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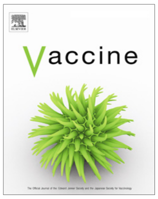

Critical role of TLR2 in triggering protective immunity with cyclophilin entrapped in oligomannose-coated liposomes against *Neospora caninum* infection in mice

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ABSTRACT

Neospora caninum is an intracellular protozoan parasite closely related to *Toxoplasma gondii. N. caninum* is thought to be a major cause of abortion in cattle worldwide. Given the current situation of drastic economic losses and a lack of efficient control strategies against such parasites, the challenge to develop potent vaccine candidates and technologies remains. We investigated the immune stimulating activity of *N. caninum* cyclophilin (NcCyp) with and without a formulation with oligomannose-coated-liposomes (OML) as the potential adjuvant. NcCyp-OML activated NF-κB in RAW 264.7 cells and triggered interleukin (IL)-12p40 production from murine peritoneal macrophages. In BALB/c mice, immunization with NcCyp-OML was associated with the production of specific antibodies (IgG1 and IgG2a). The specific antibody (IgG1) against naked NcCyp was also observed after the challenge infection, but it was significantly lower than those of NcCyp-OML. Moreover, significant cellular immune responses were induced, including spleen cell proliferation and interferon-gamma production. The immunization of mice with NcCyp-OML, and to lesser extent with naked NcCyp, induced significant protection against challenge with a lethal dose of *N. caninum* compared with the PBS control group. This protection was associated with a higher survival rate, slight changes in body weight, and lower clinical score of mice. In addition, the significant protective efficacy of NcCyp-OML was confirmed in another mouse strain, male C57BL/6 mice. The current study revealed the marked contribution of Toll-like receptor 2 (TLR2) to the protective immunity triggered by NcCyp-OML because higher numbers of $TLR2^{-/-}$ mice succumbed to a lethal dose of *N. caninum* compared with C57BL/6 mice. Moreover, prominent spleen cell proliferation and IFN-γ production was induced in NcCyp-OML-immunized mice by a TLR2-dependent mechanism.

1. Introduction

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

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

Professional antigen presenting cells (APCs) such as dendritic cells and macrophages also play a vital role in triggering immune responses against invading pathogens by presenting antigens to major histocompatibility complex molecules (MHC) class I and II, which activates lymphocytes, leading to antigen-specific acquired

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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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immunity along with immunologic memory [7]. This event is mediated by pattern recognition receptors including Toll-like receptors (TLRs), which are highly expressed by APCs. TLRs recognize many pathogens by matching conserved pathogen-associated molecular patterns [8]. Previous reports revealed that mice lacking TLR2 exhibited a higher parasite burden than wild-type (WT) mice against *N. caninum* and *T. gondii* infections [9,10]. Moreover, the TLR2 is involved in innate and adaptive immunity against *N. caninum* in murine cells [11]. The activation of TLRs is strongly correlated to activation of the signal transduction of nuclear factor kappa-B, the major integral component of immune responses and protection against microbial infections [12,13]. Moreover, the chemokine receptor, CCR5, was reported to be associated with the induction of protective immunity in mice infected with *N. caninum*. Mice lacking the CCR5 gene were more vulnerable to *N. caninum* infection [14], possibly because interactions of CCR5 with parasite-derived cyclophilin elicits the migration and activation of innate immune cells [5,15]. Oligomannose-coated-liposomes (OML) are used as a delivery system and potential adjuvant in vaccine development against several pathogens of protozoan origin [5].

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

2. Materials and methods

2.1. Ethical statement

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

2.2. Animals

Six-to-seven-week-old female BALB/c mice were purchased from Clea Japan (Tokyo, Japan) and used for vaccination experiments. Male and female C57BL/6 mice at 7-weeks of age were purchased from Clea Japan and used for the vaccination study. The macrophage assay was conducted using cells isolated from male mice. C57BL/6-background TLR2-knockout (TLR$2^{-/-}$) mice were a kind gift from Dr. Satoshi Uematsu and Dr. Shizuo Akira (Osaka University, Osaka, Japan) [18].

2.3. RAW 264.7 cell lines and NF-κB analysis

The NF-κB secreting alkaline phosphatase reporter cell line generated from RAW 264.7 cells (NF-κB/SEAP cells) was obtained from Novus Biological Inc. (Littleton, CO, USA). The cells were seeded in 96-well plates (2×10^5 in 100 μL/well) and incubated at 37 °C for 4 h to allow adherence of the cells to the bottom of the plate. The NcCyp, NcCyp-OML, and GST-OML proteins, and LPS as a positive control and medium alone as a negative control, were added to the cells, and then the plates were incubated at 37 °C for 24 h. The procedures for expression and purification of recombinant proteins and OML were described in Supplemental Information file. The culture supernatant from NF-κB/SEAP cells was collected and the levels of secreted alkaline phosphatase were measured using a SEAP reporter assay kit (Novus) according to the manufacturer's instructions. Further details of the culture of NF-κB/SEAP cells, expression and purification of recombinant proteins, and preparation of OML can be found in the Supplemental Methods in the Supplemental Information.

2.4. Macrophages and IL-12p40 production

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

2.5. Immunization and infection

Different mouse strains (BALB/c and C57BL/6), genders (male and female), and genetic backgrounds (wild-type and TLR$2^{-/-}$) were used in this study to investigate the immunoprophylactic properties of NcCyp. Mice were injected subcutaneously with recombinant NcCyp, NcCyp-OML, and GST-OML, or with PBS alone (each 100 μL) three times at 14-day intervals. To inoculate the same molecular number of the antigens, the inoculum was calculated by molar number based on the molecular weight of each antigen (NcCyp; 20.5 kDa, GST; 27 kDa). Each mouse was immunized with 25 pmol from each antigen (NcCyp-OML; 0.51 μg, NcCyp; 0.51 μg, GST-OML; 0.68 μg). In the BALB/c mouse model, only female mice were used (12 mice per group from 2 independent trials). In the C57BL/6 mouse model, immunizations were administered to male and female wild type or TLR$2^{-/-}$ mice (males: 14 mice per group except for the NcCyp-immunized TLR$2^{-/-}$ group (13 mice) from 2 independent trials; females: 7 per group from one trial). Two weeks after the third immunization, mice were infected with 1×10^6 tachyzoites of the *N. caninum* Nc-1 strain via the intraperitoneal route. The mouse survival rates, body weight, and clinical scores were monitored for 32 and 70 days after challenge for BALB/c and C57BL/6 mice, respectively. Serum (20 μL) was collected from mice via the tail veins at −2, 7, 21, and 35 days after the first immunization, and at 7 dpi to investigate the dynamics of specific antibodies generated against NcCyp, using an indirect enzyme-linked immunosorbent assay (ELISA). At the indicated times, serum and brain samples were collected from all surviving mice after they were euthanized. Further details of parasite preparation, clinical score observation, and ELISAs can be found in the Supplemental Methods in the Supplemental Information.

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

Fourteen days after the third immunization, spleens were removed from immunized and PBS-inoculated mice ($n = 4$/group) under aseptic conditions. The spleen cells were placed into 96-well plates (3×10^5/100 μL/well) and stimulated with recombinant NcCyp, concanavalin A (ConA; Sigma-Aldrich, St Louis, MO) as the positive control, or with stimulant-free medium as the negative control. The cells were incubated for 48 h at 37 °C in 5% CO_2. A 100-μl aliquot of culture supernatant was collected and assayed for cytokines (IL-4, IL-10 and IFN-γ). At the same time, the proliferation of spleen cells was measured by a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Further details of the collection of splenocytes and stimulation indices can be found in the Supplemental Methods in the Supplemental Information.

2.7. Statistical analyses

Statistical analyses were performed using a one- or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group comparisons. The significance of differences in mouse survival was analyzed by the χ^2 test. All statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Immune stimulating activity of NcCyp

Responses in the NF-κB signaling pathway in RAW 264.7 cells secreting NF-κB dependent SEAP, and IL-12p40 production from naturally isolated peritoneal macrophage of mice against stimulation with NcCyp were investigated (Fig. 1). Regarding the NF-κB pathway, a statistically significant difference against mock treatment (negative control) was only detected for high concentrations of NcCyp-OML (100 nM) and LPS (positive control) (Fig. 1A). In the peritoneal mouse macrophages, Il-12p40 production was observed in the treatment with LPS (10 ng/mL) and NcCyp-OML at concentrations of 10 nM and 100 nM, but not naked NcCyp- or GST-OML-treated cells (Fig. 1B). These results suggest NcCyp-OML has immune-stimulating activity.

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

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

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

The immunization of mice with NcCyp-OML increased their survival rate (83.3%), and attenuated the severity of infection as confirmed by lower changes in clinical scores and body weight compared with all other groups, especially the control groups (survival rate; 16.7% in PBS-injected mice, 25.0% in GST-OML-injected mice) (Fig. 4). When compared with control groups, protective efficacy

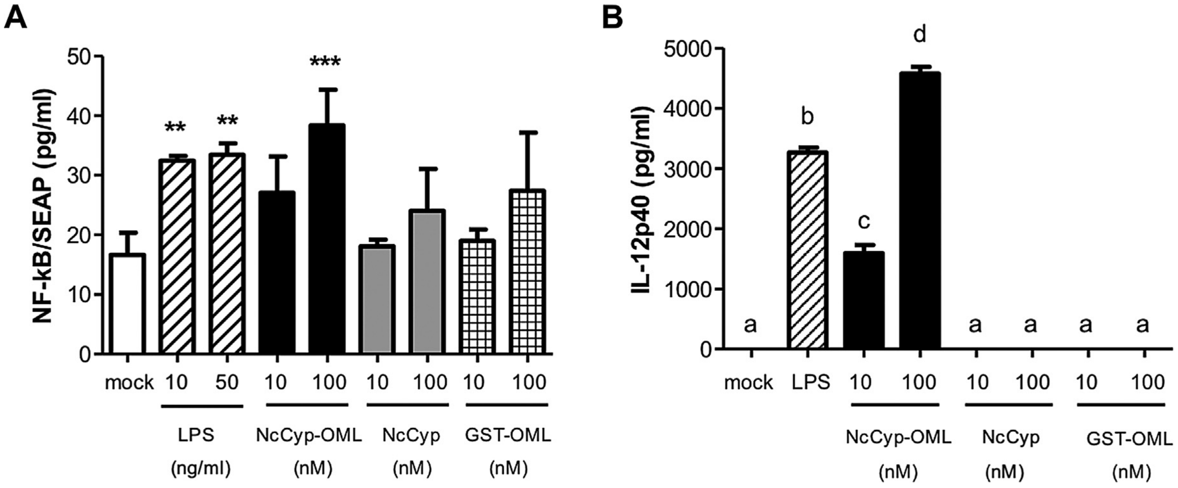

Fig. 1. NF-κB activation and cytokine production. (A) NF-κB/SEAP cells were treated with LPS (10 and 50 ng/mL), medium only (mock), and recombinant proteins of NcCyp and GST (10 and 100 nM) with or without OML. Stimulated cells were incubated for 24 h to measure secreted alkaline phosphatase levels.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

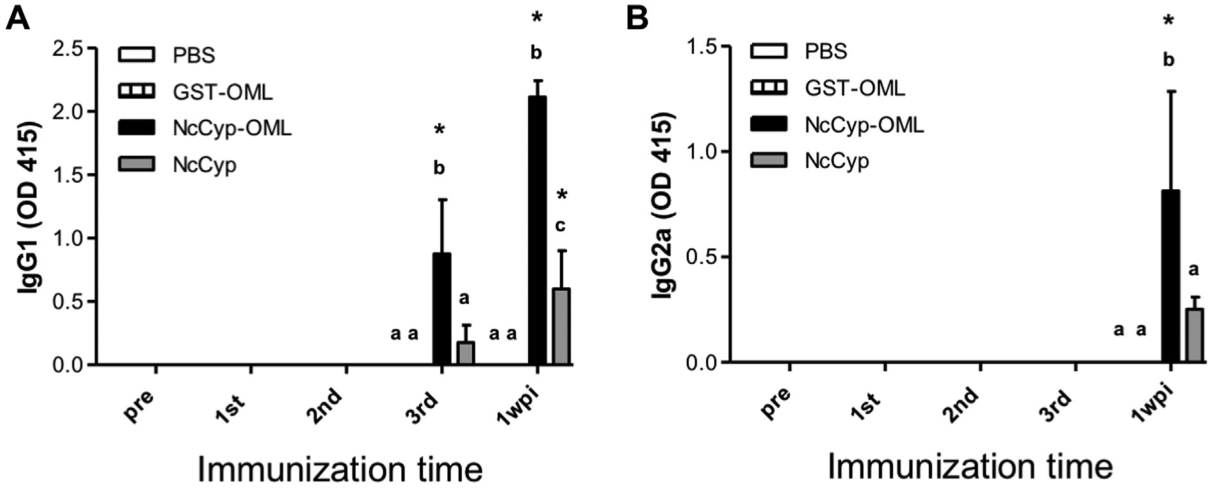

Fig. 2. Production of specific antibodies against NcCyp in female BALB/c mice. Mice were immunized with 25 pmol of NcCyp-OML, NcCyp, GST-OML, or PBS alone via the subcutaneous route and then challenged with a lethal dose (1×10^6) of *N. caninum* tachyzoites via an intraperitoneal route. Sera were collected from all mouse groups 2 days before immunization and tested for NcCyp-specific IgG1 and IgG2a antibodies using indirect ELISAs. The mean optical density (OD) was determined at a wavelength of 415 nm. Each bar represents the mean ± standard deviation for mice in each group ($n = 6$). The results are representative of one trial from two repeated experiments with similar results. The different letters above the bars in the graphs indicate statistically significant differences among the same immunization group by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$). *, statistically significant differences were observed compared with day 0 after the first immunization in the same immunized group by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$).

was also observed in mice immunized by NcCyp alone determined by their higher survival rate (66.7%) and lower clinical score. Furthermore, parasite burden in brain was lower in NcCyp-OML-immunized group than all other groups, although it was statistically significant against the control PBS-inoculated mice (Fig. S3A).

3.3. Protective efficacy of NcCyp in C57BL/6 mice and the role of TLR2

The ability of NcCyp-OML and NcCyp to generate specific antibodies or to induce cellular immunity was investigated in the C57BL/6 mouse model to reveal the mechanism of protection. In contrast to the results observed in BALB/c mice, no detectable levels of anti-NcCyp antibodies (IgG1 or IgG2c) were observed in C57BL/6 mice (Fig. 5 and S1). Spleen cell proliferation was only observed in NcCyp-OML-immunized wild-type mice but not in TLR2$^{-/-}$ mice when stimulated with rNcCyp at 50 µg/ml (Fig. 6A). Additionally, increased IFN-γ production was predominant in NcCyp-OML-immunized wild-type mice when stimulated with rNcCyp at 50 µg/ml (Fig. 6B). Spleen cell proliferation and IFN-γ production was determined in all groups with ConA stimulation as a positive control. These results suggest NcCyp-OML induces cellular immunity in male C57BL/6 mice, and might be partially dependent on TLR2.

Next, we investigated the protective efficacy of NcCyp with and without OML using male and female C57BL/6 mice. TLR2$^{-/-}$ mice were used to evaluate the role of TLR2 in the induction of immunity and in conferring protection (Figs. 7, S2 and S3). Using male mice, NcCyp-OML protected wild-type mice (survival rate: 85.7%) against infection with *N. caninum* compared with the PBS treated wild-type mice (survival rate: 28.6%) (Fig. 7A). This effect was markedly abrogated when the same antigen was inoculated in TLR2$^{-/-}$ mice (survival rate: 35.7%) (Fig. 7A). There were minimal changes in clinical score and body weight in the wild-type mice immunized with NcCyp-OML (Fig. 7B, C). Similar tendencies regarding the protective efficacy of NcCyp with and without OML were observed in female mice (Fig. S2). No tangible differences in cerebral parasite burden among all groups of male or female wild-type and TLR2KO mice were observed because of low number of survived mice of control group (Fig. S3B, C).

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

A recent study suggested a role for TLR2 in vaccine development against *N. caninum* was targeted, although no tangible progress has been achieved. The TLR2 ligand OprI (a bacterial lipoprotein) exerts strong adjuvant activity when combined with the *N. caninum*

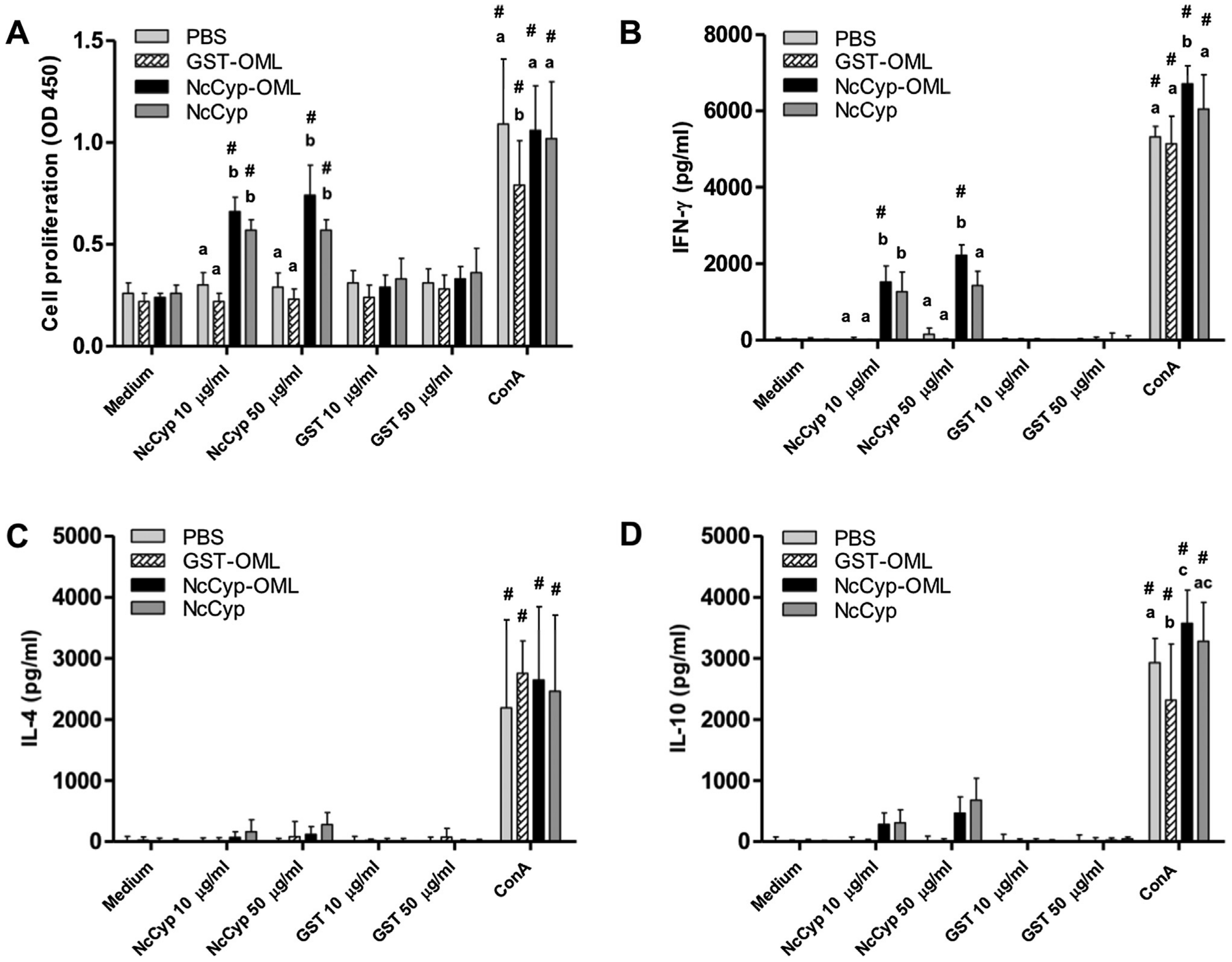

Fig. 3. Splenocyte proliferation and cytokine production in female BALB/c mice. Spleens were dissected aseptically from immunized mice 2 weeks after the third immunization with PBS, NcCyp-OML, NcCyp alone, or GST-OML. Cells were stimulated with Concanavalin A (ConA, 5 μg/mL), NcCyp, GST, or without any stimulator (Medium) for 48 h. (A) Cell proliferation, (B) IFN-γ, (C) IL-4, and (D) IL-10 production. Each bar represents the mean ± standard deviation ($n = 4$ for all groups). The different letters above the bars in the graphs indicate statistically significant differences among groups with the same stimulation by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$). #, Statistically significant differences were observed compared with medium only in the same immunized group by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$).

chimeric protein Mic3-1-R, but no protection was reported in mice [26]. Activation of TLR2 by vaccine inoculation via any route including mucosal tissues in the digestive tract, the main entrance for infection by *N. caninum* cysts, induces generalized protection. This effect is triggered by the homing of T cells and dendritic cells to the gut mucosa via MyD88-dependent TLR2 signals [27]. Such an approach has achieved variable success in vaccine development for other pathogens such as *Leishmania major* and *Brugia malayi* [28], *Mycobacterium tuberculosis* [29], and influenza virus [30]. Nevertheless, our study has provided the first direct evidence for the contribution of TLR2 in protective immunity against *N. caninum* infection. The immunization of C57BL/6 mice with NcCyp-OML conferred substantial protection against challenge with *N. caninum*. Strikingly, this protection was dramatically decreased in TLR2$^{-/-}$ mice, indicating the contribution of TLR2 to the induction of protective immunity. This protection was associated with Th1 or cell-mediated immunity because cell proliferation and IFN-γ production of spleen cells is dependent on TLR2.

Based on the high immune stimulatory effect of NcCyp-OML compared with NcCyp alone or other control stimulants, we believe NcCyp-OML has the potential to protect host animals from infection with *N. caninum*. Notably, treatment of macrophages with NcCyp-OML induced IL-12p40 production via the activation of NF-κB signals, indicating the immune-stimulating activity of NcCyp-OML. Several studies have reported a role for the TLR2/NF-κB-signaling axis in eliciting immune responses triggered primarily via cytokine production [31,32]. Because macrophages are powerful effector immune cells against *N. caninum* infection [33], the immune regulatory effects of NcCyp-OML will be an important factor in controlling infection.

Few reports have revealed the efficacy of OML via the manipulation of cell receptors and signaling pathways. The mannose-binding C-type lectin receptor, SIGNR1, was reported to be a physiological receptor for OML during its uptake by macrophages. The activation of SIGNR1 was associated with IL-12 production via NF-κB signaling [34]. Moreover, OMLs were reported to induce extracellular signal-regulated kinase activation in a murine macrophage cell line via the phosphorylation of Src family kinases and enhancement of the PI3K/Akt pathway [35]. In a more recent study, IFN-γ production by spleen cells from mice immunized with OML was highly dependent on TLR4 but not TLR2, using knockout mice for each receptor. However, neither the removal of

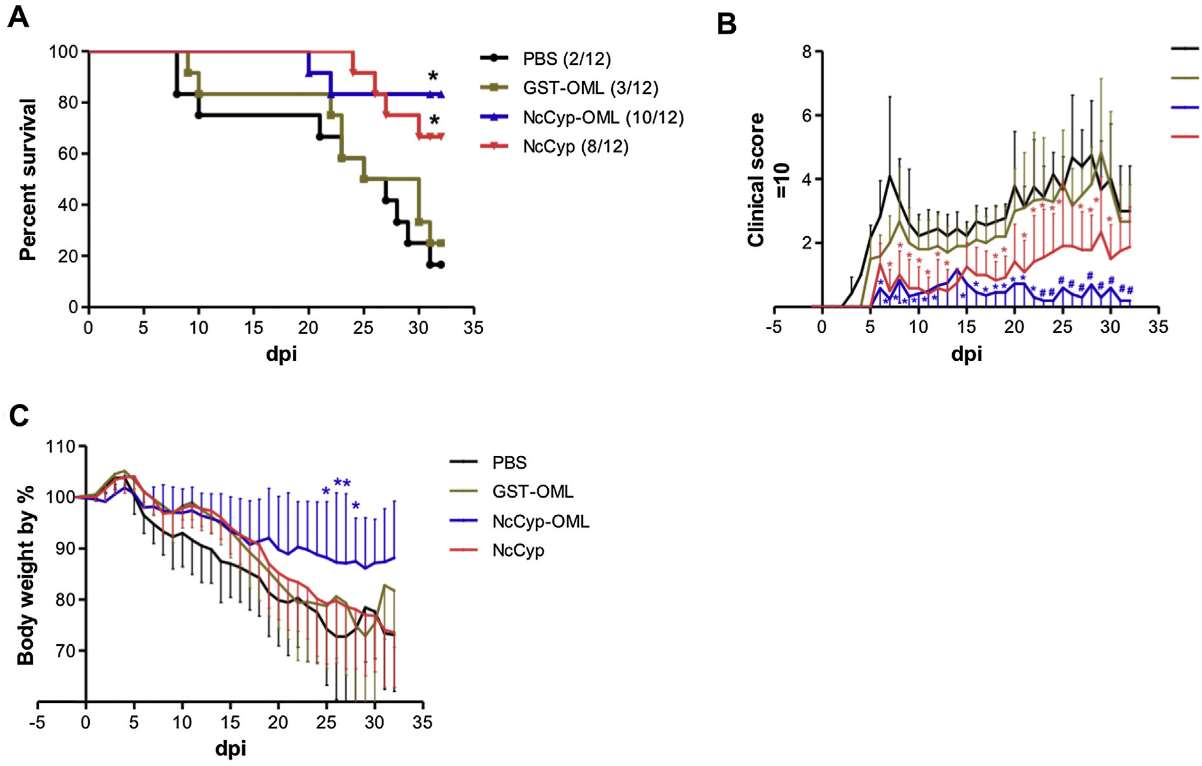

Fig. 4. Clinical parameters and protective indices in immunized female BALB/c mice. After the third immunization, all mice (6 per group) were intraperitoneally challenged with a lethal dose (1×10^6) of *N. caninum* tachyzoites. (A) The survival rates (surviving mice/total mice) were calculated from two pooled independent experiments: PBS; 2/12 (16.7%), GST-OML; 3/12 (25%), NcCyp-OML; 10/12 (83.3%) and NcCyp; 8/12 (66.7%). *, the differences were significant between the PBS- and GST-OML-injected groups and those of NcCyp-OML or NcCyp-immunized groups by χ^2 test ($P < 0.05$). Changes in the clinical score (B) and body weight (C) were calculated as the means ± standard deviation of clinical score and body weight values of all mice in a group from −2 to +32 dpi. The significance in change in body weight or clinical score was determined by two-way ANOVA plus Tukey–Kramer *post hoc* analysis ($P < 0.05$). Asterisk (*) refers to a significant difference in the test groups (NcCyp-OML and NcCyp alone) compared with either control group (PBS and GST-OML). #, Significant difference among the test groups.

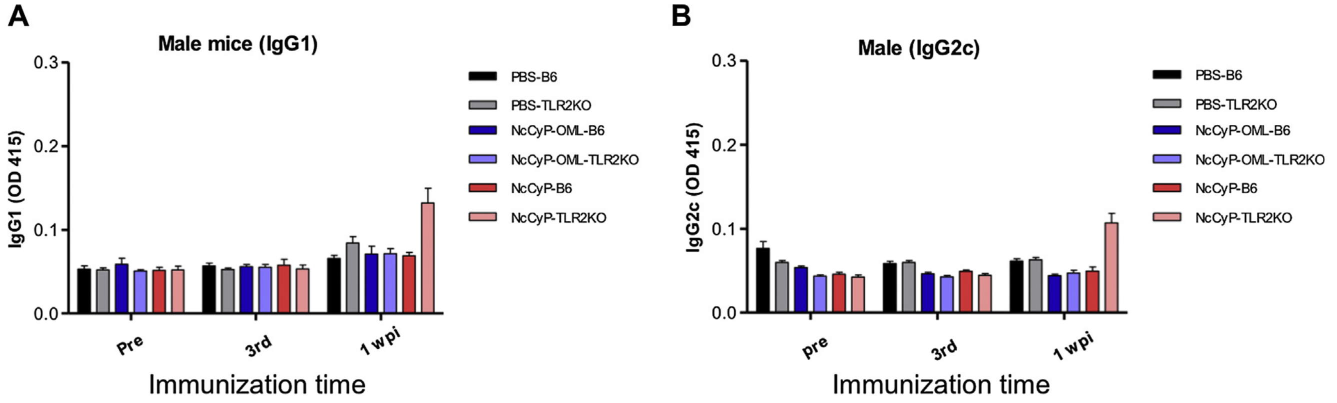

Fig. 5. Specific antibody response against NcCyp in male C57BL/6 and TLR2$^{-/-}$ mice. Sera were collected from all mice in each group (NcCyp-OML, NcCyp, GST-OML, or PBS alone) 2 days before immunization and 1 week after the third immunization to test for NcCyp-specific IgG1 (A) and IgG2c antibodies (B) using indirect ELISA. In addition, sera were collected from these mice 1 week after challenge with a lethal dose (1×10^6) of *N. caninum* tachyzoites to estimate the boosting effect of immunization. The mean optical density (OD) was determined at a wavelength of 415 nm. Each bar represents the mean ± standard deviation for mice in each group ($n = 6$). Data are representative of one trial from two repeated experiments with similar results. No statistically significant differences were observed among the immunization groups as determined by one-way ANOVA plus Tukey–Kramer *post hoc* analysis ($P < 0.05$).

TLR2 nor TLR4 affected the production of IL-12 after mouse macrophages were treated with OML [36]. Our results were similar to those obtained in the aforementioned study regarding TLR2-independent IL-12 production from macrophages (Fig. S4).

Interactions between TLR2 and NcCyp-OML are important for the induction of protection against infection in mice by immunization with NcCyp-OML. When NcCyp-OML is administered, it is preferentially taken up by macrophages and/or other APCs [37]. The delivered antigen is then processed and its peptides are presented to T-cells by MHC class I and class II molecules, leading to the activation of T-cell subsets responsible for antigen-specific immune responses. IL-12 production was observed in TLR2$^{-/-}$ macrophages treated with NcCyp-OML, indicating that TLR2 was not involved in APC activation. Instead, TLR2 may contribute to the induction of acquired immunity triggered by immunization with NcCyp-OML. The synergistic effect between OML and NcCyp

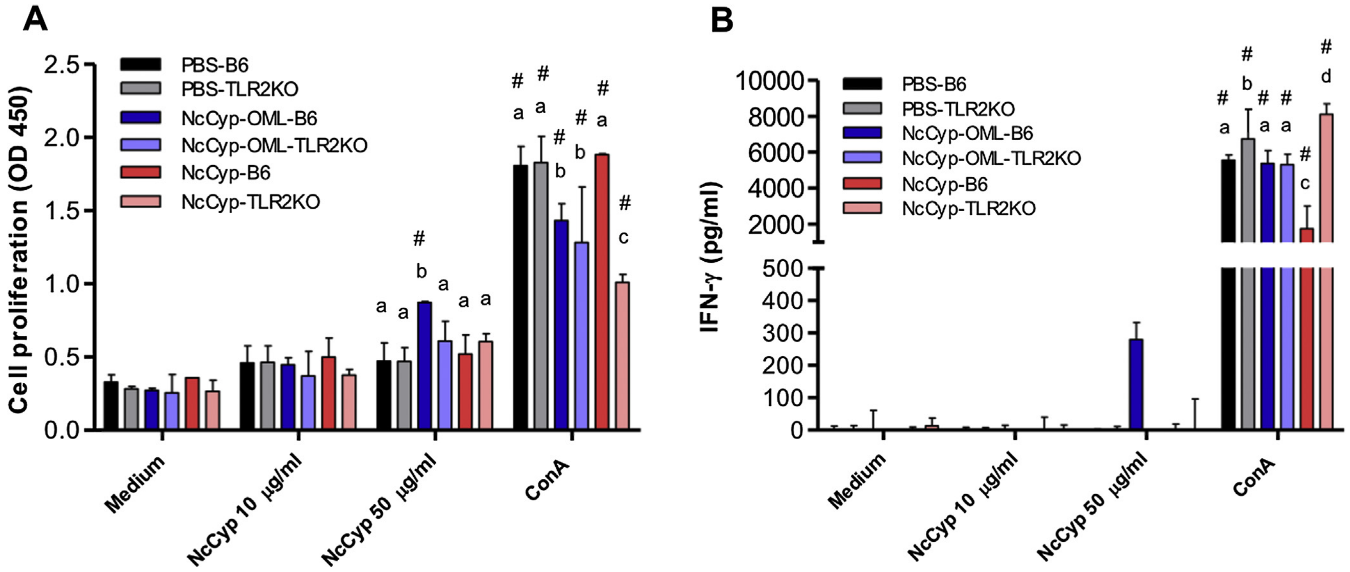

Fig. 6. Splenocyte responses in male C57BL/6 and TLR2$^{-/-}$ mice. At 2 weeks after the last immunization, spleens were dissected from wild-type and TLR2$^{-/-}$ male mice immunized with PBS, NcCyp-OML, or NcCyp alone. Cells were seeded in plates and stimulated with Concanavalin A (Con A, 1 µg/mL), NcCyp, or without any stimulator (Medium) for 48 h. For cell proliferation (A) and IFN-γ production (B), each bar represents the mean ± standard deviation ($n = 4$ for all groups). The different letters above the bars in the graphs indicate statistically significant differences among groups with the same stimulation by two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$). #, Statistically significant differences were observed compared with medium alone in the same immunized group with two-way ANOVA and Tukey–Kramer *post hoc* analysis ($P < 0.05$).

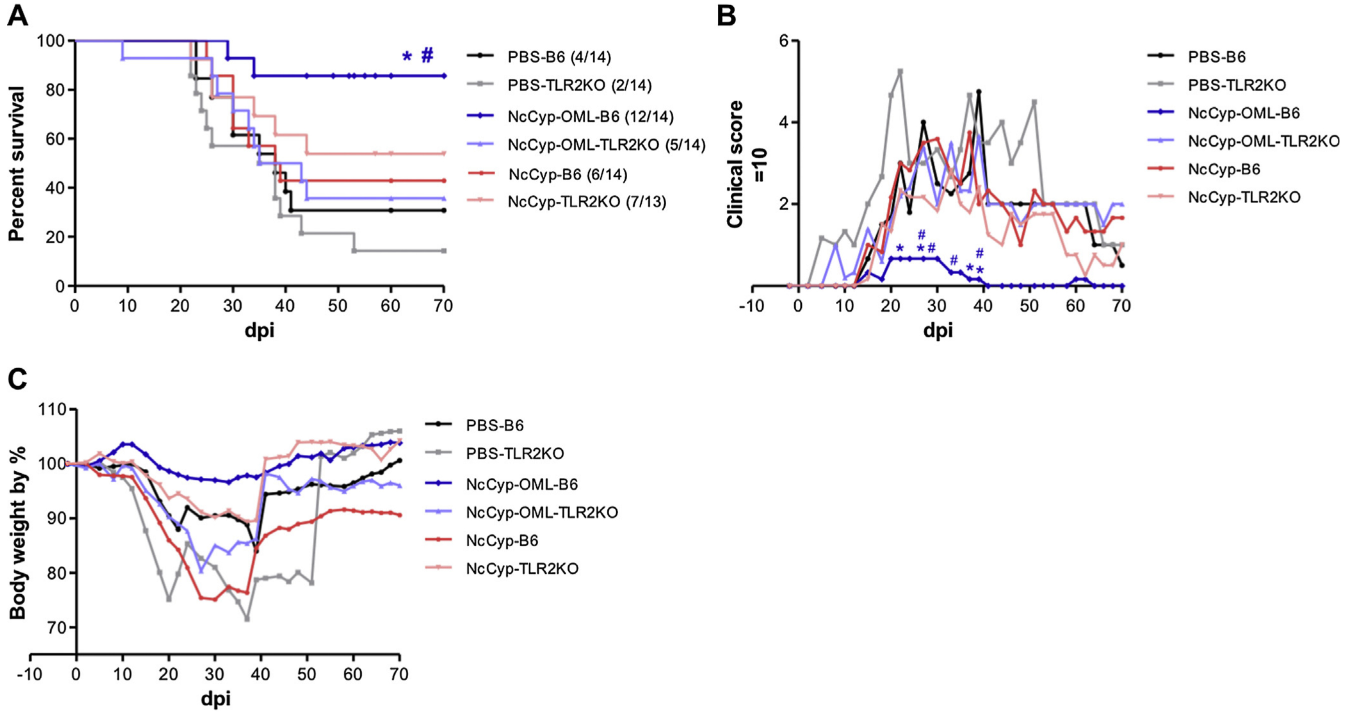

Fig. 7. Survival rate and clinical parameters in male C57BL/6 and TLR2$^{-/-}$ mice. (A) To determine the survival rate, wild type (WT) or TLR2$^{-/-}$ (KO) C57BL/6 male mice were immunized (14 mice per group except for the NcCyp-TLR2$^{-/-}$ group (13 mice) from 2 independent trials). After the third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

is crucial for the potent immunogenicity and protective efficacy of NcCyp-OML. In conclusion, our data suggest that TLR2 has a crucial role in NcCyp-OML-conferred protection against *N. caninum* infection of mice. Proficiency in triggering immune response and protective effect for NcCyp, in particular those formulated in OML, is a promising aspect for further evaluation against the vertical transmission of *N. caninum*. In addition, role of TLR2 should be investigated to reveal its effect in pregnant mouse model.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.01.005.

References

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

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

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

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

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

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

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

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

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

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

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

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

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

[14] Abe C, Tanaka S, Nishimura M, Ihara F, Xuan X, Nishikawa Y. Role of the chemokine receptor CCR5-dependent host defense system n *Neospora caninum* infections. Parasit Vect 2015;8:5.

[15] Kameyama K, Nishimura M, Punsantsogvoo M, Ibrahim HM, Xuan X, Furuoka H, et al. Immunological characterization of *Neospora caninum* cyclophilin. Parasitol 2012;139:294–301.

[16] Tuo W, Zhao Y, Zhu D, Jenkins MC. Immunization of female BALB/c mice with *Neospora* cyclophilin and/or NcSRS2 elicits specific antibody response and prevents against challenge infection by *Neospora caninum*. Vaccine 2011;29:2392–9.

[17] Basto AP, Leitão A. Targeting TLR2 for vaccine development. J Immunol Res 2014;2014:619410.

[18] Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficint mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol 2000;165:5392–6.

[19] Zhang H, Nishikawa Y, Yamagishi J, Zhou J, Kojima N, Yokoyama N, et al. *Neospora caninum:* application of apical membrane antigen 1 encapsulated in the oligomannose-coated liposomes for reduction of offsprings mortality from infection in BALB/c mice. Exp Parasitol 2010;125:130–6.

[20] Nishikawa Y, Zhang H, Ikehara Y, Kojima N, Xuan X, Yokoyama N. Immunization with oligomannose-coated liposome-entrapped dense granule protein 7 protects dams and offspring from *Neospora caninum* infection in mice. Clin Vaccine Immunol 2009;16:792–7.

[21] Nishimura M, Kohara J, Kuroda Y, Hiasa J, Tanaka S, Muroi Y, et al. Oligomannose-coated liposome-entrapped dense granule protein 7 induces protective immune response to *Neospora caninum* in cattle. Vaccine 2013;31:3528–35.

[22] Aguado-Martínez A, Basto AP, Leitão A, Hemphill A. *Neospora caninum* in non-pregnant and pregnant mouse models: cross-talk between infection and immunity. Int J Parasitol 2017;47:723–35.

[23] Chen X, Oppenheim JJ, Howard OM. BALB/c mice have more CD4+CD25+ T regulatory cells and show greater susceptibility to suppression of their CD4+CD25- responder T cells than C57BL/6 mice. J Leukoc Biol 2005;78:114–21.

[24] Ferreira BL, Ferreira ÉR, de Brito MV, Salu BR, Oliva MLV, Mortara RA, et al. BALB/c and C57BL/6 mice cytokine responses to *Trypanosoma cruzi* infection are independent of parasite strain infectivity. Front Microbiol 2018;9:553.

[25] Scotland RS, Stables MJ, Madalli S, Watson P, Gilroy DW. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. Blood 2011;118:5918–27.

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

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

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

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

[30] Shepardson KM, Schwarz B, Larson K, Morton RV, Avera J, McCoy K, et al. Induction of antiviral immune response through recognition of the repeating subunit pattern of viral capsids is toll-like receptor 2 dependent. MBio 2017;8:e01356–e1417.

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

[32] Zhang G, Ghosh S. Toll-like receptor-mediated NF-B activation: a phylogenetically conserved paradigm in innate immunity. J Clin Invest 2001;107:13–9.

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

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

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

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

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