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Vaccination with the recombinant chimeric antigen recNcMIC3-1-R induces a non-protective Th2-type immune response in the pregnant mouse model for *N. caninum* infection

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ABSTRACT

The major route of transmission of *Neospora caninum* in cattle is transplacentally from an infected cow to its progeny. Therefore, a vaccine should be able to prevent both the horizontal transmission from contaminated food or water and the vertical transmission. We have previously shown that a chimeric vaccine composed of predicted immunogenic epitopes of NcMIC3, NcMIC1 and NcROP2 (recNcMIC3-1-R) significantly reduced the cerebral infection in BALB/c mice. In this study, mice were first vaccinated, then mated and pregnant mice were challenged with 2×10^6 *N. caninum* tachyzoites at day 7–9 of pregnancy. Partial protection was only observed in the mice vaccinated with a tachyzoite crude protein extract but no protection against vertical transmission or cerebral infection in the dams was observed in the group vaccinated with recNcMIC3-1-R. Serological and cytokine analysis showed an overall lower cytokine level in sera associated with a dominant IL-4 expression and high IgG1 titers. Thus, the Th2-type immune response observed in the pregnant mice was not protective against experimental neosporosis, in contrary to the mixed Th1-/Th2-type immune response observed in the non-pregnant mouse model. These results demonstrate that the immunomodulation that occurs during pregnancy was not favorable for the protection against *N. caninum* infection conferred by vaccination with recNcMIC3-1-R.

1. Introduction

The coccidian parasite *Neospora caninum* is reported as a leading cause of abortion in cattle, thus bovine neosporosis represents an important veterinary health problem and is of high economical significance [1–3]. The transplacental transmission from an infected dam to the fetus accounts for the vast majority of new infections [4,5]. Therefore, immunization with a vaccine that would protect against both horizontal and vertical transmission appears to be the most cost-efficient method for the control of bovine neosporosis [6,7]. At present time, there is no vaccine capable of inducing protective immunity against endogenous transplacental transmission in cattle, although better understanding of the disease have given indications on the possible strategies to follow for the development of an efficient vaccine [8–10]. The only commercialized vaccine against bovine neosporosis was based on tachyzoite extract, but it induced only partial protection against abortion [11] and did not prevent vertical transmission [12]. The mouse model has been widely used for the development of vaccines against neosporosis for that it provides valuable proof-of-concept data (reviewed in [10,13–15]). We have reported earlier that vaccination of mice with recNcMIC1 [16], recNcMIC3 [17] or recNcROP2 [18] reduced the cerebral infection upon challenge infection and that the combination of the three antigens in a single-dose vaccine showed protection in both pregnant and non pregnant mouse models [19]. Based on those findings, mice were vaccinated with chimeric recombinant proteins composed of predicted immunorelevant domains of NcMIC1, NcMIC3 and NcROP2, and protection against challenge infection with *N. caninum* tachyzoites was assessed in non-pregnant mice. One of those chimeric antigens, named recNcMIC3-1-R, provided a high level of protection against disease and cerebral infection [20].

In this paper, we report on vaccination trials employing recNcMIC3-1-R in the pregnant mouse model. As a control, vaccination was also carried out with crude *N. caninum* tachyzoite antigens. We demonstrate that recNcMIC3-1-R exhibits a lack of protection against transplacental transmission and cerebral infection in the dams, which could be related to different outcomes of the immune responses in pregnant and non-pregnant mouse models.

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2. Materials and methods

Unless otherwise stated, all cell culture reagents were supplied by Invitrogen (Paisly, UK) and chemicals were purchased from Sigma (St. Louis, MO, USA).

2.1. Culture and purification of parasites

N. caninum tachyzoites of the Nc1 isolate [21] were maintained by serial passages in Vero cells using standard procedures [20]. Tachyzoites were harvested by repeatedly passaging infected cells through a 25 G-needle, and liberated parasites were purified on Sephadex G25 PD-10 columns (GE Healthcare, Buckinghamshire, England) [22], and they were counted in a Neubauer chamber using trypan blue stain exclusion. For challenge infections, parasite numbers were adjusted to 2×10^7/ml in RPMI 1640 medium and tachyzoites were used for infection experiments (100 μl/mouse) immediately.

2.2. Preparation of N. caninum crude protein extract

N. caninum tachyzoites were purified as described in the previous section and adjusted to 1×10^8 parasites/ml in sterile PBS. They were then disrupted by performing 4 freeze/thaw cycles using a dry-ice/methanol bath and a 37 °C water bath followed by sonication for 5×1 min at 57 W (Branson Sonifier cell disruptor, Skan AG, Allschwill, Switzerland). The parasites were observed microscopically to ensure that complete disruption had occurred and the protein concentration was measured using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA) and acetylated BSA as a standard. Just prior to vaccination, the lysate was vortexed and sonicated until a homogenous suspension was obtained.

2.3. Preparation of recNcMIC3-1-R

The recombinant chimeric protein recNcMIC3-1-R was obtained as described in [20]. Briefly, *in silico* predicted putative immunogenic epitopes of NcMIC1, NcMIC3 and NcROP2 were amplified from *N. caninum* cDNA and assembled as a single gene. The protein was expressed in One Shot® BL21 (DE3) chemically competent *Escherichia coli* (Invitrogen) and purified by Ni^{2+}-chelate chromatography under denaturing conditions. After dialysis, the protein was dried in Speedvac and resuspended in PBS. The protein concentration was measured using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA) and acetylated BSA as a standard.

2.4. Vaccination trial, clinical monitoring and sample collection

Six weeks old BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained under conventional day/night cycle housing conditions as required by the animal welfare legislation of the Swiss Veterinary Office. They were used for experimentation after 2 weeks of acclimatization. Females were randomly allocated into three groups of twenty animals each. Each group was vaccinated three times by intraperitoneal (i.p.) injection at 2 weeks interval as follows: group 1 (non-infected) was injected with 100 μl PBS, group 2 (SAP) was injected with 5 μg saponin in 100 μl PBS (adjuvant control), group 3 (MIC3-1-R) was injected with 10 μg recNcMIC3-1-R (freshly purified as described in [20]) and group 4 (crude extract) was vaccinated with 10 μg *N. caninum* lysate (equivalent to approximately 1×10^6 parasites). Just prior to use, recNcMIC3-1-R and the crude protein extract were suspended at the appropriate concentrations in saponin adjuvant, sonicated for 2×30 s at 57 W and extensively vortexed. Two weeks after the third immunization, synchronization of oestrus by the Whitten effect [23] followed by mating for three nights was performed [19]. Seven days after mating (day 7–9 of pregnancy), all mice from groups 2 to 4 were challenged i.p. with 2×10^6 freshly purified Nc-1 tachyzoites. The mice were then housed separately to rear their pups and all animals were checked for clinical signs on a daily basis until day 34 post partum. Data on the fertility rate (percentage of pregnant mice/group), litter size, neonatal mortality (pups born dead or that died within the three first days post partum) and postnatal mortality (pups dead between day 4 and 34 post partum) were collected. The pups were weighted every second day from day 14 onwards. Pregnant mice were weighted on a daily basis until delivery, and abortion was detected by sudden and important weight loss. Mice exhibiting clinical signs of neosporosis (ruffled coat, apathy, neurological and walking disorders), were euthanized at the onset of these signs. On day 34 post-partum, all surviving dams and pups were sacrificed by CO_2 euthanasia. Upon euthanasia, blood was drawn by cardiac puncture and sera were frozen at −80 °C. Brains were collected using individual sterile instruments and were immediately frozen at −20 °C.

2.5. Cerebral parasite burden

Neospora-specific quantitative real-time PCR to determine the number of tachyzoites present in the cerebral tissue was performed as described by Müller et al. [24]. DNA extraction from brain tissue was performed as described [20] and concentrations were adjusted to 20 ng/μl with sterile DNase-free water. Quantitative real-time PCR was performed using the Light Cycler™ Instrument (Roche Diagnostic, Basel, Switzerland). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 parasites included in each run [24].

2.6. Serology

Individual sera taken at three distinct time points in the dams (pre-immune, post-immunization prior to infection (day 9 after the last immunization boost), and post-infection at the time of euthanasia) were analyzed for *N. caninum* specific IgG1 and IgG2a by ELISA coated with *N. caninum* lysate prepared as described in Section 2.2. The ELISAs were performed with a 1:100 dilution of the sera [20]. Serum from a mouse experimentally infected with Nc-1 that had reacted positively against *N. caninum* lysate in ELISA and serum from a non-infected mouse were used as positive and negative control, respectively.

2.7. Serum cytokine levels

Serum levels of IFN-γ, IL-4, IL10 and IL12(p70) were measured by means of multiplex bead immunoassay using the MILLIPLEX®MAP Mouse Cytokine/Chemokine kit (Millipore Corp., Billerica, MA, USA). Undiluted post-immunization and post-infection sera were pooled per group and analyzed in triplicate according to the manufacturer's protocol. Microtiter filter plates were run on Luminex instruments (Bio-Plex™ 200 system, Bio-Rad) and cytokine concentrations were calculated with the Bio-Plex Manager software [20]. When cytokine concentration was below the detection limit, an arbitrary value corresponding to the detection limit of undiluted samples (provided by the kit manufacturer) was used for analysis.

2.8. Statistical analysis

Dam survival and pup mortality were analyzed according to Kaplan–Meier and the survival curves between groups were