Protection against Lethal *Neospora caninum* Infection in Mice Induced by Heterologous Vaccination with a *mic1 mic3* Knockout *Toxoplasma gondii* Strain[†]

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***Neospora caninum* and *Toxoplasma gondii* are closely related, obligate intracellular parasites infecting a wide range of vertebrate hosts and causing abortion and neonatal morbidity and mortality. Several lines of evidence suggest that cross immunity between these two pathogens could be exploited in the design of strategies for heterologous vaccination. We assessed the ability of an attenuated strain of *T. gondii* ("mic1-3KO strain") conferring strong protection against chronic and congenital toxoplasmosis to protect mice against lethal *N. caninum* infection. Mice immunized with mic1-3KO tachyzoites by the oral and intraperitoneal routes developed a strong cellular Th1 response and displayed significant protection against lethal heterologous *N. caninum* infection, with survival rates of 70% and 80%, respectively, whereas only 30% of the nonimmunized mice survived. We report here the acquisition of heterologous protective immunity against *N. caninum* following immunization with a live attenuated mic1-3KO strain of *T. gondii*.**

Neospora caninum and *Toxoplasma gondii* are closely related apicomplexans displaying extensive morphological and genetic similarity (37). These cyst-forming coccidians of the family Sarcocystidae (11) cause similar disorders in different animals (15). Despite controversial documentation on their phylogenetic relationship (30), molecular (31) and biological studies have shown that these species have followed different evolutionary paths and have different life cycles and host preferences. Canids are the definitive hosts of *N. caninum*, which causes neosporosis, a major disease of cattle, whereas felids are the definitive hosts of *T. gondii*, which causes toxoplasmosis, a major disease of sheep, goats, and humans (10). Both parasites are responsible for important economic losses in livestock production through neonatal mortality and abortion. *T. gondii* also causes congenital neuropathology and opportunistic infections in immunocompromised humans (41), but there is no conclusive evidence to suggest that *N. caninum* can infect humans (29).

Previous clinical and diagnostic studies have shown that specific antibodies directed against *N. caninum* or *T. gondii* cross-react in serological and immunohistochemical tests, suggesting a possible convergence of immune responses during infections with *T. gondii* and *N. caninum* (32, 38). It has recently been shown that antibodies directed against *N. caninum* antigens

inhibit host cell invasion by both these parasites (22, 43). Similarly, the specific cellular responses stimulated upon experimental infections with *N. caninum* are also stimulated *in vitro* by *T. gondii* antigenic lysate (21, 26). Consistent with these findings, CD8⁺ T cells specific for *N. caninum* have been shown to protect mice against lethal *T. gondii* infection (19). The existence of cross-reactive epitopes between *N. caninum* and *T. gondii* antigens is supported by the high level of sequence identity between conserved proteins (13). A number of cross-reactive antigens have been identified in the micronemes, rhoptries, and dense granules of tachyzoites and in bradyzoites (2, 3, 28, 43). All these observations suggest that the conserved antigenicity between *N. caninum* and *T. gondii* might represent a rational basis for the development of efficient vaccines for the control of both parasitic diseases.

A vaccine based on dead *N. caninum* tachyzoites is currently available for prophylaxis; this vaccine is thought to confer about 46% protection against *N. caninum*-induced abortion in cattle (36). However, in a number of countries, this vaccine has not been licensed, since more complete scientific documentation is required to authorize the use of a vaccine against *N. caninum* (8). The need for a more effective vaccine against transplacental infection in cattle is therefore of the utmost importance. Live vaccines are thought to induce complete protective immunity against *N. caninum* infection. In vaccination trials with the mouse model, the use of *N. caninum* tachyzoite crude extract as the immunogen resulted in an absence of protection against parasite-related neurological illness and death (5, 27). Such vaccinations have also proved ineffective for the prevention of abortion in cattle, even in the presence of adjuvants (42).

Given that protective immunity against intracellular patho-

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TABLE 1. Immunization protocol and survival of mice after lethal challenge^a

Immunization	Route	Dose (no. of parasites)	% Survival ^b (mean survival ^c) after challenge with:	
			5 × 10 ⁷ NC-1 parasites	100 RH parasites
<i>mic1</i> -3KO strain	Oral i.p.	1 × 10 ⁴ 20	80* (25.00 ± 10.54)	100*** (30.00 ± 0.0)
			70 (22.30 ± 12.40)	100*** (30.00 ± 0.0)
NC-1 strain	Oral i.p.	1 × 10 ⁶	70 (21.70 ± 13.37)	30 (11.60 ± 12.74)
		1 × 10 ⁶	0 (3.300 ± 1.418)	0 (2.600 ± 2.066)
Control			30 (13.70 ± 11.28)	0 (7.000 ± 1.155)

^a Twenty-four mice were immunized in each group. On day 30, four mice in each group were killed, and the remaining 20 mice were assigned to two groups. Each group was challenged by intraperitoneal injection with a lethal dose of either homologous or heterologous tachyzoites 50 days after immunization. Mouse survival was monitored daily for 30 days. The data shown are representative of two independent experiments.

^b *, $P < 0.05$; ***, $P < 0.0001$, chi-square test comparing mortality rates between immunized mice and nonimmunized control mice.

^c Mean number of days that animals remained alive ± standard deviation.

gens such as *T. gondii* and *N. caninum* involves T-cell-mediated immunity (12, 21) and that experimental evidence of protection against *N. caninum* transplacental transmission has been shown to involve high levels of gamma interferon (IFN-γ) production (17, 42), we propose an innovative approach based on heterologous vaccination.

Taking into consideration the antigenic similarities between *N. caninum* and *T. gondii*, we used an attenuated strain of *T. gondii* as a heterologous vaccine against *N. caninum*. A mutant RH strain of *T. gondii* tachyzoites lacking the *mic1* and *mic3* genes was constructed in our laboratory, the “*mic1*-3KO strain.” The disruption of these two genes, both of which code for proteins involved in tachyzoite adhesion and invasion, greatly decreases virulence in mice (7). Vaccination with the *mic1*-3KO strain provides strong protection against chronic and congenital toxoplasmosis in mice through the induction of strong humoral and Th1 cellular immune responses (18). In this study, we used this attenuated strain as a heterologous vaccine. Our results provide evidence for protection against lethal *N. caninum* infection. This protection was associated with strong cross-reactive humoral and Th1 cellular immune responses, overcoming the biological and antigenic differences between the two species.

MATERIALS AND METHODS

Chemicals. All biochemical reagents were purchased from Sigma-Aldrich, and culture reagents were purchased from Gibco Invitrogen.

Parasites and parasite antigens. The NC-1 strain of *N. caninum* used in this study was generously provided by S. Romand and P. Thulliez (Laboratoire de la Toxoplasmose, Institut de Puériculture, Paris, France). The attenuated *mic1*-3KO strain of *T. gondii* was constructed in our laboratory by Ceredo et al. (7) and was derived from the highly virulent type I RH strain of *T. gondii*. These strains were used for antigen preparation and mouse infections throughout this study.

Tachyzoites of the NC-1, *mic1*-3KO, and RH strains were grown in human foreskin fibroblast (HFF) cells (Hs27; American Type Culture Collection CRL-1634) at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin under an atmosphere containing 5% CO₂. The culture medium was replaced with serum-free DMEM 24 h before tachyzoites were harvested. Extracellular parasites were harvested and counted in a hemocytometer. DMEM was added to the desired final concentration immediately before mouse infection. The desired parasite load was adjusted to a final volume of 200 µl for oral and 250 µl for intraperitoneal inoculations.

Extracts of *N. caninum* and *T. gondii* were used as antigens for serological enzyme-linked immunosorbent assay (ELISA) tests, *in vitro* stimulation of splenocytes, and rabbit immunizations. Briefly, extracellular tachyzoites were washed, resuspended in phosphate-buffered saline (PBS), and subjected to three

freeze/thaw cycles followed by three rounds of sonication at 60 W for 60 s in a Bioblock 72442 Vibra Cell sonicator, with 5 min of cooling on ice between cycles. The resulting suspension was centrifuged at 2,000 × g for 15 min at 4°C, and the protein concentration in the supernatant was determined using protein assay dye reagent (500-0006; Bio-Rad Laboratories), using bovine serum albumin (BSA) as the protein standard. An extract of uninfected HFF cells was prepared in the same way for use as an additional control. The antigens were stored at -80°C until use.

Mice and experimental design. Six- to 8-week-old female outbred Swiss OF1 mice were obtained from Elevage CER Janvier (Le Genest-St-Isle, France). Mice were assigned to groups of 24 animals and were immunized by intraperitoneal injection (i.p.) or oral inoculation (*per os* [p.o.]) with freshly harvested *T. gondii* or *N. caninum* tachyzoites or were left untreated (Table 1). Mice were bled 30 days after immunization, and sera were tested by ELISA for IgG antibodies against *Toxoplasma* and *Neospora* antigens. On the same day, four of the mice in each group were killed and their spleens were removed for proliferation and cytokine assays. Fifty days after immunization, all groups of mice, including the nonimmunized ones, were split into two equal subgroups, one of which was then challenged intraperitoneally with a lethal dose of homologous parasites whereas the other was challenged intraperitoneally with a lethal dose of heterologous live parasites (Table 1). An additional group of healthy animals was left uninfected. Mouse survival was monitored daily for 30 days after challenge, and the survivors were killed 80 days after immunization. Their brains and spleens were collected for further analyses. Two independent experiments were carried out according to the same protocol. The experimental protocol was approved by our institutional standing ethic committee for animal research according to the European directives (86/609/CEE).

ELISA. Serum samples from immunized and control mice were analyzed for IgG antibodies in ELISA with *T. gondii* and *N. caninum* antigens. Maxisorp (Nunc) microtiter plates were coated by incubation overnight at 4°C with 10 µg/ml antigen in 50 mM sodium carbonate buffer (pH 9.6). The plates were then washed with 0.05% Tween 20 in PBS (PBS-T). Nonspecific binding sites were blocked by incubation with 4% BSA in PBS for 2 h at room temperature (RT). The plates were then incubated with mouse serum diluted 1:100 in PBS-T for 1 h at RT. Plates were washed five times with PBS-T, and bound antibodies were then labeled by incubation with alkaline phosphatase-conjugated anti-mouse IgG at a 1:10,000 dilution in PBS-T for 1 h at RT. The plates were washed, and bound alkaline phosphatase activity was evaluated with 1 mg/ml *p*-nitrophenylphosphate in 1 M diethanolamine buffer (pH 9.8). The optical density at 405 nm (OD₄₀₅) of each sample was determined in a Wallac Victor² 1420 multilabel counter (Perkin Elmer Life Sciences).

Western blotting. *T. gondii* and *N. caninum* tachyzoites were solubilized in 1% Triton X-100 buffer and heated in Laemmli sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.005% bromophenol blue, 100 mM dithiothreitol [DTT] in 62.5 mM [pH 6.8] Tris-HCl) at 100°C for 5 min. The proteins present in the lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels and were transferred onto nitrocellulose membranes with 0.45-µm pores. The nitrocellulose membranes were saturated by incubation overnight with 5% nonfat milk powder in TNT buffer (140 mM NaCl, 0.05% [vol/vol] Tween 20 in 15 mM [pH 8] Tris-HCl) and were then incubated with mouse or rabbit serum, diluted 1:100 and 1:2,000, respectively, in 5% nonfat milk powder in TNT. Bound antibodies were detected by incubation with an anti-mouse or anti-rabbit IgG antibody conjugated to alkaline phosphatase, at a

dilution of 1:10,000 in 5% nonfat milk powder in TNT. Alkaline phosphatase activity was detected with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system.

Rabbit sera were obtained after subcutaneous immunization with NC-1 or *mic1*-3KO antigens in the presence of Freund's complete adjuvant followed by three additional injections of parasite antigens alone. Rabbits were bled 80 days after the last immunization.

In vitro T-cell proliferation. Control and immunized mice were killed after immunization or at the end of the experiment after challenge (80 days postimmunization), and their spleens were removed for proliferation and cytokine assays. Erythrocytes were removed from spleen cell suspensions by hypotonic lysis. The spleen cells were then washed with PBS and suspended at a density of 5×10^6 cells/ml in prewarmed PBS supplemented with 0.1% BSA. Cells were labeled by incubation with 2 μ M carboxyfluorescein succinimidyl ester (CFSE) for 10 min at 37°C. Next, 5% FBS was added, and the cells were incubated for 5 min on ice and washed three times in ice-cold RPMI 1640 medium.

The splenocytes were immediately suspended in RPMI 1640 medium supplemented with 5% FBS, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin. They were used to seed flat-bottomed 96-well microtiter plates (Costar) in duplicate at a density of 5×10^5 cells per well in 200 μ l culture medium containing 10 μ g/ml *T. gondii* antigens, *N. caninum* antigens, concanavalin A (positive control), or HFF cell extract or without antigen (negative control). The plates were incubated for 3 or 6 days under an atmosphere containing 5% CO₂ at 37°C. Cultures were then harvested, washed twice in PBS, fixed with 1% paraformaldehyde, and analyzed with a FACScan flow cytometer with the acquisition of a minimum of 200,000 events for each sample. CellQuest software (Becton Dickinson) was used to analyze the collected fluorescence-activated cell sorter (FACS) data. The lymphocyte population was gated on the basis of forward-scatter and side-scatter properties. The CFSE intensity of gated cells was determined by histogram analysis. The peak position of unstimulated cells was used to establish a cutoff point for antigen-stimulated proliferation. The percentage of total proliferating cells (Tp) obtained was used for further analysis (25).

Cytokine analysis. Spleen cells were cultured in duplicate as described above for the proliferation assay. Cell culture supernatants were collected at various time points, from 24 h to 96 h, and were assayed for IFN- γ , interleukin 2 (IL-2), IL-4, and IL-10 production, using commercial ELISA kits according to the manufacturer's instructions (OptEIA set; BD Pharmingen, San Diego, CA). Cytokine concentrations were determined by reference to standard curves generated with known amounts of mouse recombinant IFN- γ , IL-2, IL-4, and IL-10.

Statistics. The nonparametric Mann-Whitney test was used for the comparison of ODs for IgG, proliferative responses, and cytokine levels within the GraphPadInstat 2 software program. Kaplan-Meier survival curves were analyzed by log rank tests, and survival times were compared with chi-squared tests and the use of contingency tables within the GraphPadInstat 2 software program.

RESULTS

Humoral immune response after immunization. One month after immunization with *T. gondii* or *N. caninum* tachyzoites, we carried out ELISA to assess the seroconversion of mice for each vaccinal strain. We also assessed the presence of cross-reactive antibodies against *N. caninum* and *T. gondii* antigens by ELISA and Western blotting.

(i) Mice immunized with *mic1*-3KO. All the mice immunized with *mic1*-3KO displayed strong specific antibody responses against *T. gondii* antigens in ELISA and Western blotting, regardless of the immunization route used (Fig. 1A [OD = 1.6 ± 0.8 in p.o. and 1.5 ± 0.5 in i.p. immunization groups] and B, lane 1). These responses were statistically significant compared with responses in nonimmunized controls ($P < 0.001$). Interestingly, serum samples from mice immunized with *mic1*-3KO also recognized *N. caninum* antigens in ELISA, reflecting significant cross-reaction, as indicated by the number signs in Fig. 1A (OD = 0.67 ± 0.39 in p.o. groups and 0.62 ± 0.35 in i.p. groups; $P < 0.001$ versus responses in nonimmunized controls). The amounts of antibody recognizing heterologous an-

tigens were significantly smaller than those produced in response to homologous challenge via either of the immunization routes ($P < 0.001$, represented by asterisks). Cross-reaction with *N. caninum* antigens was also observed under reducing conditions on Western blots (Fig. 1B, lane 4).

(ii) Mice immunized with NC-1. ELISA and Western blot analyses of serum samples from mice immunized with NC-1 showed high levels of specific antibodies directed against *N. caninum* in both orally and intraperitoneally immunized groups (Fig. 1C [OD = 1.62 ± 0.42 and 2.25 ± 0.52 , respectively] and D, lane 4). The strongest response was obtained with sera from i.p. immunized mice, with this response being significantly stronger than that obtained by oral immunization ($P < 0.001$). In the NC-1 i.p. immunized group, serum samples also cross-reacted with *T. gondii* antigens in ELISA and Western blotting (Fig. 1C [OD = 1.20 ± 0.60 ; $P < 0.01$, number signs] and D, lane 1). The cross-reactive antibody responses were significantly weaker than specific homologous responses, as shown by ELISA (Fig. 1C, $P < 0.001$, indicated by asterisks). No significant difference in the response to *T. gondii* antigens was found between nonimmunized mice and mice immunized orally with NC-1 (Fig. 1C, OD = 0.18 ± 0.07 and OD = 0.22 ± 0.17 , respectively). This route of immunization therefore does not seem to elicit the production of cross-reactive antibodies.

Thus, p.o. and i.p. immunization with *mic1*-3KO induces the production of large amounts of antibodies able to cross-react with heterologous antigens from *N. caninum* parasite extracts. However, NC-1 immunization elicited cross-reactive humoral responses only after i.p. immunization.

Cellular immune response after immunization. Four mice from each of the immunized and control groups were killed 30 days after immunization, and their spleen cells were assayed for proliferation and cytokine secretion *in vitro* after stimulation with *T. gondii* or *N. caninum* antigens.

(i) Mice immunized with *mic1*-3KO strain. Flow cytometry assessment of CFSE dye dilution was used to evaluate splenocyte proliferative responses. High levels of specific T-cell proliferation were recorded in mice immunized with the *mic1*-3KO strain in both the orally and intraperitoneally immunized groups after stimulation with *T. gondii* antigens (Fig. 2A) ($45.32\% \pm 7.77\%$ and $54.59\% \pm 5.66\%$, respectively; $P < 0.001$ compared with results for nonimmunized mice). As expected, cross-reactive proliferation was also observed *in vitro* in the presence of *N. caninum* antigens in T cells from animals immunized with the *mic1*-3KO strain via either the p.o. or i.p. route (Fig. 2A, $21.29\% \pm 8.22\%$ and $41.32\% \pm 5.08\%$ respectively; $P < 0.001$, indicated by number signs). These responses were significantly weaker than those induced by homologous stimulation in both groups (Fig. 2A, $P < 0.001$; represented by asterisks).

We also studied the cytokine secretion profile of these animals *in vitro*. Immunization with *mic1*-3KO induced high levels of IFN- γ secretion in animals immunized by the oral or intraperitoneal route, as shown following stimulation with *T. gondii* antigens (Fig. 2B, 247.0 ± 183.2 ng/ml and 287.6 ± 114.8 ng/ml, respectively). No significant differences were observed between these two groups. In the same way, large amounts of IFN- γ were produced after heterologous stimulation of these cells with *N. caninum* antigens in both p.o. and i.p. immunized mice (Fig. 2B, 154.3 ± 127.50 ng/ml and 167.1 ± 104.6 ng/ml,

FIG. 1. Humoral responses after immunization. (A and C) Serological ELISA results for mice immunized with the *mic1-3KO* (A) or NC-1 (C) strain. Mice were bled on day 30 after immunization, and serum samples were tested for IgG against *T. gondii* and *N. caninum* antigens. The results for each group are represented as means $\pm$ SD ($n = 24$). Significant differences between heterologous responses and responses in nonimmunized controls are indicated by number signs. Mann-Whitney test: ###, $P < 0.001$. Significant differences between heterologous and homologous responses are indicated by asterisks. Mann-Whitney test: ***, $P < 0.001$. The data shown are representative of two independent experiments. (B and D) Western blot analysis of serum samples from mice immunized with *mic1-3KO* (B) or NC-1 (D) tachyzoites. *T. gondii* and *N. caninum* lysates were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Sera were from mice immunized with live tachyzoites by the i.p. route (lanes 1 and 4), nonimmunized mice (lanes 2 and 5), or rabbits immunized with parasite lysate (3 and 6).

respectively; $P < 0.001$ indicated by number signs). Interestingly, the levels of IFN- γ secretion in *mic1-3KO* strain-immunized mice were statistically similar after homologous and heterologous stimulation (see also Fig. S3 in the supplemental material).

The high level of IFN- γ production induced by *mic1-3KO* immunization was accompanied by marked IL-2 production and an absence of IL-4 secretion in response to *T. gondii* antigens. A similar Th1 cytokine profile was obtained in response to heterologous stimulation *in vitro* with *N. caninum* antigens (see Fig. S1A and S1B in the supplemental material).

Immunization with the *mic1-3KO* strain triggered strong production of the regulatory cytokine IL-10 in both p.o. and i.p. immunized mice (Fig. 2C, 8.45 ± 3.07 ng/ml and 6.20 ± 1.21 ng/ml, respectively). *N. caninum* antigens also induced the secretion of IL-10 through cross-reactive responses (Fig. 2C, 5.13 ± 1.31 ng/ml and 4.99 ± 1.82 ng/ml in p.o. and i.p. immunized mice, respectively; $P < 0.0001$ compared with results for nonimmunized mice). IL-10 secretion differed significantly between the specific and cross-reactive responses only for orally immunized mice (Fig. 2C, $P < 0.01$, indicated by asterisks).

(ii) **Mice immunized with NC-1.** The stimulation of spleen cells from NC-1-immunized mice with *N. caninum* antigens induced high rates of proliferation in both orally and intraperitoneally immunized groups (Fig. 2D, $36.33\% \pm 9.84\%$ in p.o.-immunized mice and $48.63\% \pm 10.75\%$ in i.p. immunized mice; $P < 0.001$ compared with results for nonimmunized mice). The level of proliferation stimulated by cross-reaction *in vitro* with *T. gondii* antigens was higher than that obtained *in vitro* in the presence of *N. caninum* (Fig. 2D, $47.81\% \pm 1.52\%$ in p.o.-immunized mice and $57.03\% \pm 15.13\%$ in i.p. immunized mice; $P < 0.001$, indicated by number signs). Compared with specific stimulation, this difference was significant in mice immunized p.o. ($P < 0.01$, indicated by asterisks in Fig. 2D) but was not significant in mice immunized i.p.

We also studied the cytokine secretion profiles of these animals. *N. caninum* antigenic stimulation *in vitro* induced high levels of IFN- γ secretion in mice immunized by the oral or intraperitoneal route (Fig. 2E, 85.38 ± 51.14 ng/ml and 100.20 ± 59.54 ng/ml, respectively). This cytokine was also produced after heterologous stimulation with *T. gondii* antigens (Fig. 2E, 31.33 ± 5.39 ng/ml in the p.o. group and 51.43 ± 20.34 ng/ml in the i.p. group; $P < 0.001$ compared with results

FIG. 2. Cellular responses after immunization. Four mice per group were killed 30 days after immunization (*mic1*-3KO strain [A to C] or NC-1 [D to F]), and their spleen cells were stimulated *in vitro* with *T. gondii* and *N. caninum* antigens. Cells were labeled with CFSE for the analysis of *in vitro* proliferation (A and D). The culture supernatants were harvested and assayed for IFN- γ (B and E) or IL-10 (C and F) secretion. The results for each group are expressed as the means $\pm$ SD ($n = 4$). Significant differences between heterologous responses and the responses of nonimmunized controls are indicated by number signs. Mann-Whitney test: ###, $P < 0.001$. Significant differences between heterologous and homologous responses are indicated by asterisks. Mann-Whitney test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The data shown are representative of the results for two independent experiments.

for nonimmunized mice, indicated by number signs). However, following homologous stimulation, significantly more IFN- γ was secreted by either p.o. or i.p. immunized mice compared with heterologous stimulation (Fig. 2E, $P < 0.01$ and $P < 0.05$, respectively, indicated by asterisks). We compared IFN- γ production after immunization with the *mic1*-3KO and NC-1 strains. Interestingly, animals immunized with *mic1*-3KO secreted more IFN- γ in response to *N. caninum* antigens *in vitro* than did animals immunized with NC-1 in response to *N. caninum* antigens (Fig. 2B and E, 154.30 ± 127.50 ng/ml versus 85.38 ± 51.64 ng/ml in p.o. groups and 167.10 ± 104.6 ng/ml versus 100.20 ± 59.54 ng/ml in i.p. groups); however, this difference was not significant.

Immunization with NC-1 also induced IL-2 production in

response to homologous and heterologous stimulation *in vitro* with *N. caninum* and *T. gondii* antigens, respectively, but no IL-4 was detected (see Fig. S1C and Fig. S1D in the supplemental material). Animals immunized with NC-1 produced large amounts of IL-10 in response to *N. caninum* antigens (Fig. 2F, 4.87 ± 1.43 ng/ml and 4.80 ± 1.13 ng/ml in the p.o. and i.p. groups, respectively). Significant cross-reactive IL-10 secretion was also observed in both p.o. and i.p. NC-1-immunized mice (Fig. 2F, 6.11 ± 1.13 ng/ml and 5.69 ± 2.13 ng/ml, respectively; $P < 0.001$ compared with results for nonimmunized mice, indicated by number signs). No differences between homologous and heterologous stimulation were observed in p.o. and i.p. immunized mice.

Thus, our results show that the immunization of mice with

FIG. 3. Survival rates after lethal challenge. Ten mice were immunized per group, and 50 days after immunization, mice were infected by intraperitoneal injection of lethal doses of NC-1 (A) or RH (B) tachyzoites. Mouse survival was monitored daily for 30 days. The results shown are representative of two independent experiments. See also Fig. S4 in the supplemental material.

the *mic1*-3KO strain triggers a strong IFN- γ response accompanied by the production of large amounts of the regulatory cytokine IL-10. A similar cytokine response was obtained after NC-1 immunization, except that less IFN- γ was produced. No significant difference in cytokine production was observed between animals immunized orally and intraperitoneally for either parasite. Cross-reactive cell responses were observed in mice immunized with the *mic1*-3KO strain and in mice immunized with NC-1, demonstrating the feasibility of spleen cell cross-activation by *N. caninum* and *T. gondii* in mice. Cross-reactive responses appeared to be parasite dependent, since no responses were observed in cultures grown without parasite antigen (RPMI or HFF controls; see Fig. S2 in the supplemental material) or in cultures of stimulated and nonstimulated spleen cells from nonimmunized mice.

Protection of immunized mice against lethal challenge. Having demonstrated that the *mic1*-3KO strain induced a strong Th-1 cross-reactive immune response against *N. caninum*, we evaluated the strength of the protection afforded by this immunization against lethal doses of NC-1 tachyzoites and compared it with that afforded by NC-1 immunization.

(i) Mice challenged with NC-1. Fifty days after immunization, we injected a lethal dose of NC-1 tachyzoites i.p. into five groups of mice (Table 1). Mice immunized orally or intraperitoneally with the *mic1*-3KO strain were protected against this lethal heterologous challenge and presented survival rates of 80% and 70%, respectively on day 30 postinfection (Table 1 and Fig. 3A). In the groups of animals immunized with the NC-1 strain (homologous protection), two different situations were observed: animals immunized by the oral route were protected against the lethal challenge with a survival rate of 70% on day 30 postinfection, whereas animals immunized intraperitoneally were particularly susceptible to the challenge and all the animals died. Only 30% of nonimmunized control mice survived the lethal infection, with deaths occurring within 9 days following lethal injection, (Table 1 and Fig. 3A; see also Fig. S4 in the supplemental material).

(ii) Mice challenged with RH. We also evaluated the protection conferred by *mic1*-3KO and NC-1 immunization against a lethal dose of *T. gondii*. Both groups of mice immunized with *mic1*-3KO parasites by the oral or intraperitoneal route were fully protected against lethal challenge. All protected mice survived for 30 days and were then killed for further analysis (Table 1 and Fig. 3B). Conversely, the two groups of mice

previously immunized with NC-1 tachyzoites were extremely susceptible to RH challenge: all mice immunized i.p. died, and only 30% of the mice immunized orally survived. Deaths were recorded very early, from day 1 to day 5 after injection of the lethal dose in both groups (Table 1 and Fig. 3B). Finally, all the animals in the nonimmunized group died between 6 and 9 days after infection.

Thus, oral immunization with *mic1*-3KO provided protection as strong as that from homologous immunization with the NC-1 strain against *N. caninum* lethal infection (survival rates of 80% versus 70% respectively; $P < 0.05$ in *mic1*-3KO strain-immunized animals compared with results for nonimmunized mice). Intraperitoneal immunization with the *mic1*-3KO strain also provided high levels of heterologous protection (70% survival). In addition, *mic1*-3KO strain immunization provided complete homologous protection against a highly virulent *T. gondii* strain ($P < 0.001$ in both the p.o. and i.p. groups compared with results for nonimmunized mice).

Cellular immune response in surviving mice. Having demonstrated the protection against heterologous infection, we assessed the cytokine profile involved in the mechanism of protection against NC-1 or RH tachyzoites by killing animals 30 days after challenge with the lethal dose and analyzing cytokine secretion in spleen cells.

(i) Mice challenged with NC-1. We focused on the mechanism of heterologous protection induced during NC-1 infection after immunization with the *mic1*-3KO strain. We analyzed the levels of cytokines produced after encounters with the two parasites (immunization and challenge) and compared them with the values obtained 30 days after immunization. In mice immunized with the *mic1*-3KO strain, there was no significant difference in IFN- γ levels before and after *N. caninum* challenge (Fig. 4A, 154.3 ± 127.5 ng/ml versus 156.3 ± 122.1 ng/ml in the p.o. group and 167.1 ± 104.69 ng/ml versus 98.6 ± 35.2 ng/ml in the i.p. group). A nonsignificant increase in IFN- γ production after *N. caninum* infection was observed in mice previously immunized with NC-1 by the p.o. route (Fig. 4A, 85.4 ± 51.1 ng/ml versus 187.1 ± 118.5 ng/ml). Conversely, IL-2 production increased considerably after *N. caninum* challenge in mice previously immunized with *mic1*-3KO (Fig. 4B, 100.1 ± 34.9 pg/ml versus 241.4 ± 83.4 pg/ml in the p.o. group and 212.3 ± 83.6 pg/ml versus 373.1 ± 144.7 pg/ml in the i.p. group; $P < 0.001$ and $P < 0.05$, respectively, indicated by asterisks). In mice immunized p.o. with NC-1, IL-2 levels were

FIG. 4. Cytokine responses in surviving mice. On day 30 after challenge, the mice were killed and their spleen cells were isolated and stimulated *in vitro* with *N. caninum* and *T. gondii* antigens. (A to C) IFN- γ (A), IL-2 (B), or IL-10 (C) secretion after NC-1 challenge. (D to F) IFN- γ (D), IL-2 (E), or IL-10 (F) secretion after RH challenge. The results for each group are expressed as the means $\pm$ SD; n = number of surviving mice in each group. Significant differences in the responses obtained before and after challenge are indicated by asterisks. Mann-Whitney test: *, $P < 0.05$; ***, $P < 0.001$. The data shown are representative of the results of two independent experiments.

lower after *N. caninum* challenge than after immunization (Fig. 4B, 288.8 ± 156.1 pg/ml before and 192.5 ± 97.2 pg/ml after challenge).

The regulation of these Th-1 cellular immune responses was achieved, to some extent, by IL-10 secretion. Mice immunized by the oral route produced similar amounts of IL-10 after lethal challenge and immunization. Conversely, IL-10 secretion in mice immunized i.p. with *mic1*-3KO decreased after *N. caninum* infection (Fig. 4C, 5.09 ± 1.69 ng/ml before and 2.60 ± 0.38 ng/ml after challenge; $P < 0.001$). Finally, IL-4 production was not induced after *N. caninum* challenge in any of the groups of mice (data not shown).

(ii) Mice challenged with RH. Animals surviving the injection of a lethal dose of RH had similar levels of IFN- γ and IL-2 secretion after challenge and after immunization (Fig. 4D and E). Markedly lower levels of IL-10 secretion after lethal challenge than after immunization were observed in animals immunized with *mic1*-3KO tachyzoites by the oral route (Fig. 4F, 8.45 ± 3.08 ng/ml versus 3.63 ± 1.49 ng/ml; $P < 0.001$, indicated by asterisks).

DISCUSSION

In this study, we have demonstrated that heterologous immunization with an attenuated *T. gondii* strain induced protec-

tive immunity against the closely related pathogen *N. caninum*. Previous studies have provided evidence of humoral and cellular cross-reactivity between *T. gondii* and *N. caninum* in different hosts (16, 19, 21, 32), but the extent to which *T. gondii* immunization could protect against subsequent *N. caninum* infection has never before been investigated using the mouse model. Whereas in a pregnant-ruminant model of *N. caninum* infection, a *T. gondii* vaccinal strain failed to induce protection against *N. caninum*-induced abortion (16), mice infected with *N. caninum* were protected against lethal toxoplasmosis (19). The present report is the first to show that *T. gondii* (*mic1*-3KO strain) can protect against *N. caninum* lethal infection in mice.

The murine model of *N. caninum* infection is widely used for analysis of the immune responses and protection obtained in vaccination trials (34). Although *N. caninum* natural infection in the murine host is less prevalent than *T. gondii* infection (14), it represents an important model for the initial validation of the concept of protection induced by new vaccine candidates, yet protection results obtained with murine models should be interpreted with the utmost care (34). Oral immunization with *N. caninum* tachyzoites has recently been shown to be a valuable system for the establishment of specific immune responses in immunocompetent mice. Oral immunization with tachyzoites appears to be very close to one of the natural routes of *N. caninum* infection but with a parasitic stage more easily obtained than the oocyst (39). The infectivity of *T. gondii* tachyzoites administered by the oral route was originally documented by Dubey et al., who successfully established infection with 1,000 extracellular tachyzoites (9). We undertook a series of pilot experiments to determine effective immunization doses for *mic1*-3KO and NC-1 tachyzoites by the oral route in groups of female Swiss mice (data not shown). We then carried out immunization trials to study their capacity to induce protection against heterologous or homologous lethal challenge following both intraperitoneal and oral immunization. The *mic1*-3KO strain induced heterologous and homologous protective immune responses whether administered orally or intraperitoneally. Unexpectedly, only the oral immunization with the NC-1 strain induced homologous protection; however, it is remarkable that the early deaths observed in mice immunized with NC-1 were too soon after the challenge to be caused by the parasites and probably reflect the development of an adverse response in immunized mice against the challenge. However, we did not conduct a postmortem examination of these mice to study the immunological mechanism associated with these deaths.

We found that *T. gondii mic1*-3KO strain immunization triggered a strong Th1 immune response that was found to be in correlation with high rates of protection against an *N. caninum* lethal challenge, with 80% and 70% survival after p.o. and i.p. immunization, respectively. The heterologous protection induced by a commercial strain of *T. gondii* was previously studied by Innes et al. using a ruminant model for congenital infection with *N. caninum*. This vaccination failed to protect against an *N. caninum* challenge at 90 days of gestation (16). The authors discuss the fact that under their challenge conditions, the immunity generated against the antigens common between *T. gondii* and *N. caninum* was insufficient to protect against fetal death, but if their challenge had been milder, it

might allow detection of a biological effect. Indeed, even if heterologous protection was not reached during this vaccination trial, various indications of the induction of heterologous immunity following immunization were evident.

We evaluated the heterologous cellular immune response in protected *mic1*-3KO strain-immunized animals and showed that high IFN- γ and IL-2 levels were present in mice that controlled the lethal infection. Interestingly, IFN- γ secretion remained at the same high levels before and after NC-1 lethal challenge, whereas IL-2 secretion presented an important increase during *N. caninum* infection. Another interesting finding in mice surviving an *N. caninum* lethal challenge was the difference in IL-10 secretion between groups immunized with the *mic1*-3KO strain by the p.o. and i.p. routes. This regulatory cytokine was significantly downregulated in mice previously immunized by intraperitoneal injection, whereas no such downregulation was observed in mice immunized by the oral route. However, protection levels were similar despite these differences in the response. This finding suggests that different regulatory mechanisms may be triggered by these two routes of immunization.

In accordance with this, two different situations were observed after homologous challenge in the groups of animals immunized with the NC-1 strain: animals immunized by the oral route were capable of controlling the lethal infection, whereas animals immunized intraperitoneally died. The early deaths observed within 5 days postchallenge were likely due to an adverse response to the challenge rather than to parasite activity. Interestingly, levels of cytokines produced in response to *N. caninum* antigens were similar in animals immunized by either the oral or the intraperitoneal route with the NC-1 strain (IFN- γ , IL-2, IL-10, and IL-4); the only difference found between these two groups of animals was a stronger humoral response in mice immunized i.p. than in mice immunized p.o. In this last group, the protection was associated with an increase in IFN- γ production after NC-1 challenge, whereas the IL-2 levels remained low. In contrast to the oral immunization, the intraperitoneal immunization with NC-1 increased the susceptibility of immunized animals to *N. caninum* infection compared with nonimmunized controls. Interestingly, increased susceptibility in terms of mortality has already been found in immunized mice challenged with sublethal doses of *N. caninum*. Depending on the mouse strain used, this susceptibility was associated either with a Th2 immune response in BALB/c mice (27) or with an excessive proinflammatory response in C57BL/6 mice (35). Furthermore, immunization with irradiated *N. caninum* tachyzoites has been shown to induce either protection or lethal susceptibility depending on both the route of immunization (intraperitoneal or intradermal, respectively) and the mouse strain used (BALB/c or C57BL/6, respectively) (33, 40). Contrary to our results, it has been recently shown that intraperitoneal inoculation of BALB/c mice with live attenuated *N. caninum* tachyzoites protects against *N. caninum* intraperitoneal lethal challenge. This protection was associated with a modulated Th1 immune response, showing the importance of a balanced response to avoid immunopathology during *N. caninum* infection (4). It is evident that the differential induction of immune mediators associated with the immunization route and with the parasite itself determines the development of protective immunity. Teixeira et al. compared the immune response elicited by *N. caninum* after intraperitoneal

or intragastric immunization and found very similar levels of IFN- γ and IL-10 mRNA production, in agreement with our results (39). After intraperitoneal and intragastric immunization, both effector and T-regulatory CD4⁺ CD25⁺ cells were found to be expanded in the spleen of immunized mice (39). Accordingly, different regulatory cell types can be triggered in the site of immunological encounter in order to activate regulatory mechanisms that can account for the development of protective immunity in relation to the immunization route. For instance, the intestinal mucosa can stimulate a noninflammatory Th17 response that has been shown to be important in mucosal protective immunity (1, 20). Further studies of systemic and mucosal immune responses during *N. caninum* infection, including the study of the regulation of local responses at different time points after infection, are needed, although these studies are beyond the scope of this report on heterologous immunity.

In the study described here, we also found high mortality rates in mice immunized with NC-1 tachyzoites after lethal RH challenge. As mentioned above, it is noteworthy that in our study, 60% of mice immunized intraperitoneally with NC-1 died in the first 24 h after the RH challenge; these deaths are too soon to be caused by the parasite and are more indicative of an adverse response against the challenge in immunized mice. Lindsay et al. showed that immunization of mice with *N. caninum* provides no protection against pathogenic *T. gondii* strains; however, cross-protection may not have been observed in these studies due to the large numbers of parasites used for challenge (2.5×10^4) (23, 24). On the contrary, Kasper and Khan showed that *N. caninum* protects A/J mice against lethal challenge with low doses (5×10^2) of RH tachyzoites (19). These mice produced significant quantities of IL-12 and IFN- γ shortly after infection with *N. caninum*, and similar amounts of IFN- γ were produced when their splenocytes were stimulated with either *T. gondii* or *N. caninum* antigens (21). By contrast, we observed that splenocytes from Swiss mice immunized either i.p. or p.o. with *N. caninum* secreted less IFN- γ when stimulated with *T. gondii* than when stimulated with *N. caninum* antigens. This difference can reflect the involvement of the genetic background of the animals in the acquisition of heterologous protection. This result may also suggest the activation of cross-reactive T cells immunodominant for *N. caninum* antigens. These cross-reactive memory cells induced by *N. caninum* immunization might prevent the development of more-effective high-affinity T cells responding to the normally immunodominant *T. gondii* epitopes (6).

In conclusion, we demonstrate here that the immunization of mice with the *mic1*-3KO strain protects against lethal *N. caninum* and *T. gondii* infections. The results obtained suggest a correlation between protection and the development of cellular type Th-1 cross-reactive and specific responses, respectively. The protection obtained in this acute model of infection therefore encourages further studies focused on the protection provided by the *mic1*-3KO strain against persistent and transplacental *N. caninum* infection. This attenuated strain can also be exploited as a vector for the expression of immunogenic *N. caninum* antigens in order to strengthen their immunogenic potential.

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