Genetic manipulation of *Neospora caninum* to express the bradyzoite-specific protein NcSAG4 in tachyzoites

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SUMMARY

Neospora caninum is an apicomplexan parasite and the aetiological agent of bovine neosporosis, one of the main causes of reproductive failure worldwide. We have generated 2 independent transgenic knock-in clones, Nc-1SAG4^{c}1.1 and Nc-1SAG4^{c}2.1, that express the bradyzoite stage-specific protein NcSAG4 in the tachyzoite stage. These clones have similar growth rates *in vitro* as the wild-type (WT) strain Nc-1. Studies in a cerebral mouse model of infection revealed a slightly lower rate of detection of the transgenic strains in brains during the chronic phase of infection. However, a pregnant mouse model of infection revealed a reduction in the virulence of the Nc-1SAG4^{c}1.1 strain despite the same tachyzoite expression of Nc*SAG4* and a similar anti-NcSAG4 response displayed by mice inoculated with Nc-1 SAG4^{c}1.1 or Nc-1 SAG4^{c}2.1 parasites. This behaviour may be related to the reduced ability of the Nc-1SAG4^{c}1.1 parasites to invade host cells, which was observed in *in vitro* assays. The apparent reduction in persistence and the high growth rate of the transgenic strains, together with their constitutive expression of the protein NcSAG4, may be useful features for future immunoprophylaxis trials based on a safe live attenuated vaccine.

Key words: *Neospora caninum*, NcSAG4, knock-in strain, virulence, vaccine.

INTRODUCTION

Bovine neosporosis is one of the major causes of reproductive failure in cattle worldwide (Dubey *et al*. 2007). The aetiological agent is *Neospora caninum*, an apicomplexan coccidian that shares similar biological features with the closely related parasite *Toxoplasma gondii* (Innes and Mattsson, 2007). Transplacental transmission, which results from the reactivation of a persistent endogenous infection, is considered to be the major route of *N. caninum* transmission in cattle (Innes, 2007). Infection of a pregnant cow may lead to abortion, stillbirth or the birth of weak or healthy, but congenitally infected calves which can later transmit the infection to their offspring. One of the main challenges of control programmes is to prevent the establishment of a persistent infection, which enables the perpetuation of infection in herds without the participation of definitive hosts (Reichel and Ellis, 2006).

Low-virulence isolates show a reduced ability to persist in their host (lower encystation into tissue cysts in the brain, or lower presence in offspring due to reduced crossing of the placental barrier) and have been suggested as promising vaccine candidates for bovine neosporosis (Williams *et al*. 2007; Rojo-Montejo *et al*. 2009*a*). Several different approaches have been used to obtain low-virulence *Neospora* strains, including *in vitro* isolation from asymptomatic naturally infected animals (Miller *et al*. 2002; Regidor-Cerrillo *et al*. 2008; Rojo-Montejo *et al*. 2009*b*), isolation of temperature-sensitive mutants (Lindsay *et al*. 1999), gamma-irradiation of tachyzoites (Ramamoorthy *et al*. 2006; Teixeira *et al*. 2005), attenuation of virulent isolates by successive passages in cell culture (Bartley *et al*. 2006) and even genetic manipulation-based methods for the case of *T. gondii* (Dzierszinski *et al*. 2000; Cerede *et al*. 2005; Kim and Boothroyd, 2005; Saeij *et al*. 2005b; Kim *et al*. 2007) .

Additionally, bradyzoite stage-specific surface proteins seem to be involved in the persistence of bradyzoites in brain tissue in *T. gondii* infections (Kim and Boothroyd, 2005). Nonetheless, in *N. caninum* our understanding of bradyzoite stage-specific proteins is limited to NcSAG4 and NcBSR4 (Fernandez-Garcia *et al*. 2006; Risco-Castillo *et al*. 2007), whose biological functions have not yet been elucidated.

In the present study, genetic manipulation of *N. caninum* was successfully performed to generate tachyzoites that constitutively express the bradyzoite-specific antigen NcSAG4. Our hypothesis consists of avoiding the establishment of the chronic infection by the transgenic parasites generated, following the approach previously suggested by Kim and Boothroyd (2005). The early exposure of NcSAG4 to

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the immune system at the beginning of the infection, with the subsequent development of a specific immune response, might lead to the clearance of all the parasites, avoiding the tissue cyst formation. Both the transgenic Nc-1 SAG4^{c} strains and the wild-type (WT) isolate, Nc-1 WT, were characterized *in vitro* and their virulence was evaluated in both cerebral and pregnant mouse models of infection.

MATERIALS AND METHODS

Neospora caninum *culture and generation of NcSAG4-constitutive transgenic strains (Nc-1 SAG4^{c})*

N. caninum tachyzoites were grown in MARC-145 cell culture, according to standard procedures (Perez-Zaballos *et al*. 2005). Generation of the Nc-1 SAG4^{c} knock-in parasites was performed in a clonal hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) deficient mutant of *N. caninum* (Nc-1 *hxgprt*-, gift from Dr P. Bradley). The Nc*SAG4*-carrying vector was constructed in a pBluescript plasmid (Kim and Boothroyd, 2005). Promoter sequences from the *T. gondii* tachyzoite-specific gene *GRA1* (p*GRA1*) were used to express Nc*SAG4* in the tachyzoite stage. The full-length Nc*SAG4* coding region was amplified by PCR with the following primers: 5′GGGCATGCATGAGAA-AAGCGCCTTCTTTCCCAGG3′ and 5′GCCCC-TTAATTAACCCTTATATCGAACTCAGTGC3′. Amplified products were digested with *Nsi*I and *Pac*I and cloned in between the indicated promoter and the *GRA2* downstream sequence in the plasmid vector, which also contained a copy of the *HXGPRT* gene, driven by the dihydrofolate reductase promoter (p*DHFR*) in the reverse orientation (Kim and Boothroyd, 2005). Next, restriction enzyme-mediated integration (REMI) was applied to optimize the plasmid insertion rate into the genome. The plasmid was linearized with *Not*I and transfected in the presence of *Not*I by electroporation into 10^{7} Nc-1 *hxgprt*-tachyzoites (Donald and Roos, 1993). Stably transfected tachyzoites were selected with mycophenolic acid and xanthine (50 μg/ml each) (Sigma, St Louis, MO, USA) in a HFF cell monolayer.

SDS-PAGE, Western blot and immunofluorescence analysis of Nc-1 SAG4^{c}

The expression of NcSAG4 in transgenic and Nc-1 WT tachyzoites and in a control of *in vitro*-induced bradyzoites (Risco-Castillo *et al*. 2004) was analysed by Western blot following standard methods, using a polyclonal rabbit antiserum developed against the recombinant NcSAG4 protein (Fernandez-Garcia *et al*. 2006). Immunofluorescence assays were performed by labelling infected monolayers with the NcSAG4 polyclonal antisera followed by the incubation with a goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA). Micrographs were taken using a digital camera (Nikon Digital Sight DS-L1) connected to an inverted fluorescence microscope (model TE200 Nikon 100X oil-immersion objective).

Nucleic acid isolation and PCR

An RNeasy Kit (Qiagen, Hilden, Germany) was used to extract RNA from transgenic and Nc-1 WT tachyzoites and the control of *in vitro*-induced bradyzoites (Risco-Castillo *et al*. 2004). cDNA was synthesized by RT-PCR as described previously (Fernandez-Garcia *et al*. 2006). Primers specific for the Nc*SAG4*, Nc*SAG1* and *18S* ribosomal RNA (Nc*18sR*) genes (Fernandez-Garcia *et al*. 2006) were used to perform a relative quantification by quantitative real-time PCR to compare Nc*SAG4* transcript levels (Collantes-Fernandez *et al*. 2002). All samples were processed in triplicate and the results were expressed as *x*-fold induction compared to Nc-1 WT, calculated using the $2^{-\Delta\Delta Ct}$ formula (Aguado-Martinez *et al*. 2009; Livak and Schmittgen, 2001). A DNeasy Blood and Tissue Kit (Qiagen) was used to extract DNA from tachyzoite cell cultures used to estimate tachyzoite proliferation rates by absolute quantification by quantitative real-time PCR. A Realpure Genomic DNA Purification System (Durviz, Valencia, Spain) was used to extract DNA from mouse tissues. The presence of parasite DNA was detected using a nested PCR to amplify the ITS-1 region of *N. caninum* (Buxton *et al*. 1998).

Tachyzoite-bradyzoite switch assay

In vitro stage conversion for all the strains evaluated, Nc-1 SAG4^{c} strains and Nc-1 WT and Nc-Liv (conversion control), was performed by the addition of sodium nitroprusside as described previously (Risco-Castillo *et al*. 2004). At 3, 5, and 7 days post-stress induction, the tachyzoite-bradyzoite conversion rates were assessed using a double-immunofluorescence assay (Risco-Castillo *et al*. 2004; Rojo-Montejo *et al*. 2009*b*). The experiment was repeated in triplicate in 2 independent assays and chi-square and Fisher *F*-tests were employed for data analysis.

Invasion assay

Invasion assays were performed for both transgenic strains and the Nc-1 WT. MARC-145 monolayers were infected with purified *N. caninum* tachyzoites and incubated for 4 h at 37 °C with 5% CO_2. The invasion rate was estimated by a double immunofluorescence assay in which a rabbit polyclonal anti-*Neospora* tachyzoite antibody (Alvarez-Garcia *et al*.

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2007) followed by an anti-rabbit Alexa Fluor 594 conjugated antibody were used to label extracellular tachyzoites. The cells were then permeabilized and incubated again with the same primary antibody, followed by an anti-rabbit Alexa Fluor 488 conjugated antibody to stain intracellular tachyzoites. The assays were carried out in quadruplicate, and each experiment was repeated 4 times. The chi-square and Fisher *F*-tests were employed for data analysis.

Proliferation assay

A proliferation assay was performed with the transgenic strains and the Nc-1 WT isolate. MARC-145 cells were grown and infected with purified tachyzoites, which were allowed to invade host cells for 4 h. Samples were recovered at different time-points (4 h, 8 h, 20 h, 32 h, 44 h, 56 h and 68 h post-infection, h.p.i.) and the proliferation rate was estimated by quantitative real-time PCR. Each condition was tested in triplicate in 3 independent assays. Regression analyses were run on log-transformed data to determine the functional dependence of cell number on time (h.p.i.) (Sundermann and Estridge, 1999).

Pathogenicity studies

Cerebral mouse model of infection. *N. caninum* distribution during acute infection and persistence in the chronic phase was evaluated in a cerebral mouse model of infection (Collantes-Fernandez *et al.* 2006). BALB/c mice (Harlan Interfauna Ibérica, Barcelona, Spain) were injected with 2 different doses (2×10^6 and 10^7) of transgenic or WT *Neospora* strains or PBS. Mice were sacrificed with CO_2 at different time-points (days 7, 14 and 28), and tissue samples were recovered. *N. caninum* DNA was detected by PCR in lungs and brain. Humoral immune responses were evaluated using serum.

Pregnant mouse model of infection. A pregnant BALB/c mouse model of infection (Lopez-Perez *et al.* 2006, 2008; Whitten, 1957) was employed to examine whether constitutive expression of NcSAG4 affects the vertical transmission of parasites during pregnancy and/or the pup's mortality and morbidity. Pregnant females were infected subcutaneously 7 days after conception with 2×10^6 tachyzoites from transgenic strains, WT strains or PBS and then followed for 64 days. The following parameters were evaluated: parasite presence and humoral response in the dams, body weight (from day 14 to day 42 post-partum (PP), every 2 days), hebdomadal mortality (from birth until day 2), post-natal mortality (from day 2 to day 50 PP), vertical transmission rate (by using nested PCR in brain and lung tissues) and humoral response in the pups. Dams and pups were sacrificed with CO_2 gas on day 50 PP to evaluate humoral immune responses in the offspring without the interference of colostral antibodies (Appleby and Catty, 1983). Mortality was analysed by the Kaplan-Meier survival method (Bland and Altman, 1998) and log-rank test was applied to compare survival curves (Bland and Altman, 2004). Vertical transmission was analysed by the chi-square and Fisher *F*-tests.

Humoral immune responses from experimental mouse infections

Levels of *N. caninum*-specific IgG2a and IgG1 isotypes in the serum were determined using a soluble *N. caninum* tachyzoite antigen ELISA (Collantes-Fernandez *et al.* 2006). Antibody responses that developed against the NcSAG4 protein were evaluated by employing recombinant NcSAG4 (rNcSAG4) protein in an ELISA assay (Aguado-Martinez *et al.* 2008). Data were analysed by a one-way ANOVA followed by Duncan's Multiple Range test.

RESULTS

Generation of SAG4-constitutive mutants (Nc-1 SAG4^c)

Two independent clones expressing the NcSAG4 protein in the tachyzoite stage, Nc-1 SAG4^{c}1.1 and Nc-1 SAG4^{c}2.1, were selected from stable populations of transfected tachyzoites for further analysis. Constitutive expression and surface localization of NcSAG4 protein in the transgenic parasites were confirmed by Western blot and immunofluorescence (Fig. 1A and B). Furthermore, both transgenic clones showed similar and substantial levels of Nc*SAG4* transcript (1893-fold induction for Nc-1 SAG4^{c}1.1, 1655-fold induction for Nc-1 SAG4^{c}2.1 and 429-fold induction for the *in vitro*-induced bradyzoite control, compared to Nc-1 WT tachyzoites).

In vitro *studies, tachyzoite-bradyzoite switch, invasiveness and proliferation rates*

Transgenic and Nc-1 WT parasites exhibited a similar low rate of tachyzoite-bradyzoite conversion ($P>0{\cdot}05$, χ^2, Fisher *F*-tests; Fig. 2A). In addition, similar invasion rates were observed for both Nc-1 SAG4^{c}2.1 and WT tachyzoites, whereas the Nc-1 SAG4^{c}1.1 strain showed a significantly reduced invasion capacity in all the assays performed ($P<0{\cdot}05$, Student's *t*-test) (Fig. 2B). Finally, no significant differences were observed in the proliferation rates between the 3 strains ($P>0{\cdot}006$, $r^2\geqslant0{\cdot}95$) (Fig. 2C). The generation time was approximately 9–11 h for both transgenic and WT parasites.

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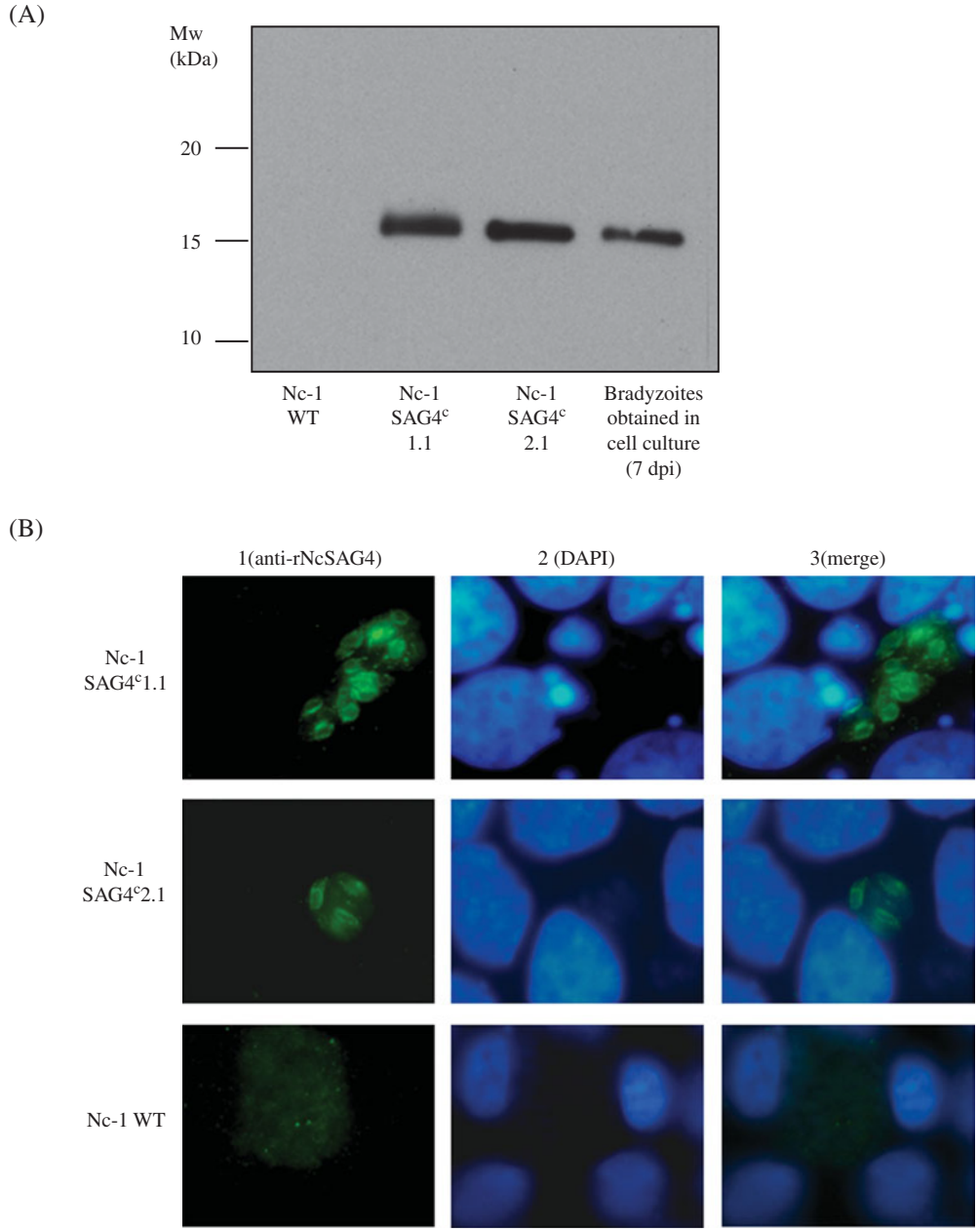

Fig. 1. Expression of NcSAG4 in transgenic strains. (A) Western blot analysis of NcSAG4 protein expression in transgenic clones, WT strains and bradyzoite extract (positive control). Zoites were counted using Trypan blue staining and the same number of parasites was used for each extract. (B) Immunofluorescence images of transgenic and WT parasites. Panels 1: parasites recognized by polyclonal anti-NcSAG4 antibodies (green). Panels 2: host cell nuclear material detected by 4′,6-diamidino-2-phenylindole (DAPI) staining. Panels 3: merge of recognized parasites and host cell nucleus.

In vivo *studies*

Cerebral mouse model of infection. Mice did not show clinical neosporosis or succumb to infection during the follow-up period. *N. caninum* infection was dose dependent, with higher doses resulting in increased parasite detection. The course of infection was also dependent on the strain used so that a higher persistence of infection was observed for Nc-1 WT-inoculated mice (Table 1). Both transgenic Nc-1 SAG4^c strains had a lower frequency of parasite detection in the brain during the chronic phase, and the parasite was mostly cleared by 28 days p.i. Nonetheless, differences between Nc-1 SAG4c1.1 and Nc-1 WT were only significant in the groups inoculated with the high dose (10^7) ($P=0{\cdot}0225$, Fisher F-tests). The differences between Nc-1 SAG4^{c}2.1 and Nc-1 WT with the same dose were not significant, but there was a trend towards significance ($P=0{\cdot}0515$, Fisher F-tests).

Concerning the humoral immune response, a specific dose-dependent response against NcSAG4 was developed only in groups inoculated with transgenic Nc-1 SAG4^c tachyzoites. IgG1 and IgG2a

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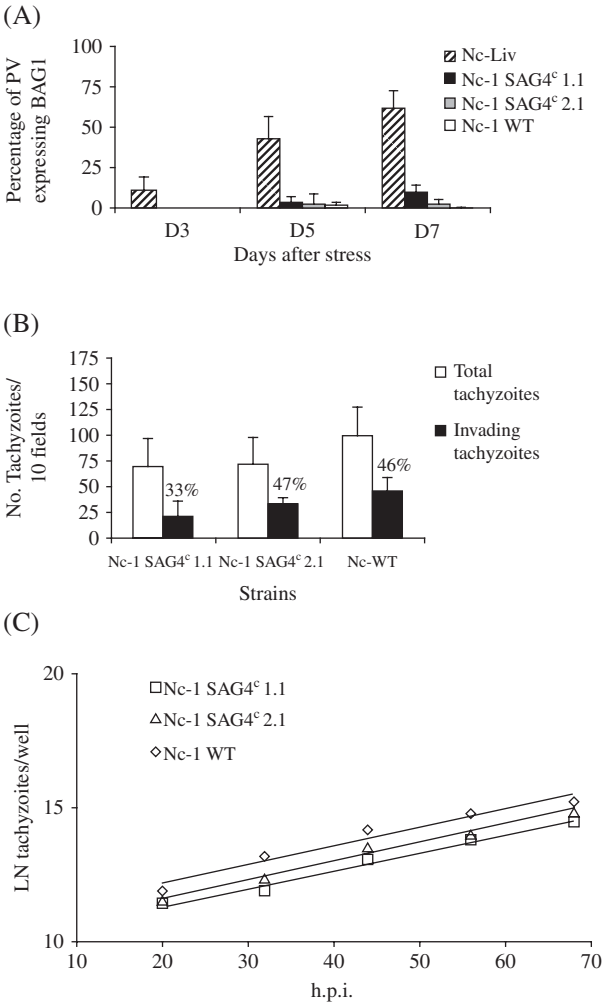

Fig. 2. *In vitro* assays. (A) Tachyzoite-bradyzoite switch assay. Bars represent the mean percentage of parasitophorous vacuoles expressing BAG1 for the different strains from day 3 to day 7 after stress. (B) Host-cell invasion assay. White columns indicate both intracellular and extracellular tachyzoites interacting with the host cell, and black columns indicate intracellular parasites only. The invasion rate is shown as a percentage for each strain. (C) Proliferation assay. Lines represent the linear regression (LN transformed) of tachyzoite number as a function of time. Slopes indicate the growth rate. Figures represent a pool of all the individual data sets.

responses were also dose dependent at each time-point studied in transgenic Nc-1 SAG4^{c}-inoculated mice ($P<0{\cdot}0001$, one-way ANOVA, Duncan's post-test) but not for Nc-1 WT-inoculated mice on days 14 and 28 p.i. (data not shown).

Pregnant mouse model of infection. When the presence of the parasite was investigated in dam's brains, 18% (3/16) of Nc-1 WT-infected mice and 40% (2/5) of Nc-1 SAG4^{c}2.1-infected mice tested positive. In contrast, all dams (0/17) from Nc-1 SAG4^{c}1.1-infected mice were PCR-negative. IgG1 and IgG2a responses showed similar levels for all inoculated dams ($P>0{\cdot}05$, one-way ANOVA). However, only dams inoculated with the transgenic parasites developed a specific immune response against the NcSAG4 protein ($P<0{\cdot}0001$, one-way ANOVA, Duncan's post-test) (data not shown).

Concerning pups, the results showed that an infection during gestation with Nc-1 WT and Nc-1 SAG4^{c}2.1 resulted in a delayed weight gain from day 20 to day 42 PP ($P<0{\cdot}05$, one-way ANOVA and Duncan's post-test) (data not shown). In contrast, pups born from mice infected with Nc-1 SAG4^{c}1.1 and PBS remained healthy throughout the experiment. No significant differences in percentage of hebdomadal mortality were observed ($P=0{\cdot}5289$, χ^2) (Table 2). During the neonatal period, several pups born from Nc-1 WT and Nc-1SAG4^{c}2.1-infected dams showed clinical signs of infection (a delay in the hair coat development, rough hair coat and neurological signs) (Table 2), all of which were PCR-positive for parasites in the brain. In addition, the survival percentage of the Nc-1 SAG4^{c}1.1-inoculated mice was significantly higher compared to the Nc-1 WT and Nc-1SAG4^{c}2.1-infected groups ($P=0{\cdot}0213$ and $P=0{\cdot}0032$, Log-rank test, respectively) (Fig. 3A). When transplacental transmission was evaluated, Nc-1 WT and Nc-1SAG4^{c}2.1-infected dams transmitted the infection, respectively, to a 100% and 87·5%. In contrast, in the Nc-1 SAG4^{c}1.1-infected dams, *N. caninum* DNA was only detected in the lung of 1 pup ($P<0{\cdot}0001$, χ^2) (Table 2). Neonates from the Nc-1 WT and Nc-1SAG4^{c}2.1-infected dams developed higher IgG1 and IgG2a antibody titres compared to pups born from Nc-1SAG4^{c}1.1-infected dams ($P<0{\cdot}0001$, one-way ANOVA, Duncan's post-test) (Fig. 3B).

DISCUSSION

In this study, for the first time, transgenic *N. caninum* tachyzoites that constitutively express the bradyzoite-specific antigen NcSAG4 were generated. The approach we followed might have 2 important applications. Firstly, the successful genetic manipulation accomplished in *Neospora* may lead to additional cell biology and gene function studies, which have been widely performed in *Toxoplasma* (Bohne *et al*. 1998; Dzierszinski *et al*. 2000; Huynh *et al*. 2004; Cerede *et al*. 2005; Kim and Boothroyd, 2005). Secondly, generating marked parasites that are unable to persist is the main challenge for safe live vaccines and to differentiate between naturally infected and vaccinated animals.

Homologous and stable expression of *N. caninum* genes has been successfully accomplished in generating NcSAG4 knock-in strains (tachyzoites that constitutively express the bradyzoite stage-specific protein NcSAG4) through HXGPRT selection and tested using immunofluorescence and Western blot (Matrajt *et al*. 2002; Saeij *et al*. 2005*b*; Kim *et al*.

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Table 1. Detection of *Neospora caninum* DNA by nested PCR in lung and brain samples from BALB/c mice inoculated with 2×10^6 or 10^7 transgenic Nc-1 SAG4[c] or WT tachyzoites

2×10^6 tachyzoites Day p.i.[b]	Nc-1 SAG4[c] 1.1[a]		Nc-1 SAG4[c] 2.1		Nc-1 WT	
	Lung	Brain	Lung	Brain	Lung	Brain
7	2/5 (40%)	0/5 (0%)	2/6 (33·3%)	1/6 (16·7%)	7/11 (63·7%)	2/11 (18·2%)
14	0/8 (0%)	1/8 (12·5%)	0/6 (0%)	2/6 (33·3%)	1/8 (12·5%)	3/8 (37·5%)
28	0/7 (0%)	0 / 7 (0%)	0/19 (0%)	2/19 (10·5%)	0/23 (0%)	3/23 (13%)
10^7 tachyzoites Day p.i.[b]	Nc-1 SAG4[c] 1.1		Nc-1 SAG4[c] 2.1		Nc-1 WT	
	Lung	Brain	Lung	Brain	Lung	Brain
7	3/8 (37·5%)	0/8 (0%)	2/6 (33·3%)	1/6 (16·7%)	11/14 (78·6%)	1/14 (7·14%)
14	1/8 (12·5%)	3/8 (37·5%)	0/6 (0%)	2/6 (33·3%)	2/14 (14·3%)	7/14 (50%)
28	0/8 (0%)	0/8 (0%)	0/6 (0%)	0/6 (0%)	0/14 (0%)	7/14 (50%)

[a] Fractions represent number of PCR-positive mice/total number of mice tested by nested PCR (percentage).
[b] Day post-infection.

Table 2. Litter size, hebdomadal mortality, post-natal mortality and vertical transmission in pups of pregnant BALB/c mice inoculated with transgenic Nc-1 SAG4[c] tachyzoites, WT tachyzoites or PBS buffer

Groups	Litter size[a]	Hebdomadal mortality[b]	Post-natal mortality[c]	Post-natal morbidity[d]	Vertical transmission[e]
Nc-1SAG4[c] 1.1	5·5 ± 1·70	11/88 (12·5%)	4/77 (5·2%)	0/77 (0%)	1/73 (1·4%)
Nc-1SAG4[c] 2.1	5·8 ± 0·84	6/29 (20·7%)	6/23 (26·1%)	2/23 (8·7%)	11/22 (50%)
Nc-1 WT	5·7 ± 1·58	15/91 (16·5%)	13/75 (17·1%)	15/75 (20%)	42/85 (50%)
PBS	5·6 ± 1·68	12/185 (6·5%)	2/173 (1·2%)	0/173 (0%)	0/78 (0%)

[a] Average ± S.D.
[b] Number of full-term dead pups from birth to day 2/total number of born pups (percentage).
[c] Number of dead pups from day 2 to the end of the experiment/total number of pups born alive (percentage).
[d] Number of pups that showed clinical symptoms/total number of pups born alive (percentage).
[e] Number of PCR-positive pups/total number of pups tested (percentage). Vertical transmission was calculated only for the samples analysed, some samples could not be collected due to cannibalism of sick or dead pups by dams.

2007). Others have previously employed the selectable marker dihydrofolate reductase (Beckers *et al*. 1997) and used phleomycin selection in transfection (Howe and Sibley, 1997) of heterologously expressed *T. gondii* genes in *N. caninum*, suggesting the suitability of *Toxoplasma* vectors for use in *Neospora*. In our study, NcSAG4 was expressed under the tachyzoite Tg*GRA*1 promoter because no specific *Neospora* promoters have been described. Similar levels of Nc*SAG4* mRNA transcript were observed in both knock-in strains (Nc-1 SAG4[c]1.1 and Nc-1 SAG4[c]2.1), validating the ability to use the Tg*GRA1* promoter for gene expression in *N. caninum* tachyzoites, as has been demonstrated previously (Howe *et al*. 1997). However, the Nc*SAG4* gene was selected as the target gene for genetic manipulation to obtain strains that are unable to persist in the host brain because it has been speculated that the stage-specific expression of major surface antigens by the parasite plays a crucial role in immune system evasion after the dissemination of *T. gondii* tachyzoites (Kim and Boothroyd, 2005). Moreover, the NcSAG4 protein has 2 interesting features that suggest that it may play a role in stage-conversion pathways. First, the protein is expressed in the initial steps of bradyzoite differentiation, as evidenced by *in vitro* (Fernandez-Garcia *et al*. 2006) and *in vivo* assays (Aguado-Martinez *et al*. 2009). Secondly, NcSAG4 is able to induce a strong humoral immune response in naturally infected cattle (Aguado-Martinez *et al*. 2008).

When *in vitro* phenotypes were investigated, there were no significant differences in the generation times or tachyzoite-bradyzoite conversion rates between the 2 transgenic strains and the WT isolate. Growth rate is a common virulence characteristic

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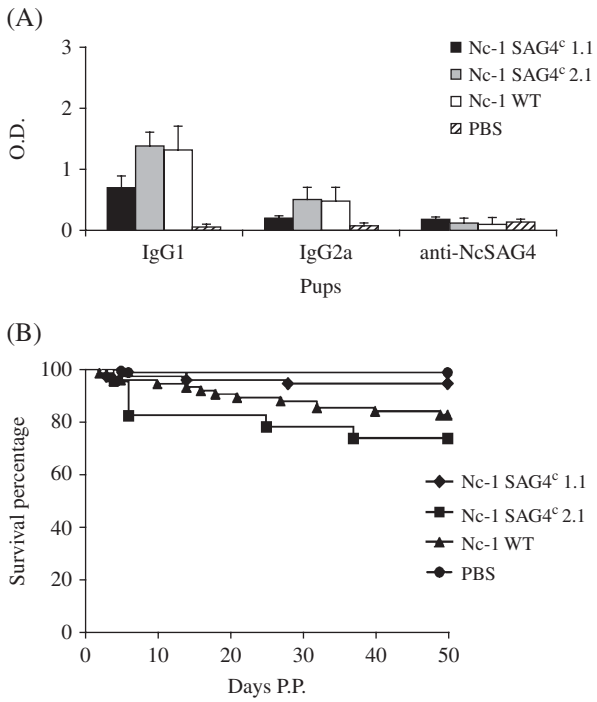

Fig. 3. *In vivo* assays. (A) Antibody detection. Bars represent the mean absorbance of anti-*Neospora caninum* IgG1 and IgG2a isotypes and anti-NcSAG4 antibodies from BALB/c dams inoculated with *N. caninum* tachyzoites from the different strains or PBS in pups born. (B) Kaplan–Meier survival curves for neonates born to dams inoculated with different *N. caninum* tachyzoite strains or PBS. Curves represent the percentage of surviving animals over a period of 50 days p.p. Vertical steps downward correspond to days p.p. when a mouse was found dead or was sacrificed.

in protozoan pathogens, and differences between isolates have been previously described in both *N. caninum* (Schock *et al*. 2001; Perez-Zaballos *et al*. 2005; Rojo-Montejo *et al*. 2009*b*;) and *T. gondii* (Saeij *et al*. 2005*a*). All strains in the present study exhibited an elevated growth rate, which is a crucial feature for strains that are going to be used as live vaccines and, consequently, large-scale propagation. Additionally, the tachyzoite-bradyzoite switch leads to parasite persistence in the brain, probably due to an efficient evasion of the immune system by developmentally expressed proteins (Kim and Boothroyd, 2005; Kim *et al*. 2007; Saeij *et al*. 2008). In our study, low conversion rates were observed in transgenic and WT strains. Nonetheless, Nc-1 WT tachyzoites are able to convert into bradyzoites *in vivo,* evidenced by the detection of antibodies against NcSAG4 and Nc*SAG4* transcripts in brains of mice infected with this strain (Collantes-Fernandez *et al*. 2006; Aguado-Martinez *et al*. 2009).

However, a reduction in the invasion rate was observed for the Nc-1 SAG4^{c}1.1 strain, which may be related to its reduced virulence observed in the pregnant mouse model of infection. Regidor-Cerrillo *et al*. (2010 and *unpublished observations*) have found an association between reduced invasion rates and decreased virulence in several Spanish *Neospora* isolates following inoculation into pregnant mice. Moreover, Saeij *et al*. (2005*a*) reported a higher reinvasion rate for virulent type I *T. gondii* strains compared to the less-virulent type II and III strains. This association has also been observed in modified strains (Dzierszinski *et al*. 2000; Cerede *et al*. 2005). A reduction in invasiveness may offer the immune system valuable additional time to mount a defence, facilitating the clearance of parasites and avoiding parasite dissemination.

Persistence and virulence of transgenic and WT strains was also studied in *in vivo* assays, employing cerebral (Collantes-Fernandez *et al*. 2006) and pregnant (Lopez-Perez *et al*. 2006, 2008) mouse models of infection. In the cerebral mouse model of infection, parasite distribution in tissues during the acute and chronic phases of infection was in agreement with previous observations (Collantes-Fernandez *et al*. 2006). However, parasites were detected in brain tissue on day 28 p.i. for both the high and low doses in WT-inoculated mice, whereas parasite clearance was observed in all the doses of transgenic parasite-inoculated groups, except for 1 (low dose of Nc-1 SAG4^{c}2.1 inoculated group). This finding may be explained by the early expression of NcSAG4 during tachyzoite-bradyzoite conversion (Fernandez-Garcia *et al*. 2006) and the strong anti-NcSAG4 immune response induced in mice inoculated with the transgenic strains.

Although persistence in a *Neospora* infection can be studied in non-pregnant mice, those animals are highly resistant to infection, and differences between strains are more difficult to observe (Pereira Garcia-Melo *et al*. 2010). Pregnant mice, however, are very susceptible to *Neospora* infection, where transmission and neonatal mortality parameters provide clear evidence of phenotypic differences (Regidor-Cerrillo *et al*. 2010). In the present study, reduced virulence of Nc-1 SAG4^{c}1.1 was evidenced by data corresponding to different parameters, such as the almost complete absence of transplacental transmission and a lower mortality and morbidity rates in pups, compared to the other transgenic strain and the WT strain. Moreover, the low antibody titres detected in pups born to dams inoculated with Nc-1 SAG4^{c}1.1 parasites provides evidence for a lower level of parasite exposure.

The different *in vitro* and *in vivo* (pregnant mouse model of infection) results provided evidence for a different behaviour between the Nc-1 SAG4^{c}1.1 and Nc-1 SAG4^{c}2.1 strains, proving the Nc-1 SAG4^{c}1.1 strain to be less virulent than the other strain. The similar levels of Nc*SAG4* transcript and protein expression for both transgenic strains, together with a strong specific humoral immune response against NcSAG4 protein developed by transgenic strain-inoculated mice, suggest that the constitutive expression of NcSAG4 in the tachyzoite stage may

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not be responsible for this low virulence of the Nc-1 SAG4^{c}1.1 strain and led us to consider random plasmid integration as being responsible for the reduced ability of Nc-1 SAG4^{c}1.1 to invade host cells. In addition, Aguado-Martinez *et al*. (2010) performed *in vitro* studies employing monoclonal antibodies and corroborated that the novel NcSAG4 protein, when expressed on the tachyzoite surface, is not involved in the host cell invasion process.

In summary, we have engineered *N. caninum* tachyzoites, for the first time, to constitutively express NcSAG4 protein using stable transfection methods. These transgenic parasites exhibit a lower persistence in mouse brains and may be good alternatives for a safe live vaccine in future immunoprophylaxis trials. In addition, the elevated immune response generated against NcSAG4 is a worthy value for the development of marked vaccine. Nonetheless, the safety of these genetically modified strains should be corroborated in a bovine model (Rojo-Montejo *et al*. 2009*a*). Finally, the reduced capacity of the Nc-1 SAG4^{c}1.1 strain to be vertically transmitted to offspring is an interesting feature and further work should be done to identify the position of the plasmid integration in the genome of this transgenic strain.

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