Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Interferon-γ-dependent protection against *Neospora caninum* infection conferred by mucosal immunization in IL-12/IL-23 p40-deficient mice

Pedro Ferreirinha [a,b], Ricardo Fróis-Martins [a,b], Luzia Teixeira [c,d], António Rocha [c,e], Manuel Vilanova [a,b,c], Alexandra Correia [a,b,f,*]

[a] *I3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal*
[b] *IBMC – Instituto de Biologia Molecular e Celular, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal*
[c] *ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal*
[d] *UMIB – Unidade Multidisciplinar de Investigação Biomédica, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal*
[e] *CECA/ICETA – Centro de Estudos de Ciência Animal, Universidade do Porto, Praça Gomes Teixeira, Apartado 55142, 4051-401 Porto, Portugal*
[f] *DGAOT, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal*

ARTICLE INFO

Article history:
Received 16 February 2018
Received in revised form 17 June 2018
Accepted 27 June 2018
Available online xxxx

Keywords:
Neospora caninum
Intranasal vaccination
IL-12/23 p40
Interferon-gamma
Antibodies

ABSTRACT

We have recently demonstrated the effectiveness of an intranasal immunization approach against *Neospora caninum* infection in immunosufficient mice. Generated evidence indicated that antibodies could be mediating the observed protection. We similarly immunized IL-12/IL-23 p40 chain-deficient (*Il12b*$^{-/-}$) mice, which have impaired cellular immunity, to further explore the host protective mechanism conferred by the used immunization strategy. The immunized mice presented lower parasitic burdens after intraperitoneal infection with *N. caninum* and also had elevated levels of parasite-specific antibodies. However, passive immunization with antibodies purified from immunized donors conferred only limited protection to infected *Il12b*$^{-/-}$ recipients. Despite their intrinsic IL-12 deficiency, the immunized *Il12b*$^{-/-}$ mice mounted a parasite-specific immune response that was mediated by interferon-γ (IFN-γ). Neutralization of IFN-γ in the immunized mice abrogated the observed protective effect of the immunization. These results show altogether that the used immunization strategy overcome the cellular immunity defect of *Il12b*$^{-/-}$ mice and conferred protection from *N. caninum* infection. The observed protective effect was predominantly mediated by IFN-γ and to a lesser extent but non-negligibly by IgG antibodies. These results also highlight that in a host with compromised cellular immunity, the immune response against intracellular pathogens could be markedly boosted by immunization.

1. Introduction

Neospora caninum is an obligate intracellular apicomplexa protozoan that can infect a wide range of mammals of which cattle is the economically relevant host [1]. Cattle infection with *N. caninum* is associated with high economic losses due to an increased abortion rate observed in infected animals [2]. Although vaccination is estimated to be most effective strategy to control neosporosis, no commercial vaccine effective against this parasitic disease is currently available [3]. As *N. caninum* is an obligate intracellular protozoan, it could be expected that Th1-type cell-mediated immunity would be essential for parasite control. Indeed, previous studies have shown that mice defective in the IL-12/IFN-γ axis were lethally susceptible to this parasite [4–9]. Nevertheless, B-cell deficient mice displayed marked susceptibility to *N. caninum* infection, suggesting that antibodies could also have a host protective role [10]. In that line, several studies reported that *in vitro* infection of host cells by *N. caninum* was impaired by antibodies specific to parasite antigens mediating attachment to and invasion of host cells [11–17].

We have recently reported that intranasal (i.n.) immunization using a *N. caninum* antigen extract and CpG adjuvant conferred long lasting protection against neosporosis established via the gastrointestinal tract [18]. As both intestinal IgA and serum IgG raised by immunization displayed *in vitro* effector function by agglutinating parasites and decreasing host cell parasitic burden, we hypothesized that antibodies could be mediating the observed protection [18,19].

IL-12 is a heterodimeric cytokine formed by polypeptide chains p40 and p35, that in its immunologically active form is designated

* Corresponding author at: IBMC – Instituto de Biologia Molecular e Celular, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal.
E-mail address: alexandra.correia@ibmc.up.pt (A. Correia).

https://doi.org/10.1016/j.vaccine.2018.06.060
0264-410X/

as IL-12 p70. IL-12 p40 chain may also associate with IL-23p19 to form IL-23 [20]. IL-12/IL-23 p40-deficient (*Il12b*$^{-/-}$) mice have impaired cellular immunity [21] and are lethally susceptible to *N. caninum* infection [9]. Taking into account these features, we used *Il12b*$^{-/-}$ mice as model to assess the role of systemic parasite-specific IgG antibodies, generated by immunization, in protection against neosporosis. Here infection was established by the intraperitoneal route, to overcome the effect of the intestinal barrier and of locally produced IgA. The obtained results showed that in the *Il12b*$^{-/-}$ background, the used mucosal immunization approach still induced a Th1-type immune response, which contributed to protection.

2. Materials and methods

2.1. Animals

Female or *Il12b*$^{-/-}$ mice in the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and bred under specific pathogen-free conditions at the animal facility of Instituto de Ciências Biomédicas Abel Salazar (ICBAS). Housing and nesting materials were provided as enrichment. Experiments were approved by the institutional board responsible for animal welfare at ICBAS (document 109/2015) and by the competent national authority (documents 0420/000/000/2010 and 0421/000/000/2016).

2.2. Growth of parasites and preparation of tachyzoite lysates and cell-membrane extracts

N. caninum tachyzoites (Nc1 isolate) were kept by serial passages in VERO cells cultures and isolated as previously described [8]. Parasite concentration in cell suspensions was determined in a hemocytometer. Whole parasite sonicates lysates (NcS) and *N. caninum* antigen extracts enriched in membranar proteins (NcMP) were prepared and analyzed accordingly to previously described methods [19].

2.3. Immunizations and tissue sample collection

Mice, 8–10 weeks-old, were randomly distributed into 2 groups. Animals were immunized i.n. twice with three-week interval under light isoflurane anesthesia with 20 μl of PBS containing 10 μg of CpG ODN 1826 (VacciGrade, Invivogen, San Diego, CA) (CpG group) or with PBS containing 30 μg of NcMP plus 10 μg of CpG ODN 1826 (NcMP/CpG group). Three weeks after the boost immunization, mice were either sacrificed by cervical dislocation upon isoflurane anesthesia for organ collection or i.p. challenged with 1×10^4 *N. caninum* tachyzoites, respectively. Infected mice were similarly sacrificed three and seven days after infection. Spleens and mesenteric lymph nodes (MLN) were collected for analysis of the elicited immune response. The brain and lungs were collected and stored at −20 °C until DNA extraction. Serum was collected from all infected mice for detection of NcMP-specific antibodies.

2.4. In vivo IFN-γ neutralization

Neutralization of IFN-γ was performed 12 h before the i.p. parasitic challenge by i.v. administration of 500 μg of anti-IFN-γ mAb (clone R4-6A2) or rat IgG1 isotype control (clone HRPM), both from BioXcell (West Lebanon, NH, USA). Mice were sacrificed 7 days after infection. Brains were collected and stored frozen at −20 °C for DNA extraction.

2.5. Antibody detection

Serum titres of NcMP-specific IgG, IgG1 and IgG2c were quantified by ELISA as previously described [19], using respective alkaline phosphatase-coupled goat anti-mouse antibodies (all from Southern Biotechnology Associates, Birmingham, USA).

2.6. Purification of serum IgG and passive immunization

Serum samples collected from NcMP/CpG and CpG mouse groups three weeks after the boost immunization were pooled and IgG purified using a HiTrap Protein G HP purification column (GE Healthcare), according to manufacturer's instructions. Obtained IgG fractions were respectively designated IgG-NcMP and IgG-CpG.

The recovered antibodies were dialyzed against sterile PBS and the IgG concentration was adjusted to 1.5 mg/ml before stored at −20 °C. The NcMP-specific antibody titres of the IgG-NcMP and IgG-CpG preparations were 1.559×10^9 and below the detection limit, respectively, as determined by ELISA. Passive immunization was performed by intravenous (i.v.) injection of 200 μg IgG-CpG per mouse (IgG-CpG group) or 200 μg IgG-NcMP (IgG-NcMP group). Twelve hours following IgG transfer, mice were i.p. infected with 1×10^4 *N. caninum* tachyzoites. Mice were sacrificed seven days after infection and the brains were collected and stored at −20 °C for DNA extraction.

2.7. In vitro cell cultures and cytokine detection

To assess cytokine production, spleens aseptically collected from mice sacrificed at specific time-points were homogenized and red blood cells were lysed. Recovered splenocytes were suspended in RPMI-1640 (Sigma), supplemented with 10% fetal calf serum (Biowest), HEPES (10 mM), penicillin (200 IU/ml) and streptomycin (200 μg/ml) (all from Sigma) and β-mercaptoethanol (0.1 μM) (Merk, Darmstadt, Germany) (RPMI), plated (5×10^5/well) in triplicate per animal in round-bottom 96-well plates (Nunc) and stimulated with NcS (60 μg/ml) for 3 days at 37 °C and 5% CO_2. Non-stimulated conditions were set to assess basal cytokine production. The concentration of IFN-γ and IL-4 in cell culture supernatants were respectively quantified with Mouse IFN-γ and IL-4 ELISA Ready-Set-Go!® (eBioscience, San Diego, CA) kits, according to manufacturer's instructions.

2.8. DNA extraction and real-time PCR analysis

DNA was extracted from the brain of infected mice as previously described [22]. Briefly, brains were weighted and homogenized. Samples were incubated overnight at 55 °C in a solution containing 1% SDS and 1 mg/ml Proteinase K (Sigma). DNA was extracted by the phenol (Sigma)-Chlorophorm (Merk) method followed by ammonium acetate/ethanol precipitation. Parasite burden in the brains of infected mice was assessed by quantitative real-time PCR (qPCR) analysis of parasitic DNA performed as previously described [23]. In all runs, parasite burden was determined by interpolation of a standard curve performed with DNA isolated from *N. caninum* tachyzoites, ranging from 10 to 10×10^{-4} ng of parasitic DNA (2 to 2×10^5 parasites), included in each run. Data were analyzed in the Rotor gene 6000 software v1.7 (Corbett life science) and expressed as log10 parasites per g of tissue.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad prism, Version 7.0 (GraphPad Software, Inc., La Jolla, CA). In scatter dot graphs a horizontal bar indicates the mean for each group. Column graphs

Please cite this article in press as: Ferreirinha P et al. Interferon-γ-dependent protection against *Neospora caninum* infection conferred by mucosal immunization in IL-12/IL-23 p40-deficient mice. Vaccine (2018), https://doi.org/10.1016/j.vaccine.2018.06.060