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

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**Keywords**: *Neospora caninum*; intranasal vaccination; IL-12/23 p40; Interferon-gamma; antibodies

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**Highlights**

- immunization can boost the immune response of *Il12b*$^{-/-}$ mice against *N. caninum*

- antibodies alone confer limited protection against *N. caninum* infection

- IFN-γ is vital in vaccination-induced protection against *N. caninum* in *Il12b*$^{-/-}$ mice

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## 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-$\gamma$ axis were lethally susceptible to this parasite [4-9]. Nevertheless, B-cell deficient mice also 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 for 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 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 susceptibility 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 $1 \times 10^4$ *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 ºC 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 ºC 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 ºC. The NcMP-specific antibody titres of the IgG-NcMP and IgG-CpG preparations were $1.559 \times 10^9$ 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 $1 \times 10^4$ *N. caninum* tachyzoites. Mice were sacrificed seven days after infection and the brains were collected and stored at -20 ºC 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 ºC 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 ºC 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 $10\times10^{-4}$ ng of parasitic DNA (2 to $2\times10^5$ parasites), included in each run. Data were analyzed in the Rotor gene 6000 so ware 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 5.0 (GraphPad Software, Inc., La Jolla, CA). In scatter dot graphs a horizontal bar indicates the mean for each group. Column graphs represented the mean values. Statistical analysis between groups was done using unpaired Student's *t*-test or analysis of variance (ANOVA) as indicated in figure legends.

## 3. Results

### *3.1. Reduced parasitic burden in immunized Il12b$^{-/-}$ mice infected with N. caninum*

Mucosal immunization of *Il12b*$^{-/-}$ mice with NcMP plus CpG adjuvant raised the titers of NcMP-specific serum IgG, of both IgG1 and IgG2c isotypes. In sham-immunized controls no serum parasite-specific IgG was detected (Fig. 1A). Moreover, higher levels of IFN-γ were detected in culture supernatants of NcS-stimulated splenocytes and MLN cells obtained from the immunized mice comparatively to controls. IL-4 levels were found near the detection limit for all assessed groups (Fig. 1B). These results indicate that despite having a compromised IL-12/IFN-γ axis, *Il12b*$^{-/-}$ mice mounted a Th1-type response in response to the i.n. immunization. Having confirmed the effectiveness of immunization, mice were infected i.p. with *N. caninum* and the parasitic burden was assessed in the lungs and brain at days 3 and 7 after the parasitic challenge, respectively. As shown in Fig. 2A, immunized mice clearly presented lower parasitic burdens than sham-immunized controls. In several immunized animals, no parasitic DNA was detected both in the lungs and brain. In the immunized mice the levels of NcMP-specific IgG were elevated after the parasitic challenge, with a preponderant IgG2c production (Fig. 2B). Splenocytes from 3-day infected immunized mice responded *ex vivo* to parasite antigen stimulation by producing IFN-γ whereas this was not observed in controls. Splenocytes from 7-day infected mice of the CpG and NcMP/CpG groups responded to *in vitro* NcS stimulation by producing IFN-γ to similar levels. The levels of IL-4 detected in culture supernatants were low in cultures of 3-day infected mice splenocytes. In the cultures of splenocytes from 7-day infected mice, IL-4 levels increased but were not different between groups (Fig. 2C). These results altogether show that the

206 used intranasal immunization strategy induced parasite-specific IgG antibodies
207 and the production of IFN-γ by *Il12b*$^{-/-}$ mice and conferred protection against
208 infection with *N. caninum* established by the i.p. route.

209

210 *3.2. Passive immunization confers limited host protection against N. caninum*
211 *challenge*

212 To determine whether the IgG antibodies raised by immunization could be
213 mediating the protective effect observed in the infected mice, IgG-NcMP and IgG-
214 CpG were respectively transferred into naïve *Il12b*$^{-/-}$ recipients that were infected
215 i.p. 12 h upon the passive immunization. As shown in Fig. 3, a reduction in
216 parasitic burden was observed in mice that received IgG-NcMP in comparison
217 with controls transferred with IgG-CpG. However, the protective effect was slight
218 and less marked than the one induced by active immunization. This result
219 indicates that IgG induced by immunization did not *per se* confer the protection
220 observed in the i.n.-immunized mice.

221

222 *3.3. IFN-γ production in infected immunized Il12b*$^{-/-}$ *mice mediates protection*

223 As IgG antibodies only partially mediated the protective effect induced by
224 the immunization, the contribution of IFN-γ to protection was assessed using a
225 specific mAb to neutralize this cytokine. As shown in Fig. 4A, the protective effect
226 induced by immunization was markedly impaired in mice receiving IFN-γ-
227 neutralizing mAb. IFN-γ neutralization also raised the parasitic burden in controls
228 sham-immunized with CpG. These results implicate IFN-γ in the protective effect
229 induced by immunization in the *Il12b*$^{-/-}$ mice. IL-18 is a well-known IFN-γ-inducing

230 factor [24]. However, antibody-mediated neutralization of IL-18 did not affect the
231 production of IFN-γ in *in vitro* NcS-stimulated mononuclear splenocytes obtained
232 from the immunized *Il12b*$^{-/-}$ mice (Supplementary material 1). As shown in Figure
233 4B, neutralization of IFN-γ 12 h prior to infection did not significantly affect the
234 levels of parasite-specific IgG2c. However, the importance of IFN-γ in inducing
235 the IgG2c response during immunization is evidenced by the absence of parasite-
236 specific antibodies of this isotype in immunized IFN-γ deficient mice
237 (Supplementary material 2).

## 4. Discussion

Previous studies have demonstrated the essential role of the IL-12/IFN-γ axis in host resistance against *N. caninum* infection [4-9]. This could be expected taking into account that this protozoan is an obligate intracellular parasite. Nevertheless, we previously showed *in vitro* that parasite-specific antibodies raised by immunization agglutinated *N. caninum* tachyzoites and reduced parasitic burden in infected macrophages [18, 19]. Therefore, we hypothesized that in immunized mice antibodies could contribute to the protective effect. B cell-deficient mice are highly susceptibility to infection caused by *N. caninum* [10], also indicating a possible role for antibody production in protection against this parasite. To further explore this hypothesis, we immunized and infected *Il12b*$^{-/-}$ mice, which have impaired cellular immunity but a normal B cell compartment [21]. The i.p. route was chosen to exclude a role of mucosal IgA in protection. Immunized *Il12b*$^{-/-}$ mice presented parasite-specific IgG levels similar to those previously detected in similarly immunized WT mice [18]. This indicates that absence of IL-12/IL-23 did not significantly impact IgG production induced by the i.n. immunization. IgG production independent of IL-12/IL-23 signaling, elicited by i.n. immunization, has been also reported by others using an alternative antigen and adjuvant [25]. In another study, *Il12b*$^{-/-}$ mice of the same background (C57BL/6) as used here, immunized subcutaneously with a parasite antigen plus CpG adjuvant produced IgG at the same level as wild-type mice [26]. However, the isotype profile was biased towards IgG1, contrasting our observation in the NcMP/CpG mice, where a preponderant production of IgG2c was still detected in response to immunization. This discrepancy may result from and highlight specific immune mechanisms elicited by mucosal immunization.

263 The hypothesized protective role of IgG in *N. caninum* infected hosts was
264 confirmed here *in vivo* in passively immunized animals. Passive immunization
265 with antibodies has previously been shown to mediate protection in mice infected
266 with the closely related protozoan *Toxoplasma gondii*, likely by inhibiting parasite
267 penetration into host cells [27, 28] or by promoting parasite intracellular killing by
268 macrophages [29, 30]. However, the protective effect of antibody observed here
269 was limited as it caused only a small reduction in the parasitic burden. As *Il12b*$^{-/-}$
270 mice have an impaired production of IFN-γ when infected with *N. caninum* [31], a
271 protective role of antibodies in promoting intracellular killing may also depend on
272 an intact capacity to produce this cytokine. Nonetheless, even in an
273 immunosufficient recipient, transfer of immune serum raised in *Il12b*$^{-/-}$ mice failed
274 to confer protection against *Plasmodium berghei* sporozoite infection [32]. The
275 observed limited protection conferred by antibodies altogether with the lethal
276 susceptibility of B cell-deficient mice to *N. caninum* [10] may also indicate that B
277 cells participate in host protection against *N. caninum* by further mechanisms
278 than antibody production such as providing co-stimulatory ligands for T cells [33]
279 or by producing pro-inflammatory cytokines [34].

280 Taking into consideration the IL-12/IL-23-deficient phenotype, it was
281 surprising that IgG2c was the predominant isotype in the serum of the immunized
282 *Il12b*$^{-/-}$ mice, due to the importance of IFN-γ in IgG2c production [35]. A possible
283 explanation may reside in a direct effect of used CpG adjuvant in B cells, driving
284 Toll-Like Receptor 9 (TLR9) dependent IgG2c class-switch [36]. CpG may also
285 induce IFN-γ production by NK cells via TLR9. However, this effect was shown to
286 also require concomitant IL-15 and IL-18 [37], that act in combination with IL-12
287 [38], which would be prevented in the *Il12b*$^{-/-}$ background. The elevated levels of

IgG2c antibodies induced by immunization were still detected in infected mice in which IFN-γ was neutralized by specific mAb. This shows that IgG2c-switched B cells, as a consequence of immunization, do not need IFN-γ produced in the course of acute infection to sustain the production of antibodies of this isotype. In accordance with the IgG isotype profile, production of IFN-γ was higher in cultures of parasite antigen-stimulated spleen and MLN cells obtained from immunized mice. The stimulatory effect of CpG in IFN-γ production is well-known [39-41] and, as we show here, it can also occur in the absence of IL-12. As CpG can also promote the production of IL-18 [42], this cytokine may be a possible candidate for the induction of IFN-γ production in immunized mice. Indeed, generation of IFN-γ-mediated memory responses and host protection in the absence of endogenous IL-12 has already been described following infection with other protozoa [32, 43, 44] in a process dependent on IL-18 [43]. However, as neutralization of this cytokine did not affect *in vitro* parasite-antigen-driven IFN-γ production by splenocytes of immunized *Il12b*$^{-/-}$ mice, this hints that other cytokines may be more important in promoting the production of IFN-γ in response to immunization. IL-12 upregulates the expression of the IL-18 receptor on cells producing IFN-γ [45], and this may have limited the effect of IL-18 in the IL-12-deficient mice splenocyte cultures. A role for IL-18 in the *in vivo* differentiation of Th1-type cells triggered by immunization cannot however be ruled out. Also, very low levels of IL-4 were detected in both immunized and control mice splenocyte cultures. Infection of IL-12-deficient mice with *Leishmania* without increased IL-4 production has been reported in previous studies [44].

The protective effect of the used immunization approach observed here was similar to the one previously obtained in intragastrically infected wild type mice [18, 19]. Although no direct comparison can be made, since infection route and inoculum were distinct, *Il12b*$^{-/-}$ immunized animals generally presented lower or no detectable brain parasitic burden, as previously observed. Neutralization of IFN-γ markedly increased the parasitic burden in immunized mice to values similar to the ones detected in sham-immunized controls receiving either isotype control or IFN-γ neutralizing mAb. This result confirmed the major role of IFN-γ in host protection in the i.n. immunized *Il12b*$^{-/-}$ mice. As CD4$^{+}$ and CD8$^{+}$ T cells as well as NKT cells have been shown to produce IFN-γ in *N. caninum* infected hosts [23, 31], it would be interesting to assess in future studies the particular contribution of these T cell populations to the protective effect induced by immunization in the immunodeficient host used here.

Altogether, the obtained results excluded a major role of IgG antibodies in protecting from systemic *N. caninum* infection and emphasized the main role of IFN-γ in the protective mechanism elicited by the used i.n. immunization with NcMP and CpG. Moreover, our results show that the used mucosal immunization can induce systemic protection against *N. caninum* that was effective in i.p. infected mice harboring a mutation that compromises Th1-type immunity. This indicates that vaccination would be worth exploring as a host protective strategy against intracellular parasites in hosts with depressed cell-mediated immunity.

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**Conflicts of interest**

The authors declare no conflicts of interest.

**Author contribution**

Pedro Ferreirinha, Luzia Teixeira, Manuel Vilanova, Alexandra Correia conceived and designed the experiments; Pedro Ferreirinha, Ricardo Fróis-Martins, Alexandra Correia performed the experiments; Pedro Ferreirinha, Manuel Vilanova, Alexandra Correia analyzed the data; Pedro Ferreirinha, António Rocha, Manuel Vilanova, Alexandra Correia wrote the manuscript.

## References


[1] McAllister MM. Diagnosis and Control of Bovine Neosporosis. Vet Clin North Am Food Anim Pract. 2016;32:443-63.

[2] Reichel MP, Alejandra Ayanegui-Alcerreca M, Gondim LF, Ellis JT. What is the global economic impact of Neospora caninum in cattle - the billion dollar question. Int J Parasitol. 2013;43:133-42.

[3] Horcajo P, Regidor-Cerrillo J, Aguado-Martinez A, Hemphill A, Ortega-Mora LM. Vaccines for bovine neosporosis: current status and key aspects for development. Parasite Immunol. 2016;38:709-23.

[4] Khan IA, Schwartzman JD, Fonseka S, Kasper LH. Neospora caninum: role for immune cytokines in host immunity. Exp Parasitol. 1997;85:24-34.

[5] Baszler TV, Long MT, McElwain TF, Mathison BA. Interferon-gamma and interleukin-12 mediate protection to acute Neospora caninum infection in BALB/c mice. Int J Parasitol. 1999;29:1635-46.

[6] Nishikawa Y, Tragoolpua K, Inoue N, Makala L, Nagasawa H, Otsuka H, et al. 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. 2001;8:811-6.

[7] Ritter DM, Kerlin R, Sibert G, Brake D. Immune factors influencing the course of infection with Neospora caninum in the murine host. The Journal of parasitology. 2002;88:271-80.

372 [8] Teixeira L, Botelho AS, Batista AR, Meireles CS, Ribeiro A, Domingues HS,
373 et al. Analysis of the immune response to Neospora caninum in a model of
374 intragastric infection in mice. Parasite Immunol. 2007;29:23-36.

375 [9] Mineo TW, Benevides L, Silva NM, Silva JS. Myeloid differentiation factor 88
376 is required for resistance to Neospora caninum infection. Vet Res. 2009;40:32.

377 [10] Eperon S, Bronnimann K, Hemphill A, Gottstein B. Susceptibility of B-cell
378 deficient C57BL/6 (microMT) mice to Neospora caninum infection. Parasite
379 Immunol. 1999;21:225-36.

380 [11] Nishikawa Y, Xuan X, Nagasawa H, Igarashi I, Fujisaki K, Otsuka H, et al.
381 Monoclonal antibody inhibition of Neospora caninum tachyzoite invasion into host
382 cells. International Journal for Parasitology. 2000;30:51-8.

383 [12] Naguleswaran A, Cannas A, Keller N, Vonlaufen N, Schares G, Conraths FJ,
384 et al. Neospora caninum microneme protein NcMIC3: secretion, subcellular
385 localization, and functional involvement in host cell interaction. Infection and
386 Immunity. 2001;69:6483-94.

387 [13] Keller N, Naguleswaran A, Cannas A. Identification of a Neospora caninum
388 microneme protein (NcMIC1) which interacts with sulfated host cell surface
389 glycosaminoglycans. Infect Immun. 2002;70:3187-98.

390 [14] Cho J-HH, Chung W-SS, Song K-JJ, Na B-KK, Kang S-WW, Song C-YY, et
391 al. Protective efficacy of vaccination with Neospora caninum multiple
392 recombinant antigens against experimental Neospora caninum infection. The
393 Korean journal of parasitology. 2005;43:19-25.

[15] Naguleswaran A, Alaeddine F, Guionaud C, Vonlaufen N, Sonda S, Jenoe P, et al. Neospora caninum protein disulfide isomerase is involved in tachyzoite-host cell interaction. International Journal for Parasitology. 2005;35:1459-72.

[16] Haldorson GJ, Stanton JB, Mathison BA, Suarez CE, Baszler TV. Neospora caninum: antibodies directed against tachyzoite surface protein NcSRS2 inhibit parasite attachment and invasion of placental trophoblasts in vitro. Exp Parasitol. 2006;112:172-8.

[17] Srinivasan S, Mueller J, Suana A, Hemphill A. Vaccination with microneme protein NcMIC4 increases mortality in mice inoculated with Neospora caninum. The Journal of parasitology. 2007;93:1046-55.

[18] Ferreirinha P, Correia A, Teixeira-Coelho M, Osorio H, Teixeira L, Rocha A, et al. Mucosal immunization confers long-term protection against intragastrically established Neospora caninum infection. Vaccine. 2016;34:6250-8.

[19] Ferreirinha P, Dias J, Correia A, Perez-Cabezas B, Santos C, Teixeira L, et al. Protective effect of intranasal immunization with Neospora caninum membrane antigens against murine neosporosis established through the gastrointestinal tract. Immunology. 2014;141:256-67.

[20] Teng MW, Bowman EP, McElwee JJ, Smyth MJ, Casanova JL, Cooper AM, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. Nat Med. 2015;21:719-29.

[21] Magram J, Connaughton SE, Warrier RR, Carvajal DM, Wu CY, Ferrante J, et al. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity. 1996;4:471-81.

[22] Nishikawa Y, Inoue N, Xuan X, Nagasawa H, Igarashi I, Fujisaki K, et al. Protective efficacy of vaccination by recombinant vaccinia virus against Neospora caninum infection. Vaccine. 2001;19:1381-90.

[23] Correia A, Ferreirinha P, Botelho S, Belinha A, Leitao C, Caramalho I, et al. Predominant role of interferon-gamma in the host protective effect of CD8(+) T cells against Neospora caninum infection. Sci Rep. 2015;5:14913.

[24] Okamura H, Kashiwamura S, Tsutsui H, Yoshimoto T, Nakanishi K. Regulation of interferon-gamma production by IL-12 and IL-18. Curr Opin Immunol. 1998;10:259-64.

[25] Bielinska AU, Makidon PE, Janczak KW, Blanco LP, Swanson B, Smith DM, et al. Distinct pathways of humoral and cellular immunity induced with the mucosal administration of a nanoemulsion adjuvant. J Immunol. 2014;192:2722-33.

[26] Rosa DS, Bastos KR, Bargieri DY, Tzelepis F, Nomizo A, Russo M, et al. Role of interferon-gamma during CpG oligodeoxynucleotide-adjuvanted immunization with recombinant proteins. Vaccine. 2007;25:6007-17.

[27] Cha DY, Song IK, Lee GS, Hwang OS, Noh HJ, Yeo SD, et al. Effects of specific monoclonal antibodies to dense granular proteins on the invasion of Toxoplasma gondii in vitro and in vivo. Korean J Parasitol. 2001;39:233-40.

[28] Sayles PC, Gibson GW, Johnson LL. B cells are essential for vaccination-induced resistance to virulent Toxoplasma gondii. Infect Immun. 2000;68:1026-33.

[29] Hauser WE, Jr., Remington JS. Effect of monoclonal antibodies on phagocytosis and killing of Toxoplasma gondii by normal macrophages. Infect Immun. 1981;32:637-40.

[30] Anderson SE, Jr., Bautista SC, Remington JS. Specific antibody-dependent killing of Toxoplasma gondii by normal macrophages. Clin Exp Immunol. 1976;26:375-80.

[31] Teixeira L, Marques RM, Ferreirinha P, Bezerra F, Melo J, Moreira J, et al. Enrichment of IFN-gamma producing cells in different murine adipose tissue depots upon infection with an apicomplexan parasite. Sci Rep. 2016;6:23475.

[32] Romero JF, Ibrahim GH, Renggli J, Himmelrich H, Graber P, Corradin G. IL-12p40-independent induction of protective immunity upon multiple Plasmodium berghei irradiated sporozoite immunizations. Parasite Immunol. 2007;29:541-8.

[33] Teixeira L, Marques A, Meireles CS, Seabra AR, Rodrigues D, Madureira P, et al. Characterization of the B-cell immune response elicited in BALB/c mice challenged with Neospora caninum tachyzoites. Immunology. 2005;116:38-52.

[34] Bao Y, Liu X, Han C, Xu S, Xie B, Zhang Q, et al. Identification of IFN-gamma-producing innate B cells. Cell Res. 2014;24:161-76.

[35] Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, et al. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature. 1988;334:255-8.

[36] Dosenovic P, Adori M, Adams WC, Pedersen GK, Soldemo M, Beutler B, et al. Slc15a4 function is required for intact class switch recombination to IgG2c in response to TLR9 stimulation. Immunol Cell Biol. 2015;93:136-46.

462 [37] Souza-Fonseca-Guimaraes F, Parlato M, de Oliveira RB, Golenbock D,
463 Fitzgerald K, Shalova IN, et al. Interferon-gamma and granulocyte/monocyte
464 colony-stimulating factor production by natural killer cells involves different
465 signaling pathways and the adaptor stimulator of interferon genes (STING). J Biol
466 Chem. 2013;288:10715-21.

467 [38] Lauwerys BR, Renauld JC, Houssiau FA. Synergistic proliferation and
468 activation of natural killer cells by interleukin 12 and interleukin 18. Cytokine.
469 1999;11:822-30.

470 [39] Christian B, Gan Z, Folkert S, Takeshi K, Dennis MK. CpG DNA as a vaccine
471 adjuvant. Expert review of vaccines. 2011;10:499-511.

472 [40] Toussi DN, Massari P. Immune Adjuvant Effect of Molecularly-defined Toll-
473 Like Receptor Ligands. Vaccines (Basel). 2014;2:323-53.

474 [41] Srivastava A, Gowda DV, Madhunapantula SRV. Mucosal vaccines: a
475 paradigm shift in the development of mucosal adjuvants and delivery vehicles.
476 APMIS. 2015;123:275-88.

477 [42] Gould MP, Greene JA, Bhoj V, DeVecchio JL, Heinzel FP. Distinct
478 modulatory effects of LPS and CpG on IL-18-dependent IFN-gamma synthesis.
479 J Immunol. 2004;172:1754-62.

480 [43] Muller U, Kohler G, Mossmann H, Schaub GA, Alber G, Di Santo JP, et al.
481 IL-12-independent IFN-gamma production by T cells in experimental Chagas'
482 disease is mediated by IL-18. J Immunol. 2001;167:3346-53.

483 [44] Hernandez MX, Barcante TA, Vilela L, Tafuri WL, Afonso LC, Vieira LQ.
484 Vaccine-induced protection against Leishmania amazonensis is obtained in the
485 absence of IL-12/23p40. Immunol Lett. 2006;105:38-47.

[45] Ahn HJ, Maruo S, Tomura M, Mu J, Hamaoka T, Nakanishi K, et al. A mechanism underlying synergy between IL-12 and IFN-gamma-inducing factor in enhanced production of IFN-gamma. J Immunol. 1997;159:2125-31.

**Fig. 1.** Mucosal immunization induces the production of *N. caninum*-specific IgG and IFN-γ. (A) Parasite-specific IgG, IgG1 and IgG2c isotype levels in the serum of mice immunized twice i.n. with NcMP plus CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG), as indicated, 3 weeks after boost immunization. Data is presented as $\log_{10}$ of the antibody titres. Results correspond to pooled data of two independent experiments with a total number of 12-14 mice per group. Each dot represents an individual mouse. Bars correspond to the mean value in each group. Numbers above bars correspond to the IgG1/IgG2c ratio. BDL - below detection limit; (B) IFN-γ and IL-4 concentration in the supernatants of mesenteric lymph nodes (MLN) or splenocytes cell cultures unstimulated or stimulated for 3 days with NcS. Cells were isolated from the spleens and MLN of mice 3 weeks upon the last of two i.n. with NcMP and CpG (NcMP/CpG) or sham-immunized with CpG (CpG). Results correspond to one representative experiment out of two independent experiments. Number of samples per group: CpG n=4; NcMP/CpG n=4. Stimulated cells were plated in triplicates per mouse per condition. Each dot represents the mean concentration of triplicate samples per assessed condition with cells from each individual mouse. Bars correspond to the mean value in each group. (unpaired *t*-test ** $p < 0.01$; *** $p < 0.001$).

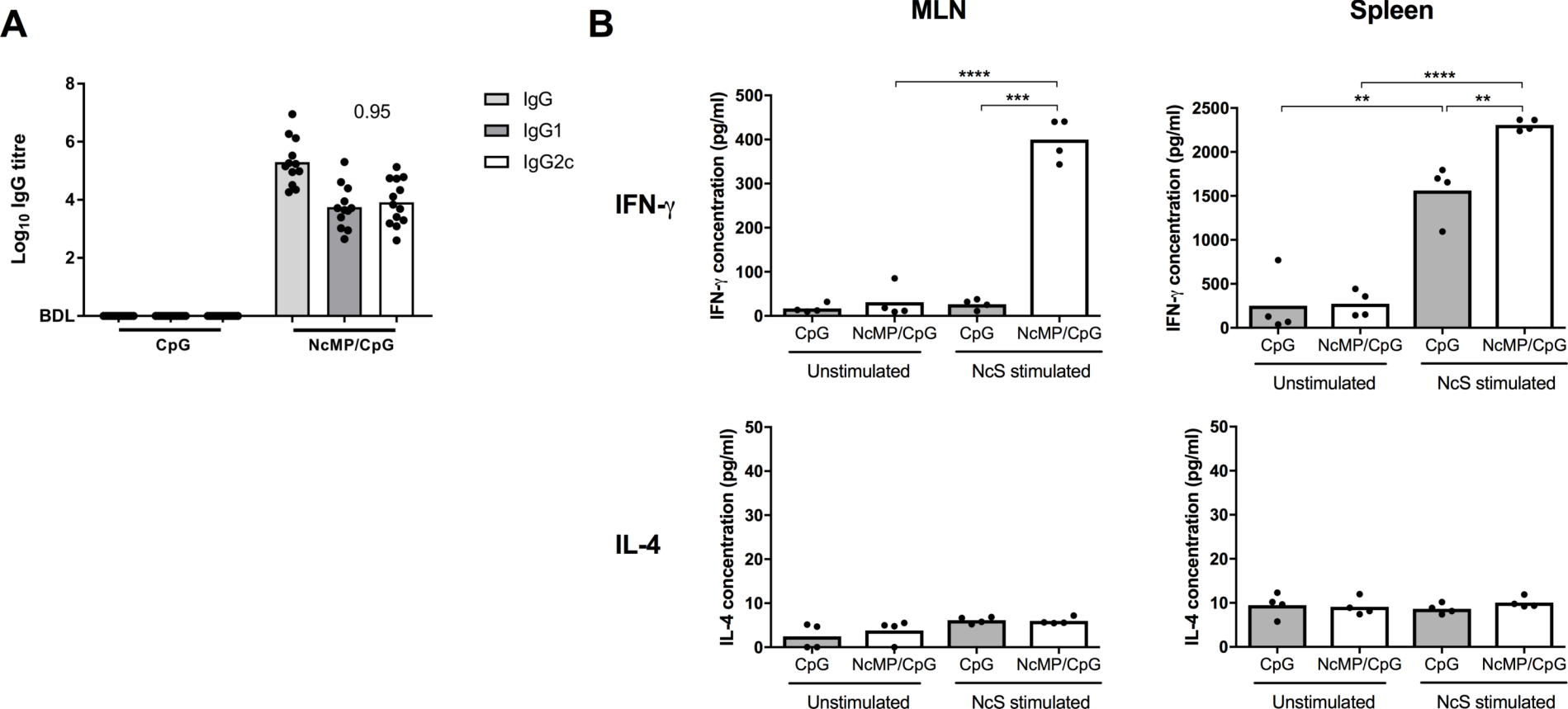
**Fig. 2.** Protective effect of NcMP/CpG immunization against *N. caninum* infection in i.p. challenged *Il12b*$^{-/-}$ mice. (A) Parasitic load assessed by qPCR three days (Lungs) or one week (Brain) upon i.p. challenge with $1 \times 10^4$ *N. caninum* tachyzoites in mice immunized with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG alone (CpG). Data is presented as log10 of the

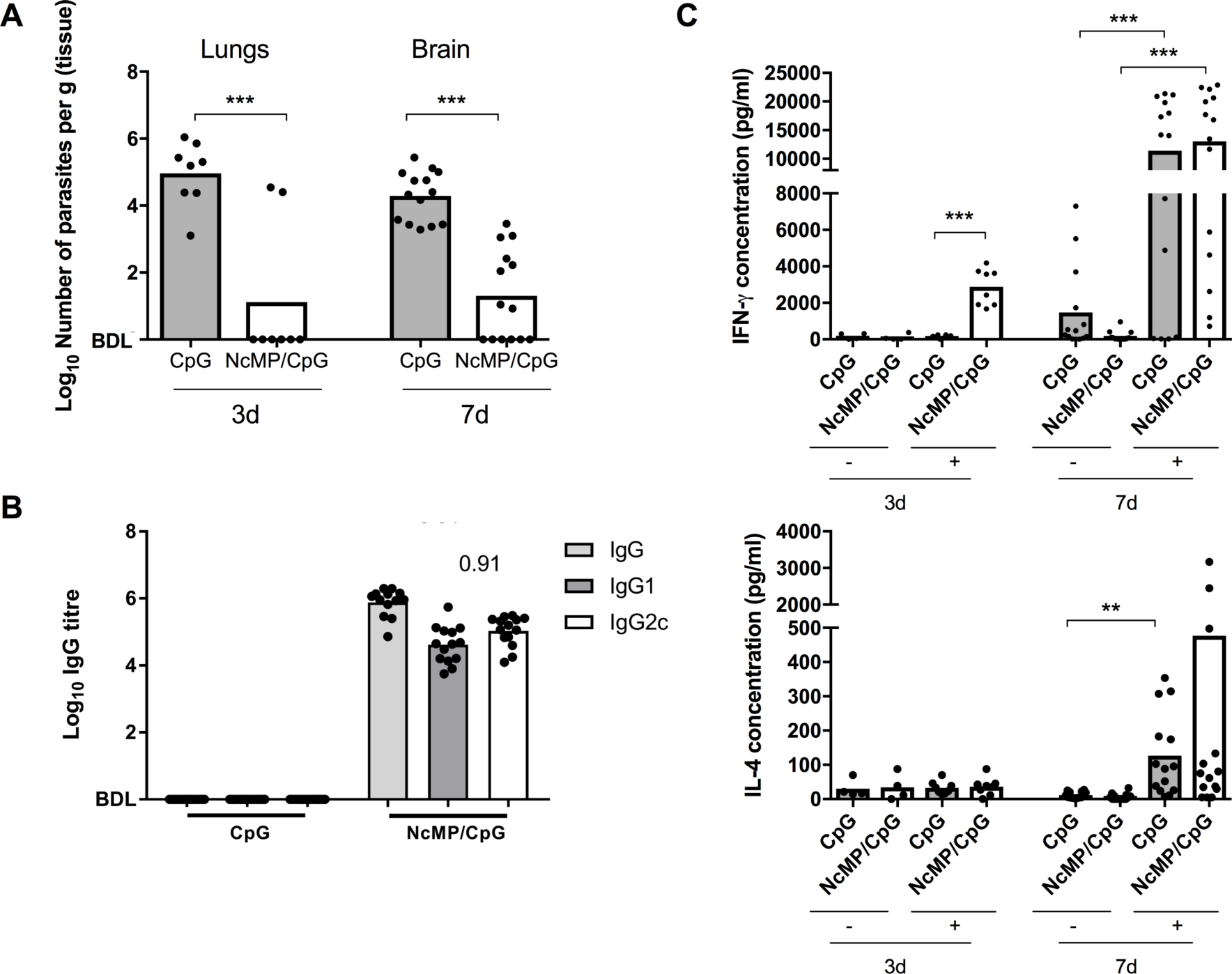
number of parasites per gram of tissue; n = 8 per group (Lungs, day 3 upon infection); n = 14 per group (Brain, day 7 upon infection). Each symbol represents an individual mouse. Bars correspond to the mean value in each group; (unpaired *t*-test ***p < 0.001); (B) Parasite-specific IgG, IgG1 and IgG2c isotype levels in the serum of immunized mice (NcMP/CpG) and controls (CpG), as indicated, 7 days after i.p. infection with $1 \times 10^4$ *N. caninum* tachyzoites. Data is presented as $\log_{10}$ of the antibody titres. Results correspond to pooled data of two independent experiments with a total number of 14 mice per group. Each dot represents an individual mouse. Bars correspond to the mean value in each group. Numbers above bars correspond to the IgG1/IgG2a ratio. BDL - below detection limit; (C) IFN-$\gamma$ and IL-4 concentration in the supernatants of splenocyte cell cultures unstimulated (-) or stimulated for 3 days with NcS (+). Cells were isolated from the spleens of immunized mice (NcMP/CpG) or controls (CpG), 3 and 7 days after i.p. infection with $1 \times 10^4$ *N. caninum* tachyzoites. Results correspond to pooled data of two independent experiments with a total number of mice per group of 8 (3 days) or 14 (7days). Each dot represents an individual mouse. Bars correspond to the mean value in each group (unpaired *t*-test *** p < 0.001).

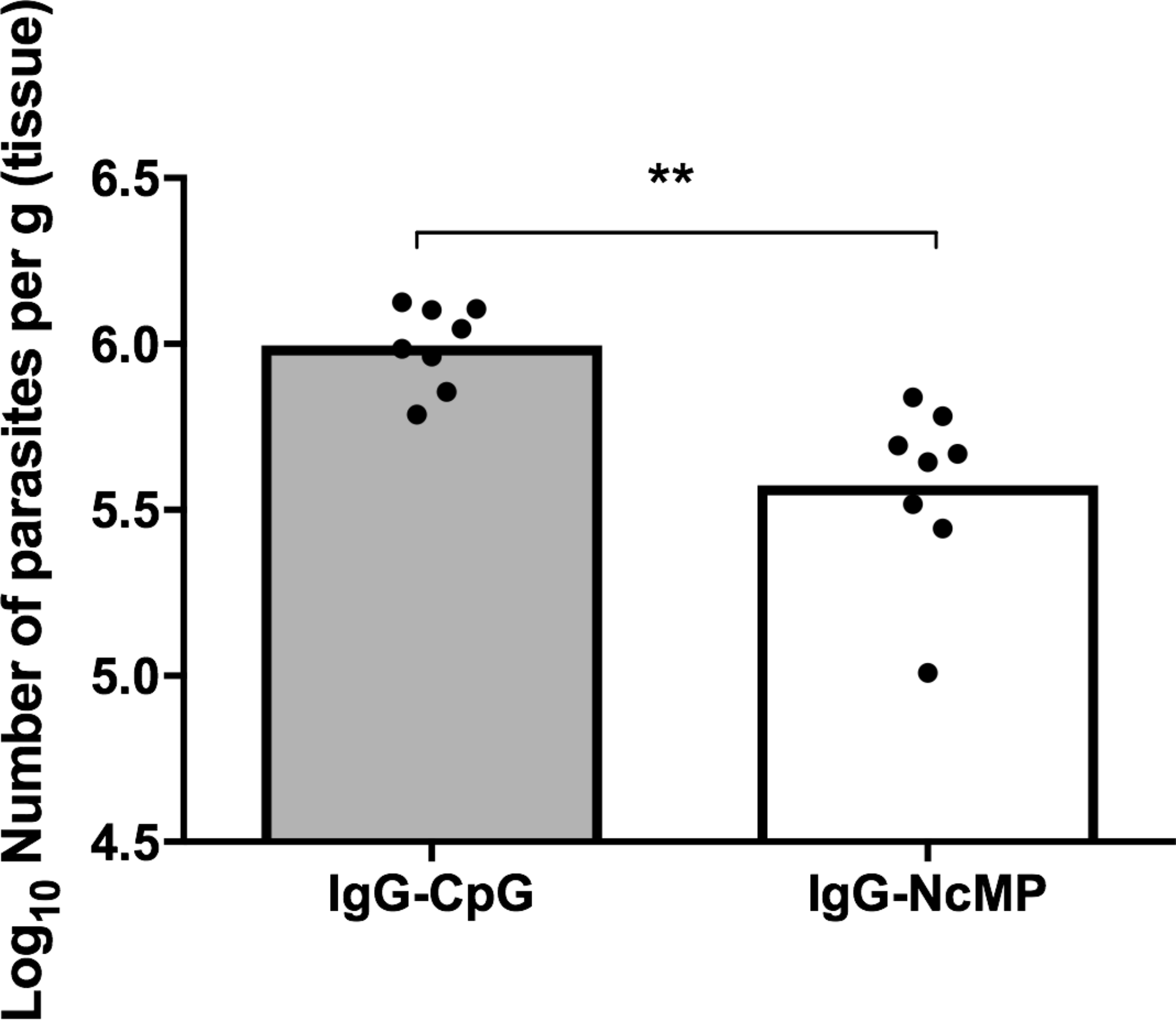
**Fig. 3.** Passive immunization confers limited protection against *N. caninum* i.p. challenge. Parasitic load assessed by qPCR in mice passively immunized with IgG-CpG or IgG-NcMP, as indicated, and subsequently challenged i.p. with $1 \times 10^4$ *N. caninum* tachyzoites. Data is presented as $\log_{10}$ of the number of parasites per gram of tissue, collected 7 days upon the i.p. challenge. Results correspond to pooled data of two independent experiments with a total number of 8 mice per

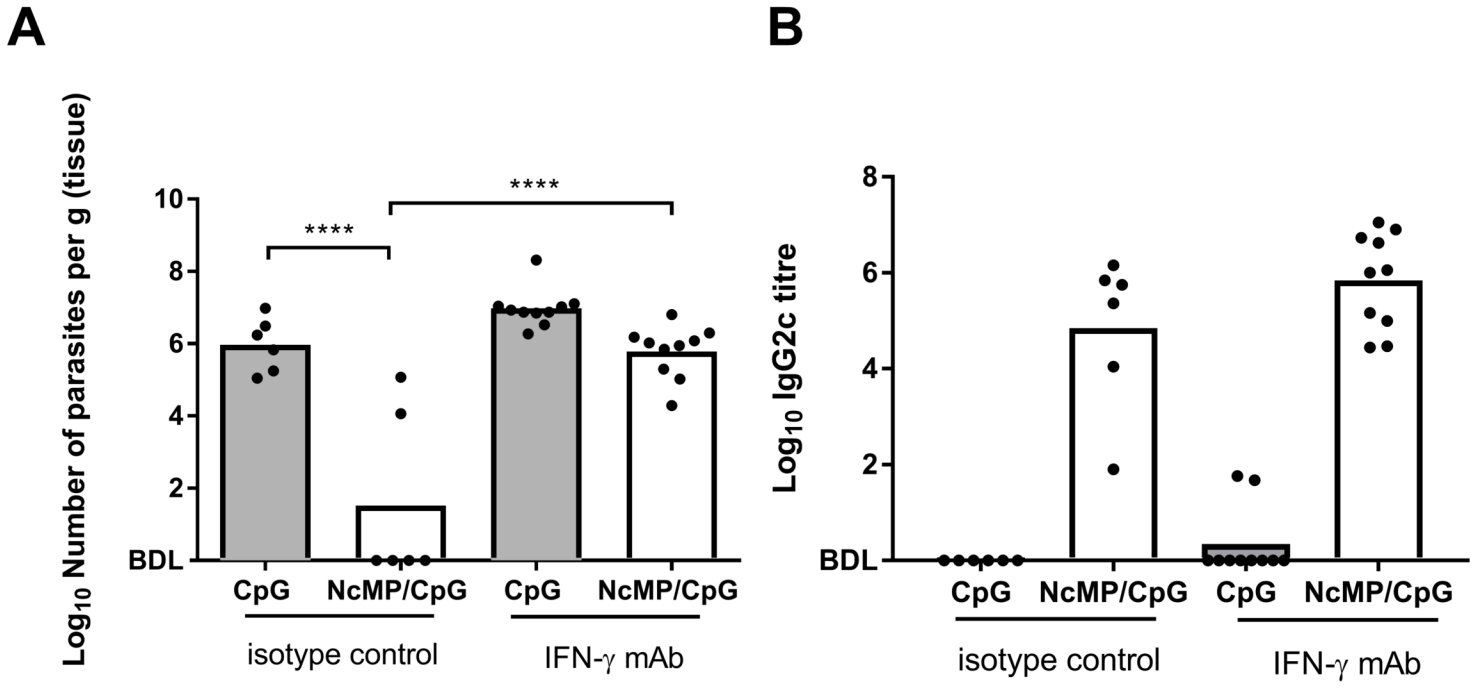
group. Each dot represents an individual mouse. Bars correspond to the mean value in each group (unpaired *t*-test ∗∗ $p < 0.01$).

**Fig. 4.** IFN-γ neutralization abrogates protection conferred by immunization. (A) Parasitic load assessed by qPCR in the brain of immunized (NcMP/CpG) or sham-immunized (CpG) mice, 7 days after i.p. challenge with $1 \times 10^4$ *N. caninum* tachyzoites, treated with IFN-γ-specific mAb (IFN-γ mAb) or isotype control, as indicated, 12 h prior to the i.p. infection. Results correspond to pooled data of two independent experiments with a total number of mice per group of 6 (isotype control) and of 10 (IFN-γ mAb). Each dot represents an individual mouse. Bars correspond to the mean value in each group; (Two-way ANOVA followed by multiple comparison test; ∗∗ $p < 0.01$; ∗∗∗∗ $p < 0.0001$). BDL - below detection limit. (B) Parasite-specific IgG2c levels in the serum of immunized mice (NcMP/CpG) and controls (CpG), as indicated, of the same groups as above. Data is presented as log10 of the antibody titres. Bars correspond to the mean value in each group. BDL - below detection limit.

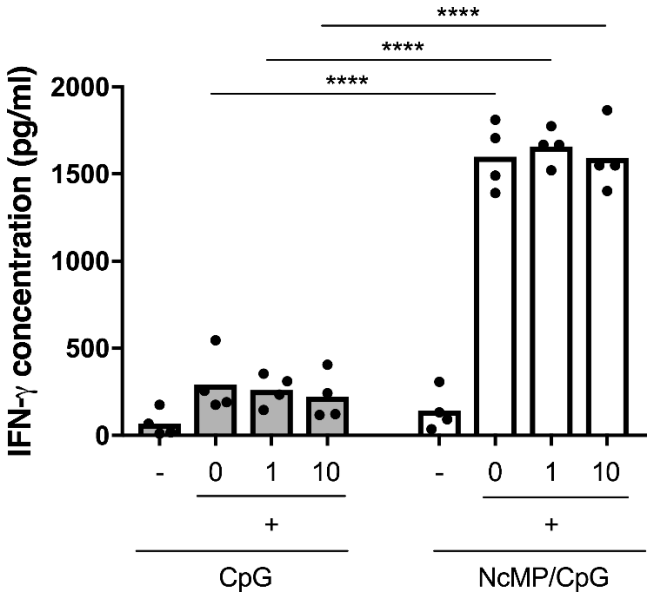

A
Log10 IgG titre
8
6
4
2
BDL
0.95
IgG
IgG1
IgG2c
CpG
NcMP/CpG
B
MLN
Spleen
IFN-γ
IL-4
IFN-γ concentration (pg/ml)
IL-4 concentration (pg/ml)
****
***
**
CpG
NcMP/CpG
Unstimulated
NcS stimulated


A
Lungs
Brain
Log10 Number of parasites per g (tissue)
8
6
4
2
BDL
***
***
CpG
NcMP/CpG
CpG
NcMP/CpG
3d
7d
B
Log10 IgG titre
8
6
4
2
BDL
0.91
IgG
IgG1
IgG2c
CpG
NcMP/CpG
C
IFN-γ concentration (pg/ml)
25000
20000
15000
10000
8000
6000
4000
2000
0
***
***
***
CpG
NcMP/CpG
CpG
NcMP/CpG
CpG
NcMP/CpG
CpG
NcMP/CpG
-
+
-
+
3d
7d
IL-4 concentration (pg/ml)
4000
3000
2000
500
400
300
200
100
0
**
CpG
NcMP/CpG
CpG
NcMP/CpG
CpG
NcMP/CpG
CpG
NcMP/CpG
-
+
-
+
3d
7d


Log10 Number of parasites per g (tissue)
6.5
6.0
5.5
5.0
4.5
**
IgG-CpG
IgG-NcMP


A
B
Log10 Number of parasites per g (tissue)
10
8
6
4
2
BDL
****
****
CpG
NcMP/CpG
CpG
NcMP/CpG
isotype control
IFN-γ mAb
Log10 IgG2c titre
8
6
4
2
BDL
CpG
NcMP/CpG
CpG
NcMP/CpG
isotype control
IFN-γ mAb

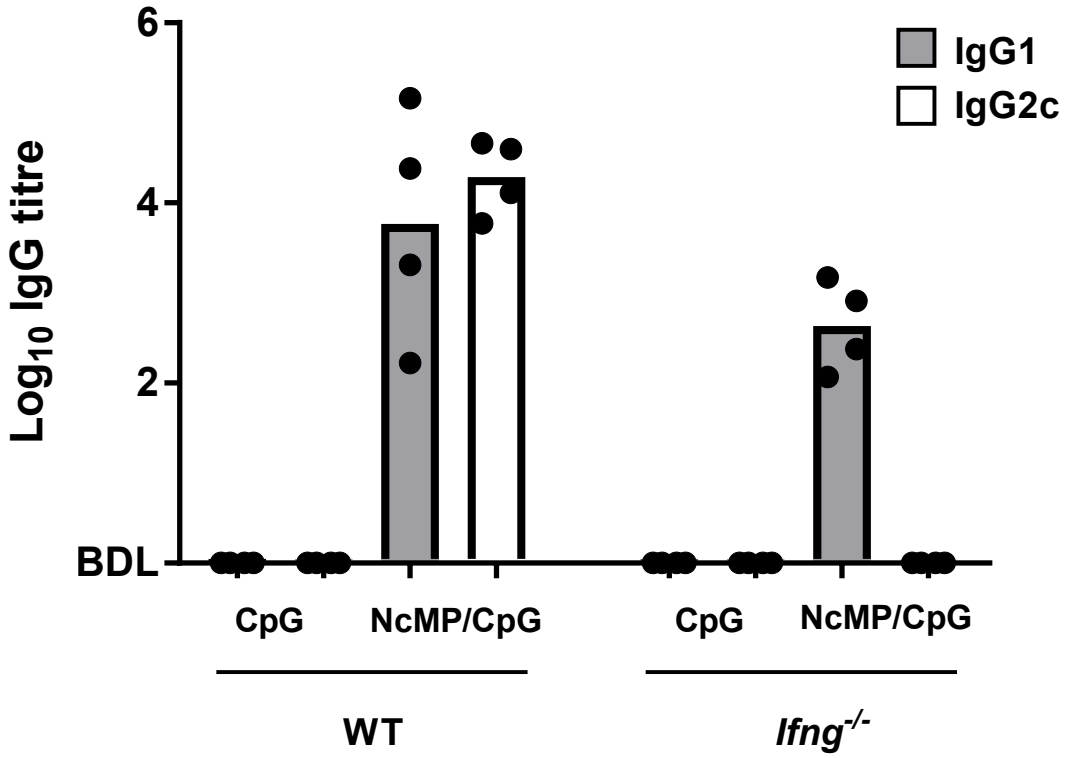
**Supplementary material 1**. IL-18-neutralizing mAb did not affect parasite-antigen stimulated FN-γ production by splenocytes of immunized *Il12b*$^{-/-}$ mice.




IFN-γ concentration in the supernatants of splenocyte cell cultures unstimulated (-) or stimulated for 3 days with *N. caninum* sonicates (NcS) (+) in the absence (0) or presence of 1 or 10 μg/ml of anti-IL-18 mAb (1 and 10, respectively). Cells were isolated from the spleens of *Il12b*$^{-/-}$ immunized mice (NcMP/CpG) or controls (CpG), 7 days after i.p. infection with $1 \times 10^4$ N. caninum tachyzoites. Bars correspond to the mean value in each group; (One-way ANOVA and Tuckey's multiple comparison test; **** $p < 0.0001$). Neutralization of IL-18 was done using anti-mouse IL-18 mAb (1 and 10 μg/ml), purified from culture supernatants of SK113AE-4 hybridoma (kindly provided by Prof. Irmgard Förster, Institut für Umweltmedizinische Forschung, University of Düsseldorf gGmbH)

using a HiTrap™ protein G HP column (GE Healthcare, Sweden). Anti-IL-18 mAb was added concomitantly with NcS. Controls were similarly treated with mouse IgG1 isotype control.

**Supplementary material 2**. Parasite-specific IgG1 and IgG2c antibody levels in willd-type or IFN-γ-deficient C57BL/6 mice immunized with NcMP plus CpG.




*Neospora caninum* sonicates-specific IgG1 and IgG2c levels detected by ELISA in the serum of wild-type or IFN-γ-deficient (*Ifng*$^{-/-}$) C57BL/6 mice immunized twice i.n. with *N. caninum* membrane protein extracts NcMP plus CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG), as indicated, 3 weeks after boost immunization. Data is presented as $\log_{10}$ of the antibody titres. Each dot represents an individual mouse. Bars correspond to the mean value in each group. BDL - below detection limit.