Macrophage Depletion Prior to *Neospora caninum* Infection Results in Severe Neosporosis in Mice

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We observed that murine macrophages showed greater activation and increased interleukin 6 (IL-6), IL-12p40, and interferon gamma (IFN-γ) production during *Neospora caninum* infection. Many macrophages migrated to the site of infection. Furthermore, macrophage-depleted mice exhibited increased sensitivity to *N. caninum* infection. This study indicates that macrophages are required for achieving protective immunity against *N. caninum*.

*Neospora caninum* was first identified as a *Toxoplasmagondii*-like parasite that causes encephalomyelitis and myocarditis in dogs in Norway in 1984 (1). It was classified as a distinct species in 1988 (2). *N. caninum* is a cause of neosporosis, which leads to abortion, neonatal mortality, and congenital infection in cattle and neuromuscular pathology in dogs (3). The ability to survive infection is dependent upon interferon gamma (IFN-γ), the major mediator of resistance against *N. caninum* (4, 5). While IFN-γ-producing CD4+ and CD8+ T cells are required for the acquisition of protective immunity against parasitic infection (6), different types of innate cells may also exert distinct roles in achieving protective immunity. Generally, innate cells, such as natural killer (NK) cells and NKT cells, play essential roles as primary effector cells at the interface between the host and *N. caninum* (7, 8). Moreover, it is well known that the rapid recruitment of macrophages and dendritic cells (DCs) to sites of infection can enhance innate immune responses against *N. caninum* (9). However, these cells may also transport intracellular pathogens, such as *N. caninum* and *T. gondii*, away from the sites of primary infection and facilitate parasite propagation throughout the host (10, 11). However, the role of macrophages in generating protective immunity against *N. caninum* is not well characterized. Therefore, we investigated macrophage activation and the effects of macrophage depletion in *N. caninum*-infected mice.

To test the role of macrophages in protective immunity, mice were infected i.p. with 1 × 10^6 *N. caninum* tachyzoites. At 0, 5, and 10 days postinfection (dpi), mice were euthanized under anesthesia and peritoneal exudate cells were harvested by lavage with 5 ml ice-cold PBS. Peritoneal cells were stained with anti-CD11b MAb and examined by flow cytometry. The absolute number of CD11b+ cells was calculated as follows: absolute cell number = total host cell number × (% CD11b+ cells/100) × (% gated cells by flow cytometry/100). The number of peritoneal CD11b+ cells (monocytes and macrophages) significantly increased at 5 dpi (Fig. 2A), suggesting that migration of macrophages to the site of infection plays a crucial role in achieving protective immunity against *N. caninum*. To investigate whether macrophages play an important role during infection in vivo, macrophages were depleted by two i.p. injections of 300 μl clodronate or PBS liposomes (clodronate encapsulated liposome [Clodrosome]; Haarlem, the Netherlands), administered 3 days and immediately prior to infection of mice with 1 × 10^6 *N. caninum* tachyzoites. Liposomes were handled according to the manufacturer’s instructions. The macrophage depletion was determined by flow cytometry by
staining cells from peritoneal exudates of mice at 3 dpi with CD11b and F4/80 MAbs (Fig. 2B). All mice were monitored for survival and clinical signs of neosporosis, such as head tilting, limb paralysis, circling behavior, and febrile responses (e.g., a starchy stiff coat). In response to N. caninum infection, macrophage-depleted mice showed higher sensitivity, which was associated with early death (Fig. 2C). Neurological signs, including circling motion, head tilting, and leg paralysis, were observed in nine and three mice of PBS liposome-treated and clodronate liposome-treated animals, respectively. The mice did not show particular clinical signs during acute death. In addition, we detected the parasite DNA in the blood of mice treated with clodronate liposome at 1 dpi (clodronate treated, 4 positive in 13 mice; PBS treated, 0 positive in 12 mice).

This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the Obihiro University of Agriculture and Veterinary Medicine. The Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine approved the study protocol. All surgeries were performed while the mice were under isoflurane anesthesia, and all efforts were made to minimize suffering.

In this study, we determined that macrophages play a crucial role in generating protective immunity against N. caninum infection. N. caninum infection triggered IL-12 production and major histocompatibility complex (MHC) class II expression, resulting in the development of parasite-specific CD4+ cytotoxic T lymphocytes (5, 12). Cytotoxic T cells eliminate N. caninum-infected
cells (12). On the other hand, IFN-γ produced from T cells and NK cells stimulates nitric oxide production in the macrophages associated with inhibition of parasite growth (5). Therefore, upon infection, macrophage activation and cytokine production might control parasites at the site of infection and allow the survival of the mice.

Innate immunity is also important to understanding of the host-parasite interactions. T. gondii possesses several unique molecules for stimulating immune responses and cell migration in the host (13). While profilins are actin-binding proteins that in T. gondii stimulate innate immunity in mice by binding Toll-like receptors on DCs, leading to release of inflammatory cytokines, N. caninum profilin also elicited strong IFN-γ and IL-12 responses (14). In addition, innate immune recognition of the excreted/secreted antigens of N. caninum triggered monocytic cell migration to the site of infection in a C-C chemokine receptor 5 (CCR5)-dependent manner (10). Moreover, N. caninum cyclophilin induces CCR5-dependent migration of murine and bovine cells (15). Although the ability of T. gondii to attract, invade, and survive inside immune cells (T cells, DCs, and macrophages), along with the migratory properties of DCs and macrophages that allow parasite dissemination around the host, have been reported (16), the role of immune cell migration is still unclear in N. caninum infection. Thus, study of parasite-derived ligands and host receptors may provide important information to understand host survival and parasitism. Further work will be required to dissect the mechanism of macrophage migration response in host-factors and/or parasite molecules.

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