



Protective immunity against *Echinostoma caproni* in rats is induced by *Syphacia muris* infection

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ABSTRACT

Syphacia muris (Nematoda: Oxyuridae) is a ubiquitous nematode that commonly infects rats in the laboratory and can interfere with the development of unrelated biological assays. In this context, we analysed the effect of a patent *S. muris* infection in Wistar rats on a superimposed infection with the intestinal trematode, *Echinostoma caproni* (Trematoda: Echinostomatidae). The results indicate that in the rats, infection with *S. muris* induces an immunity against a subsequent infection with *E. caproni*, although each parasite occupies different niches in the host. *Echinostoma caproni* worm recovery was significantly decreased in the rats primarily infected with *S. muris* and, at 3 and 4 weeks post-primary infection, the rats primarily infected with *S. muris* were refractory to the challenge infection with *E. caproni*. We observed that the main alterations induced by *S. muris* in the niche of *E. caproni* (ileum) that may be the cause of the resistance are: (i) a local antibody response with elevated levels of mucosal IgA, IgE, IgG, IgG1 and IgG2a that cross-react with *E. caproni* antigens; (ii) development of a biased Th17/Th2 phenotype; and (iii) changes in the glycosylation of ileal mucins. This indicates that *S. muris* induces distant alterations to the ileum of rats affecting the development of other helminth species. Apart from the interest of these results in the study of the interactions between helminths in a single host, it has been demonstrated that pinworm infections may interfere in non-related experiments.

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1. Introduction

Syphacia muris (Nematoda: Oxyuridae) is a ubiquitous nematode that commonly infects laboratory rodents. The direct transmission of this gastrointestinal nematode by contaminated food, water and bedding facilitates transmission in animal holdings. This results in a continual re-exposure to the parasite, making the control of pinworms quite difficult and rodent colonies are frequently infected (Taffs, 1976). *Syphacia muris* inhabits the caecum and the colon of the host, where the female matures within 7–8 days (Stahl, 1961; Taffs, 1976). Mature females migrate from the intestine mainly at midday and deposit the eggs at the perianal region, which develop into infective larvae. Although pinworm parasites are usually regarded as symptomless, heavy infections have been associated with rectal prolapse, mucoid enteritis and intestinal impaction and intussusception (Taffs, 1976). Apart from the interest of pinworm infections in relation to animal health care, these parasites can interfere in the development of protocols and later the interpretation of the final results of non-related experiments which has often been underestimated. Infection with pinworms can alter the immune response and influence the susceptibility of

rodents to other intestinal helminths (Keeling, 1961; Stahl, 1961; Michels et al., 2006). Michels et al. (2006) reported that an outbreak of *Syphacia obvelata* altered the outcome of an unrelated experiment. These authors showed that *S. obvelata*-infected mice immunised against ovalbumin elicited more severe anaphylactic shock than did pinworm-free animals (Michels et al., 2006). Furthermore, rodents infected with pinworms terminate self-tolerance and enhance neonatal induction of a Th2-associated autoimmune disease and T-cell memory (Agersborg et al., 2001). All of these facts highlight the importance of maintaining mice for use as experimental models under pinworm-free conditions, which necessitates the development of methods for the control of these parasites and a better understanding of their interactions with the host and other parasites or pathogens.

Echinostoma caproni (Trematoda: Echinostomatidae) is an intestinal trematode that does not undergo tissue migration in its definitive host. Following infection of the definitive host with *E. caproni*, the metacercariae excyst in the duodenum and the juvenile parasites migrate to the ileum where they attach to the mucosa by the ventral sucker (Toledo et al., 2009). *Echinostoma caproni* has been thoroughly used as an experimental model to study factors determining the rejection of intestinal helminths (Toledo and Fried, 2005). This parasite has a broad spectrum of definitive laboratory hosts, although its compatibility may vary depending on the

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host species (Toledo et al., 2009). Moreover, in rats *E. caproni* induces acute infections in which the parasite is retained until 6–7 weeks p.i. (Toledo et al., 2004).

In our laboratory, it was observed that, although both parasites occupy different niches within the host, infection of Wistar rats with *S. muris* induced a protective immunity against a superimposed experimental infection with *E. caproni*. The protective effect of the *S. muris* infection was evident from the first week post-primary infection (ppi) with the nematode, but finally the rats became completely refractory to the superimposed infection with *E. caproni*. Herein, we analyse the antagonistic interaction between both parasites and the alterations induced by the primary infection with *S. muris* that may determine the subsequent resistance to *E. caproni*. The results obtained are of importance for the study of immunomodulation induced by helminths and, in general, for any experimental study involving laboratory rodents which may be infected with pinworms.

2. Materials and methods

2.1. Animals

Female Wistar rats weighing 80–100 g were used in the study. The animals were maintained under standard conditions with food and water available ad libitum. The study was approved by the institutional committee for animal care at the University of Valencia (Spain), in compliance with the European Agreement of Strasbourg.

2.2. Infection procedures

2.2.1. Infection with *S. muris*

Infection and recovery of *S. muris* were done as described by Sotillo et al. (2012). Briefly, eggs of *S. muris* used for infections were collected from the caeca of naturally infected albino rats (Wistar) maintained at barrier facilities. The caeca were sliced open and submerged in a gauze mesh at the mouth of a conical flask for 1 to 2 h at 37 °C to allow the worms to migrate out. After washing in 0.65% NaCl, worms were crushed and their eggs were isolated by passage through 70 µm nylon cell strainers. Rats were infected orally with 500 eggs each via a stomach tube and the adults of *S. muris* were recovered from the caecum and colon after necropsy of the animals. Positivity ante-mortem was determined coprologically or by Cellophane Tape Test.

2.2.2. Infection with *E. caproni*

The strain of *E. caproni* and the infection procedures have been described previously (Fujino and Fried, 1993; Trelis et al., 2011). Encysted metacercariae of *E. caproni* were removed from kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* snails and used to infect Wistar rats. Rats were each infected by stomach tube with 100 metacercariae of *E. caproni*. Positivity ante-mortem was determined coprologically.

2.3. Infection trials

A total of 85 Wistar rats were involved in the challenge trials. Animals were randomly separated into four groups. Group 1 contained 10 helminth-naïve rats that were necropsied on day 0 to generate tissues for the time zero data point. Group 2 contained 40 rats. A total of 20 of these animals were infected with 500 eggs of *S. muris*. At each week p.i., five animals were necropsied to generate tissues from *S. muris*-infected rats. The other 20 animals belonging to this group remained uninfected; five animals were sacrificed weekly and used as negative controls. Group 3 contained

a total of 25 animals. Twenty of the rats in this group were each infected with 500 eggs of *S. muris*. At 1, 2, 3 and 4 weeks p.i. with *S. muris*, five animals were each challenged with 100 metacercariae of *E. caproni*. The additional five rats belonging to this group were simply infected with 100 metacercariae of *E. caproni* and used as controls for *E. caproni* worm recovery. All of the animals were necropsied at 2 weeks p.i. with *E. caproni* and the worms per host recovered and counted. Group 4 included a total of 10 animals. Five of these animals were each infected with 500 eggs of *S. muris*, whereas the other five animals were left uninfected and used as controls for the study of serum antibodies. At each week p.i., blood samples were taken from each animal in this group by cardiac puncture under anaesthesia. After clotting of the blood overnight at 4 °C, serum was separated from the clot by centrifugation. The serum samples were stored at –20 °C until use.

For each experiment the same batch of eggs and/or metacercariae were used.

2.4. Antigen preparation

Adult worms were collected from the caecum and colon (*S. muris*) and ileum (*E. caproni*) of experimentally infected rats 4 weeks p.i. After thorough washing with PBS, (pH 7.4), the worms were homogenised in a culture medium of PBS containing 0.8 mM phenylmethylsulphonyl fluoride (PMSF, Sigma, USA), 100 U penicillin (Sigma), and 100 µg/ml streptomycin (Sigma). After initial centrifugation at a low speed to remove larger particles, the supernatant was centrifuged at 15,000g for 30 min at 4 °C and the supernatant was collected. The protein content was measured by the Bio-Rad (Hercules, California, USA) protein assay and adjusted to 1 mg/ml using an ultrafiltration membrane (YM-3, Millipore, Bell-erica, Massachusetts, USA). The antigens were stored at –20 °C until use.

2.5. Intestinal lavages

The presence of antibodies in the intestine of rats infected with *S. muris* was investigated in the animals of group 2 and the intestinal secretions were collected using the method described by Sotillo et al. (2008). Briefly, at each time p.i., five rats were killed and the intestine removed. Three ml of a solution of 0.1 mg/ml of soybean trypsin–chymotrypsin inhibitor (Sigma) in 50 mM EDTA (Sigma) was flushed through the intestine which was massaged gently before recovery of the fluid. The samples were then centrifuged at 700g for 10 min before addition of 30 µl of 100 mM PMSF in 95% ethanol. The samples were clarified by centrifugation at 3,000g at 4 °C for 15 min, after which 20 ml of PMSF and 20 ml of 1% sodium azide (Sigma) were added to the supernatant. The samples were stored at –20 °C until use.

2.6. Indirect ELISA for antibody detection

In order to detect specific antibodies for *S. muris* and/or *E. caproni*, an indirect ELISA was carried out as described by Toledo et al. (2003) with some modifications. This method was used to detect IgM, IgA, IgG, IgG1 and IgG2a in sera and intestinal samples of rats experimentally infected with *S. muris*. Optimal dilutions of antibodies and sera were determined by checkerboard titrations. Polystyrene microtiter plates (Nalgene, Naperville, Illinois, USA) were coated overnight at 4 °C with 100 µl/well of a 30 µg/ml solution of antigens in 0.1 M carbonate coating buffer, pH 9.6. The plates were washed three times with PBS with 0.05% Tween 20 (PBST). Uncoated sites were blocked with 5% non-fat dry milk in PBST. After incubation for 1 h at 37 °C, the plates were emptied and sera and intestinal samples at an adequate dilution in PBST were dispensed into each well. The plates were incubated for 2 h at room

temperature (RT) and then washed five times with PBST. One hundred microlitres of horse radish peroxidase-conjugated goat anti-rat IgM (OEM concepts, USA), goat anti-rat IgA (Nordic, Immunological Laboratories, The Netherlands), goat anti-rat IgG (ICN Biomedicals, Aurora, Ohio, USA), goat anti-rat IgG1 (Nordic), and goat anti-rat IgG2a (Nordic) at adequate dilutions in PBS were added to each well and incubated for 2 h at RT. The plates were washed as described above and 100 µl of the substrate solution (10 µl of 30% (w/v) hydrogen peroxide, 25 ml of 0.1 M citrate buffer, pH 5.0, 10 mg of o-phenylenediamine hydrochloride) were added and incubated in the dark at RT. The enzyme reaction was stopped with 50 µl per well of 3 N HCl. The plates were read at 492 nm in a Bio-Rad 550 microplate ELISA reader.

2.7. Total RNA extraction

At each week p.i., five rats belonging to group 2 were necropsied. Total RNA was extracted from excised spleen, Peyer's patches and mesenteric lymph nodes (MLNs) and from full-thickness sections of ileum at the sites where the worms were located. Total RNA from each tissue obtained from rats and mice was isolated using a Rapid Total RNA Purification Kit (Marligen Biosciences Inc., USA) according to the manufacturer's instructions. The cDNA was synthesised using a SuperScript III First-Strand Synthesis System (Invitrogen, USA).

2.8. Real-time PCR and relative quantification analysis

For quantitative PCR, 40 ng of total RNA reverse transcribed to cDNA were added to 10 µL of TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2×), 1 µL of the specified TaqMan® Gene Expression Assay and water, to a final reaction volume of 20 µL. Reactions were performed on the Abi Prism 7000 (Applied Biosystems, USA), with the following thermal cycler conditions: initial setup of 2 min UNG activation at 50 °C, followed by 10 min at 95 °C, 40 cycles of 15 s denaturation at 95 °C and 1 min of anneal/extension at 60 °C each. Samples were amplified in a 96-well plate. On each plate the endogenous control, samples and the negative control were analysed in triplicate. All TaqMan® Gene Expression primers and probes, both for cytokines and mucins, except those for IL-6, were designed by Applied Biosystems and offered as Inventoried Assays. The assay ID details, the housekeeping β-actin and cytokine mRNAs are shown in [Supplementary Table S1](#). Each assay contains two unlabelled primers and one 6-FAMTM dye-labelled, TaqMan® MGB probe. Primers and fluorescent FAM-labelled probes of rat IL-6 were designed in-house from published sequences using the PrimerExpress software from Applied Biosystems (Trelis et al., 2011) ([Supplementary Table S1](#)).

The cycle threshold (Ct) value was calculated for each sample, housekeeping gene and uninfected control. To normalise for differences in efficiency of sample extraction or complementary DNA synthesis, β-actin was used as a housekeeping gene. To estimate the influence of infection in the expression levels, a comparative quantification method ($2^{-\Delta\Delta Ct}$) was used (Livak and Schmittgen, 2001). The method is based on the fact that the difference in threshold cycles (ΔCt) between the gene of interest and the housekeeping gene is proportional to the relative expression level of the gene of interest. The fold change in the target gene was normalised to β-actin and relative to the expression at time zero (uninfected animals) to generate relative quantification of expression levels (Klein, 2002).

2.9. Histology

Histopathological responses to *S. muris* infection in the ileum of rats were evaluated during the weeks p.i. using the methodology

described previously (Toledo et al., 2006a,b). At each time p.i., five rats from group 2 were necropsied and ileal sections of 0.7–1.0 cm in length were obtained from each animal and fixed in 4% buffered formalin. After embedding in paraffin wax, serial 4 µm sections were cut from each tissue block. Intestinal sections were stained with H&E, Giemsa, Alcian Blue and Toluidine Blue. Cells were counted in a total of 50 villi. All cell counts were expressed as the number of cells per villus-crypt unit (VCU). Results are expressed as the mean number of cells per VCU ± S.E.

2.10. Immunohistochemistry and immunofluorescence microscopy

Levels of glycosylation in the ileum of rats infected with *S. muris* were determined using the avidin-peroxidase technique on formalin-fixed sections of the intestinal samples. Briefly, tissue sections were deparaffinised and rehydrated. A blocking solution of 5% BSA (Roche, Switzerland) and Triton™ X-100 (Sigma–Aldrich) in PBS was applied for 1 h. After washing, slides were incubated with biotinylated lectins (*Dolichus biflorus*, DBA; *Triticum vulgaris*, WGA; *Ulex europaeus* agglutinin I, UEA-I; *Sambucus nigra*, SNA; and *Ara-chis hypogaea*, PNA) at the adequate dilution for 1.5 h. After washing, the sections were incubated with the avidin-peroxidase conjugate diluted 1:1,000 in PBS with Triton™ X-100 (0.2%) for 2 h, followed by repeated washings. Next, the slides were incubated with goat antibody anti-peroxidase conjugated with fluorescein (Jackson ImmunoResearch, USA) at a dilution 1:200 in PBS for 1 h. Finally, the sections were mounted using the Fluoromount procedure (Sigma–Aldrich). In order to determine potential autofluorescence or unspecific binding, negative controls were used. They were processed as described above with the exception of the incubation with lectins. The slides were counterstained using DAPI. The histological staining was quantified as described previously by Rinaldi et al. (2011) and was ranked using (–) for absent, (+) for just detectable, (++) for positive and (+++) for strongly positive.

The slides were analysed by multitracking on a FV10I confocal microscope (Olympus). A total of five slides were analysed for each time point.

2.11. Statistical analysis

To determine statistical significance of the effect of time p.i. and the infection with *S. muris* in the different parameters studied, a two-factor ANOVA with interactions was used. Moreover, when a significant interaction was detected, the Bonferroni *t*-test of the difference between means was performed as a post hoc analysis to confirm the significance of the differences. Differences in mean values were considered statistically significant at $P < 0.05$. Prior to analyses, data were log transformed to achieve normality and verified by the Anderson–Darling Test.

3. Results

3.1. Primary infections with *S. muris* induced resistance to superimposed infections with *E. caproni*

All of the rats in group 3 that were exposed to 500 eggs of *S. muris* became infected and the infection lasted until the end of the experiment. All of the *S. muris* adult worms were confined to the colonic and caecal regions, without invading the small intestine. Similarly, all of the control rats, without primary infection with *S. muris*, became infected with *E. caproni*, showing recovery rates from 31 to 56 (43.3 ± 4.0) worms/animal ([Table 1](#)). However, the results obtained from challenge infections clearly showed that primary infection with *S. muris* induced a progressive status of resistance against a superimposed *E. caproni* infection as shown

Table 1

Recovery data obtained in Wistar rats primarily exposed to 500 eggs of *Syphacia muris* and then challenged with 100 encysted *Echinostoma caproni* metacercariae at different weeks post primary infection.

Primary infection	Weeks between primary infection and challenge infection	Challenge infection	Rats infected with <i>E. caproni</i> (rats infected/exposed)	<i>E. caproni</i> worm recovery at 2 weeks post-primary infection range (mean $\pm$ S.E.)
–	0	<i>E. caproni</i>	5/5	31–56 (43.3 $\pm$ 4.0)
<i>S. muris</i>	1	<i>E. caproni</i>	5/5	19–30 (24.7 $\pm$ 1.5) ^a
<i>S. muris</i>	2	<i>E. caproni</i>	2/5	15–29 (21.9 $\pm$ 1.9) ^a
<i>S. muris</i>	3	<i>E. caproni</i>	0/5	0
<i>S. muris</i>	4	<i>E. caproni</i>	0/5	0

^a Significant differences compared with rats that were not exposed to *S. muris* ($P < 0.05$).

in Table 1. Although all of the rats that were challenged with *E. caproni* metacercariae 1 week ppi became infected, worm recovery was significantly lower than that observed in control animals ($P < 0.05$). The percentage of rats that became infected was reduced to 40% in those rats that were challenged at 2 weeks ppi and worm recovery significantly decreased with respect to controls ($P < 0.05$). The rats that were challenged at 3 and 4 weeks ppi were found to be completely refractory to the superimposed *E. caproni* infection (Table 1). All of the *E. caproni* adult worms recovered were confined to the ileum.

3.2. *Syphacia muris* infection did not affect the cell populations in the ileum of experimentally infected rats

The potential changes in the cell populations in the ileum of rats induced by the infection with *S. muris* were studied in the animals of group 2. A total of five sections were analysed for each rat.

Histological examination did not show changes in the ileal populations of eosinophils, mucosal mast cells, goblet cells, neutrophils or mesenteric inflammatory cells in relation with the infection with *S. muris* (Supplementary Fig. S1).

3.3. *Syphacia muris* infection induced significant levels of mucosal antibodies that cross-reacted with *E. caproni*

The generation of specific antibody responses in the sera of rats experimentally infected with *S. muris* was studied by indirect ELISA on the animals of group 4. Our results demonstrated that *S. muris* does not elicit significant antibody responses (IgA, IgM, IgE, IgG, IgG1, IgG2a) in the sera of experimentally infected rats (data not shown).

In contrast, the infection with *S. muris* induced a rapid ileal antibody response as demonstrated in the animals of group 2 (Fig. 1). The levels of all IgG classes rapidly increased compared with naïve

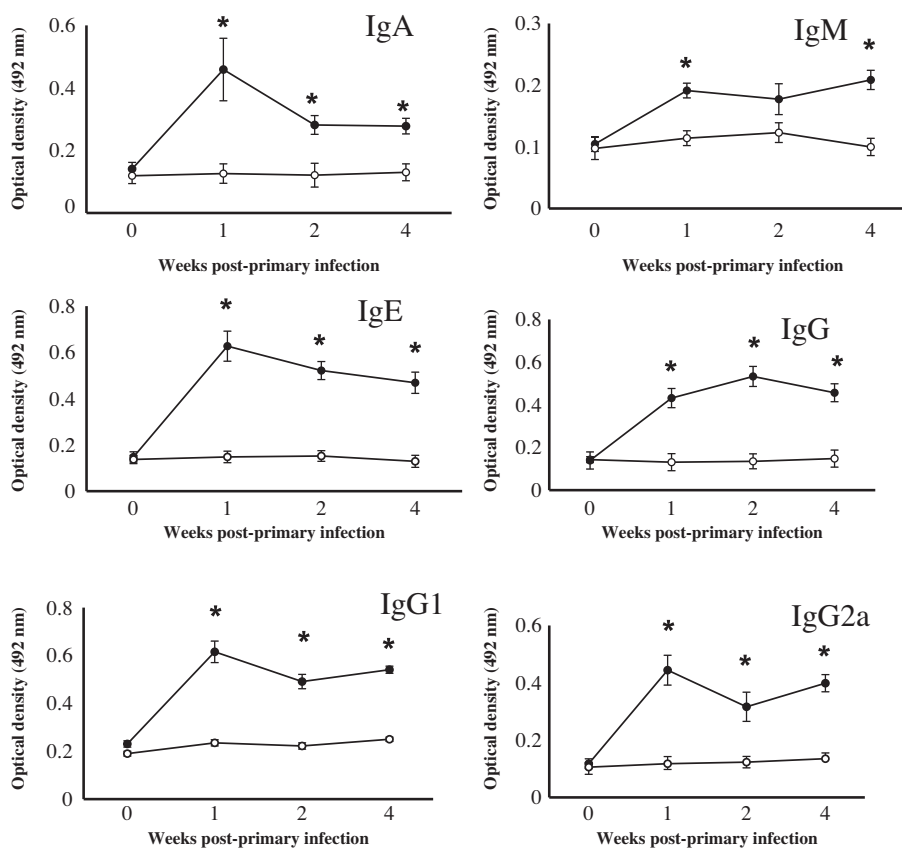


Fig. 1. Presence of antibodies in the intestine of rats, primarily infected with *Syphacia muris*, recognising *S. muris* antigens by indirect ELISA. Mean O.D. values of control ($\circ$) ($n = 20$, five per time point) and infected animals ($\bullet$) ($n = 20$, five per time point) of group 2 over the course of the experiment. Vertical bars represent the S.E. Asterisks represent significant differences with respect to uninfected control animals ($P < 0.05$).

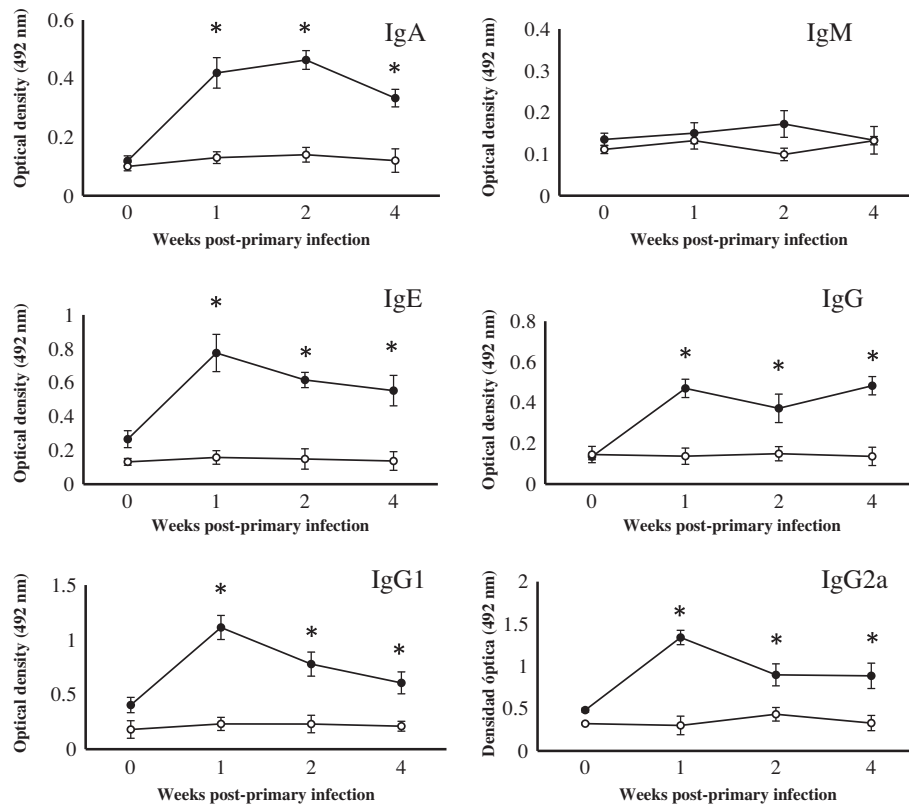


Fig. 2. Presence of antibodies in the intestine of rats, primarily infected with *Syphacia muris*, recognising *Echinostoma caproni* antigens by indirect ELISA. Mean O.D. values of control (○) ($n = 20$, five per time point) and infected animals (●) ($n = 20$, five per time point) of group 2 over the course of the experiment. Vertical bars represent the S.E. Asterisks represent significant differences with respect to uninfected control animals ($P < 0.05$).

animals and were significantly elevated from 1 week ppi. The IgM response was the weakest and significantly elevated levels with respect to controls were detected at 1 and 4 weeks ppi. In the remainder of the Ig classes, the levels were significantly higher than those observed in the controls for each week of the study (Fig. 1).

In order to analyse the potential role of the antibodies generated by a primary infection with *S. muris* on the resistance against a subsequent infection with *E. caproni*, we studied cross-reactivity of the anti-*S. muris* local antibodies with *E. caproni* (Fig. 2). The results showed that all of the Ig classes analysed strongly reacted with *E. caproni* antigens, with the exception of IgM. Interestingly, the reactivity was apparently greater than that observed against *S. muris* antigens (Fig. 2).

3.4. *Syphacia muris* induced an increase in the levels of several cytokines in different tissues of experimentally infected rats

We also detected that *S. muris* primary infections induced changes in the levels of cytokines in several tissues. Figs. 3 and 4 show the profile of the cytokines for which we detected differences in the levels with respect to controls for each tissue studied. No increase was detected in the regulation of remaining cytokines (Supplementary Figs. S2–S5).

The profiles observed in the spleen, MLNs and Peyer's patches were rather heterogeneous. In the spleen, significant overexpression, with respect to naïve rats, of IFN- γ and eotaxin/CCL11 were observed during the complete course of the experiment. Moreover transient elevations, compared with controls, of the levels of IL-4 (1 and 3 weeks ppi), IL-5 (1, 2 and 3 weeks ppi) and IL-13 (3 weeks ppi) were detected (Fig. 3A). In the MLNs, a strong development of a Th17 phenotype with increasing of IL-17 during the complete

experiment was observed and IL-23 (at 1 and 3 weeks ppi). Moreover, the level of eotaxin/CCL11 was significantly more elevated than controls during the complete experiment. The levels of IL-6 were momentarily higher in infected animals than in controls (1 week ppi) (Fig. 3B). In the Peyer's patches, rapid but transient increases, with respect to non-infected rats, in the levels of IL-13 and transforming growth factor (TGF)- β were detected at 1 week ppi. In contrast the levels of TNF- α were elevated for each week of the experiment (Fig. 3C).

Considering that *E. caproni* inhabits the ileum without invading other tissues, the responses involving ileal tissue appear to be more relevant in explaining the resistance generated by a primary infection with *S. muris*. In the ileum, a strong Th17 response during the first 3 weeks ppi was observed, with elevated levels with respect to control animals, of IL-17, IL-23 and TGF- β . IL-6 also showed increased expression during the first 3 weeks ppi. Moreover, the levels of eotaxin/CCL11 remained elevated during the complete experiment. Sporadic rises compared with controls of IL-4 (1 and 2 weeks ppi) and IL-5 (2 and 3 weeks ppi) were observed (Fig. 4).

3.5. *Syphacia muris* infection did not induce changes in the expression of mucin genes in the ileum of experimentally infected rats

Mucins produced by goblet cells have often been related to the rejection and/or the development of intestinal helminths. To analyse the effect of these mucins in resistance to *E. caproni* induced by *S. muris*, we studied the relative expression of several mucin-related genes (MUC1, MUC2, MUC3a, MUC4, MUC13, MUC15, MUC16, MUC20) in the ileum of the rats, primarily infected with *S. muris*, belonging to group 2. The results obtained indicated that none of the mucin genes studied showed upregulation during the first 4 weeks ppi in the ileum of rats (Supplementary Fig. S6).

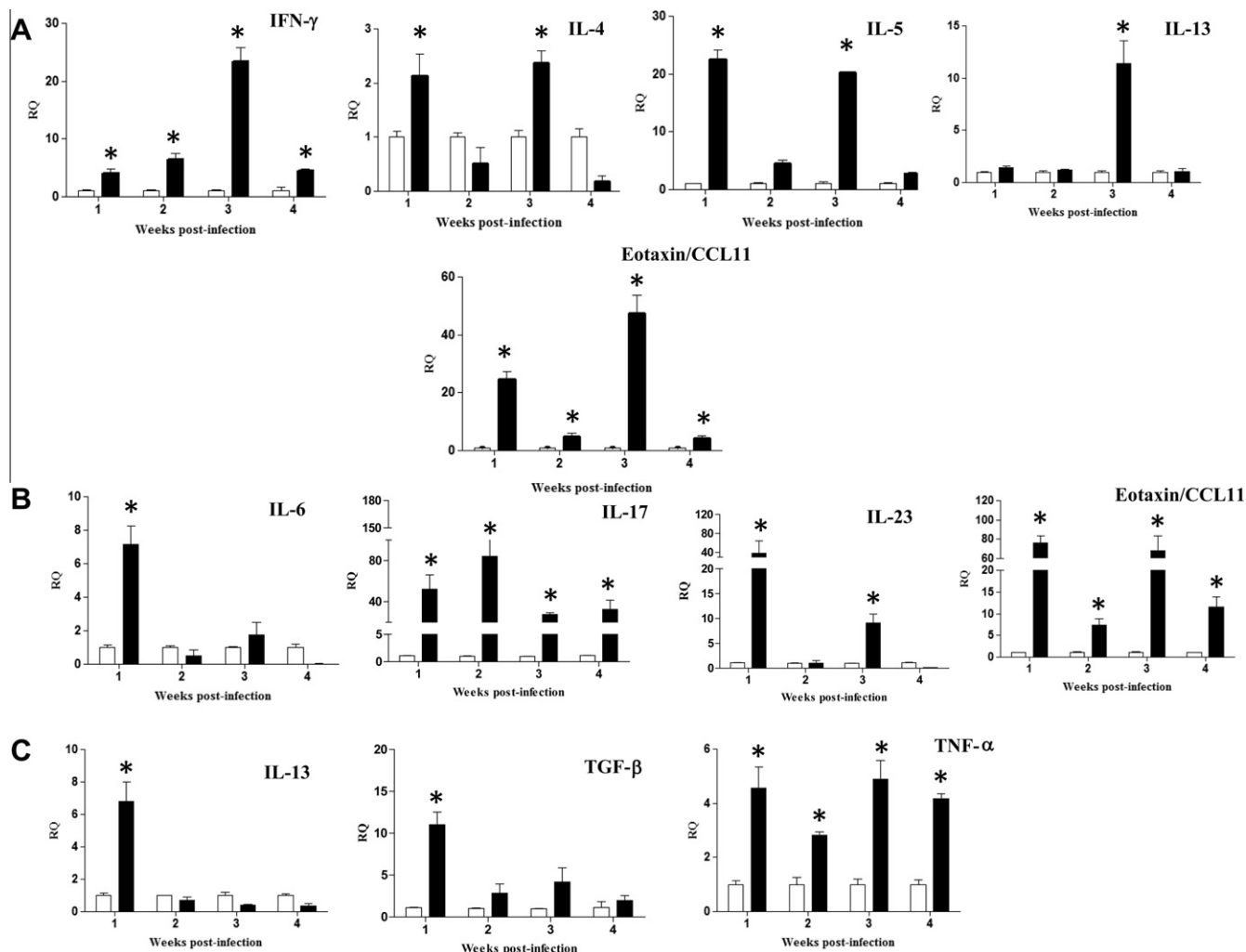


Fig. 3. Expression of cytokine mRNA in the spleen (A), mesenteric lymph nodes (B) and Peyer's patches (C) of rats primarily infected with *Syphacia muris* (black bars) ($n = 20$, five per time point) for those cytokines for which statistical differences with respect to uninfected rats (white bars) ($n = 20$, five per time point) were detected. The relative quantities (RQ) of cytokine expression are shown after normalisation with β -actin and standardisation of the relative amount against a day 0 sample. Vertical bars represent the S.E. Asterisks represent significant differences between primarily infected and uninfected rats ($P < 0.05$). The values for the remainder of the cytokines are shown in [Supplementary Figs. S2–S4](#).

3.6. *Syphacia muris* induced changes in the glycosylation patterns of mucin in the ileum of rats

Although we detected neither upregulation of mucin genes nor goblet cell hyperplasia, the patterns of mucin glycosylation may be of importance in the clearance of intestinal parasites. Therefore, to evaluate the mucin glycoconjugates in the ileum of rats in response to primary infection with *S. muris*, immunofluorescence labelling for glycans was performed and the relative staining was quantified at 2 and 4 weeks ppi (Fig. 5 and Table 2). The most striking differences were observed in samples stained with DBA and WGA. Using DBA lectin, only a few goblet cells within the ileal epithelium became stained in the uninfected animals and in those necropsied at 2 weeks ppi (Fig. 5). In contrast, most of the goblet cells in the ileum of rats were stained at 4 weeks ppi when the animals were resistant to superimposed infection with *E. caproni*. A similar pattern was observed using WGA lectin (Fig. 5). We did not detect staining of goblet cells at 0 and 2 weeks ppi, whereas at 4 weeks ppi most of the goblet cells in the ileum became stained (Fig. 5). In contrast, using UEA-I, SNA and PNA lectins, the glycosylation pattern was similar between control and infected animals

([Supplementary Fig. S7](#)). Although goblet cells became stained using UEA-I and SNA, the quantity was similar between control and infected rats. Using PNS, a low level of staining was observed in all of the samples.

We also observed differences in the thickness of the glycocalyx using DBA, WGA and UEA-I lectins. Although differences were detected using DBA, the great degree of variability observed intra- and inter-groups prevented further consideration of this issue. In contrast, the observations using WGA and UEA-I were more homogeneous (Fig. 6). Immunofluorescence microscopy using WGA lectin showed a progressive increase in the mucosal thickness over the course of *S. muris* infection (Fig. 6A). Using UEA-I the glycocalyx thickness abruptly reduced at 4 weeks ppi (Fig. 6B and C).

Overall, lectin histochemistry analysis suggested that the mucins produced after *S. muris* infection in rats were altered in their glycosylation and glucosamine, galactose or galactosamine residues were increased as a consequence of the primary infection. Moreover, the composition of the glycocalyx also appeared to be altered by *S. muris* infection, leading to a reduction in fucosylated residues that appeared to be replaced by galactosamine residues.

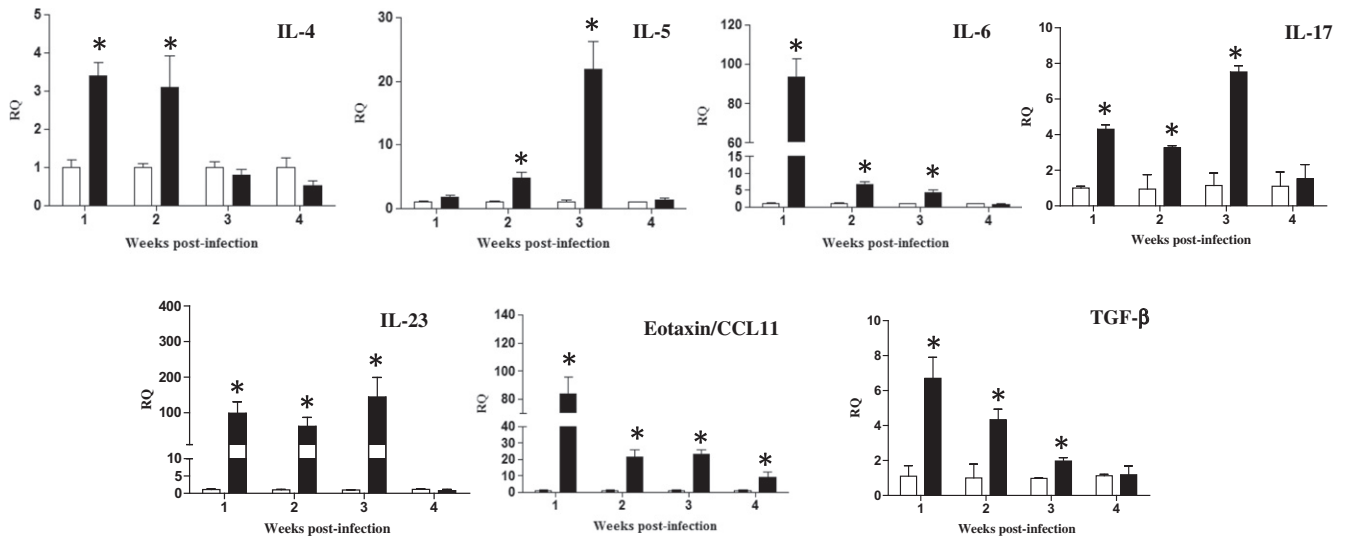


Fig. 4. Expression of cytokine mRNA in the ileum of rats primarily infected with *Syphacia muris* (black bars) ($n = 20$, five per time point) for those cytokines for which statistical differences with respect to uninfected rats (white bars) ($n = 20$, five per time point) were detected. The relative quantities (RQ) of cytokine expression are shown after normalisation with β -actin and standardisation of the relative amount against a day 0 sample. Vertical bars represent the S.E. Asterisks represent significant differences between primarily infected and uninfected rats ($P < 0.05$). The values for the remainder of the cytokines are shown in [Supplementary Fig. S5](#).

4. Discussion

This study showed that the response generated in rats by *S. muris* is not limited to the immediate site of infection, reaching at least the ileum. Furthermore, this distant response is able to regulate and even generate resistance against other helminths, such as *E. caproni*, in the small intestine. The resistance appears as early as 1 week ppi with *S. muris* manifested in a reduction of *E. caproni* worm recovery and resistance was complete from 3 weeks ppi until the end of the experiment at 4 weeks ppi. Although we cannot exclude other mechanisms of regulation, the different niche occupied by each parasite species within the host or the alterations induced by *S. muris* infection in the ileum of infected rats confirms that resistance relies on the immune response to *S. muris* infection. In fact, it was observed that enhanced resistance is associated with a significant mucosal antibody response, changes in the glycosylation of mucins and is correlated with significant Th17 responses in the ileum of rats.

Although it is well known that infections with gastrointestinal nematodes, including members of the genus *Syphacia*, are characterised by the initiation of immunomodulatory responses (Maizels et al., 2004; Michels et al., 2006), the immune responses generated by *Syphacia* spp. have been little studied. In mice the related species, *S. obvelata*, induces a transient Th2-type response which leads to a dominant type 2 cytokine upregulation with elevated expression of IL-4, IL-5, IL-13 and IFN- γ in the spleen and MLNs (Michels et al., 2006). Furthermore, elevated levels of IgG, mainly IgG1, IgA and IgM have been found in the sera of *S. obvelata*-infected mice (Sato, 1993; Sato et al., 1995; Michels et al., 2006; Perec and Okulewicz, 2006). The results obtained herein in relation to the cytokine profile in the spleen and MLNs are consistent with those reported in mice infected with *S. obvelata*. We also showed that in rats, *S. muris* infection induced significant Th17 responses characterised by upregulation of IL-17 and IL-23 (in the MLN). In contrast, the presence of specific antibodies in the sera of *S. muris*-infected rats was not detected.

Because *E. caproni* inhabits the ileum without invading other tissues, the alterations induced by *S. muris* infections at this level were analysed in detail. Although *S. muris* adult worms are con-

fined to the caecum and colon, the mucosal response is not limited to the immediate site of infection. The dissemination of mucosal responses against gastrointestinal nematodes throughout the intestine was previously demonstrated after multiple challenge infections (Bendixsen et al., 1995). Recently, Menzies et al. (2010) detected differential expression of oxidant, anti-oxidant and mucus-related genes in different areas of the gut after primary challenge with *Trichostrongylus colubriformis* or *Haemonchus contortus*. Furthermore, Su et al. (2011) found that *Heligmosomoides polygyrus*, which resides in the duodenum, produced a significant increase in colonic epithelial permeability.

Consequently, we analysed several alterations induced by the *S. muris* infection in the ileum of rats that has been commonly correlated with expulsion and/or resistance to gastrointestinal helminths. In this context, local cell population responses and regulation of locally produced mucins do not appear to be involved in the development of resistance against superimposed infections with *E. caproni*. Mastocytosis, eosinophilia, neutrophilia and goblet cell hyperplasia are characteristic features in infections with gastrointestinal helminths (Khan and Collins, 2004; Artis, 2006; Toledo et al., 2006a,b). However, this study has shown that *S. muris* infection does not elicit significant changes in the populations of these cells in the ileum of rats. In the same manner, several studies have shown that changes in the regulation of the goblet cell-produced mucins induced by intestinal helminths have an important role in development of the worms (Yamauchi et al., 2006; Menzies et al., 2010; Hasnain et al., 2011a, b). Herein, neither upregulation of either secreted gel-forming (MUC2) nor membrane bound mucins (MUC1, MUC3a, MUC4, MUC 13, MUC15, MUC16, MUC20) in the ileum of rats primarily infected with *S. muris* were observed.

In this study, the main alterations induced by *S. muris* infections in the ileum of rats that may be involved in the enhanced resistance to *E. caproni* were: (i) local antibody response with elevated levels of mucosal IgA, IgE, IgG, IgG1 and IgG2a that cross-reacted with *E. caproni*; (ii) changes in the glycosylation of mucins; and (iii) development of a biased Th2/Th17 phenotype.

A central role for antibodies in providing protective immunity has been recently identified in secondary infections with *H. polygyrus* (McCoy et al., 2008; Wojciechowski et al., 2009; Liu et al.,

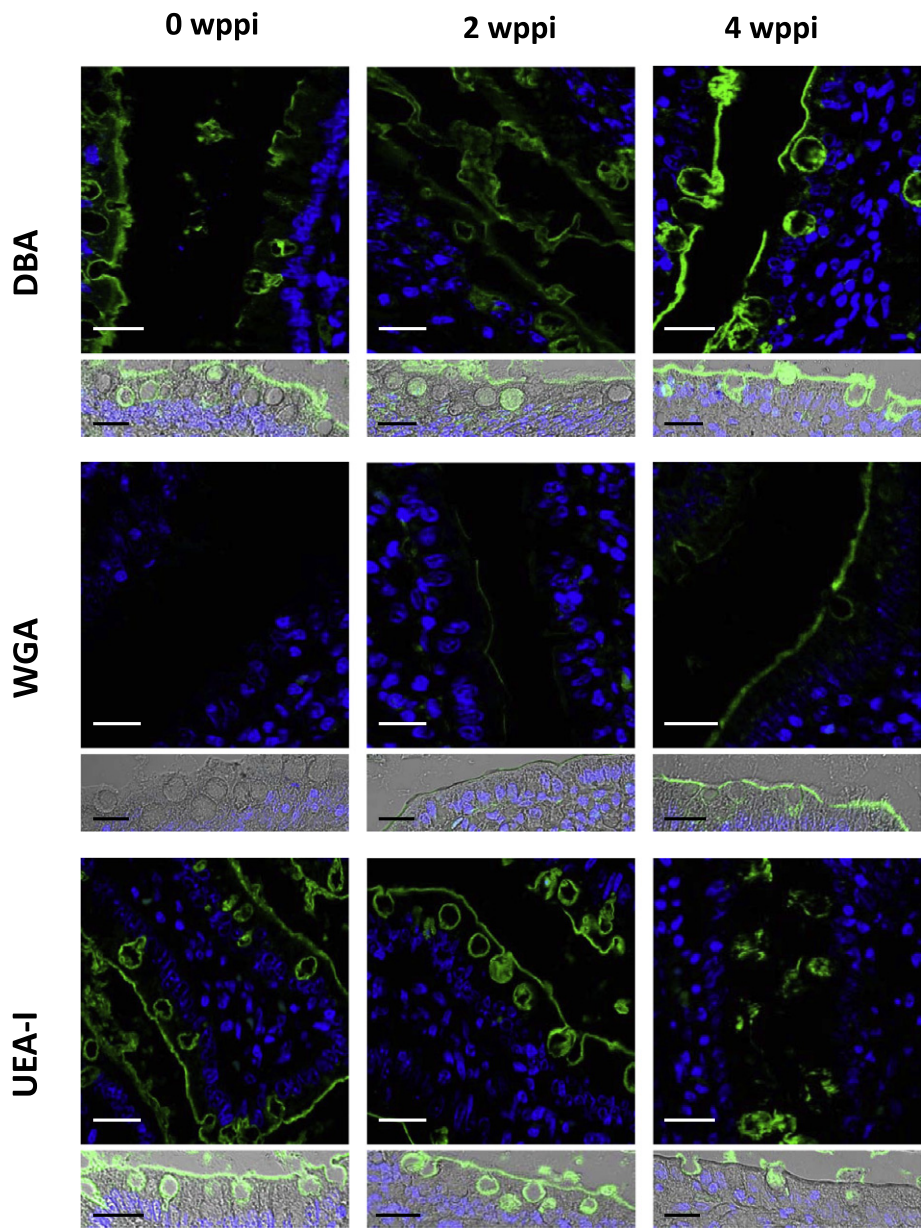


Fig. 5. Images of immunofluorescence and transmission superposed microscopy of ileal tissue from rats primarily infected with *Syphacia muris* at different weeks post-primary infection (wppi) stained with lectins (*Dolichus biflorus*, DBA; *Triticum vulgaris*, WGA; *Ulex europaeus* agglutinin I, UEA-I) (green, FITC; blue, DAPI) (Scale bar: 20 μ m).

Table 2

Quantitative scores of immunofluorescent staining of rat ileal tissue with lectins on weeks 0, 2 and 4 after primary infection (wppi) with *Syphacia muris*.

Lectin	Recognises	0 wppi	2 wppi	4 wppi
<i>Dolichus biflorus</i> (DBA)	GalNAc α 1,3(LFuc α 1,2)Gal β 1,3/4GalNAc β 1-	+	+	+++
<i>Triticum vulgaris</i> (WGA)	GlcNAc β 1,4	-	+	++
<i>Ulex europaeus</i> agglutinin I (UEA-I)	α -L-Fucosyl terminals	+++	+++	++
<i>Sambucus nigra</i> (SNA)	NeuAc α 2,6Gal=NeuAc β 2,6GalNAc	+	+	+
<i>Arachis hypogea</i> (PNA)	Gal β 1,3GalNAc α 1->Gal β 1,4GlcNAc β 1-	-	-	-

-, absent; +, just detectable; ++, positive; +++, strongly positive.

2010). Primary infections in mice were related to changes in jejunal physiology, including IL-4- and IL-13-regulated changes to the intestinal epithelial cell function and smooth muscle cells (Shea-Donohue et al., 2001; Zhao et al., 2003). In subsequent infections, the development of sexually mature worms was prevented and

the parasites were quickly rejected (Morimoto et al., 2004). In the last mentioned study, protective immunity was entirely dependent on the antibodies, mainly IgA and IgG1, that arose from previous *H. polygyrus* infections. Antibodies affected early larval worm migration and development during the tissue-dwelling

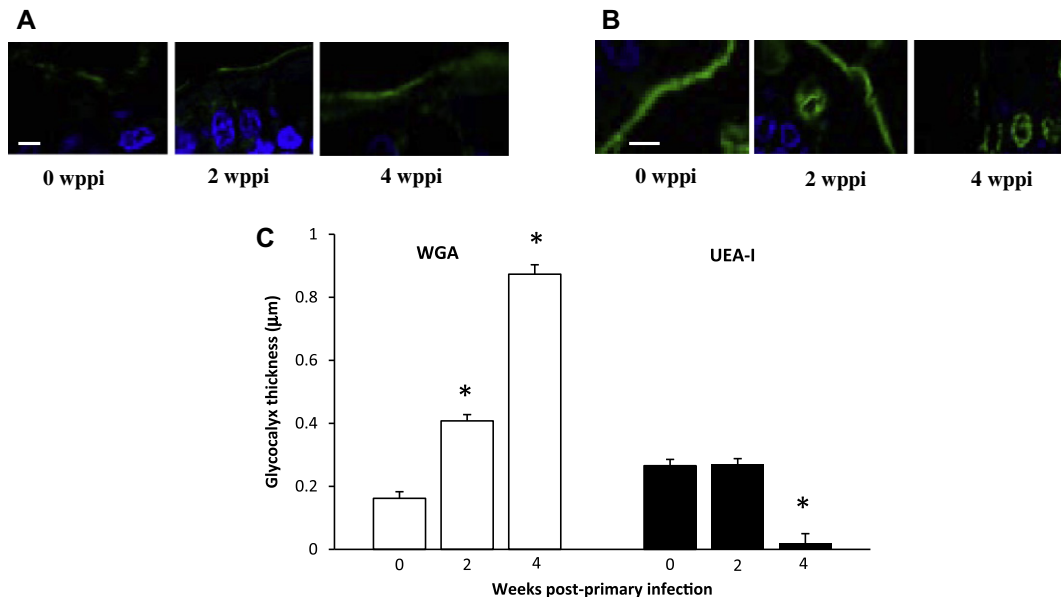


Fig. 6. Changes in the glycocalyx thickness of rats primarily infected with *Syphacia muris* at different weeks post-primary infection (wppi). Immunofluorescent staining using lectins (A) (*Triticum vulgaris*, WGA) (Scale bar: 5 μm); (B) *Ulex europaeus* agglutinin I (UEA-I) (Scale bar: 10 μm); and (C) quantitation of the changes observed with WGA (white bars) and UEA-I (black bars). Vertical bars represent the S.E. Asterisks represent significant differences with respect to uninfected rats ($P < 0.05$) ($n = 15$, five per time point).

phase (McCoy et al., 2008; Liu et al., 2010). Although it is known that antibodies have little or no impact on the clearance of primary *E. caproni* infections (Toledo et al., 2006a,b, 2009; Sotillo et al., 2007), in superimposed infections the mechanism necessary for the generation of resistance differs from that required for the expulsion of luminal dwelling adult worms. In our experiments, the antibody-mediated immunity was targeted against juvenile worms immediately after excystation. It was found that *S. muris* infection in rats induced an antigen-specific response leading to elevated levels of IgA, IgM, IgE, IgG1 and IgG2a in the ileum. Furthermore, a high degree of cross-reactivity with *E. caproni* antigens was found for all the Ig classes except for IgM, suggesting that those may be involved in resistance to *E. caproni*. These antibodies could affect juvenile worms, inhibiting their development, attachment and/or establishment in the intestine of *S. muris*-infected rats.

The glycosylation status of the terminal chains of mucins might have an important role in the establishment and or rejection of the intestinal helminths, even in the absence of goblet cell hyperplasia or upregulation of mucins (Ishikawa, 1994; Maruyama et al., 2002; Yamauchi et al., 2006). The pattern of glycosylation is essential to determine the barrier properties of the mucus gel and participate in processes such as the hydration of mucus retaining antibodies or entrapping the worms or molecules derived from them, facilitating worm expulsion (Singh and Hollingsworth, 2006; Thornton et al., 2008). Previous studies have shown that the mucins and their patterns of glycosylation are essential for the expulsion of *Echinostoma* spp. from rodents and are mediated by goblet cell hyperplasia and increased levels of N-acetyl-D-galactosamine (GalNAc) and N-acetyl-D-glucosamine (GlcNAc) (Fujino and Fried, 1993; Fujino et al., 1996). Although we did not observe goblet cell hyperplasia, this is consistent with the results obtained herein in relation to the mucin glycosylation pattern. It was found that resistance against superimposed *E. caproni* infections was associated with elevated levels of GalNAc and GlcNAc residues within the ileal niche. Strong staining and labelling for DBA and WGA lectins in the ileum of rats were observed concomitantly with the refractory status in rats. In fact, we also observed an increase in the glycocalyx

thickness using the WGA lectin, together with a decrease using the UEA-I lectin, which suggest that infection with *S. muris* induces a replacement of the fucose by galactosamine chains. These findings suggest that an increase in glucosamine, galactose or galactosamine terminal chains induced by the primary *S. muris* infection are involved in resistance against *E. caproni* superimposed infections.

Recent studies have shown that early rejection of *E. caproni* infection is mediated by the expansion of the local Th2 lineage with upregulation of IL-5, IL-6 and IL-13, concomitantly with the Th17 lineage at the expense of Th1. Moreover, the upregulation of TGF-β and IL-10 suggest an important role for the Treg lineage in this response (Sotillo et al., 2011; Trelis et al., 2011). In this context, IL-13 appears to be essential in its ability to bind the IL-4 receptor α (IL-4Rα) activating signal transducer and activator of transduction 6 (STAT6) which may induce physiological alterations including changes in the glycosylation of mucins. Herein, it was observed that primary infection with *S. muris* induces a similar profile of cytokines in the ileum of rats with a predominance of Th17 responses. The Th17 responses may facilitate the expulsion of *E. caproni* adult worms. Trelis et al. (2011) showed that rejection of *E. caproni* infections in rats is mainly mediated by IL-13 and is based on an increase of the intestinal motility and changes in the quantity and quality of mucus secretion. Fu et al. (2010) demonstrated that IL-17 significantly increases intestinal contractility in mice experimentally infected with *Trichinella spiralis*. In this way, the Th17 responses observed in *S. muris*-infected rats may well contribute to the generation of resistance against *E. caproni*. Although the upregulation of IL-4 in the ileum of rats infected with *S. muris* was relatively transient, it may well contribute to generating an environment responsible for the refractory status in rats. It has been shown that IL-4 or IL-13 can activate epithelial cells to induce upregulation of polypeptide N-acetylgalactosaminyltransferases and N-acetylglucosaminyltransferase and, consequently, the GalNAc and GlcNAc incorporation pattern of mucin tandem repeat peptides (Beum et al., 2005; Kanoh et al., 2008).

Resistance against superimposed *E. caproni* is not dependent upon expression or mucin genes or on the magnitude of the goblet

cell populations. In contrast, the cytokine profile, the antibody response and qualitative alterations in glycosylation of mucins as a consequence of the *S. muris* primary infection appear to be involved in the prevention of attachment of *E. caproni* in the challenged rats.

In summary, we showed that the response generated in rats by *S. muris* is not limited to the immediate site of infection. Furthermore, this distant response is able to regulate and even generate resistance against other helminths, such as *E. caproni*, in the small intestine. These features are of particular interest considering that *S. muris* is a common parasite of laboratory rodents and its presence may compromise the outcome of unrelated experiments.

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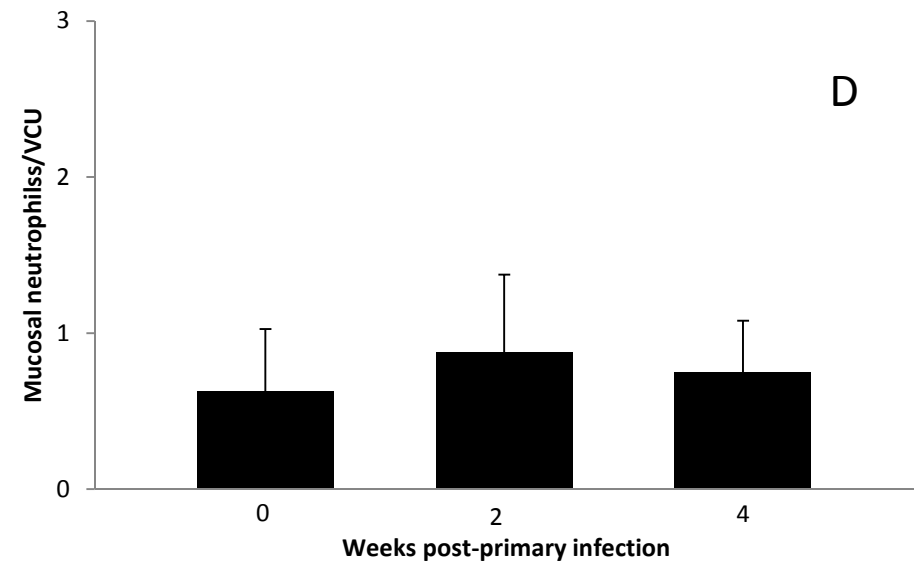
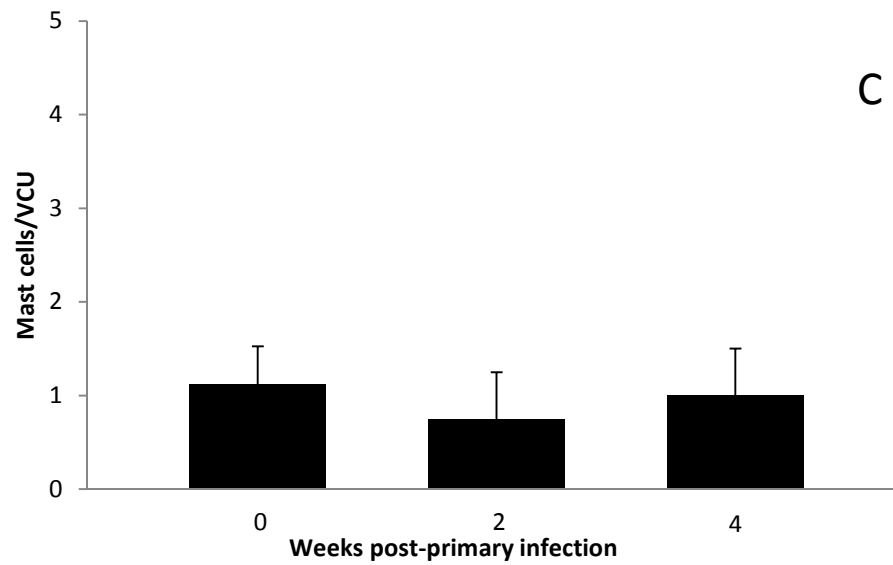
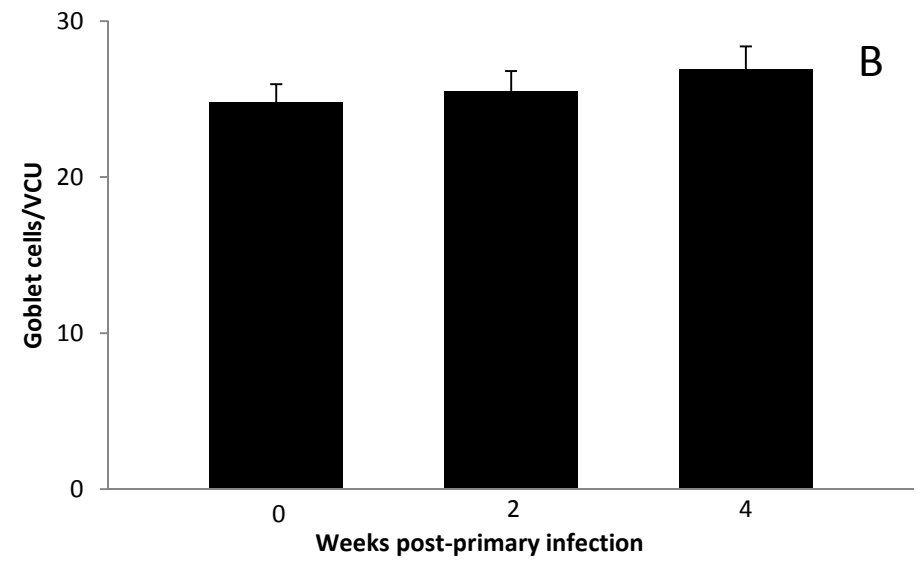
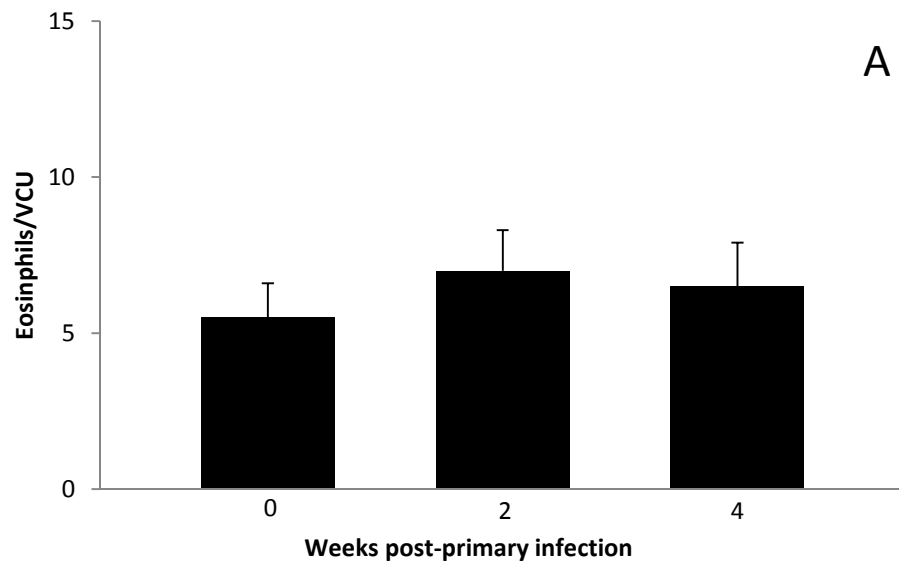
Appendix A. Supplementary data

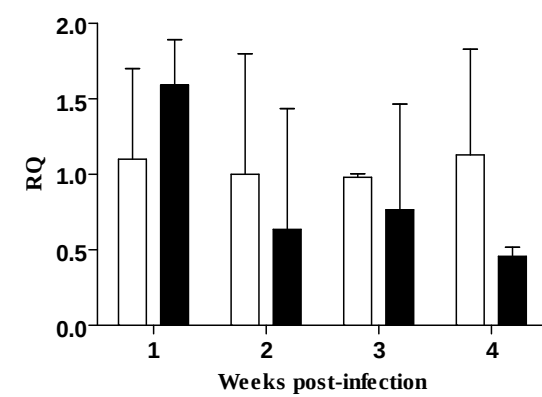
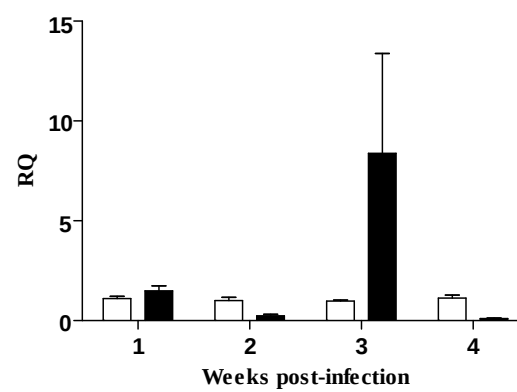
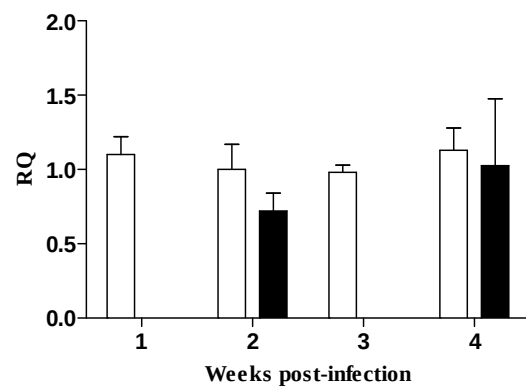
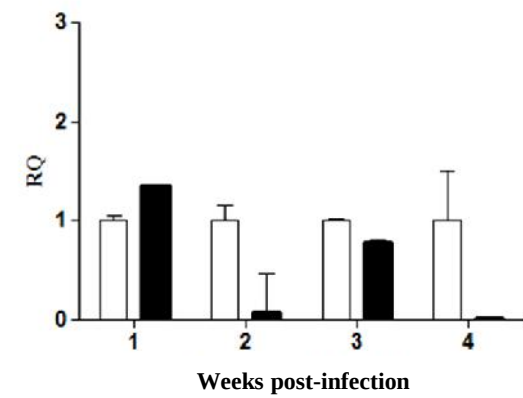
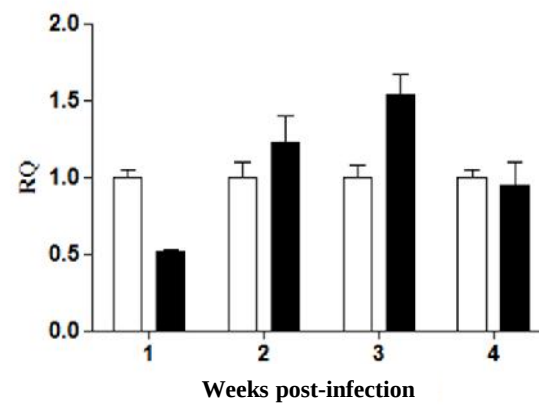
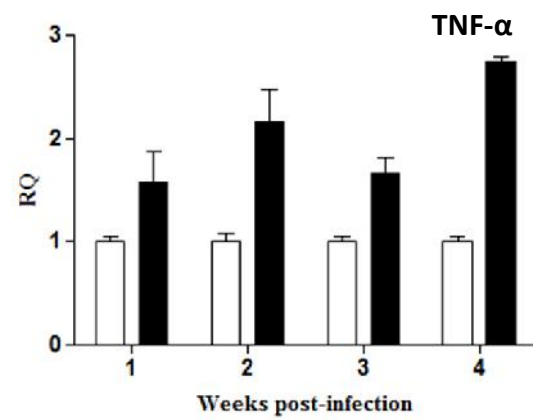
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2012.12.009>.

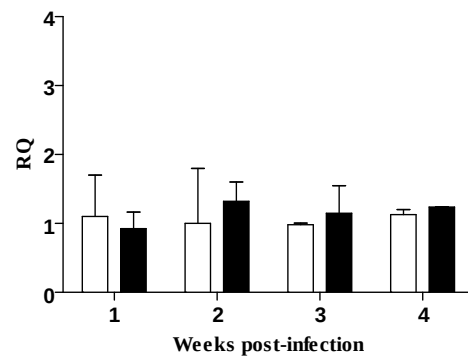
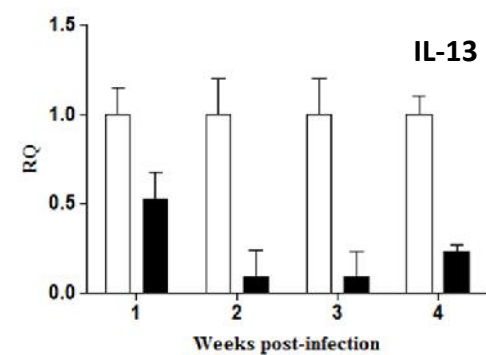
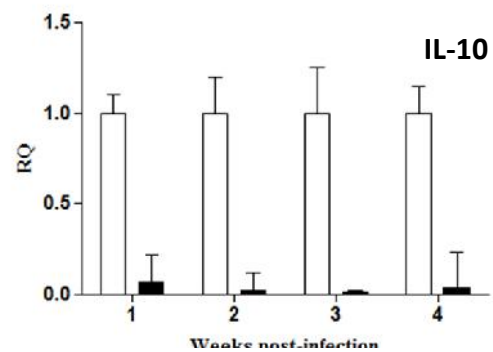
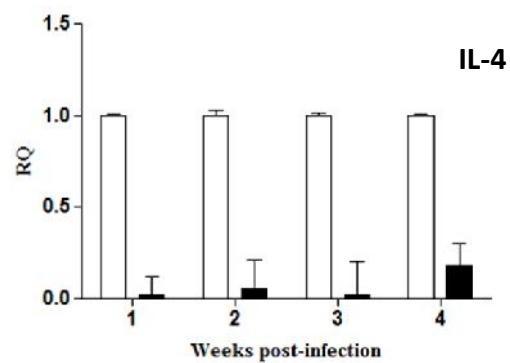
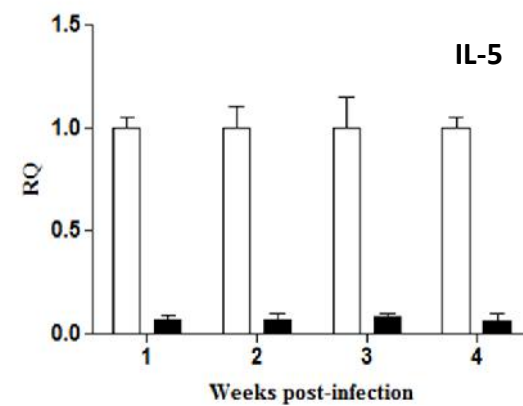
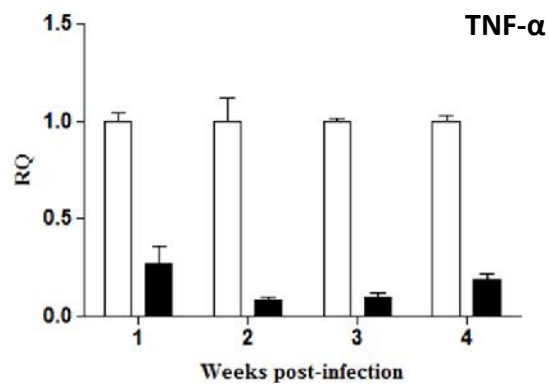
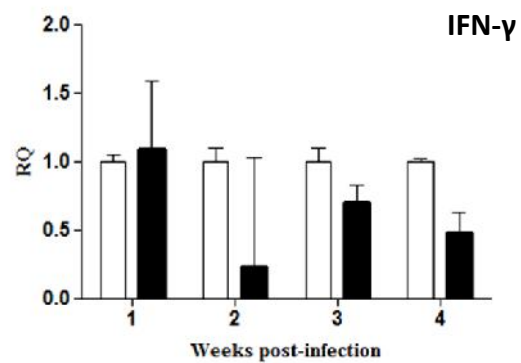
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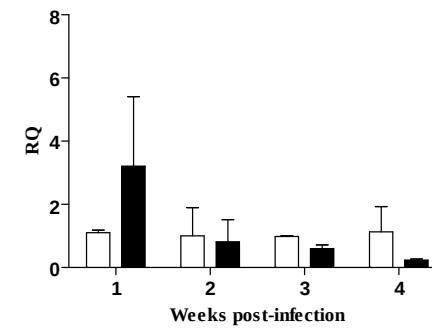
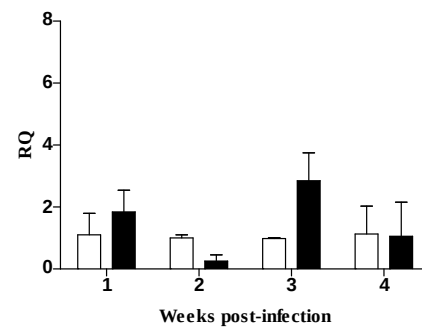
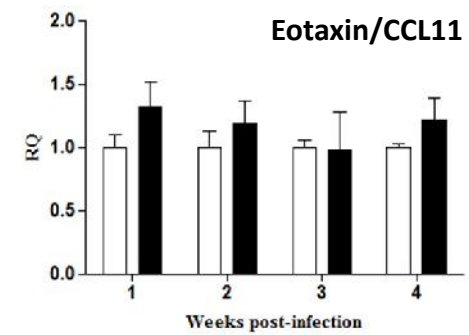
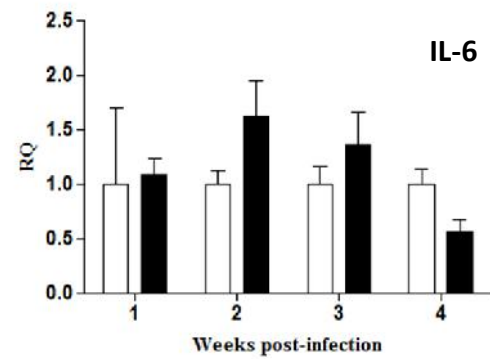
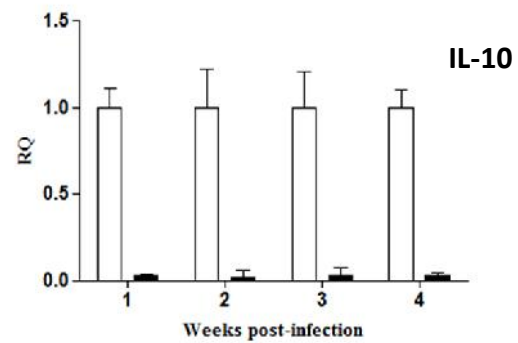
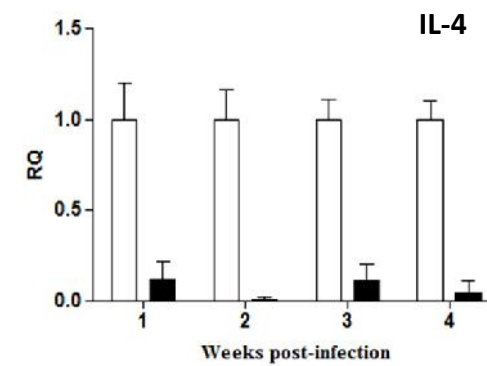
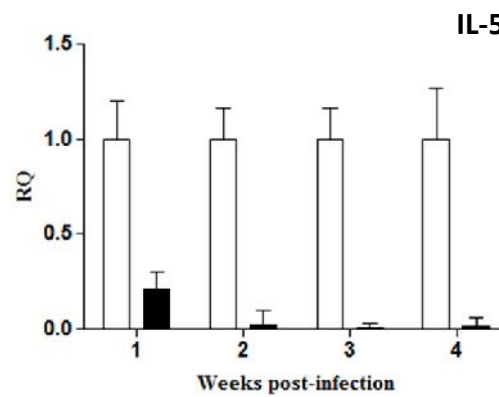
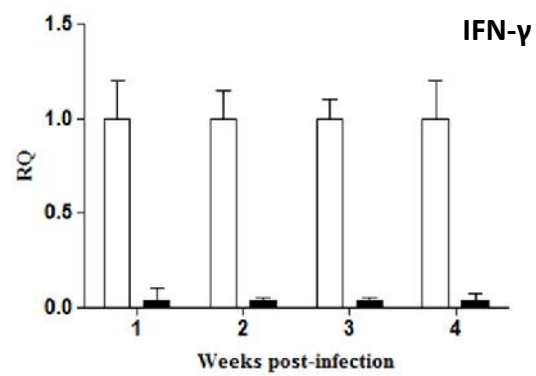
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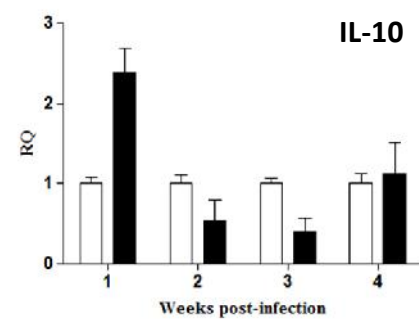
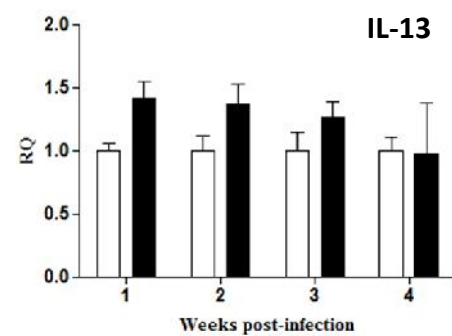
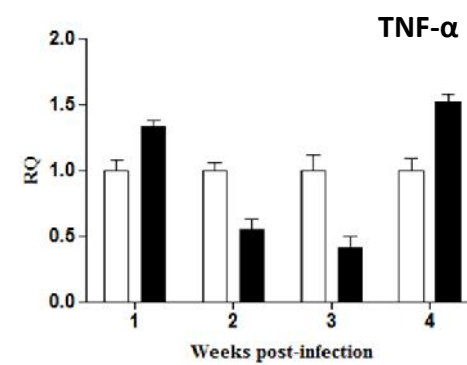
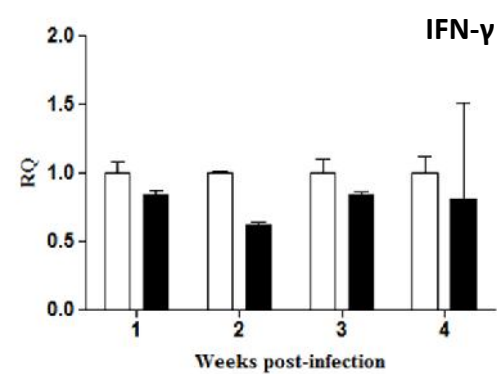
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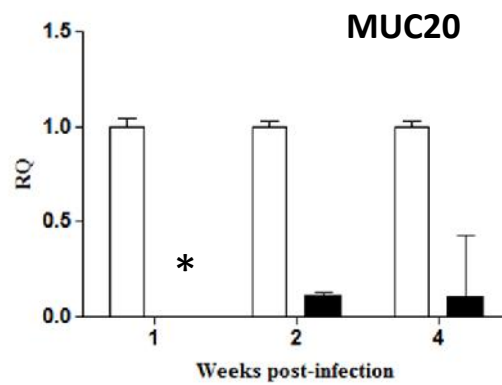
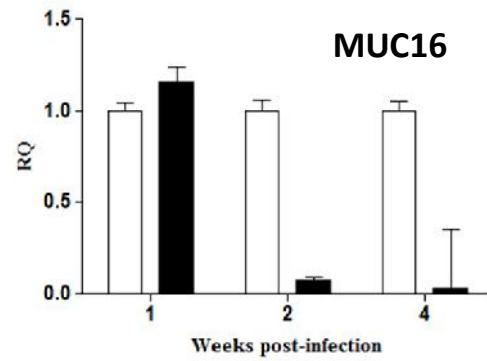
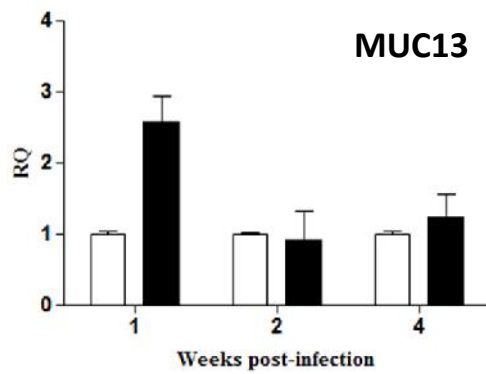
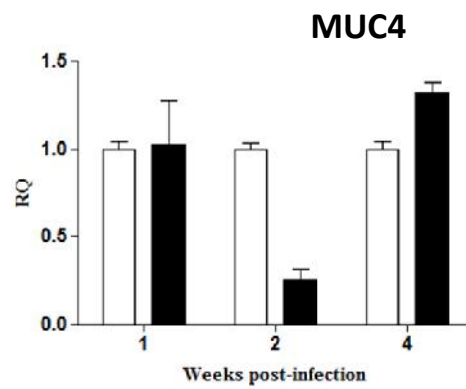
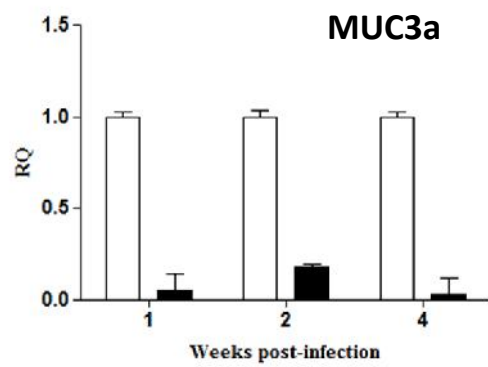
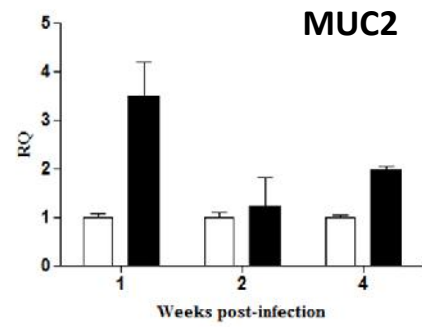
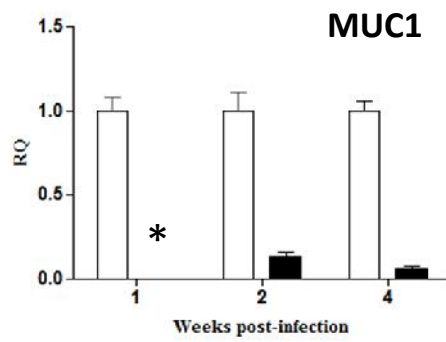


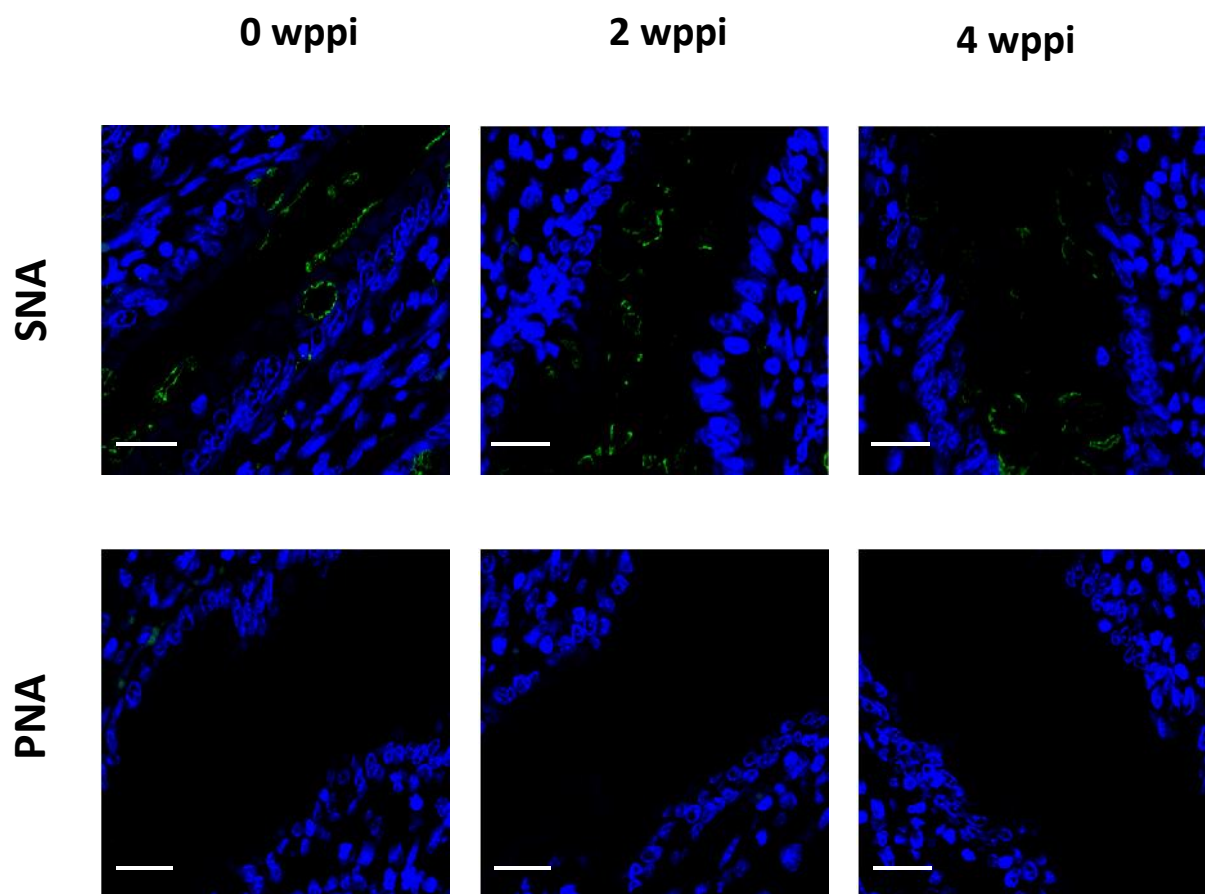












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Supplementary Table S1

Applied Biosystems Inventoried and custom assays used for β -actin, cytokines and mucin genes.

Genes	Assay ID Details
β -actin	Rn00667869_m1
Cytokines	
IL-4	Rn99999010_m1
IL-5	Rn01459975_m1
IL-6 ^a	5'-ACCCACAACAGACCAGTATATACCA-3' (sense) 5'-CATTTCCAAGATCTCCCTGAGAACA-3' (antisense) 5'-CACAAGTCGGAGGCTTA-3' (probe)
IL-10	Rn00563409_m1
IL-13	Rn00587615_m1
IL-17a	Rn01757168_m1
IL-23a	Rn00590334_m1
IFN- γ	Rn01420317_m1
TGF- β	Rn00572010_m1
TNF- α	Rn99999017_m1
Eotaxina/CCL11 ^b	Rn00569995_m1
Mucins	
MUC1	Rn01462585_m1
MUC2	Rn01498197_m1
MUC3a	Rn01481134_m1
MUC4	Rn01475265_m1
MUC13	Rn00709919_m1
MUC15	Rn01747580_m1
MUC16	Rn01448094_m1
MUC20	Rn01474963_m1

^aCustom assay

^bChemokine (C-C motif) ligand 11