IMMUNOLOGICAL CHARACTERIZATION OF *NEOSPORA CANINUM* CYCLOPHILIN

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ABSTRACT

Neosporosis is caused by an intracellular protozoan,Neospora caninum, which is an important pathogen of dogs and cattle. In this study, we analyzed Neospora caninum cyclophilin 18 protein (NcCyp18), which regulates proliferation and migration of murine macrophages and lymphocytes. From our study, NcCyp18 showed the activity of macrophage proliferation in a CCR5-independent manner and activity of spleen cell proliferation in a CCR5-dependent manner. NcCyp18 showed the activity of macrophage migration in a CCR5-independent manner and activity of spleen cell migration in a CCR5-dependent manner. Moreover, recombinant NcCyp18 induced the production of the nitric oxide and IL-12 p40. The data suggest that NcCyp18 recruits host cells and enhances the growth of host cells at the site of infection for maintenance of interaction between the parasite and host.

KEYWORDS: Neosporosis, *Neospora caninum*, NcCyp18,

INTRODUCTION

Neospora caninum is a coccidial protozoan parasite that infects a large range of mammals including dogs, cats, mice, and cattle. Morphologically, *N. caninum* appears indistinguishable from *Toxoplasma gondii.* Although they are closely related parasites structurally, genetically, and immunologically, caution should be used in making generalizations about *N.caninum* based on the biology of *T.gondii* because neosporosis and toxoplasmosis are biologically distinct diseases [1]. Although there have been no reported cases of this infection in humans, it shows clinical signs such as stillbirth and abortion in cattle and neuromuscular in dogs. The parasite is followed by invasion of the placenta and fetus. Cattle and a wide range of other warm-blooded animals can act as intermediate hosts and the dog is the definitive host. This agent may infect many cells types including neural cells, vascular endothelial cells, myocytes, hepatocytes, renal cells, alveolar, macrophages and placental trophoblasts [2].

Cell-mediated immunity is expected to have a major role in protection against *N. caninum* infection [5]. The cellular response to this parasite can be characterized by the induction of antigen-specific T cells and is mediated by the production of IL-12 and IFN-γ [4]. Cellular immune responses were assessed by lymphocyte proliferation test and IFN-γ production [7]. Cyclophilin was discovered for its peptidyl-prolylcis-transisomerase

immunophilin activity and its high binding affinity to cyclosporine, an immunosuppressant drug commonly used to prevent graft rejection. *Neospora* cyclophilin18 (NcCyp18) is major The characterization of immunostimulatory molecule is very important when attempting to control the parasite. The aim of this study is to characterize the function of NcCyp18 component of the parasite responsible for the induction of IFN-γ production by peripheral blood mononuclear cells and antigen- specific $CD4^+$ T cells [9].

MATERIALS AND METHODS

***N.caninum* tachyzoite propagation:** *N.caninum* (Nc-1 strain) tachyzoites were cultured on vero cells in Eagle's medium essential medium (Sigma) supplemented with 8% heat-inactive fetal bovine serum (FBS) and 50 µg/ml kanamycin at 37^0C in 95 % air and 5% CO_2 atmosphere. For the purification of parasites, tachyzoites and host cell debris were washed in PBS, and the final pellet was re-suspended in PBS and passed through a 27-gauge needle and after the procedure, it was filtered by a 5 µm-pore filter (Millipore).

Experimental animals: BALB/c female mice, 6-8 weeks of age were obtained from Clea Japan. CCR5 knockout (CCR5 KO) mice were purchased from the Jackson Laboratory.

Construction and expression of recombinant NcCyp18 (rNcCyp18): Parasite cDNA was synthesized from RNA isolated with Trizol reagent using a SuperScript first-strand synthesis system for reverse transcription-PCR. To clone recombinant NcCyp18 without the signal peptide, one set of oligonucleotide primers that included a *Bam*HI restriction enzyme site (boldface type) in the forward primer (5' T**AG GAT CC**A TGG AAA ACG CCG GAG TCC AG 3') and an *Eco*RI site (boldface type) in the reverse primer (5'GC**G AAT TC**T TAC AAC AAA CCA ATG TCC GT 3') was designed. PCR products were digested with *Bam*HI and *Eco*RI and then ligated into the glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* expression vector pGEX-4T1, which been digested with the same set of restriction enzymes (pGEX-NcCyp18). The nucleotide sequences of the plasmid were analyzed with a model ABI 3100 DNA sequencing machine (Applied Biosystems).

NcCyp18 were expressed as GST fusion proteins in *E. coli* DH5α cells (Takara, Bio, Inc.). GST tags of the rNcCyp18 were removed by thrombin protease (GE Healthcare) according to manufacturer's instructions. Proteins were dialyzed in PBS and purified with Detoxi-Gel endotoxin-removing gel in order to remove endotoxins. The purity and quantity of the proteins were detected as a single band by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie brilliant blue staining. The protein concentration was measured using a BCA protein assay kit.

Production of anti-NcCyp18 serum: Seventy five micrograms of the rNcCyp18 with Freund's complete adjuvant (Sigma) was intraperitoneally injected into five BALB/c mice. Following immunizations were performed with same antigen in Freund's incomplete adjuvant and injected in peritoneal cavity of mice on 14, 28 and 42 days.

An indirect enzyme linked immunosorbent assay (ELISA): Ninety six-well plates were coated with 0.1 µM of purified recombinant NcCyp18 and glutathione S-transferase (GST) control and incubated for overnight at 4^0C with carbonate-bicarbonate buffer (pH 9.6). After blocking with phosphate buffered saline (PBS) containing 3% skim milk (PBS-SM) for 1 hour at 37^0C, plates were washed 1 time with PBS containing 0.05% Tween 20 (PBS-T), and 50 µl of serum samples diluted in 1:500 with PBS-SM were added to duplicate wells. Plates were incubated at 37^0C for 1 hour. After washing 6 times with PBS-T, plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG diluted at 1:4000 with PBS-SM at 37^0C for 1 hour. Plates were washed 6 times, then substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2 and 0.3 mg/ml 2,2'–azide-bis[3-ethylbenzthiazoline-6-sulforinc acid] (Sigma) was added to each well in 100 µl aliquots. The absorbance value at 415nm (OD_{415}) was measured after 1- hour of incubation at room temperature using an ELISA reader (Corona Microplate reader MTP- 120; Corona).

An indirect fluorescent antibody test (IFAT): Confirmation of the localization of NcCyp18 in the parasite was explored using coverslips of confluent vero cell infected with *N. caninum* tachyzoites. The coverslips were collected 24 hours after parasite inoculation, washed twice with PBS, and then fixed with 3% paraformaldehyde in PBS. After washing twice with PBS, the cells were permeabilized with 0.3% Triton X-100 in PBS for 5 min at room temperature. After washing, the

coverslips were incubated with 3% bovine serum albumin (BSA) in PBS at room temperature for 30 min. The coverslips were incubated with anti-NcCyp18 mouse serum diluted 1:100 in 3% BSA in PBS for 30 min at room temperature. After washing three times with PBS, the coverslips were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (Sigma) diluted 1:200 in 3% BSA in PBS for 30 min at room temperature and then washed again. The coverslips were placed onto a glass slide coated with Mowiol (Calbiochem). The slides were examined using a fluorescence microscope.

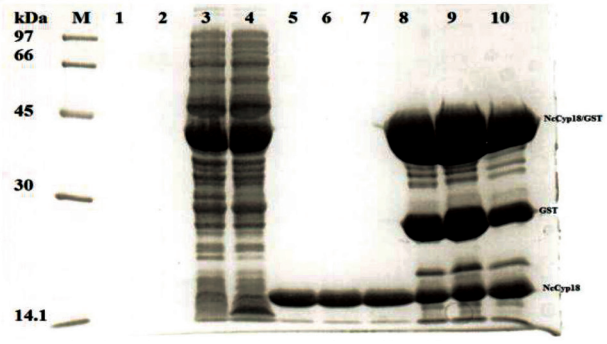
Monolayer cultures of peritoneal macrophages: Mouse peritoneal macrophages were collected from wild-type mice and CCR5 KO mice at 4 days after intraperitoneal injection with 1 ml of 4.05% brewer modified BBL™ thioglycolate medium (Becton Dickinson), by peritoneal washing with 5 ml of cold PBS. After harvesting, the cells were centrifuged at 800 x *g* for 10 min and suspended in RPMI-1640 medium or Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS.

Lymphocyte and macrophage proliferation assay: The macrophages and spleen cells were then added to 96-well-tissue culture plate at 5 Ч 10^5 cells/ well. Macrophages and spleen cells were cultured with rNcCyp18 for 24 and 48 hours, respectively. The proliferation was measured using a cell counting kit-8 (Dojindo Laboratories) and optical density was determined at 450 nm (Corona Electric).

Migration assays: Cell migration was assessed using 24-well chemotaxis chambers and inserts with 8-μM pore polycarbonate filters (Falcon, Beckton Dickinson). Macrophages or spleen cells (1 Ч 10^6) from B6 and CCR5 KO mice suspended in 0.4 ml of RPMI-1640 supplemented with 10% FBS, were applied to the upper compartment of the chamber. In the lower chamber, NcCyp18 and MIP-3α (R&D Systems) in 1.2 ml RPMI-1640 supplemented with 10% FBS, were added as chemoattractants. The cells that migrated into the lower compartment were counted by means of the microscope. Each treatment was performed in triplicate. Results were expressed as the percentage of macrophages or spleen cells in the lower compartment in relation to the number of total macrophages or spleen cells used in the experiment as follows: The percentage of migrating cells = (No. of cells in the lower chambers / total cell No) Ч 100.

RESULTS

SDS- PAGE of rNcCyp18 and production of mouse sera against rNcCyp18: A specific band 18 kDa of the rNcCyp18 without GST was detected in SDS –PAGE using 15% acrylamide gel. an indirect ELISA test was performed to confirm the production of antibodies against rNcCyp18. The result showed that mouse antisera against rNcCyp18 were produced successfull y.



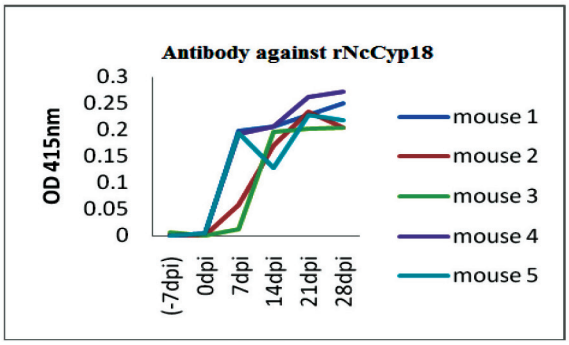


FIG. 1: SDS-PAGE of rNcCyp18. M: molecular marker, lanes 1 and 2: 1st supernatant of culture medium, lanes 3 and 4: pellet after sonication, lanes 5, 6 and 7: purified rNcCyp18, lanes 8, 9 and 10: rNcCyp18 with glutathione sepharose 4B beads.

FIG. 2: The ELISA results of antibodies against rNcCyp18. Antibody production in mice serum started to increase from the day of imminization.

Localization of NcCyp18: It was shown that rNcCyp18 localized in endoplasmicreticulum of *N. caninum* tachyzoites.

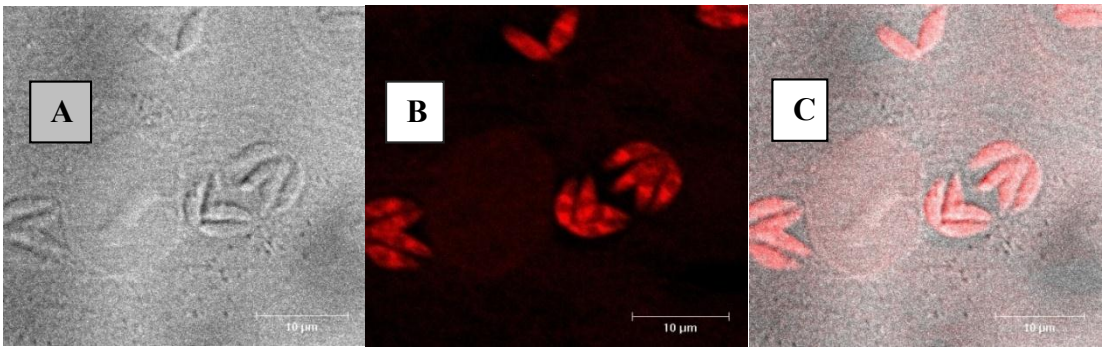


FIG. 3. Localization of rNcCyp18. Vero cells were infected with tachyzoites of Nc-1 strain followed by immunofluorescence labeling. NcCyp18 localized within endoplasmic reticulum. (Scale bar = 10μm)

Effect of NcCyp18 on proliferation of spleen cells:
The mitogenic biological effects of NcCyp18 were evaluated on spleen cells. The highest dose of NcCyp18 (50 μg/ml) had significant effects on the proliferation of these cells (FIG. 4A). Significant proliferative responses were observed after stimulation with 50 μg/ml of NcCyp18 in wild-type spleen cells compared with CCR5 KO spleen cells (FIG. 4B). The result from the 50 μg/ml NcCyp18 stimulation in wild- type and CCR5 KO spleen cells may give some evidence about the dependence on CCR5.

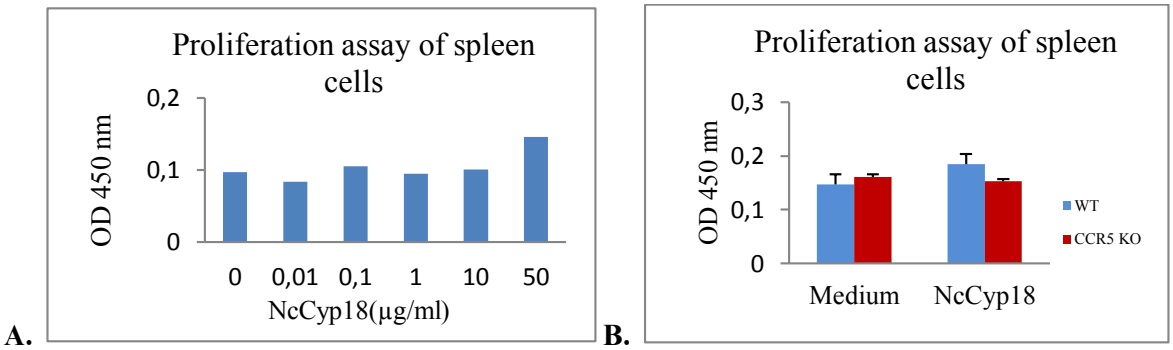


FIG. 4. (A) The cell suspensions of wild-type spleen cells were treated with 0, 0.01, 0.1, 1, 10, 50 μg/ml of rNcCyp18 and further incubated at 37^{0}C for 48 hours. (B) Spleen cell suspension of wild-type and CCR5 KO mice were treated with 50 μg/ml of rNcCyp18 and further incubated at 37^{0}C for 48 hours. The proliferation was measured using a cell counting kit-8. The value represents mean of duplicate sample (A) and the mean ± standard deviation of data from triplicate samples (B).

Effect of rNcCyp18 on migration of spleen cells:
To investigate the ability of rNcCyp18 on migration of spleen cells, wild-type cells were placed in the upper chamber and permitted to migrate to selected concentration of rNcCyp18 (FIG. 5A). The rNcCyp18 elicited a typical chemotactic response compared with the negative control. The migration of wild-type cells and CCR5 KO cells using different chemoattractants (rNcCyp18 and MIP-3α) was evaluated (FIG. 5B). This result indicated that spleen cells were migrated by NcCyp18 in a CCR-5 dependent way.

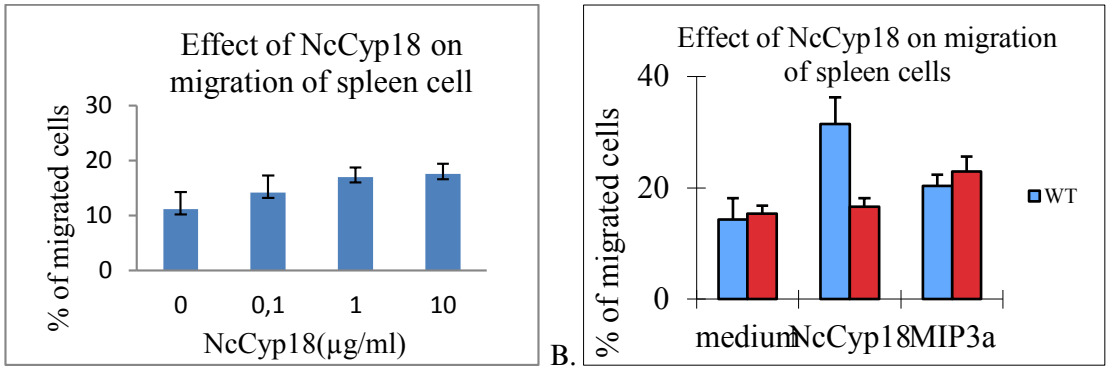


FIG. 5. (A) Spleen cells (1 Ч 10^6) from wild-type were applied to the upper compartment of the chamber. In the lower chamber, rNcCyp18 at the indicated concentration was added. (B) Spleen cells (1 Ч 10^6) from wild-type and CCR5 KO mice were applied to the upper compartment of the chamber. In the lower chamber, rNcCyp18 (50 µg/ml) and MIP-3α (0.25 µg/ml) were added. Each treatment was performed in triplicate. The cells that migrated into the lower compartment were counted by means of the microscope.

Effect of rNcCyp18 on macrophage proliferation: The mitogenic biological effects of rNcCyp18 were evaluated on peritoneal macrophages. High doses of rNcCyp18 (10 and 50 µg/ml) had minimal effects on the proliferation of these cells (FIG. 6A). Moreover, minimal proliferative responses were observed after stimulation with 50 µg/ml of rNcCyp18 in wild-type macrophages compared with CCR5 KO macrophages (FIG. 6B).

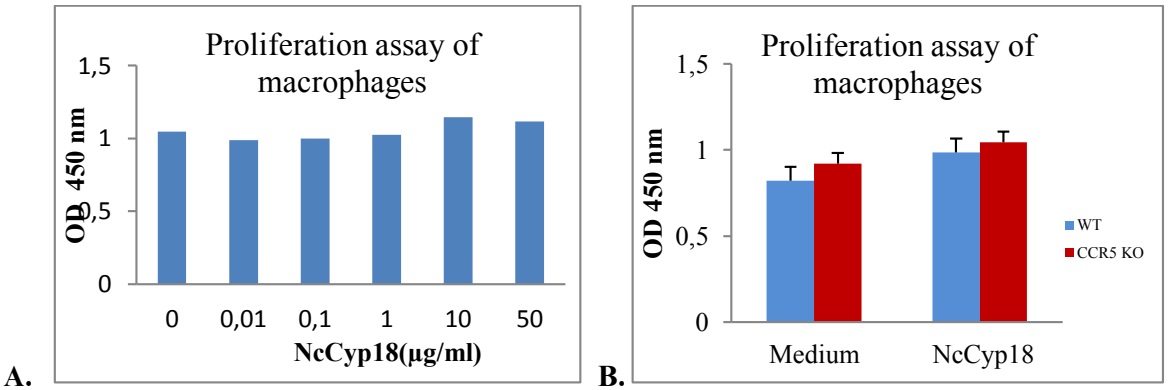
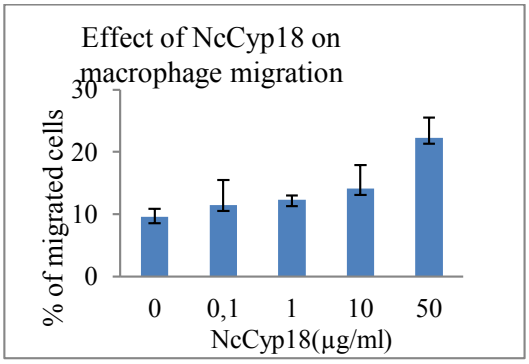


FIG. 6. (A) Macrophage of wild-type mice were treated with 0, 0.01, 0.1, 1, 10, 50 µg/ml of rNcCyp18 and further incubated at 37^0C for 24 hours. Macrophages of wild-type and CCR5 KO mice were treated with 50 µg/ml of rNcCyp18 and further incubated at 37^0C for 24 hours. The value represents the mean of duplicate samples (A) and mean ± standard deviation of data from triplicate samples (B). The proliferation was measured using a cell counting kit-8.

Effect of rNcCyp18 on macrophage migration: To investigate the ability of rNcCyp18 on macrophages migration, wild-type cells were placed in the upper chamber and permitted to migrate to selected concentrations of rNcCyp18 (FIG. 7A). The rNcCyp18 elicited a typical chemotactic response compared with the negative control (no rNcCyp18). The migration of wild-type cells and CCR5 KO cells using different chemoattractants (rNcCyp18 and MIP-3α) was evaluated (FIG. 7B). This result indicated that NcCyp18 recruited macrophages in a CCR5-independent manner.

A.



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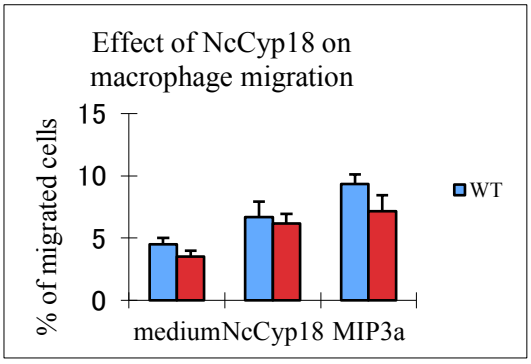

FIG. 7. Macrophages (1Ч10^6) from wild-type mice were applied to the upper compartment of the chamber. In the lower chamber, rNcCyp18 at the indicated concentration was added. (B) Macrophages (1 Ч 10^6) from wild-type and CCR5 KO mice were applied to the upper compartment of the chamber. In the lower chamber, rNcCyp18 (50 µg/ml) and MIP-3α (0.25 µg/ml) were added. The cells that migrated into the lower compartment were counted by means of the microscope. Each treatment was performed in triplicate.

DISCUSSION

Specific antibody and cell-mediated immune responses involving proliferation of cells and production of interferon (IFN)- γ have been observed in both naturally infected animals and experimentally infected with either tachyzoites or oocysts. Pro- inflammatory cytokines such as IL-2, IL-12 and IFN- γ, which are important for the generation of T helper (Th)- 1 type responses and are effective at limiting multiplication of *N. caninum*, are potentially damaging and might cause rejection or abortion of the fetus [3]. In this study, we focused on characterization of function of NcCyp18 and effects of rNcCyp18 to stimulate immune systems. NcCyp18 may play an important role in the development of host protective immunity as well as in the induction of abortion when high levels of pro-inflammatory cytokines are elicited. NcCyp18 may be crucial to the understanding of host protective immunity to *N.caninum* infection and the pathogenesis. The production of NcCyp18 from parasites may be one of the survival strategies of the parasite because infected hosts will survive by NcCyp18-stimulated immunity [9].

The cyclophilins have many intracellular functions and can be secreted [8]. Extracellular cyclophilins have demonstrated chemotactic activity in human and mouse leukocytes, including monocytes, macrophages and T-cells. Chemokine receptors and chemokine binding proteins, with the intent of evading effector immune mechanisms through the control of host cell migration [6]. From our study, rNcCyp18 shows effect on migration and proliferation of immune cells (macrophages and spleen cells). The rNcCyp18 showed the activity of macrophage proliferation in a CCR5-independent manner and activity of spleen cell proliferation in a CCR5-dependent manner. Moreover, rNcCyp18 showed the activity of macrophage migration in a CCR5-independent manner and activity of spleen cell migration in a CCR5-dependent manner. During the infection period, however immune competent cells may have ability to eliminate parasites in condition of expression of rNcCyp18. No production mediated by NcCyp18 may trigger the bradyzoite conversion. The effects of NcCyp18 on the cell migration would facilitate the parasite burden, resulted in the infection in a brain, spinal cord and muscle. The data presented herein provide new evidence for the complex interaction between *N.caninum* and the host based on the ability of NcCyp18. The present study will be important to develop the control strategy against neosporosis.

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