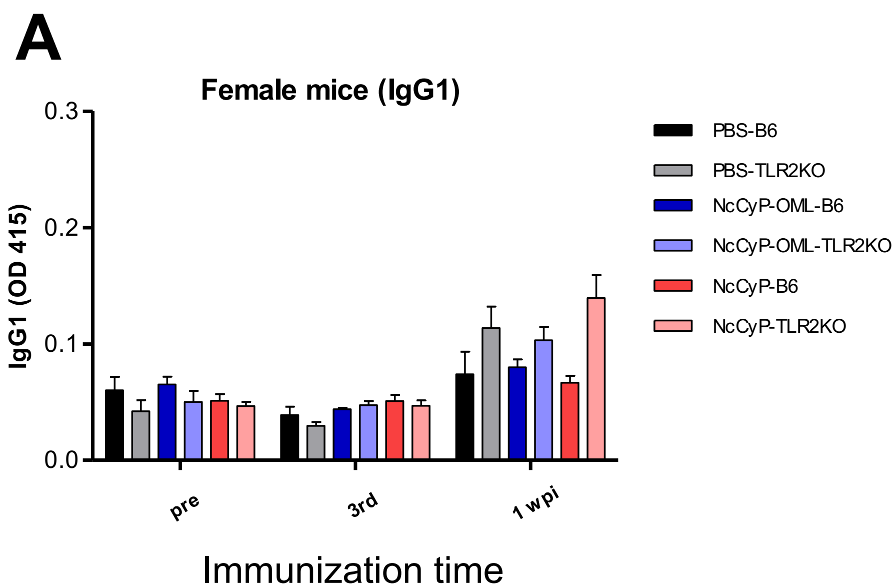
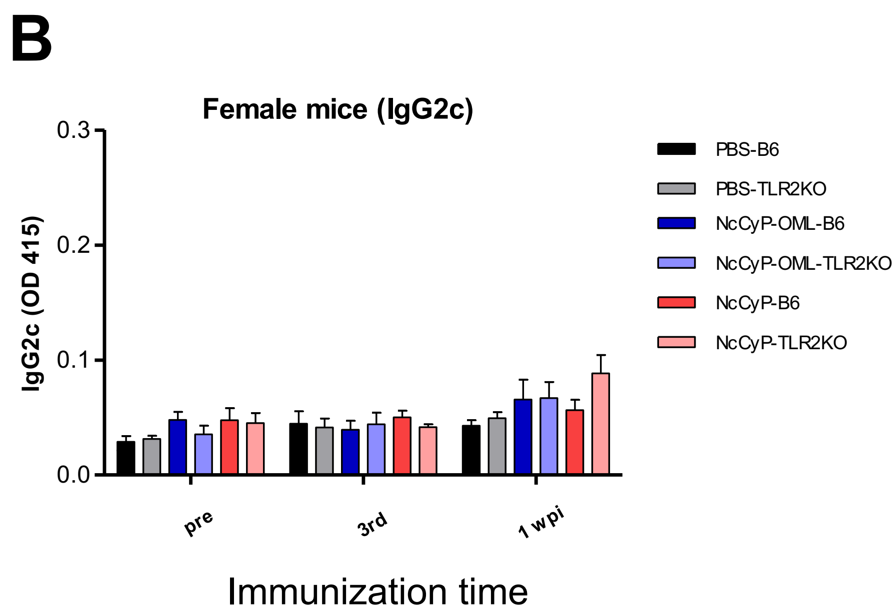
144 **Supplemental Figures**





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148 **Fig. S1. Specific antibody responses against NcCyp in female C57BL/6 and TLR2$^{-/-}$**

149 **mice.** Sera were collected from all mice in each group (7 per group from one trial)

150 (NcCyp-OML, NcCyp, GST-OML or PBS alone) 2 days before immunization and 1 week

151 after the third immunization to test for NcCyp-specific IgG1 (A) and IgG2c antibodies

152 (B) by indirect ELISA. In addition, sera were collected from the aforementioned mice

153 after 1 week of challenge with a lethal dose (1×10^6) of *N. caninum* tachyzoites to estimate

154 the boosting effect of immunization. The mean optical density (OD) was determined at a

155 wavelength of 415 nm. Each bar represents the mean ± standard deviation for mice in

156 each group. No statistically significant differences were observed among the

157 immunization groups as determined by one-way ANOVA plus Tukey–Kramer *post hoc*

158 analysis ($P < 0.05$).

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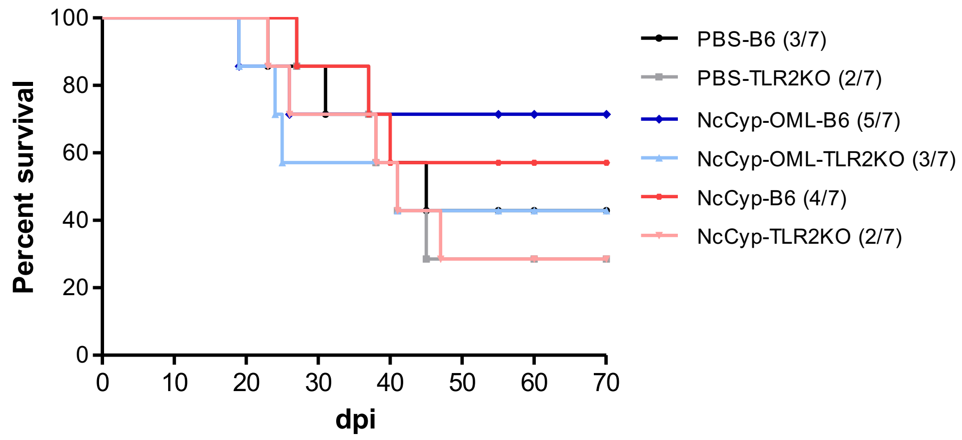

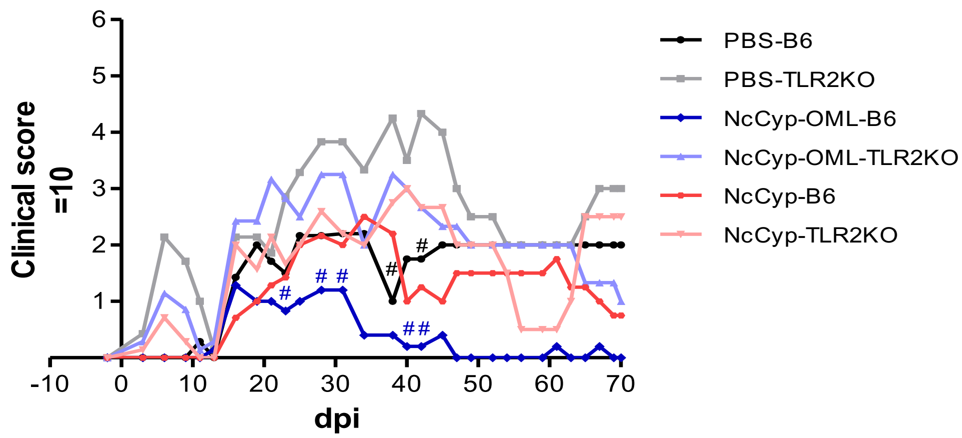

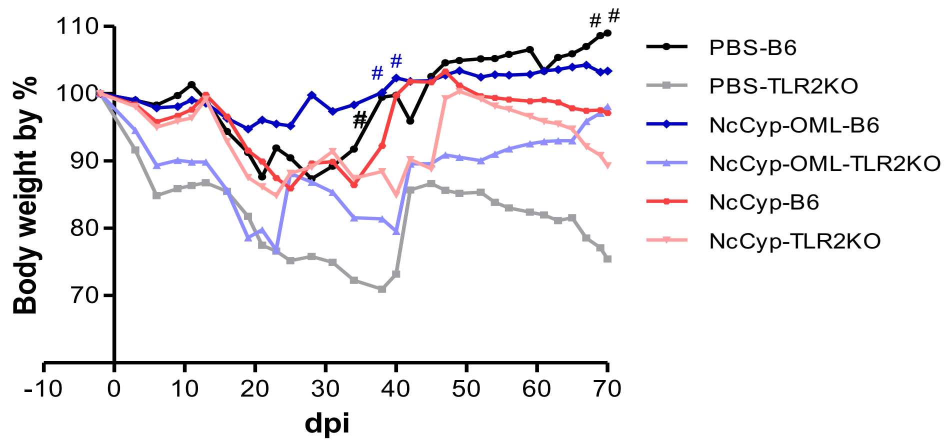

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162 **Fig. S2. Survival rate and clinical parameters in female C57BL/6 and TLR2$^{-/-}$ mice.**

163 Female C57BL/6 (WT) and TLR2$^{-/-}$ (KO) mice were immunized to investigate the

164 protective effect of NcCyp (7 per group from one trial). (A) The survival rates were

165 calculated for one trial: PBS-WT; 3/7 (42.9%), PBS-KO; 2/7 (28.6%), NcCyp-OML-WT;

166 5/7 (71.4%), NcCyp-OML-KO; 3/7 (42.9%), NcCyp-WT; 4/7 (57.7%) and NcCyp-KO;

167 2/7 (28.6%%). No significant differences were noted between wild-type and TLR2$^{-/-}$ mice

168 receiving the same vaccine antigen, or among immunized mice and the PBS control group

169 in the same mouse strain as calculated by the $\chi 2$ test ($P > 0.05$). For clinical scores (B)

170 and alterations in body weight (C), data were calculated as means ± standard deviation of

171 the values from seven mice per group from -2 until +70 dpi. Statistical significance

172 regarding body weight or clinical score was determined by two-way ANOVA plus Tukey–

173 Kramer *post hoc* analysis ($P < 0.05$). #, Significant difference between C57BL/6 and

174 TLR2$^{-/-}$ mice of the same immunization group. No significant difference was observed

175 among immunized mice and the PBS control group of the same mouse strain.

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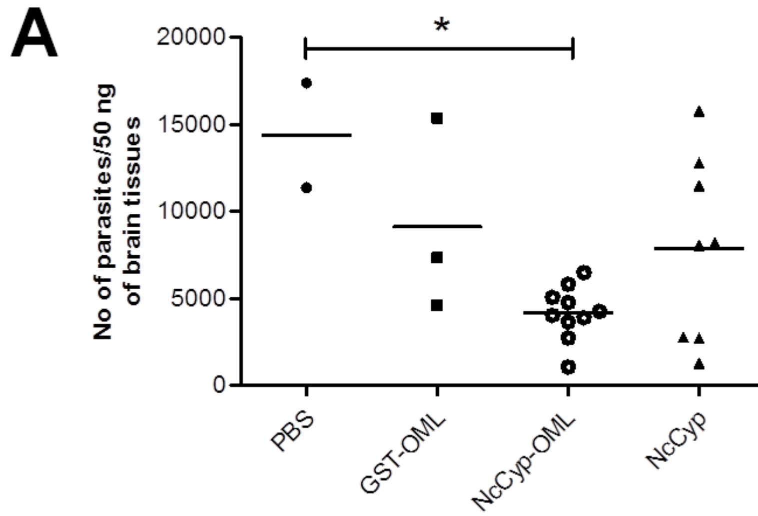

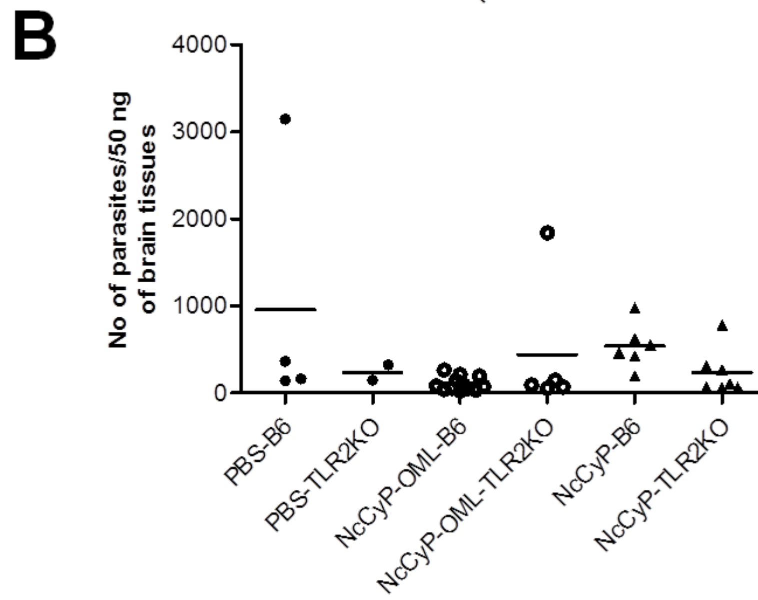

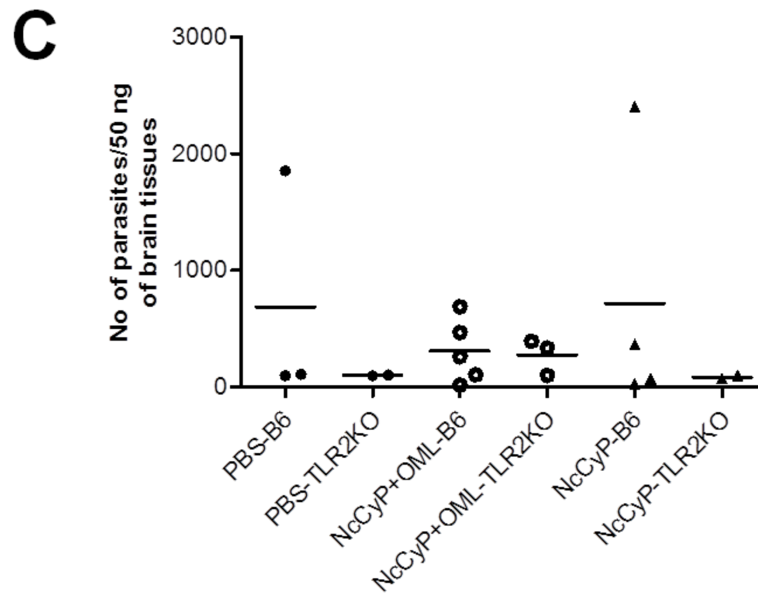

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178 **Fig. S3. Parasite burden in brains of surviving mice of BALB/c, C57BL/6 and TLR2^{-}**
179 **$^{/-}$ mice.** (A) Parasite numbers in the brains of the surviving female BALB/c mice at 32
180 dpi. The significant difference was observed only between PBS and NcCyp-OML groups
181 as determined by one-way ANOVA plus Tukey–Kramer *post hoc* analysis ($P < 0.05$).
182 Results are calculated from two pooled independent experiments (PBS; $n = 2$ out of 12,
183 GST-OML; $n = 3$ out of 12, NcCyp-OML; $n = 10$ out of 12, NcCyp; $n = 8$ out of 12). (B)
184 Parasite numbers in the brains of the surviving male C57BL/6 (WT) and TLR2$^{-/-}$ mice
185 (KO) at 70 dpi. Results are calculated from two pooled independent experiments
186 (PBS/WT; $n = 4$ out of 14, PBS/KO; $n = 2$ out of 14, NcCyp-OML/ WT; $n = 12$ out of 14,
187 NcCyp-OML/KO; $n = 5$ out of 14, NcCyp/ WT; $n = 6$ out of 14, NcCyp/KO; $n = 7$ out of
188 13). (C) Parasite numbers in the brains of the surviving female C57BL/6 (WT) and TLR2^{-}
189 $^{/-}$ mice (KO) at 70 dpi. Results are calculated from one trial (PBS/ WT; $n = 3$ out of 7,
190 PBS/KO; $n = 2$ out of 7, NcCyp-OML/ WT; $n = 5$ out of 7, NcCyp-OML/KO; $n = 3$ out
191 of 7, NcCyp/ WT; $n = 4$ out of 7, NcCyp/KO; $n = 2$ out of 7). In case of male (B) and
192 female (C), no significant differences were noted between wild-type and TLR2$^{-/-}$ mice
193 receiving the same vaccine antigen, or among immunized mice and the PBS control group
194 in the same mouse strain determined by one-way ANOVA plus Tukey–Kramer *post hoc*
195 analysis ($P < 0.05$).
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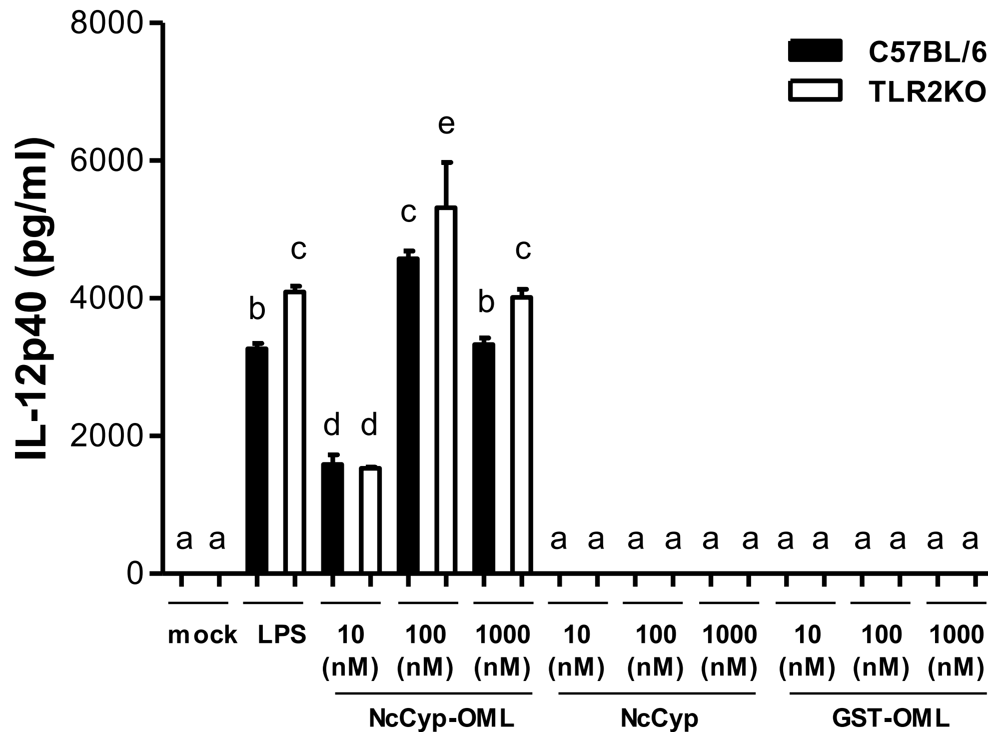

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199 **Fig. S4. Effect of TLR2 on macrophage IL-12 production.** Macrophages were isolated
200 from C57BL/6 and TLR2$^{-/-}$ mice and then treated with LPS (10 ng/mL), medium only
201 (mock), and recombinant NcCyp, NcCyp-OML, and GST-OML (10, 100, and 1000 nM)
202 for 20 h. The IL-12p40 value represents the mean ± standard deviation of triplicate
203 samples. The results are representative of two repeated experiments with similar results.
204 The different letters above the bars in the graphs indicate statistically significant
205 differences among all other groups (one-way ANOVA plus Tukey–Kramer *post hoc*
206 analysis, $P < 0.05$). NcCyp; 10 nM (0.205 μg/mL), 100 nM (2.05 μg/mL), 1000 nM (20.5
207 μg/mL), GST; 10 nM (0.27 μg/mL), 100 nM (2.7 μg/mL), 1000 nM (27 μg/mL).