The Journal of Veterinary Medical Science

REVIEW

Parasitology

Towards a preventive strategy for neosporosis: challenges and future perspectives for vaccine development against infection with *Neospora caninum*

Yoshifumi NISHIKAWA[1)]*

[1)]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

J. Vet. Med. Sci.
79(8): 1374–1380, 2017
doi: 10.1292/jvms.17-0285

Received: 25 May 2017
Accepted: 19 June 2017
Published online in J-STAGE: 7 July 2017

Abstract. Neosporosis is caused by the intracellular protozoan parasite *Neospora caninum*. This major disease-causing pathogen is responsible for inducing abortion in cattle, and these adverse events occur sporadically all over the world, including Japan. Currently, there are no vaccines on the market against infection with *N. caninum*. Because live and attenuated vaccines against *N. caninum* have had safety and effectiveness issues, development of a next-generation vaccine is urgently required. To develop a vaccine against neosporosis, my laboratory has been focused on the following: 1) understanding the host immune responses against *Neospora* infection, 2) identifying vaccine antigens and 3) developing an effective antigen-delivery system. The research strategy taken in my laboratory will have strong potential to progress current understanding of the pathogenesis of *N. caninum* infection and promote development of a novel subunit vaccine based on the specific vaccine antigen with an antigen-delivery system for controlling neosporosis.

KEY WORDS: adjuvant, cattle, *Neospora caninum*, neosporosis, vaccine

NEOSPORA CANINUM

Neospora caninum is an intracellular apicomplexan protozoan parasite with a close relationship to *Toxoplasma gondii* [5]. Since its first recognition in dogs in Norway in 1984 [4] and the description of it as a new genus and species [8], neosporosis from *N. caninum* has emerged as a serious disease in cattle and dogs worldwide. The *N. caninum* life cycle can be classified broadly into three stages: tachyzoites, bradyzoites (tissue cysts) and sporozoites (oocysts) (Fig. 1). The definitive hosts of *N. caninum* are canids, including dogs, dingoes, gray wolves and coyotes, but not foxes [10, 19, 21]. A variety of animals ranging from birds to mammals including cattle, sheep, horses and deer can act as its intermediate hosts [5, 7]. *N. caninum* can be spread by horizontal transmission of the oocysts shed by the definitive hosts and vertical transmission via the placenta in pregnant animals [5, 7]. When infected with oocysts, the sporozoites released from the gastrointestinal tract wall of the host spread throughout the whole body via parasite conversion from sporozoites to tachyzoites. Although the host immune response controls tachyzoite proliferation at each infection site, the parasites escape from the host immunity in the central nervous system and muscle tissues, resulting in them transforming into bradyzoites [22]. The bradyzoites then slowly multiply to form tissue cysts, which are another source of *Neospora* infection when they are orally ingested by another susceptible animal. Immunocompromised hosts develop severe neosporosis, while immunocompetent animals generally show no obvious clinical signs of infection. Abortion, stillbirth and neurological disease associated with *N. caninum* infection cause major economic problems in the livestock industry worldwide [6], and *Neospora*-infected cattle cannot be used for milking [6]. Vertical transmission of *N. caninum* infection can be maintained over several generations, a major factor for the expansion of the infection [41]. In fact, *Neospora* infections have a worldwide distribution, which includes Japan, and the global economic impact of this infection has been reported [40]. The total annual cost of *N. caninum* infections and abortions is estimated to range from a median US$1.1 million in the New Zealand beef industry to an estimated median total of US$546.3 million impact per annum in the USA dairy population. The estimate for the total median losses for *N. caninum* infections exceeds US$1.298 billion per annum, ranging as high as US$2.380 billion. Therefore, because vaccines against infection with *N. caninum* are not currently available, development of a safe and effective vaccine is urgently needed.

*Correspondence to: Nishikawa, Y.: nisikawa@obihiro.ac.jp

©2017 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/)

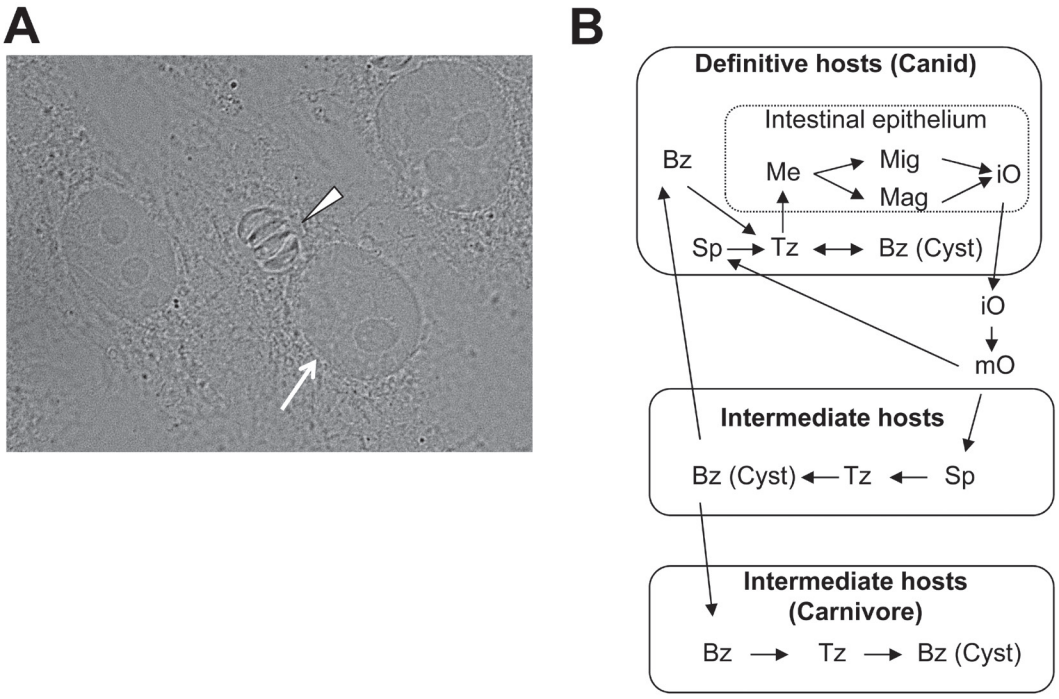

Fig. 1. The *Neospora* life cycle. (A) Phase image of *N. caninum* tachyzoites. Arrowhead: *N. caninum* tachyzoites. Arrow: Host cell nucleus. (B) Life cycle of *N. caninum*. In definitive hosts, bradyzoites or sporozoites convert to tachyzoites. Then, tachyzoites transform into merozoites, and macrogametes and microgametes in the intestinal epithelium undergo merogony as the sexual stage. Next, up to 1 million of the immature oocysts (nonsporulated oocysts) released at the intestinal lumen are shed into the environment with the host feces for 2–30 days after ingestion. The immature oocysts become mature oocysts (sporulated oocysts) outside the definitive host under suitable environmental conditions (i.e., suitable oxygen concentration, humidity and temperature) in 1–5 days. The mature oocysts can infect both definitive and intermediate hosts via horizontal transmission. Sporozoites are released from the oocysts at the duodenal lumen of the host animal and transform into tachyzoites for a multiplication phase, possibly in the mesenteric lymph nodes. The tachyzoites reach the bloodstream, and disseminate to the gravid uterus and many cell types such as those in the central nervous system (CNS), vascular endothelial cells, myocytes, hepatocytes, renal cells, alveolar macrophages and placental trophoblasts. Because of the higher multiplication rate of the tachyzoites and the inflammation response against them, they cause severe tissue lesions. To escape the host immune response, the tachyzoites start to differentiate into bradyzoites and form tissue cysts (asexual stage) in the muscle tissue and CNS during the chronic stage of the infection. The tissue cysts are an infection source for canids and other carnivores. The tissue cysts release bradyzoites into the duodenal lumen. In the intestinal epithelium, bradyzoites transform into tachyzoites. Tz: tachyzoite, Bz: bradyzoite, Sp: sporozoite, Me: merozoite, Mig: microgamete, Mag: macrogamete, iO: immature oocyst (nonsporulated oocyst), mO: mature oocyst (sporulated oocyst).

HOST-IMMUNE RESPONSES AGAINST *N. CANINUM* INFECTION

To develop an effective vaccine against *N. caninum*, systematic research on host-immune responses against this parasite is required. We can use experimental mice to analyze the immune responses and the pathology caused by this parasitic infection. In fact, parasite infiltration of the brain, lungs, liver, spleen and heart is all detectable, and reproducible results for the neurological signs and vertical transmission of the parasite are obtainable in mice [1, 12, 14, 29, 35]. Previous studies have shown that humoral and cellular immunities including a balance of T-helper 1 (Th1) and T-helper 2 (Th2) activity are required for controlling *N. caninum* infection [26, 27]. Interactions between the host cell surface and parasite protein (s) may play crucial roles during invasion of *N. caninum* into host cells. One of the candidates involved in the *N. caninum* invasion process is NcSRS2, a surface antigen of the parasite. Monoclonal antibodies (mAbs) against NcSRS2 partially inhibit *N. caninum* invasion of host cells [33]. The Th2-type immune response, with predominant production of humoral antibodies specific for parasite antigens, is capable of mediating protection against neosporosis [11]. However, transferring antibodies against NcSRS2 to mice does not inhibit the vertical transmission of *N. caninum* [28]. These results suggest that the reactions of specific antibodies alone cannot provide effective control of *N. caninum* infections. Because *N. caninum* exists in the host cell cytosol, the antibodies are not able to bind to the parasites. Nonetheless, because cellular immunity can destroy pathogen infected cells via cytotoxic T cells, this type of host immunity will contribute to the elimination of *N. caninum*-infected host cells. Interferon-gamma (IFN-γ) and interleukin-12 (IL-12), both of which are crucial cytokines for the development of Th1-type immunity (cellular immunity), are important for obtaining protective immunity against acute *N. caninum* infections. In the case of infection with *N. caninum*, the production of IL-12 and IFN-γ is detectable in host animals. Mice deficient in the IL-12 gene or the IFN-γ gene are susceptible to *N. caninum*

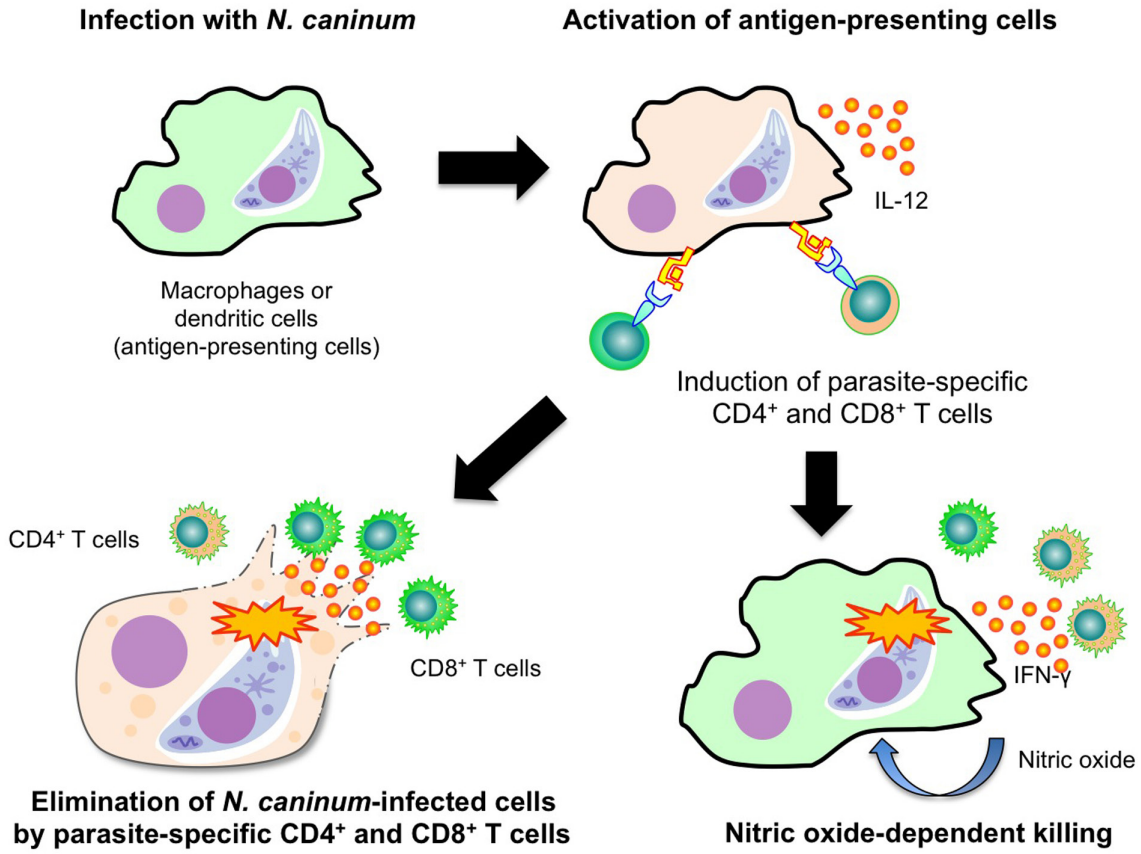

Fig. 2. Protective immunity against infection with *N. caninum*. After becoming infected with *N. caninum*, antigen-presenting cells (APCs), such as macrophages and dendritic cells, phagocytize the parasite or parasite-derived antigens. Then, activation of APCs triggers induction of parasite-specific $CD4^+$ and $CD8^+$ T cells via IL-12 production from the APCs to eliminate the *N. caninum*-infected cells. IFN-γ produced from the activated T cells stimulates nitric oxide production, and this can kill the intracellular parasites.

infection [25, 31], indicating the importance of these cytokines in host protective immunity.

In addition, macrophages play an important role in the cellular effector responses that reduce tissue parasitism and enhance host survival, because nitric oxide production by macrophages treated with IFN-γ can control the growth of *N. caninum* [31]. Moreover, macrophages can produce IL-12, a key cytokine linking the innate and adaptive compartments of the immune system. The normal production of IL-12 is initiated through recognition of highly conserved sets of molecular patterns (pathogen-associated molecular patterns, PAMPs) through pattern-recognition receptors (PRRs) [18]. However, the interaction of these PAMPs with PRRs on innate cells triggered by *N. caninum* infection has not yet been fully elucidated. Previous studies showed that engagement of Toll-like receptor 2 (TLR2), TLR3 and TLR11 triggered activation of the myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways [3, 16, 23]. The CCR5 chemokine receptor is also a key player in the immune response against *N. caninum*, because mice deficient in the CCR5 gene are susceptible to the infection [1]. Additionally, the interaction of CCR5 with parasite-derived cyclophilin triggers migration and activation of innate cells [17, 24]. Thus, MyD88- or TRIF-dependent pathways through TLRs or chemokine receptors can stimulate host immune responses against infection with *N. caninum*.

Based on these observations, the important steps in protective immunity against infection with *N. caninum* are as follows (Fig. 2):

(1) Activation of antigen-presenting cells, such as macrophages and dendritic cells.

(2) Elimination of *N. caninum*-infected cells by parasite-specific $CD4^+$ and $CD8^+$ cells.

(3) Nitric oxide-dependent killing of *N. caninum* by the IFN-γ produced by activated immune cells.

However, proinflammatory cytokines, such as IFN-γ, may also trigger severe pathology in the host. Thus, these observations suggest that a suitable balance in the induction of Th1/Th2-type immunity is crucially important to the effective control of *N. caninum* infection [27].

VACCINE DEVELOPMENT AGAINST *N. CANINUM* INFECTION

To design an effective vaccine against *N. caninum* infection, the induction of both Th1- and Th2-type immunity should be considered. For cattle, the importance of Th1-type cellular immunity has been reported as necessary for the development of

protective immunity [15]. Our previous study showed that IFN-γ production at the early stage of *N. caninum* infection played a crucial role in controlling the parasites in cattle [37]. Therefore, a strategy whereby cellular immunity is induced will be key to vaccine development for these animals.

Although preventing infection in the first place to halt its pathogenesis and abortion in cattle is the ideal way to control neosporosis, controlling abortion and vertical transmission is a practical objective. During the early stages of vaccine development, live or attenuated vaccines are tested, with the expectation that some may show partial protective efficacy [15]. Live vaccines can induce both humoral and cellular immunity, but they carry the risk of increasing the number of carrier animals for *N. caninum*. While attenuated vaccines are relatively safe, adjuvant is needed to induce effective immune responses. Previous trials have used dead parasites or antigen lysates mixed with several adjuvants, such as Havlogen (a polymer of acrylic acid cross-linked with polyallyl sucrose), Polygen (a non-particulate copolymer), a mixture of Havlogen and Bay R-1005 (a preparation of free-base synthetic glycolipids) and Montanide ISA 773 (a water-in-oil emulsion made from a mixture of metabolizable mineral oils) [2]. Although NeoGuard, a Havlogen-adjuvanted killed vaccine, was previously approved by the U.S.A. Dept. of Agriculture [15], it is not currently being sold. Thus, developing a safe and effective subunit vaccine for controlling *N. caninum* infection should be of high priority. The next subjects of discussion for *Neospora* vaccine development are as follows: 1) identifying novel antigens capable of inducing protective immunity against neosporosis and 2) developing a novel antigen delivery system.

IDENTIFICATION OF VACCINE ANTIGENS

My laboratory's strategy for identification of *N. caninum* vaccine antigens is as follows:

(1) Immune screening of *N. caninum* cDNA libraries using parasite-specific mAbs and antigen-specific polyclonal antibodies [33, 46]. The mAbs and polyclonal antibodies are generated by injecting mice with live parasites, parasite lysates or specific antigens, after which they are selected by their inhibitory effects on *N. caninum* invasion of host cells. *N. caninum* cDNA libraries are screened with the selected antibodies via incubation on nitrocellulose sheets containing phage plaques. Positive plaques are visualized using an enzyme detection system (e.g., an alkaline phosphate-conjugated antibody with a suitable substrate). The cloned insert in the plaque-purified λ phage is then subcloned into a plasmid vector for cDNA sequencing.

(2) Immune proteomics of *N. caninum* antigens using parasite-specific anti-sera [43, 46]. For target antigen determination, *N. caninum* lysates are separated using sodium dodecyl sulfate-poly acrylamide gel electrophoresis. A two-dimensional gel electrophoresis (2-DE) and protein identification by mass spectrometry are performed. The proteins separated by 1-DE and 2-DE are visualized with colloidal Coomassie brilliant blue or are transferred onto a membrane for immunoblotting. After spot image analysis, the trypsin-digested peptide mixture from the protein spot is analyzed using MALDI-TOF mass spectrometry.

(3) Screening *N. caninum* antigens using enzyme-linked immunosorbent assay (ELISA) with recombinant antigens and field sample-obtained sera [13]. The ELISA is established using *N. caninum* recombinant proteins expressed in *Escherichia coli.* The reactive antigens are detected using field samples of anti-sera from *N. caninum*-infected cattle, an important step in vaccine development against bovine neosporosis. Moreover, this approach makes correlation analysis between specific-antibody positive animals and abortion possible.

However, identifying antigens using the above-mentioned methods requires specific antibodies. Therefore, the efficacy of cellular immunity induction should be considered by further analyses. In our studies, the reactivity of candidate antigens is tested by T-cell responses using spleen cells or lymphocytes from *N. caninum*-infected animals [29, 36].

DEVELOPMENT OF AN ANTIGEN DELIVERY SYSTEM

In studies on *Neospora* vaccine development, several types of vector, such as vaccinia virus [30, 34], canine herpesvirus [32] and the *Brucella abortus* RB51 strain [38, 39, 42] have been tested as antigen delivery systems. Our previous study showed that immunizing mice with recombinant vaccinia virus expressing NcSRS2, induced cellular immunity against *N. caninum* infection. Especially, the immunizations reduced the cerebral infections and prevented vertical transmission of *N. caninum* in mice [30, 34]. These results suggest that a non-infectious antigen-delivery system that can induce cellular immunity should be efficacious.

Although virus or bacteria-based vectors can induce strong immunity, the safety of the vectors may be problematic. Therefore, the development of a safe antigen delivery system lacking a live pathogen vector is urgently needed. In one of our studies, we focused on developing a next-generation vaccine based on liposomes coated with neoglycolipids that contain oligomannose residues (OMLs) as a novel adjuvant for the induction of Th1 immune responses and cytotoxic T lymphocytes (CTLs) specific for the encased antigen [9]. The liposomes were coated with a neoglycolipid consisting of mannotriose and dipalmitoylphosphatidylethanolamine (otherwise called Man3-DPPE) [20] (Fig. 3). OMLs preferentially take up peripheral phagocytic cells and migrate into lymphoid tissues from the peripheral tissues. Importantly, in response to OML uptake, the phagocytic cells secrete IL-12 selectively and enhance the expression of costimulatory molecules, indicating their adjuvant activity. In addition, OMLs can also deliver encapsulated antigens to the major histocompatibility complex (MHC) class I and MHC class II pathways to generate antigen-specific CTLs and Th1 cells, respectively, and lipid antigen to CD1d to activate natural killer T cells. Thus, OMLs entrapped in *Neospora* antigen have potential as a novel vaccine against *N. caninum* infection.

My laboratory has identified dense granule protein 7 (NcGRA7) and apical membrane antigen 1 (NcAMA1) of *N. caninum* as candidate vaccine antigens [13, 45], and generated OMLs entrapping these antigens. The OML-entrapped NcGRA7 and NcAMA1 induced *N. caninum*-specific cellular and humoral immunity, and prevented the vertical transmission of the parasite in mice

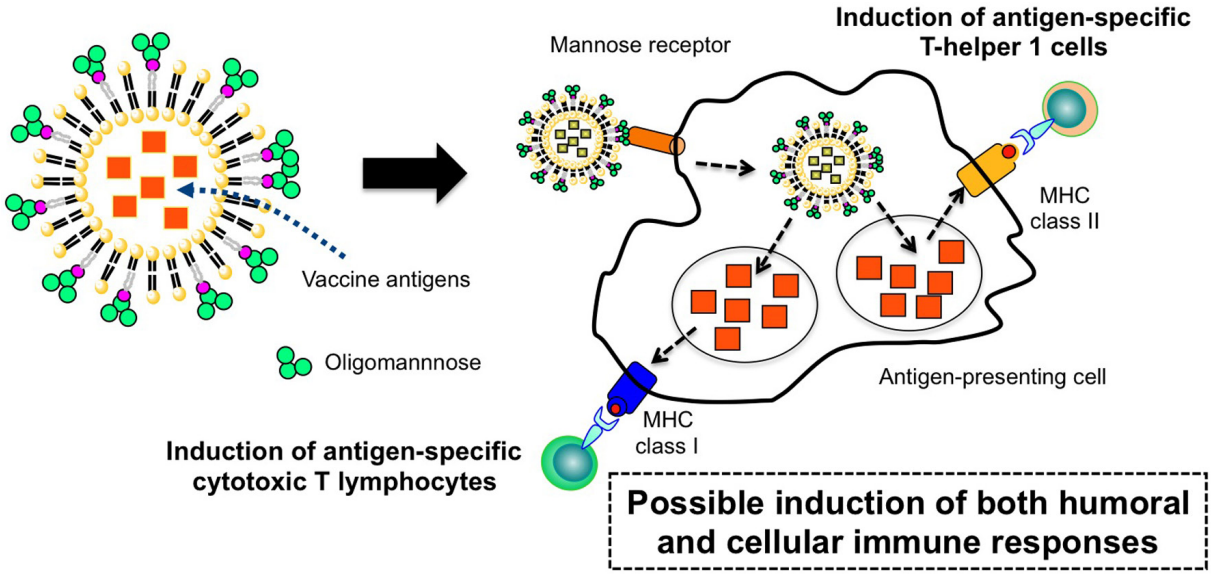

Fig. 3. Mechanism underlying the antigen-specific immune responses induced by liposomes coated with neoglycolipids containing oligomannose residues (OMLs). OML-entrapped target antigens preferentially taken up by peripheral antigen-presenting cells (APCs) in a mannose receptor-dependent manner migrate to the lymphoid tissues. In response to OML uptake, the APCs enhance the expression of costimulatory molecules, and the peptides from the encapsulated antigens are delivered to the major histocompatibility complex (MHC) class I and MHC class II pathways to generate antigen-specific cytotoxic T lymphocytes and T-helper 1 cells, respectively. Thus, OMLs can induce both humoral and cellular immune responses against the encapsulated antigens.

[29, 44]. Moreover, cellular and humoral immunity against *N. caninum* and reduced cerebral infections were observed in cattle immunized with the OML-entrapped NcGRA7 [36]. Therefore, OML can be used as a novel and effective vaccine for controlling vertical transmission and abortion from bovine neosporosis.

FUTURE DEVELOPMENTS

Previous studies have revealed the different aspects of the host–parasite relationship in pregnant cattle [15]. When cattle are experimentally infected with *N. caninum* early in gestation, death of the fetus can occur. Early in gestation, the mother is able to induce a strong cell proliferation response with production of IFN-γ against *N. caninum*. Therefore, abortion related to *N. caninum* infection may be caused by inflammation at the maternal–fetal interface, because the maternal immune responses, such as T cell reactions against infection with *N. caninum*, are induced. However, significant immunomodulation of the immune response occurs at mid-gestation, and this may trigger reactivation of the parasites in persistently infected cattle. Although the Th2 cytokine environment at the maternal–fetal interface is maintained at mid-gestation, it cannot control the *N. caninum* infection, and parasite invasion of the placenta and infection of the fetus occur. At this gestational stage, the parasite infection can trigger the death of the fetus, or the calf, both of which are congenitally infected and show some clinical signs, and they may be born as a result of vertical transmission of the disease. In late gestation, the fetal immune system matures, and it can control the infection. Therefore, the calf is congenitally infected, but otherwise healthy. Thus, the gestational age of the fetus at the time of infection is crucial in determining the severity of neosporosis.

According to what is known about the host–parasite relationship in pregnant cattle, vaccination of such cattle is not realistic. Therefore, vaccinating cattle before pregnancy is a more realistic goal for controlling neosporosis. The next objective is to evaluate the protective efficacy of the OML vaccine on abortion and vertical transmission in cattle induced by *N. caninum* infection. This research strategy will have strong potential to progress current understanding of the pathogenesis of *N. caninum* infection and promote development of a novel vaccine for controlling neosporosis. Use of this approach will shed new light on the basic biology of *N. caninum* and advance *Neospora* research relating to the activities and applications of science to clinical disease.

ACKNOWLEDGMENTS. This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS). I acknowledge the staff in my laboratory (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine) for performing the experiments and for their excellent technical assistance; namely, Dr. Naoya Kojima and Dr. Yasuhiro Kuroda (Department of Applied Biochemistry, Tokai University) for the OML study, and the staff at the Animal Research Center, Hokkaido Research Organization for the cattle experiments.

REFERENCES

1. Abe, C., Tanaka, S., Nishimura, M., Ihara, F., Xuan, X. and Nishikawa, Y. 2015. Role of the chemokine receptor CCR5-dependent host defense system in *Neospora caninum* infections. *Parasit. Vectors* **8**: 5. [Medline] [CrossRef]
2. Andrianarivo, A. G., Choromanski, L., McDonough, S. P., Packham, A. E. and Conrad, P. A. 1999. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *Int. J. Parasitol.* **29**: 1613–1625. [Medline] [CrossRef]
3. Beiting, D. P., Peixoto, L., Akopyants, N. S., Beverley, S. M., Wherry, E. J., Christian, D. A., Hunter, C. A., Brodsky, I. E. and Roos, D. S. 2014. Differential induction of TLR3-dependent innate immune signaling by closely related parasite species. *PLOS ONE* **9**: e88398. [Medline] [CrossRef]
4. Bjerkås, I., Mohn, S. F. and Presthus, J. 1984. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Z. Parasitenkd.* **70**: 271–274. [Medline] [CrossRef]
5. Dubey, J. P. and Schares, G. 2011. Neosporosis in animals—the last five years. *Vet. Parasitol.* **180**: 90–108. [Medline] [CrossRef]
6. Dubey, J. P. and Schares, G. 2006. Diagnosis of bovine neosporosis. *Vet. Parasitol.* **140**: 1–34. [Medline] [CrossRef]
7. Dubey, J. P., Schares, G. and Ortega-Mora, L. M. 2007. Epidemiology and control of neosporosis and Neospora caninum. *Clin. Microbiol. Rev.* **20**: 323–367. [Medline] [CrossRef]
8. Dubey, J. P., Carpenter, J. L., Speer, C. A., Topper, M. J. and Uggla, A. 1988. Newly recognized fatal protozoan disease of dogs. *J. Am. Vet. Med. Assoc.* **192**: 1269–1285. [Medline]
9. Fukasawa, M., Shimizu, Y., Shikata, K., Nakata, M., Sakakibara, R., Yamamoto, N., Hatanaka, M. and Mizuochi, T. 1998. Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of CD8+ cytotoxic T lymphocytes. *FEBS Lett.* **441**: 353–356. [Medline] [CrossRef]
10. Gondim, L. F., McAllister, M. M., Pitt, W. C. and Zemlicka, D. E. 2004. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* **34**: 159–161. [Medline] [CrossRef]
11. Haldorson, G. J., Mathison, B. A., Wenberg, K., Conrad, P. A., Dubey, J. P., Trees, A. J., Yamane, I. and Baszler, T. V. 2005. Immunization with native surface protein NcSRS2 induces a Th2 immune response and reduces congenital *Neospora caninum* transmission in mice. *Int. J. Parasitol.* **35**: 1407–1415. [Medline] [CrossRef]
12. Hiasa, J., Nishimura, M., Itamoto, K., Xuan, X., Inokuma, H. and Nishikawa, Y. 2012. Enzyme-linked immunosorbent assays based on Neospora caninum dense granule protein 7 and profilin for estimating the stage of neosporosis. *Clin. Vaccine Immunol.* **19**: 411–417. [Medline] [CrossRef]
13. Huang, P., Liao, M., Zhang, H., Lee, E. G., Nishikawa, Y. and Xuan, X. 2007. Dense-granule protein NcGRA7, a new marker for the serodiagnosis of *Neospora caninum* infection in aborting cows. *Clin. Vaccine Immunol.* **14**: 1640–1643. [Medline] [CrossRef]
14. Ihara, F., Nishimura, M., Muroi, Y., Furuoka, H., Yokoyama, N. and Nishikawa, Y. 2016. Changes in neurotransmitter levels and expression of immediate early genes in brain of mice infected with *Neospora caninum*. *Sci. Rep.* **6**: 23052. [Medline] [CrossRef]
15. Innes, E. A., Andrianarivo, A. G., Björkman, C., Williams, D. J. and Conrad, P. A. 2002. Immune responses to *Neospora caninum* and prospects for vaccination. *Trends Parasitol.* **18**: 497–504. [Medline] [CrossRef]
16. Jenkins, M. C., Tuo, W., Feng, X., Cao, L., Murphy, C. and Fetterer, R. 2010. *Neospora caninum*: cloning and expression of a gene coding for cytokine-inducing profilin. *Exp. Parasitol.* **125**: 357–362. [Medline] [CrossRef]
17. Kameyama, K., Nishimura, M., Punsantsogvoo, M., Ibrahim, H. M., Xuan, X., Furuoka, H. and Nishikawa, Y. 2012. Immunological characterization of *Neospora caninum* cyclophilin. *Parasitology* **139**: 294–301. [Medline] [CrossRef]
18. Kawai, T. and Akira, S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11**: 373–384. [Medline] [CrossRef]
19. King, J. S., Slapeta, J., Jenkins, D. J., Al-Qassab, S. E., Ellis, J. T. and Windsor, P. A. 2010. Australian dingoes are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* **40**: 945–950. [Medline] [CrossRef]
20. Kojima, N., Ishii, M., Kawauchi, Y. and Takagi, H. 2013. Oligomannose-coated liposome as a novel adjuvant for the induction of cellular immune responses to control disease status. *Biomed. Res. Int.* **2013**: 562924. [Medline] [CrossRef]
21. Lindsay, D. S., Dubey, J. P. and Duncan, R. B. 1999. Confirmation that the dog is a definitive host for *Neospora caninum*. *Vet. Parasitol.* **82**: 327–333. [Medline] [CrossRef]
22. McGuire, A. M., McAllister, M. M., Jolley, W. R. and Anderson-Sprecher, R. C. 1997. A protocol for the production of *Neospora caninum* tissue cysts in mice. *J. Parasitol.* **83**: 647–651. [Medline] [CrossRef]
23. Mineo, T. W., Oliveira, C. J., Gutierrez, F. R. and Silva, J. S. 2010. Recognition by Toll-like receptor 2 induces antigen-presenting cell activation and Th1 programming during infection by *Neospora caninum*. *Immunol. Cell Biol.* **88**: 825–833. [Medline] [CrossRef]
24. Mineo, T. W., Oliveira, C. J., Silva, D. A., Oliveira, L. L., Abatepaulo, A. R., Ribeiro, D. P., Ferreira, B. R., Mineo, J. R. and Silva, J. S. 2010. *Neospora caninum* excreted/secreted antigens trigger CC-chemokine receptor 5-dependent cell migration. *Int. J. Parasitol.* **40**: 797–805. [Medline] [CrossRef]
25. Mineo, T. W., Benevides, L., Silva, N. M. and Silva, J. S. 2009. Myeloid differentiation factor 88 is required for resistance to *Neospora caninum* infection. *Vet. Res.* **40**: 32. [Medline] [CrossRef]
26. Nishikawa, Y., Mikami, T. and Nagasawa, H. 2002. Vaccine development against *Neospora caninum* infection. *J. Vet. Med. Sci.* **64**: 1–5. [Medline] [CrossRef]
27. Nishikawa, Y., Inoue, N., Makala, L. and Nagasawa, H. 2003. A role for balance of interferon-gamma and interleukin-4 production in protective immunity against *Neospora caninum* infection. *Vet. Parasitol.* **116**: 175–184. [Medline] [CrossRef]
28. Nishikawa, Y., Zhang, H., Huang, P., Zhang, G. and Xuan, X. 2009. Effects of a transferring antibody against *Neospora caninum* infection in a murine model. *Vet. Parasitol.* **160**: 60–65. [Medline] [CrossRef]
29. Nishikawa, Y., Zhang, H., Ikehara, Y., Kojima, N., Xuan, X. and Yokoyama, N. 2009. Immunization with oligomannose-coated liposome-entrapped dense granule protein 7 protects dams and offspring from Neospora caninum infection in mice. *Clin. Vaccine Immunol.* **16**: 792–797. [Medline] [CrossRef]
30. Nishikawa, Y., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H. and Mikami, T. 2001. Prevention of vertical transmission of *Neospora caninum* in BALB/c mice by recombinant vaccinia virus carrying NcSRS2 gene. *Vaccine* **19**: 1710–1716. [Medline] [CrossRef]
31. Nishikawa, Y., Tragoolpua, K., Inoue, N., Makala, L., Nagasawa, H., Otsuka, H. and Mikami, T. 2001. In the absence of endogenous gamma interferon, mice acutely infected with *Neospora caninum* succumb to a lethal immune response characterized by inactivation of peritoneal macrophages. *Clin. Diagn. Lab. Immunol.* **8**: 811–816. [Medline]
32. Nishikawa, Y., Ikeda, H., Fukumoto, S., Xuan, X., Nagasawa, H., Otsuka, H. and Mikami, T. 2000. Immunization of dogs with a canine herpesvirus vector expressing Neospora caninum surface protein, NcSRS2. *Int. J. Parasitol.* **30**: 1167–1171. [Medline] [CrossRef]

33. Nishikawa, Y., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H. and Mikami, T. 2000. Monoclonal antibody inhibition of *Neospora caninum* tachyzoite invasion into host cells. *Int. J. Parasitol.* **30**: 51–58. [Medline] [CrossRef]
34. Nishikawa, Y., Inoue, N., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H. and Mikami, T. 2001. Protective efficacy of vaccination by recombinant vaccinia virus against *Neospora caninum* infection. *Vaccine* **19**: 1381–1390. [Medline] [CrossRef]
35. Nishimura, M., Tanaka, S., Ihara, F., Muroi, Y., Yamagishi, J., Furuoka, H., Suzuki, Y. and Nishikawa, Y. 2015. Transcriptome and histopathological changes in mouse brain infected with *Neospora caninum*. *Sci. Rep.* **5**: 7936. [Medline] [CrossRef]
36. Nishimura, M., Kohara, J., Kuroda, Y., Hiasa, J., Tanaka, S., Muroi, Y., Kojima, N., Furuoka, H. and Nishikawa, Y. 2013. Oligomannose-coated liposome-entrapped dense granule protein 7 induces protective immune response to *Neospora caninum* in cattle. *Vaccine* **31**: 3528–3535. [Medline] [CrossRef]
37. Nishimura, M., Kohara, J., Hiasa, J., Muroi, Y., Yokoyama, N., Kida, K., Xuan, X., Furuoka, H. and Nishikawa, Y. 2013. Tissue distribution of *Neospora caninum* in experimentally infected cattle. *Clin. Vaccine Immunol.* **20**: 309–312. [Medline] [CrossRef]
38. Ramamoorthy, S., Sanakkayala, N., Vemulapalli, R., Duncan, R. B., Lindsay, D. S., Schurig, G. S., Boyle, S. M., Kasimanickam, R. and Sriranganathan, N. 2007. Prevention of lethal experimental infection of C57BL/6 mice by vaccination with *Brucella abortus* strain RB51 expressing *Neospora caninum* antigens. *Int. J. Parasitol.* **37**: 1521–1529. [Medline] [CrossRef]
39. Ramamoorthy, S., Sanakkayala, N., Vemulapalli, R., Jain, N., Lindsay, D. S., Schurig, G. S., Boyle, S. M. and Sriranganathan, N. 2007. Prevention of vertical transmission of *Neospora caninum* in C57BL/6 mice vaccinated with *Brucella abortus* strain RB51 expressing *N. caninum* protective antigens. *Int. J. Parasitol.* **37**: 1531–1538. [Medline] [CrossRef]
40. Reichel, M. P., Alejandra Ayanegui-Alcérreca, M., Gondim, L. F. and Ellis, J. T. 2013. What is the global economic impact of *Neospora caninum* in cattle - the billion dollar question. *Int. J. Parasitol.* **43**: 133–142. [Medline] [CrossRef]
41. Schares, G., Peters, M., Wurm, R., Bärwald, A. and Conraths, F. J. 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. *Vet. Parasitol.* **80**: 87–98. [Medline] [CrossRef]
42. Vemulapalli, R., Sanakkayala, N., Gulani, J., Schurig, G. G., Boyle, S. M., Lindsay, D. S. and Sriranganathan, N. 2007. Reduced cerebral infection of *Neospora caninum* in BALB/c mice vaccinated with recombinant *Brucella abortus* RB51 strains expressing *N. caninum* SRS2 and GRA7 proteins. *Vet. Parasitol.* **148**: 219–230. [Medline] [CrossRef]
43. Zhang, H., Lee, E. G., Yu, L., Kawano, S., Huang, P., Liao, M., Kawase, O., Zhang, G., Zhou, J., Fujisaki, K., Nishikawa, Y. and Xuan, X. 2011. Identification of the cross-reactive and species-specific antigens between *Neospora caninum* and *Toxoplasma gondii* tachyzoites by a proteomics approach. *Parasitol. Res.* **109**: 899–911. [Medline] [CrossRef]
44. Zhang, H., Nishikawa, Y., Yamagishi, J., Zhou, J., Ikehara, Y., Kojima, N., Yokoyama, N. and Xuan, X. 2010. *Neospora caninum*: application of apical membrane antigen 1 encapsulated in the oligomannose-coated liposomes for reduction of offspring mortality from infection in BALB/c mice. *Exp. Parasitol.* **125**: 130–136. [Medline] [CrossRef]
45. Zhang, H., Compaore, M. K., Lee, E. G., Liao, M., Zhang, G., Sugimoto, C., Fujisaki, K., Nishikawa, Y. and Xuan, X. 2007. Apical membrane antigen 1 is a cross-reactive antigen between *Neospora caninum* and *Toxoplasma gondii*, and the anti-NcAMA1 antibody inhibits host cell invasion by both parasites. *Mol. Biochem. Parasitol.* **151**: 205–212. [Medline] [CrossRef]
46. Zhang, H., Lee, E. G., Liao, M., Compaore, M. K., Zhang, G., Kawase, O., Fujisaki, K., Sugimoto, C., Nishikawa, Y. and Xuan, X. 2007. Identification of ribosomal phosphoprotein P0 of *Neospora caninum* as a potential common vaccine candidate for the control of both neosporosis and toxoplasmosis. *Mol. Biochem. Parasitol.* **153**: 141–148. [Medline] [CrossRef]