Neospora caninum: High susceptibility to the parasite in C57BL/10ScCr mice

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Abstract

C57BL/10ScCr mice, lack Toll-like receptor 4 and a functional Interleukin-12 receptor. Taking this into account, susceptibility of these mice to Neospora caninum infection was assessed comparatively to that of immunocompetent C57BL/10ScSn mice. C57BL/10ScCr mice inoculated intraperitoneally with 5×10^5 N. caninum tachyzoites showed a high susceptibility to this parasite. All infected C57BL/10ScCr mice were dead by day 8 post-infection whereas all control C57BL/10ScSn mice survived this parasitic challenge. Immunohistochemical analysis of infected C57BL/10ScCr mice showed N. caninum tachyzoites spread in the pancreas, liver, lung, intestine, heart and brain whereas no parasites were detected in similarly infected C57BL/10ScSn controls. The higher susceptibility of C57BL/10ScCr mice to neosporosis correlates with reduced interferon-γ mRNA expression and increased IL-4 mRNA expression, comparatively to C57BL/10ScSn controls, detected in the spleen after the parasitic challenge. C57BL/10ScCr mice could thus be used as a new experimental model where to study immunobiological mechanisms associated with host susceptibility to neosporosis.
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1. Introduction

Neospora caninum is an Apicomplexa protozoan, closely related to Toxoplasma gondii, first identified as the causative agent of a neuromuscular disease in dogs (Bjerkås et al., 1984). Later, N. caninum was found to infect a wide range of animal hosts including cattle (Dubey, 2003). Neosporosis is now recognized as a major cause of bovine abortions around the world (Dubey et al., 1988) with an important negative impact in dairy industry (Dubey and Lindsay, 1996). No effective chemotherapeutic agent or vaccine is presently available to treat or prevent satisfactorily neosporosis in cattle (Haddad et al., 2005).

Severe clinical neosporosis can be induced experimentally in immunodeficient mice (Sawada et al., 1997; Nishikawa et al., 2001; Dreier et al., 1999; Lindsay and Dubey, 1989b). Such is the case of interferon-γ (IFN-γ) gene knockout mice that are highly susceptible to inoculation with N. caninum tachyzoites (Nishikawa et al., 2001). These mice, although considered good animal models to perform bioassays aimed at detecting N. caninum, are however often difficult to obtain as previously remarked (Dubey and Lindsay, 2000).

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C57BL/10ScCr (ScCr) mice are homozygous for a deletion encompassing *Tlr4* and therefore fail to express Toll-like receptor 4 (TLR4) (Poltorak et al., 1998; Poltorak et al., 2000). Moreover, these mice also exhibit an impaired responsiveness to interleukin-12 (IL-12) (Merlin et al., 2001). This ScCr mice defect is linked to a point mutation that results in the precocious termination of the transcript for the IL-12Rβ2 chain in the IL-12 receptor (IL-12R) (Poltorak et al., 2001). In contrast, the wild-type C57BL/10 ScSn (ScSn) mice are TLR4-competent and have no defective IL-12-mediated responses (Poltorak et al., 2000).

The host protective immune response to parasitic invasion, and to *N. caninum* in particular, is cell mediated and associated to T helper 1 (Th1)-type responses with INF-γ and IL-12 having a key role in immune protective mechanisms against infection with this parasite (Innes et al., 2005). Therefore, taking into account the characteristics of the ScCr strain, in the present report we aimed at evaluating the susceptibility of these mice to *N. caninum* infection.

2. Materials and methods

2.1. Mice

Male C57BL/10ScSn and C57BL/10ScCr mice (6–8 weeks old) were obtained from the Gulbenkian Institute of Science (Oeiras, Portugal). Mice were kept at the animal facilities of the Institute Abel Salazar during the experiments. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

2.2. Neospora caninum

Neospora caninum tachyzoites (NcT) (NC-1 isolate) were cultured and serially passaged in VERO cells maintained at 37 °C in Minimum Essential Medium (MEM) containing Earle's salts (Gibco: Invitrogen Corporation, Carlsbad, California) supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (200 IU/ml), and streptomycin (200 μg/ml) (all from Sigma, St. Louis, USA) in a humidified atmosphere of 5% CO_2 in air. Free parasitic forms of *N. caninum* were obtained as previously described (Hemphill, 1996) with some modifications. Infected VERO cells were cultured until the host cell monolayer was 90% destroyed. Culture supernatants and adherent cells, harvested using a cell scraper, were centrifuged at 1100*g* for 10 min. The pellet was then passed through a 25G needle and then washed three times in phosphate-buffered saline (PBS) (centrifugation at 1100*g* for 10 min). The pellet thus obtained was resupended in 3 mL of PBS and passed through a PD-10 column (Amersham Biosciences Europe GmbH, Freiburg, Germany). Parasite concentration was determined with a hemocytometer.

2.3. Challenge infections

Neospora caninum infections were performed by intraperitoneal (i.p.) inoculation of 0.5 ml PBS containing 5×10^5 tachyzoites. Control mice were similarly treated with 0.5 ml PBS.

2.4. Histopathologic examination and immunohistochemistry

Brain, heart, intestine, pancreas, lung, and liver from *N. caninum*-infected mice and controls were collected and fixed in 10% buffered formalin and sections with 3 mm gap of each organ were obtained. These were dehydrated, embedded in paraffin wax and four serial sections were cut from each block. One section was stained with haematoxylin–eosin and the others were used for immunohistochemistry, performed according to the modified avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981). Briefly, sections were dewaxed, rehydrated and immersed in 10% target retrieval solution (Dako, Carpinteria, CA, USA) and incubated in a water bath at 100 °C for 20 min. Endogenous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol (Merck, Darmstadt, Germany) for 10 min. Sections were then incubated in a moist chamber for 20 min with normal pig serum (Dako, Glostrup, Denmark) diluted 1:5 in 10% bovine serum albumin (Sigma), to eliminate non-specific staining. Excess serum was removed and the sections were incubated overnight at 4 °C, with an anti-*N. caninum* rabbit anti-serum (Lindsay and Dubey, 1989a), diluted 1:5000. Subsequently, slides were incubated for 30 min with a 1:200 dilution of biotin-labelled anti-rabbit secondary antibody (Dako, Glostrup, Denmark) and then with the avidin–biotin–peroxidase complex (Dako, Glostrup, Denmark), for further 30 min. The colour was developed by incubation with diaminobenzidine (Dako, Carpinteria, CA, USA) for 7 min. After counterstaining tissue sections with haematoxylin, slides were mounted in Entellan (Merck). A positive reaction was indicated by the presence of brown cytoplasmic staining and formalin-fixed NcT were used as positive controls. The immunohistochemistry reaction was evaluated in a semi-quantitative mode through the count of all tachyzoites present in 5 fields of high amplification (objective 40× and ocular 10×). The adopted scale for the parasite burden was established in Table 1.

2.5. RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from 1×10^6 spleen mononuclear cells of ScSn and ScCr mice of the different experimental groups using TriPure™ Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instructions. All RNA samples were recovered in 50 μl of nuclease-free H_2O and spectrophotometrically analyzed for quantity and quality. Synthesis of cDNA was then performed from 200 μg of total RNA prepared as described above in a 20 μl final volume using

Table 1
Semi-quantitative histopathological (HE staining) and immunochemical analysis for the presence of *N. caninum*, in organs of ScSn and ScCr mice at day 7 after i.p. infection with 5×10^5 tachyzoites

Mice	Mice number	Brain	Heart	Lung	Pancreas	Intestine	Liver
ScSn	1[a]	−	−	−	−	−	−
	2[a]	−	−	−	−	−	−
	3[a]	−	−	−	−	−	−
	4[a]	−	−	−	−	−	−
ScCr	1	−	+	+ +[b]	+ + + +[b]	+ +[b]	+ + + +[b]
	2	+	−	+ +[b]	+ + + +[b]	+ +	+ + + +[b]
	3	+	+	+ +[b]	+ + + +[b]	+ +[b]	+ + + +[b]
	4	+	−	+ +[b]	+ + + +[b]	+ +[b]	+ + + +[b]

−, without tachyzoites; +, 1 a 10 tachyzoites; ++, 10–30 tachyzoites; +++, 30–50 tachyzoites; ++++, >50 tachyzoites.

[a] Mice euthanized 90 days post-infection.
[b] With associated lesions.

200 U of Superscript™ RNAse H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The Polymerase Chain Reaction (PCR) program run (42 °C, 60 min; 70 °C, 15 min; 4 °C infinite) was performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Real-time PCR was then used for the quantification of hypoxanthine phosphoribosyltransferase (HPRT) or cytokine messenger RNA (mRNA) expression levels with the LightCycler FastStart DNA Master Hybridization Probes kit (Roche, Penzberg, Germany) in a LightCycler device (Roche, Penzberg, Germany). For the quantification of HPRT expression levels, the reaction was performed in a final volume of 10 µl containing 5.0 mM MgCl$_2$, 1.0 µM of each primer (Sense: 5′-GCT GGT GAA AAG GAC CTC T, antisense: 5′-CAC AGG ACT AGA ACA CCT GC), 0.2 µM of each probe (3′-fluorescein-labelled [FL] 5′-AAA GCC TAA GAT GAG CGC AAG TTG A–FL, 5′-LC-Red 640-labelled [LC] 5′-TCT GCA AAT ACG AGG AGT CCT GTT G–PH), and 1× Master Mix plus 1 µl of the synthesized cDNA. The PCR program run was as follows: (1) denaturation at 95 °C, 10 min (2) amplification in 40 cycles 95 °C, 10 s; 60 °C, 10 s; 72 °C, 11 s (3) cooling 40 °C, 2 min. The temperature transition rate was 20 °C/s in all steps. Quantification of IFN-γ expression levels was similarly performed with specific primers (sense: 5′-TGG CAA AAG GAT GGT GAC ATG, antisense: 3′-GAC TCC TTT TCC GCT TCC TGA) (TibMol, Berlin, Germany), and probes (FL TGC CAA GTT TGA GGT CAA CAA CCC ACA–FL; LC TCC AGC GCC AAG CAT TCA ATG AGC–PH) (TibMol) in MgCl$_2$ 4.0 mM and an annealing temperature of 52 °C, and extension at 72 °C, 12 s. At the end of each annealing phase fluorescence was measured in a "single" acquisition mode with the channel setting F2/F1. Quantification of IL-4 expression levels was also similarly performed with specific primers (sense: 5′-AGG TCA CAG GAG AAG GGA CG, antisense: 3′-TGC AGC TTA TCG ATG AAT CCA G) (TibMol, Berlin, Germany), and probes (FL ACC TTG GAA GCC

CTA CAG ACG AGC–FL; LC ACT CTC TGT GGT GTT CTT CGT TGC TGT G–PH) (TibMol) in MgCl$_2$ 4.0 mM and an annealing temperature of 57 °C, and extension at 72 °C, 10 s. Quantitative evaluation of fluorescence signals from the PCR products was performed with the LightCycler software (version 3.5.) and was determined by plotting the fluorescence signals vs the cycle numbers at which the signals crossed the baseline. The baseline adjustment was performed in "minimize error" mode. Samples containing 10^8 to 10^2 cDNA molecules of the respective gene were included as external standards. The correlation coefficient among the standard reactions assured linearity.

2.6. DNA isolation and PCR analysis

For DNA extraction, each organ (brain, heart, kidney, liver, lungs, and spleen) was thawed in ten time-volume of extraction buffer (0.1 M Tris–HCl, pH 9.0, 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 20 mg/ml of proteinase K at 55 °C. DNA was purified by phenol–chloroform extraction and ethanol precipitated. DNA concentration was adjusted to 20 µg/ml for PCR. DNA of uninfected organs was prepared and used as a negative control and *N. caninum* tachyzoite DNA was prepared and used as a positive control. Specific *N. caninum* DNA was amplified by PCR by a modification of a previously described method (Liddell et al., 1999). Briefly, product amplification was performed with 1 µl of template DNA, in a final volume of 10 µl containing 1.5 mM MgCl$_2$ (Roche Diagnostics, Peuzberg, Germany), 10 mM Tris–HCl, (pH 8.3), 50 mM KCl, 200 µM of dNTPs (Invitek, Berlin), 0.5 µM of each primer (forward primer Np21plus: 5′-CCC AGT GCG TCC AAT CCT GTA AC, reverse primer Np6plus: 5′- CTC GCC AGT CAA CCT ACG TCT TCT) (TIB-Molbiol, Berlin, Germany) and 1 U of DNA polymerase AmpliTaq (Roche Diagnostics). The PCR program run was as follows: (1) denaturation at 80 °C, 1 min (2) amplification in 28 cycles (including denaturation [94 °C, 45 s], annealing [63 °C, 60 s], and extension [74 °C, 90 s]) (3) final extension [74 °C, 8 min] (4) cooling 4 °C. Amplifications were made in a MWG AG Biotech Thermal Cycler (Ebersberg, Germany). The PCR products were analysed by electrophoresis in agarose gels.

2.7. Serum IFN-γ measurements

Serum IFN-γ titres in NcT-inoculated mice and controls were quantified with the Quantikine® M Murine IFN-γ ELISA kit (R & D Systems) according to manufacturer's instructions.

2.8. Statistical analysis

For the analysis of survival curves the log rank test and R software were used. For PCR experiments the level of

significance of the results was calculated by analysis of variance single factor using Microsoft Excel 2000 software.

3. Results

3.1. Survival rate and clinical signs

In order to assess the susceptibility to *N. caninum* infection, ScCr mice and fully immunocompetent ScSn mice, were inoculated i.p. with 5×10^5 NcT. As shown in Fig. 1 all NcT-challenged ScSn mice showed no apparent signs of disease and survived until the end of the experiment (90 days). The same observation was made in PBS-treated ScCr or ScSn mice controls (data not shown). In contrast, infected ScCr mice displayed rough hair coats and were reluctant to move by day 5 post-infection. At day 6, all ScCr animals were inactive and showed pelvic limb weakness. On day 7 post-infection (p.i.) three animals were found dead and a fourth died on next day (Fig. 1). These results show that ScCr mice are highly susceptible to an i.p. challenge with NcT.

3.2. Detection of parasite DNA by PCR

The presence of *N. caninum* DNA in brain, heart, pancreas, lung, and liver of infected ScSn and ScCr mice was monitored by PCR analysis on a group of 4 mice sacrificed at day 7 p.i., when all ScCr animals already presented signs of terminal disease. In the infected ScCr mice *N. caninum* DNA was detected in all analyzed organs (Fig. 2). On the contrary, no *N. caninum* DNA was detected in any of the analyzed organ samples of ScSn mice at the same day (Fig. 2). These results suggest that in contrast to ScCr mice, ScSn mice were fully competent to control this parasitic infection.

3.3. Histopathological and immunohistochemical analysis

In order to determine the existence of lesions or the presence of parasites on organs of ScCr and ScSn mice at day 7 post-infection with 5×10^5 NcT, thin sections of brain,
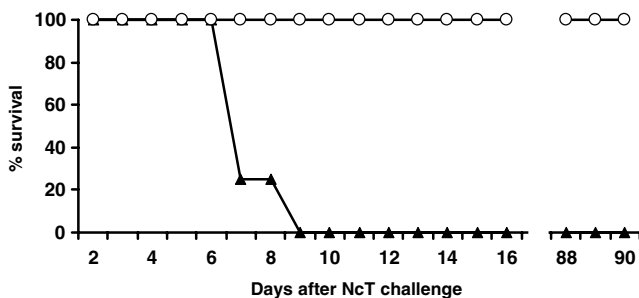
Fig. 1. High Susceptibility of ScCr mice to *N. caninum* infection. Survival rate of ScSn (open circles) or ScCr (closed triangles) mice inoculated i.p. with 5×10^5 NcT. The survival rate of ScCr mice was significantly different from that of ScSn mice (log Rank test, $\chi^2 = 7.6$ on 1 degrees of freedom, $p = 0.00582$). Four mice per group were used. This is one representative result of two independent experiments.
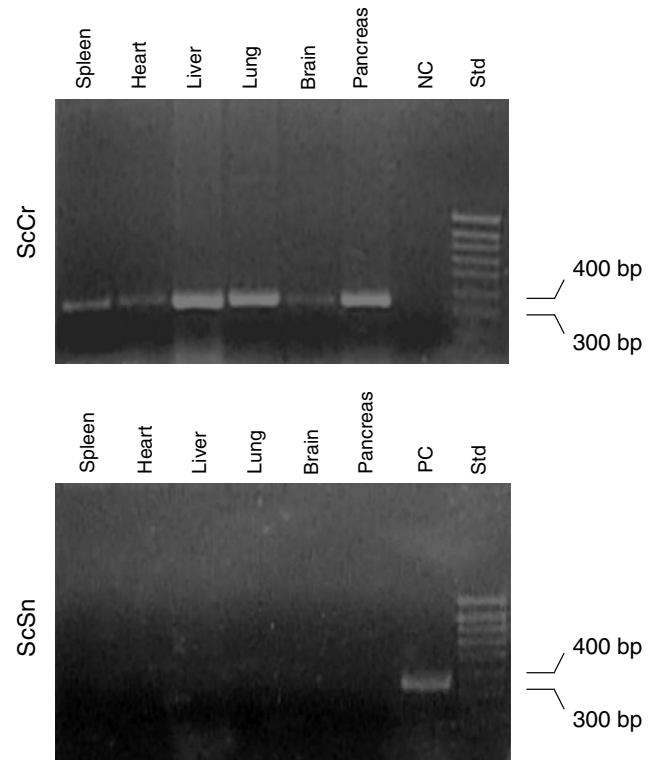
Fig. 2. Detection of parasite-specific DNA in NcT-challenged mice. Agarose gel electrophoresis of *N. caninum*-specific PCR products, of the expected band-size, amplified from DNA samples collected from the indicated organs of NcT-challenged ScCr and ScSn mice. Negative (NC) and positive (PC) control samples and DNA ladder standards (Std) were also shown. Results correspond to samples collected from one mouse of the indicated strains and are a representative example of two independent experiments performed with groups of four mice per strain.

heart, intestine, pancreas, lung, and liver were stained with HE or *N. caninum*-specific anti-serum for pathological and immunohistochemical analysis, respectively.

Results are summarized in Table 1. No lesions or parasites were observed on any organ of NcT-challenged ScSn mice. In contrast, the histopathological analysis of the infected ScCr mice showed lesions in the lung, pancreas, liver and intestine (Fig. 3). No lesions were detected in the heart and brain of these animals. All the livers of NcT-challenged mice presented a diffuse hepatitis with small necrotic areas (Fig. 3G) and tachyzoites scattered on the sinusoidal spaces (Fig. 3I). In 3 of 4 animals fibronecrotic perihepatitis was also present on the Glisson capsule (Fig. 3H). In pancreas of all infected animals, an extensive necrosis with neutrophilic, lymphocytic and plasma cells infiltration was present (Fig. 3D and E). These lesions were more conspicuous on the periphery of the organ. In these necrotic areas, small clusters of tachyzoites forming cyst-like structures were also observed (Fig. 3E). The same kind of lesions was present in adipose and connective tissue surrounding the pancreas. The lung of 3 of the 4 animals had large areas of atelectasis and pneumonia with neutrophils (Fig. 3A and B). In the remaining mouse, alveolar edema and hemorrhage was present. The small intestine of all animals showed an infiltration of neutrophils (Fig. 3K), necrosis
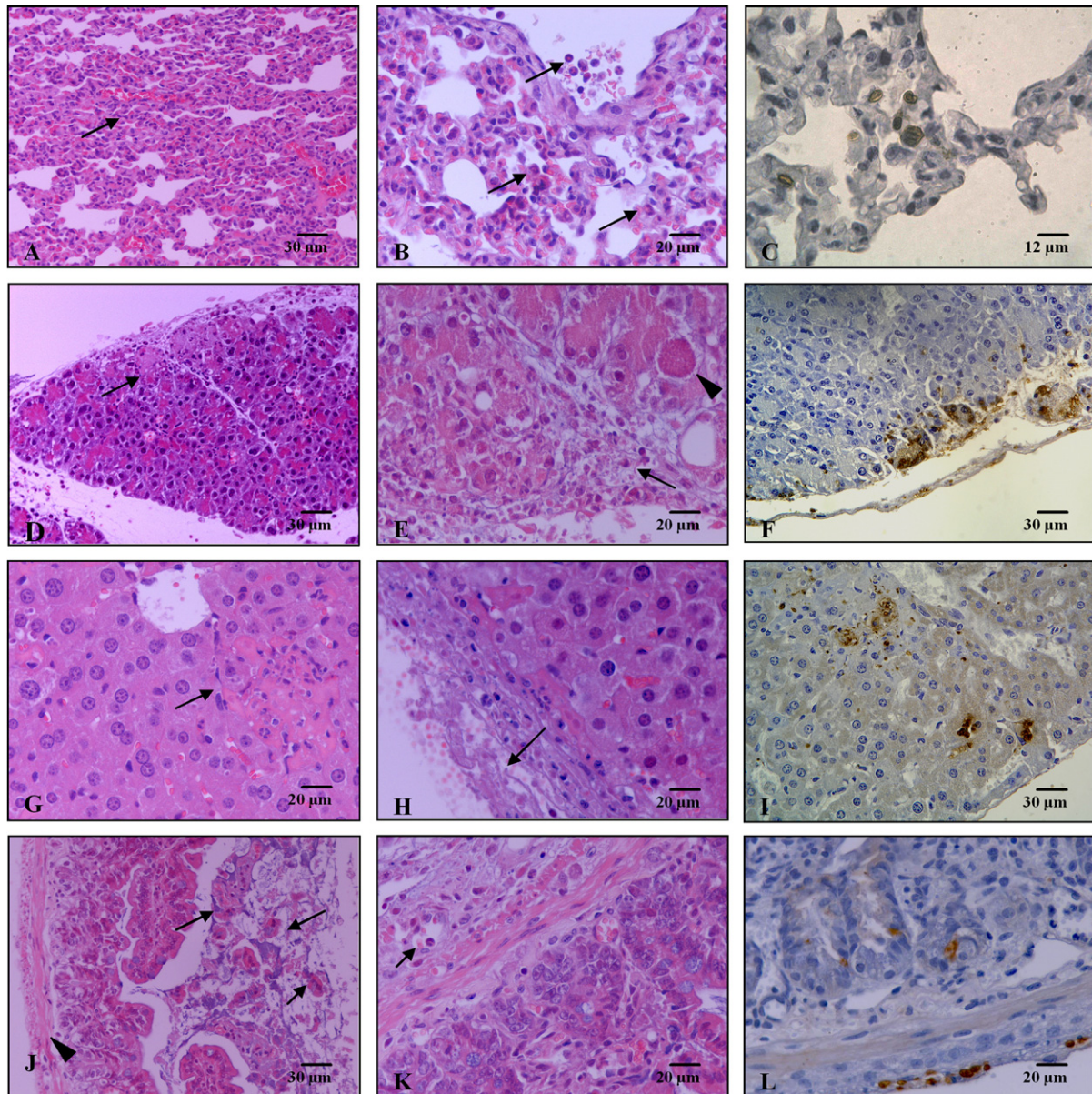
Fig. 3. Histopathology and immunohistochemistry analysis of ScCr mice organs on day 7 after infection with 5×10^5 NcT. Thin sections of lung (A–C), pancreas (D–F), liver (G–I), and intestine (J–L) of ScCr mice stained with haematoxylin–eosin or by immunohistochemistry with specific *N. caninum* anti-serum and counterstained with haematoxilyn. The lung is characterized by atalectasic areas (A—arrow) and pneumonia with neutrophil infiltration (B—arrow). In (C) tachyzoites inside macrophages are observed. In pancreas, an extensive necrotic area (D—arrow), cyst-like structures (E—arrowhead) and inflammatory infiltration (E—arrow) and several tachyzoites (F) are present, mostly in the periphery of the organ. The liver showed small necrotic areas (G—arrow) a fibrino-necrotic peri-hepatitis (H—arrow) and disseminated tachyzoites essentially in the periphery of the organ (I). In small intestine nesrosis and desquamation of the villi (J—arrows) thick membrane of fibrino-necrotic inflammation on serosal surface (J—arrowhead), infiltration of neutrophils (K—arrow) and tachyzoites in serosal surface and mucosa (L) was present. This is one representative result of two independent experiments with four mice each.

and desquamation of the villi (Fig. 3J). On the serosal surface, a fibrino-necrotic inflammation was present forming a thick membrane (Fig. 3J). These lesions were also present in the large intestine but with lower intensity (data not shown).

By immunohistochemical analysis *N. caninum* was observed in three forms: free tachyzoites, aggregates of parasites in cyst-like structures and as an antigen in the cytoplasm of phagocytic cells. This study revealed no *N. caninum* forms in ScSn mice organs. In ScCr mice, the parasite was present in most of the analyzed tissues (Table 1). Brain and heart had only a small number of free tachyzoites or rare cyst-like structures. The lung presented a moderate number of tachyzoites mostly at alveolar capillaries (Fig. 3C) and in two animals, cyst-like structures and antigen inside phagocytic cells. The pancreas and liver were the most affected organs with the parasite present essentially in the periphery of the organ (Fig. 3F and I). The small intestine presented a moderate number of tachyzoites mostly on the serosal surface but also on the mucosa (Fig. 3L).

3.4. Cytokine analysis

Since the production of IFN-γ and IL-4 have been respectively associated with host resistance or susceptibility to neosporosis (Nishikawa et al., 2001; Baszler et al., 2000), the levels of mRNA coding for these cytokines were assessed in the spleen of ScSn and ScCr mice challenged with *N. caninum.* As shown in Fig. 4, spleen IL-4 mRNA levels were found to be higher in ScCr mice challenged with NcT than in respective PBS-treated controls or similarly challenged ScSn mice, 4 days after the i.p. inoculations. In NcT-infected ScSn mice, spleen IL-4 mRNA levels were not detected above those of PBS-treated controls. As also shown in Fig. 4, ScSn mice challenged with NcT had increased levels of mRNA for IFN-γ as compared to PBS-treated controls, detected on day 4 after challenge. The levels of mRNA for IFN-γ detected in the spleen of ScSn mice at 2 and 4 days after i.p. challenge with NcT, were significantly increased as compared to those detected in similarly
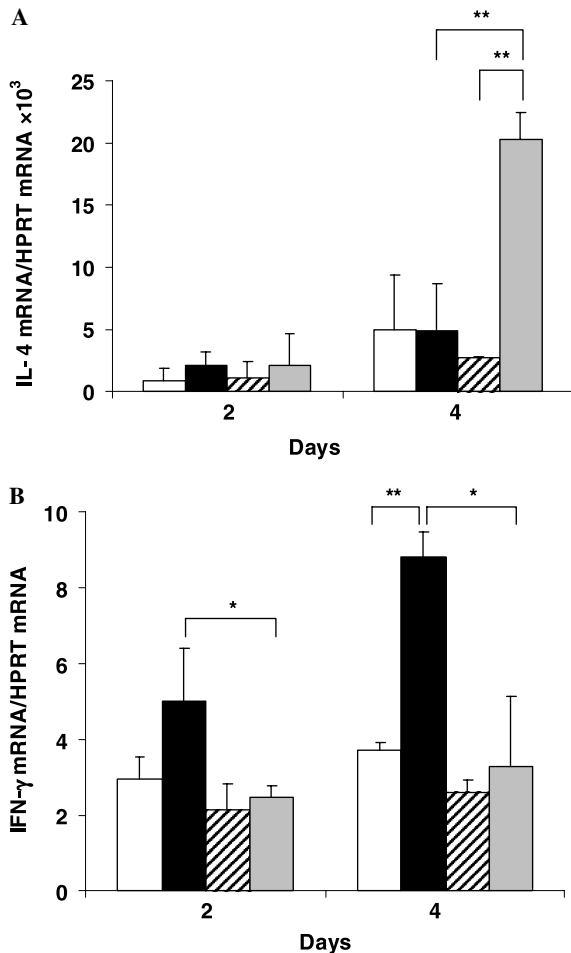
Table 2
Concentration of IFN-γ, assessed by ELISA in the sera of ScSn or ScCr mice, 2 or 5 days after i.p. treatment with PBS or with 5×10^5 NcT, as indicated

Mice	Treatment	IFN-γ pg/ml	
		Day 2	Day 5
ScSn	PBS	13 ± 10[1]	3 ± 3
	5×10^5 NcT	592 ± 11[**] [(a)], [**] [(b)]	784 ± 55[**] [(a)], [**] [(b)]
ScCr	PBS	ND	ND
	5×10^5 NcT	ND	209 ± 31[**] [(a)]

[1] Results represent the mean plus one SD of three mice per group. Statistically significant differences (**, $p < 0.01$) between NcT-challenged and PBS-treated mice on each strain (a) or between NcT-challenged mice in the two different strains (b), on the indicated days, were shown. ND, not detected; this is one representative result of two independent experiments.

challenged ScCr mice (Fig. 4). In NcT-infected ScCr mice, spleen IFN-γ mRNA levels were not detected above those of PBS-treated controls. No differences on IFN-γ or IL-4 mRNA levels were found among the different studied mice groups on day 1 after challenge (data not shown). In agreement with the increased spleen levels of IFN-γ mRNA detected in NcT-challenged ScSn mice, comparatively to similarly challenged ScCr mice, serum levels of this cytokine were also found to be higher in ScSn than in ScCr mice, 2 or 5 days after challenge with NcT (Table 2). Interestingly, as shown in Table 2, serum IFN-γ was also detected on day 5 after the parasitic challenge in ScCr mice, although at lower levels than those detected in ScCn mice. Together, these results indicate that the higher susceptibility of ScCr mice to *N. caninum* infection results from their impaired capacity to mount a protective IFN-γ-mediated immune response, reinforcing the association of host production of this cytokine with resistance to neosporosis.

4. Discussion

In the present report, we show that ScCr mice are highly susceptible to an i.p. challenge with NcT, developing lethal acute neosporosis. In contrast, ScSn mice were able to contain the infection when challenged with the same inoculum. This increased susceptibility of the ScCr mice comparatively to that of ScSn mice could be explained by either or both genetic defects carried by the ScCr strain: lack of the *Tlr4* gene (Poltorak et al., 1998) and defective *IL-12Rβ2* gene (Poltorak et al., 2001).

It was previously described that ScCr mice have an impaired IFN-γ-mediated response to protozoa (Freudenberg et al., 1991; Müller et al., 1997). This is in agreement with the results reported here that ScCr mice challenged i.p. with *N. caninum* did not show increased spleen levels of IFN-γ mRNA, a key cytokine in host resistance to *N. caninum* (Nishikawa et al., 2001; Khan et al., 1997). In contrast, similarly challenged ScSn mice responded with increased IFN-γ mRNA and were fully competent to resist the infection. This quantitative difference in the expression of IFN-γ mRNA detected in the *N. caninum*-infected ScCr, comparatively to ScSn mice, is in agreement with a similar pattern of

Fig. 4. Levels of IL-4 (A) or IFN-γ (B) mRNA normalized to HPRT mRNA, detected by real-time PCR in the spleen of ScSn mice 2 or 4 days after challenge i.p. with PBS (open bars) or with 5×10^5 NcT (closed bars) or in the same organ in ScCr mice treated i.p. with PBS (dashed bars) or with 5×10^5 NcT (grey bars). Bars represent the mean plus one SD of three mice per control or NcT-infected groups. For each assessed cytokine, this is one representative result of two independent experiments. (*$p < 0.05$; **$p < 0.01$).

IFN-γ production reported to occur in the same mice strains upon infection with another *Apicomplexa* protozoan (Freudenberg et al., 1991). Moreover, increased levels of IL-4 mRNA were also detected in the spleen of ScCr mice comparatively to ScSn mice. This is in agreement with previous studies where production of this cytokine has been associated with host susceptibility to *N. caninum* infection (Baszler et al., 2000; Long and Baszler, 2000; Quinn et al., 2004).

The impaired immune response of ScCr mice to microbial infections has been explained by their inability to respond to IL-12 (Merlin et al., 2001). Taking into account previous reports where this cytokine has been shown to mediate protection to *N. caninum* infection in mice (Khan et al., 1997; Baszler et al., 1999) the high susceptibility of ScCr mice to neosporosis reported here, is in agreement with their lack of IL-12 responsiveness. IL-12 is a well known inducer of IFN-γ production and favours the differentiation of Th1 cells (Trinchieri, 2003). In addition, IL-12 has also been shown to directly contribute for *in vitro* proliferation, expression of CD25 (and IFN-γ mRNA) by CD4$^+$CD25$^-$ T cells, even in the presence of T regulatory cells (King and Segal, 2005). Therefore, considering these activities of IL-12, it could be hypothesized that lack of a functional IL-12R could impair the activation of effector T cells in the NcT-challenged ScCr mice. Also IL-12 has been recently shown to increase IFN-γ production by IL-2 activated bovine NK cells in response to NcT (Boysen et al., 2006) and a similar host-protective mechanism, necessarily absent in the ScCr mice, can also be relevant for resistance to murine *N. caninum* infection. Production of IFN-γ by mechanisms independent of IL-12 has been described (Freundenberg et al., 2002). This could explain the detection of the former cytokine in the serum of ScCr mice, however at lower levels and later than in ScSn mice. However, taking into account the lethal susceptibility to *N. caninum* of ScCr mice, this later and lower IFN-γ production is not enough to confer protection against this parasite. This could indicate that protective immune mechanisms effective to prevent NcT infection must be elicited early after the infectious challenge.

ScCr mice also fail to express TLR4 (Poltorak et al., 1998; Poltorak et al., 2000). Therefore, their increased susceptibility to *N. caninum* could also be contributed for by this particular deficiency. This pattern recognition receptor has been shown to be relevant for the host immune defence against the protozoa *Leishmania major* (Kropf et al., 2004) and *Trypanosoma cruzi* (Oliveira et al., 2004). Also surface lipopeptidophosphoglycan of *Entamoeba hystolytica* was shown to induce the release of pro-inflammatory mediators by human macrophages (Maldonado-Bernal et al., 2005). However, signalling via TLR4 of heat shock protein 70 from the *N. caninum* closely related protozoan *T. gondii* has been implicated in mechanisms of controlling excessive inflammation triggered by this parasite (Mun et al., 2005). Further studies to be done in mice deficient in TLR4 in the absence of other genetic

defects will thus be necessary in order to determine whether this receptor plays a significant role in the course of *N. caninum* infection. In particular it would be interesting to determine in what measure the lack of TLR4 expression contributes to the severe pathology observed in the NcT-challenged ScCr mice.

The ScCr mice could thus be useful for subsequent studies on the immunobiology of *N. caninum* infection and constitute an alternative model to mice bearing more severe immunodeficiencies.

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