

Antibody and cytokine responses to *Giardia* excretory/secretory proteins in *Giardia intestinalis*-infected BALB/c mice

Juan C. Jiménez · Josette Fontaine · Colette Creusy ·
Laurence Fleurisse · Jean-Marie Grzych ·
Monique Capron · Eduardo Dei-Cas

Received: 24 December 2013 / Accepted: 16 April 2014 / Published online: 28 May 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract The humoral and cellular responses against excretory/secretory proteins and soluble extracts of *Giardia intestinalis* were evaluated in the course of experimental *G. intestinalis* infection in BALB/c mice. Production of IgG1, IgG2a, IgA, and IgE antibodies against excreted/secreted proteins and soluble extract was detected after infection by *G. intestinalis*. Specific IgA antibody against E/S proteins and soluble extract from intestinal fluids in infected mice was detected by ELISA. The Western blotting identified proteins of 30, 58, 63, and 83 kDa for IgA and IgG, respectively. High proliferation rate in vitro of spleen cell and secretion of interleukin-4 (IL-4) at 21 days p.i. after stimulation with excreted/secreted proteins and low proliferative response in the presence of soluble extract in infected BALB/c mice was observed. High production of interferon gamma

(IFN- γ) and interleukin-5 (IL-5) at the time of decreasing cyst output (14–21 days p.i.) in infected mice was recorded, suggesting the important role of these cytokines in the control of the infection. Interestingly, progressive and gradual increase of the interleukin-10 after stimulation with both preparations was recorded from 7 days until 28 days after infection, indicating the possible regulatory effect of these antigens on the immune response during *Giardia* infection. Therefore, the infection by *Giardia duodenalis* stimulates a mixed response Th1 and Th2, mainly stimulated by excretory/secretory antigens. The immunogenicity of these antigens may be a suitable for identification of the proteins related with the effective immune response in the course of infection by *G. duodenalis*.

Keyword *Giardia intestinalis* · IFN- γ · *Giardia duodenalis*

J. C. Jiménez · E. Dei-Cas
Biology and Diversity of Emerging Eukaryotic Pathogens (BDEEP),
Centre d'Infection et d'Immunité de Lille (CIIL) (IFR 142), Institut
Pasteur de Lille, INSERM U1019, CNRS UMR 8402, Université
Lille Nord de France, Lille, France

J. Fontaine · J.-M. Grzych · M. Capron
INSERM U547-Schistosomiasis, Malaria & Inflammation, Lille
Pasteur Institute (IFR-17), 1 rue du Prof-Calmette BP245,
59019 Lille, France

C. Creusy · L. Fleurisse
Service d'Anatomie et de Cytologie Pathologiques, Groupe
Hospitalier de l'Université Catholique de Lille, Lille, France

J. C. Jiménez (✉)
Institute of Immunology, Faculty of Medicine, Central University of
Venezuela, Caracas, Venezuela
e-mail: jcjimenez488@hotmail.com

E. Dei-Cas
Parasitology-Mycology Service, Centre Biology Pathology Genetics,
(CBPG) Lille-II, University Hospital Centre, 1 place Verdun,
59045 Lille, France

Introduction

Giardia lamblia (synonymes: *Giardia intestinalis* or *Giardia duodenalis*) is a flagellated protist that parasitizes upper small intestine of human and other mammals (Adam 2001). In Asia, Africa, and Latin America, about 280 million people have symptomatic giardiasis and 500,000 new cases are reported each year (Eckmann 2003; WHO-The world Health Organization Report 1996). This parasite exists in two main life forms, trophozoites and cysts; the infection occurs usually following the ingestion of water or food contaminated with the cystic forms (Belosevic and Daniels 1992). Currently, giardiasis has been recognized as a neglected disease by the World Health Organization (Savioli et al. 2006). However, the mechanism by which the immune host's system controls *G. intestinalis* infection is not known fully. In this regards, exploration of human and mouse experimental infections has revealed that host–parasite relationship is influenced by secretory IgA antibody (Heyworth and Vergara 1994;

Langford et al. 2002), T lymphocytes (Djamiatun and Faubert 1998; Heyworth et al. 1987; Hill 1990; Singer and Nash 2000; Scott et al. 2004), and cytokine activity (Jimenez et al. 2009; Scott et al. 2000; Venkatesan et al. 1996). Nevertheless, little is known about molecules related with the effective stimulation of the specific immune response in giardiasis (Palm et al. 2003). Previously, low cytokine production in Peyer's patches and spleen cells of mice infected with *Giardia muris* or *G. intestinalis* after to be stimulated with soluble extracts was detected, suggesting low immunogenic properties (Hill 1990; Venkatesan et al. 1996). However, multiple evidences in other parasitic models have shown the stimulating effect of excreted/secreted antigens (E/S-Ags) for specific immune response (Daryani et al. 2003; Goodridge et al. 2001; Holland et al. 2000). In this context, previous results on characterization and oral and systemic immunization of excretory/secretory antigens of *Giardia* have been reported (Dunn et al. 2001; Jiménez et al. 2000; Kaur et al. 1999; Kaur et al. 2001; Nash et al. 1983). These results have demonstrated the production of specific antibody response against secreted antigens. In this regards, we have demonstrated that oral administration of *Giardia* E/S Ags in BALB/c mice induced systemic and secretory specific antibody responses, and a slight inflammatory response in the small intestine (Jiménez et al. 2004). In addition, systemic immunization of BALB/c mice with excretory/secretory antigens induced a response Th2 (Jimenez et al. 2009). Therefore, the present work aimed to explore whether experimental infection in BALB/c mice with *G. duodenalis* induces specific antibody response and cytokine production after stimulation with E/S-Ags and soluble extracts. We found a significant effect on the humoral and cellular in response after infection stimulated by E-S proteins or somatic extract.

Material and methods

Mice

Specific pathogen-free, 4- to 6-week-old female, BALB/c mice (Charles Rivers, France) were used. Cages were placed in HEPA-filtered air isolators, and animal experiments were performed according to the conditions stipulated for European Specific Guidelines.

Parasite culture and infection procedure

Trophozoites of *G. intestinalis* P1 strain (Portland 1, ATCC, 30888) were cultured in growth medium TYI-S-33 for 48 h at 37 °C as described previously (Keister 1983). For infection, BALB/c mice were starved overnight before infection and were divided into four mice/cage and gavaged orally with 1×10^6 trophozoites of *G. intestinalis* in 0.2 ml phosphate

buffer saline (PBS). Twenty four mice were also inoculated orally with 0.2 ml PBS without parasites as control group from each point. Mice were returned to solid foods and water ad libitum 4–6 h postinoculation (p.i.). In parallel, another group of mice infected at the same way was also used for sampling blood by retro-orbital bleeding every 7 until 80 days in order to determine the specific serum antibody titers.

Monitoring of *G. intestinalis* infection

The presence of *G. lamblia* cysts was evaluated in pooled fecal homogenates from all inoculated mice. Every 7 days p.i., 4–7 pieces of feces from each mouse were collected, homogenized in 0.5 ml PBS in pre-weighed Eppendorf tube. Cysts number were determined at different days post-infection and expressed as number of cysts per gram of feces.

Preparation of *G. intestinalis* E/S-Ags and soluble extract

The E/S-Ags were obtained from incubated trophozoites in protein-free medium as described by Guy et al. (1991). Briefly, culture tubes with trophozoites were rinsed with warm RPMI-1640 medium (Invitrogen, Gibco, London, UK) at 37 °C to remove non-attached or dead trophozoites. Then, the parasites were incubated in protein-free RPMI medium supplemented with L-cysteine (11.4 mM) during 6 h at 37 °C. After incubation, the parasites were centrifuged at $1,000 \times g$ for 20 min at 4 °C, the supernatants were collected and concentrated threefold on Aquacide II (Calbiochem, France), filtered through a SMILLEX-GS filter (0.22 µm, Millipore, Bedford, MA, USA), and stored at –80 °C until use. The soluble extract was obtained by lysis of trophozoites in Tris–HCl 50 mM, pH 8, 0.5 % Triton X-100. Protein concentration was quantified using a protein assay kit (BCA, Pierce, Rockford, Illinois, USA).

Intestinal fluid sampling

For the measurement of secretory specific IgA antibody in infected or control mice, small intestine of infected mice, each 7 days was removed and perfused with 2 ml of PBS at 4 °C. The intestinal contents were centrifuged for 10 min at $10,000 \times g$ at 4 °C, and the supernatants were collected containing 0.1 ml of protease inhibitor cocktail (P-8215, Sigma Chemical Co., St. Louis, MO, USA) and stored at –80 °C until use.

Antibody response against excretory/secretory antigens

Antibody response was assessed using an ELISA assay as reported previously (Jiménez et al. 2004). Briefly, Immulon-96 plates (Nalgene Nunc Int, Copenhagen, Denmark) were coated with 10 µg/well of *Giardia* E/S-Ags or soluble extract

with 100 µl/well in PBS and incubated overnight at 4 °C. The plates were blocked with 1 % bovine serum albumin (BSA) for 1 h at room temperature and washed two times with PBS 0.05 % Tween 20 (PBS-T). One hundred microliters of serial twofold dilution of serum samples diluted for IgG1 and IgG2a 1:50, IgA 1:20, and IgE 1:10 of infected, and control mice in PBS-T were added, and plates were incubated overnight at 4 °C. Plates were then washed three times in PBS-T and incubated with Horseradish Peroxidase (HRP)-conjugate goat anti-mouse IgG1 (1:5,000), IgG2a (1:10,000), or IgA (1:5,000) (Southern Biotechnol, Birmingham, AL, USA) for 2 h at 37 °C. For the determination of specific IgE antibody, biotinylated rat anti-mouse IgE (PharMingen, San Diego, Cal, USA) in PBS-T (1:2,000) was used. After washing with PBS-T, streptavidin-HRP diluted in PBS-T (1:2,000) (Zymed, San Francisco, USA) was added, and the plates were incubated for 30 min at 37 °C. Plates were revealed with 100 µl/well of *o*-Phenylenediamine (OPD) (Zymed, San Francisco, USA) and read in ELISA plate reader at 490 nm. The results were expressed as endpoint titre log 2 (mean ± SD) of optical density.

Total IgE serum

Total IgE concentrations were evaluated on plates coated overnight at 4 °C with the rat monoclonal antibody anti-mouse IgE (2 µg/ml, clone R35-72; PharMingen) in carbonate buffer (100 µl/well, pH 9.6). After blocking with a 3 % bovine serum albumin (BSA) solution in PBS for 1 h at room temperature and two washes with PBS-T, 100 µl of serial dilutions of mouse IgE standard (anti-TNP; PharMingen)/well and 100 µl of diluted serum samples (1:50 in PBS-T-1 % BSA)/well were incubated overnight at 4 °C. After five washes, 100 µl of biotinylated rat anti-mouse IgE (2 µg/ml; Southern Biotechnology)/well was added and incubated for 90 min at 37 °C. Streptavidin-HRP (1:2,000; Amersham) was added. Plates were then incubated for 30 min at 37 °C and treated as described above for antibody detection. Sample OD values were converted to micrograms of IgE per milliliter by reference to the standard curve.

Western blotting

Giardia E/S-Ags containing 30 µg/well were separated in SDS-PAGE gel 10 % under reducing conditions (Laemmli 1970). The proteins were electrotransferred into a nitrocellulose membrane (NC membrane 0.45 µm, Schleicher & Schuell Keene, Dassel, Germany) for 90 min at 125 mA using a Biorad Miniprotein II apparatus (Towbin et al. 1979). Membranes were blocked with a 5 % non-fat milk solution in PBS for 2 h at room temperature and washed thrice with PBS-T. Membranes were incubated individually with pooled sera of infected or uninfected mice diluted in PBS-T (IgG1, IgG2a

1:100, IgA 1:50, and IgE 1:10) overnight at 4 °C. The membranes were washed and incubated with goat HRP-conjugated anti-mouse antibody for each isotype diluted in PBS-T (1:1,000 for IgG1 and IgG2a, 1:500 for IgA) for 1 h at 37 °C. For the detection of IgE antigens, membranes were incubated with a biotinylated rat anti-mouse IgE (PharMingen, San Diego, CA, USA) diluted at 1:250 for 2 h at 37 °C. After five washes (PBS-T), the membranes were incubated for 30 min at 37 °C in streptavidin-HRP (Zymed) 1:2,000 in PBS-T. Reactive proteins were revealed by 3, 3'-diaminobenzidine (DAB) substrate kit (Novo Castra, Newcastle, UK). For the detection of secretory IgA antibody, intestinal washes of infected or non-infected mice were diluted 1:5 in PBS-T 0.05 %, and goat HRP-conjugated IgA-anti-mouse antibody diluted in PBS-T (1:500) and incubated for 1 h at 37 °C. The strips were revealed as described above by DAB.

Culture and cellular response

Spleen cells were obtained from mice for each point and euthanatized at 0, 7, 14, 21, and 28 days after infection. The spleen cells (5×10^6 cells/ml) were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM of β-mercaptoethanol, 2 mM of glutamine, and 10 % fetal calf serum (FCS) in triplicate into 96 well flat-bottomed plates in the presence or absence of *Giardia* E/S-Ags or soluble extract (10 µg/ml) for 72 h at 37 °C, in 5 % CO₂. The supernatants were collected and stored at −80 °C for cytokine determination. Cell proliferation was assessed by thymidine incorporation assay and expressed as mean ± SD of count per minute (c.p.m.). The results were obtained from three independent experiments.

Measurement of cytokine

The IFN-γ concentration was assayed by an ELISA DuoSet kit (R&D Systems, Abingdon, UK) according to manufacturer's instruction. The cytokine IL-4, IL-5, or IL-10 were quantified by ELISA assay adapted from PharMingen protocols (Becton Dickinson, San Diego, CA, USA). Cytokine concentration in supernatants was calculated from standard curves constructed using serial dilutions of the appropriate recombinant standard for each cytokine in parallel on each plate and was expressed as picogram per milliliter.

Statistical analysis

Mann–Whitney rank sum or Student's *t* tests was used for evaluating antibody, cell proliferation or cytokine level differences, and morphometric analysis. Differences with a *p* value of *p* < 0.05 were considered significant. All the experiments were repeated at least three times.

Results

The infection was monitored by count of *Giardia* cysts number per gram of feces from mice in each point. The number of cysts per gram in stool from infected mice was increased with a peak at 14 days p.i. However, a reduction of the number of cysts was detected at 21 days p.i. being low at 28 days p.i. (Fig. 1). The presence of cysts was not detected at 40 until 70 days p.i. Finally, at day 80, no cysts were detected and no parasite was found in the stools in mice orally inoculated only with PBS.

Serum IgG antibodies

Specific IgG1 and IgG2a serum antibody responses against E/S-Ags and soluble extract were evaluated by ELISA (Fig. 2a, b). The specific IgG1 antibody response against E-S proteins and soluble extract was quite low until 30 days p.i. After that, a significant increase of titers after 35 days for E/S-Ags and soluble extract in infected mice compared to non-infected mice was detected (Fig. 2a). Specific IgG2a serum antibody against E/S Ags and soluble extract showed significant titers until 60 days p.i., as shown in the Fig. 2b. However, after 70 days p.i., the antibody titers against E/S were increased compared to non-infected mice, however, for soluble extract can reach the titers at 80 days p.i. (Fig. 2b).

Systemic and secretory IgA antibodies responses

Serum IgA antibody response against E/S-Ags or soluble extract in infected Balb/c mice started to be detected at 30 days p.i. compared to non-infected mice with a reduction of titers at

40 days after (Fig. 3a). A progressive increase of titer was observed from 50 until 80 days p.i. (Fig. 3a). The IgA antibody response against soluble extract had the same pattern, but with a low titers (Fig. 3a). Secretory IgA antibody against soluble extract showed a peak at 7 days p.i. with a progressive reduction until day 20 (Fig. 3b). After that, a second peak of minor level compared to infected mice was observed. The secretory IgA production against soluble extract showed a low detectable peak, until the end of the kinetic, with a reduction of the titers for soluble extracts was observed (Fig. 3b).

Total and specific IgE antibody in serum of infected mice

Total serum IgE was higher in infected BALB/c mice, and non-infected mice group showed basal levels of total IgE with progressive increase in the infected BALB/c mice, 21 days p.i. reaching a peak at 60 days p.i. with a reduction of concentration at the end of the kinetic (Fig. 4a). Infection with *G. intestinalis* in BALB/c mice induced production of specific IgE antibody against E/S-Ags proteins and soluble extracts (Fig. 4b). Significant levels of IgE antibody response against E/S-Ags were observed between 40 and 60 days p.i., with low titers of IgE to the soluble extract were recorded (Fig. 4b).

Reactivity by Western blot

In order to identify the E/S-Ags and soluble extract of *G. intestinalis* recognized by sera of infected mice, serum samples collected at 50 days p.i. were used (Fig. 5a). Uninfected mouse sera did not recognize proteins bands neither E/S-Ag nor soluble extracts (Fig. 5a, lanes 1 and 2). Serum IgG-, IgA-, and IgE- antibodies from infected mice reacted for

Fig. 1 Cysts output in *G. intestinalis* infected BALB/c mice (circle). Mice were orally infected with 1×10^6 trophozoites of P1 strain *G. intestinalis*. Twenty-eight days after the primary infection, a group of 20 mice were reinfected with 1×10^6 trophozoites (vertical line). Infection was monitored weekly by cyst counting in stool samples from infected mice. Data are expressed as the number of cysts per gram of feces from four mice for each data point (mean $\pm$ SD)

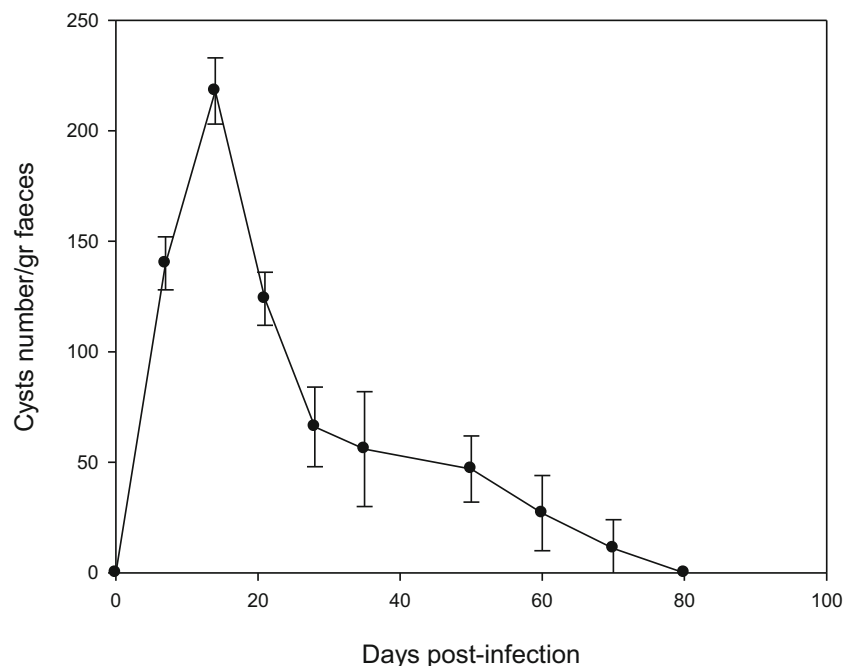
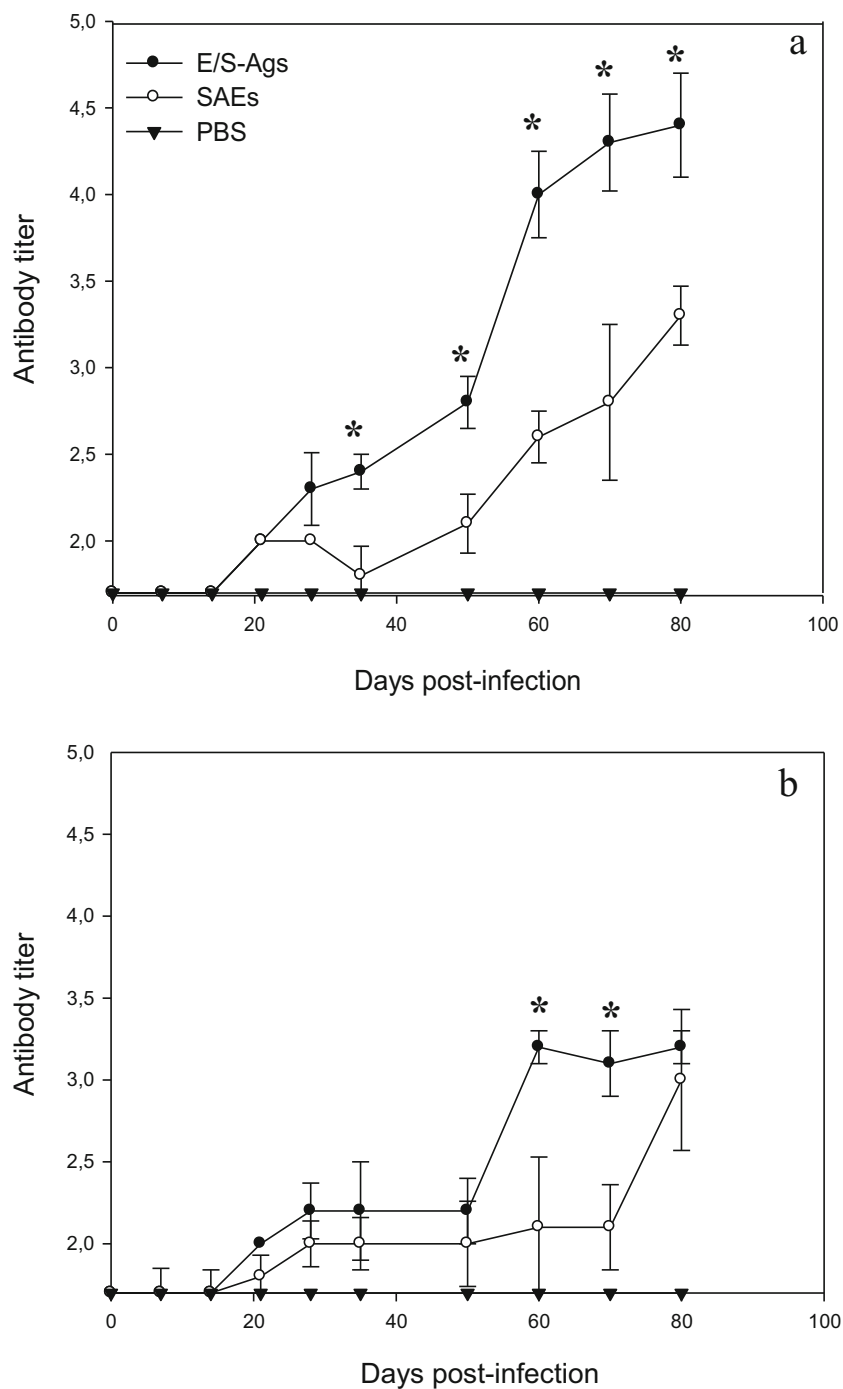


Fig. 2 Specific IgG1 **a** and IgG2a **b** antibody responses during *G. intestinalis* infection in BALB/c mice against E/S-Ags (black circle), SAE (white circle), and uninfected mice (black inverted triangle). Serum was collected every 7 days after inoculation from infected and uninfected mice. The antigen-specific antibody titer was determined by ELISA and expressed as the titers of the highest dilution whose absorbance was 0.1 than the absorbance of the sera of control mice at the same dilution from four mice per group in three individual experiments. The asterisk indicates that the mean value was significantly higher than uninfected mice group ($p < 0.05$)



E/S Ags or soluble extracts antigens. For IgG proteins of 50 and 58 kDa, serum of infected mice for E/S-Ags for all isotypes was recognized; meanwhile, for soluble extract, several bands were recognized such as 175, 153, 83, 72, 63, 36, 30, and 14 kDa, respectively (Fig. 5a, lanes 3 and 4). For specific IgA antibody, E/S-Ags proteins of 50 and 58 kDa were detected, and for soluble extract, bands of 30 and 14 kDa were observed (Fig. 5a, lanes 5 and 6). For specific IgE reactivity, one band of 58 kDa for E/S-Ags (Fig. 5a, lanes 7) and several proteins from 175 to 20 kDa for soluble extract

(Fig. 5a, lane 8) were detected. Figure 5b shows the results of secretory IgA antibody in fecal extracts against soluble extracts (Fig. 5b, lanes 1–5). In fecal extract of uninfected mice, not specific secretory IgA was detected (Fig. 5b, lane 1). In lanes 2 and 3, secretory IgA in fecal extract of infected mice (7 days p.i.) reacted with a protein of soluble extract of 30 kDa, respectively (Fig. 5b). For lanes 4 and 5, we can see reactivity for a protein of 83 kDa (14 days p.i.) in serum of infected mice 30 days p.i.

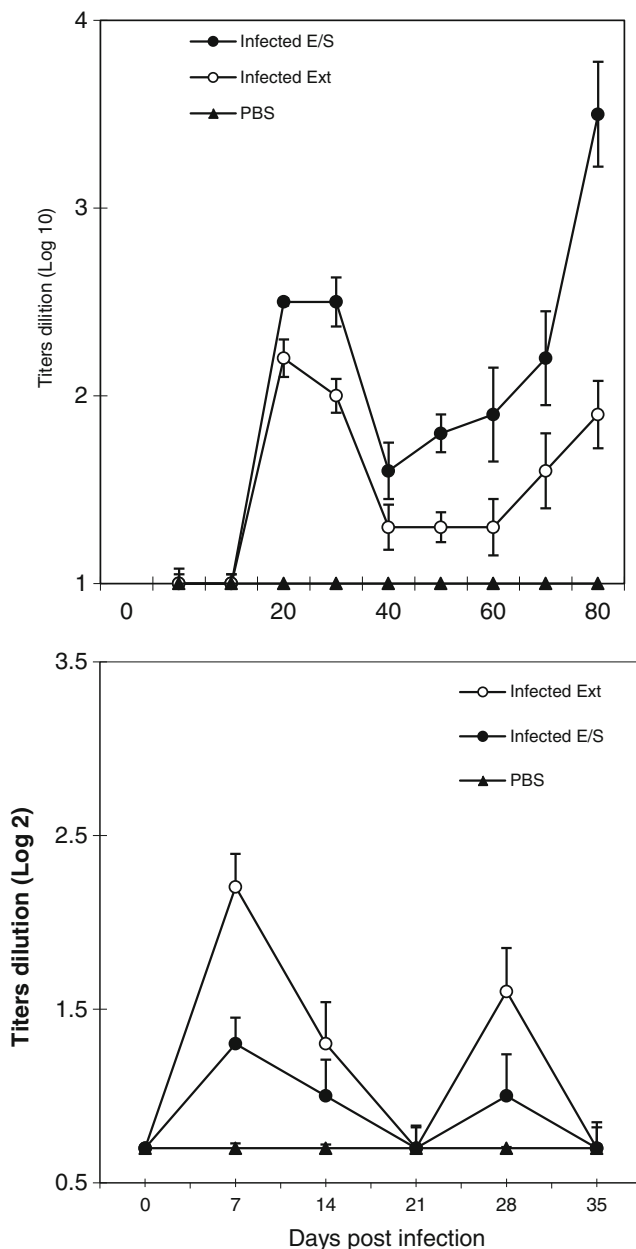


Fig. 3 Kinetics of specific IgA antibody in serum **a**, and secretory IgA in fecal extracts **b** during *G. intestinalis* infection in BALB/c mice against E/S-Ags (black circle), SAE (white circle), and uninfected mice (black inverted triangle) measured by ELISA. The results were expressed as endpoint titers of the highest dilution whose absorbance was 0.1 than the absorbance of the sera of control mice at the same dilution $\pm$ SD from four mice per group in three individual experiments. The asterisk indicates that the mean value was significantly higher than control group ($p < 0.05$)

Lymphocyte proliferation and cytokine production to *G. intestinalis* antigens

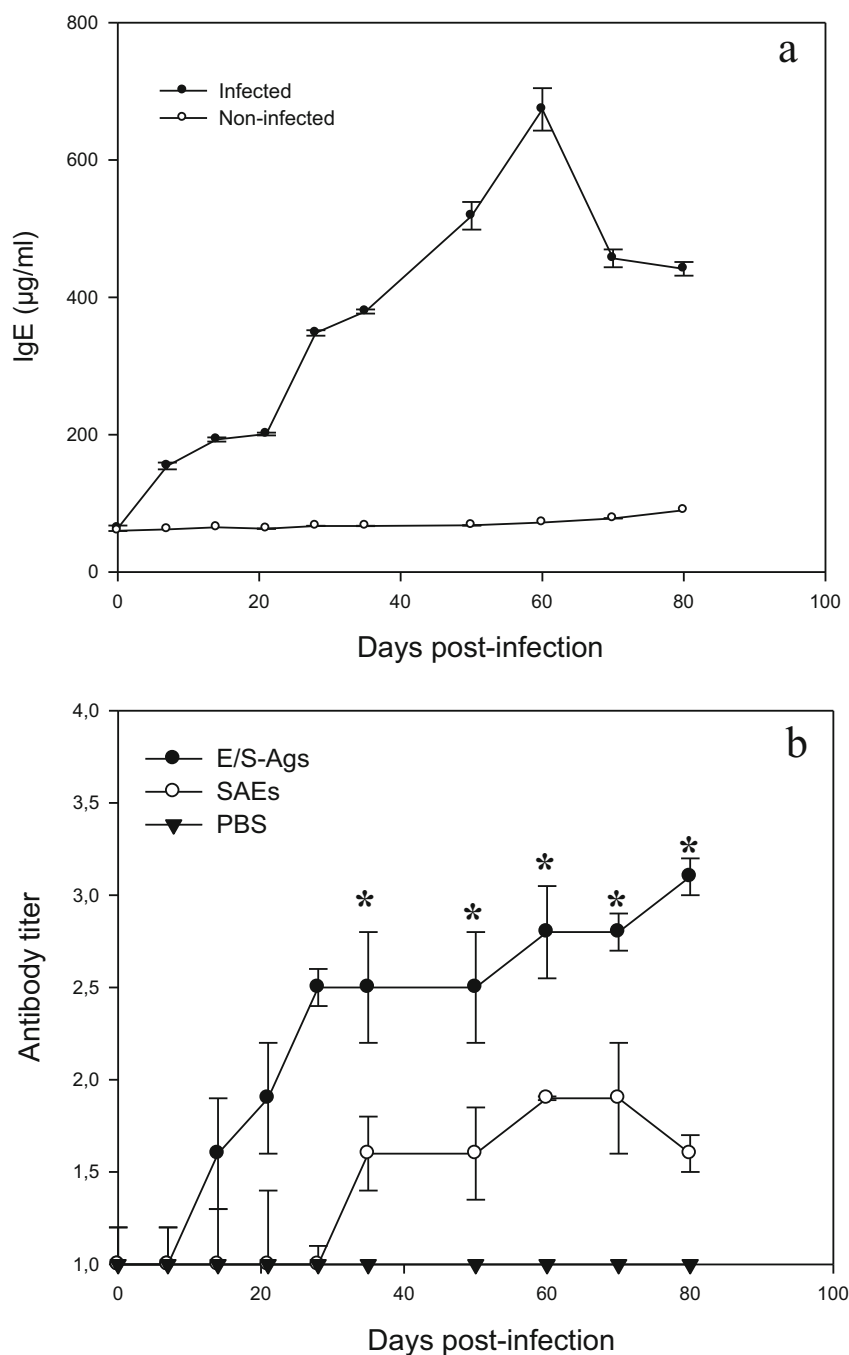
Proliferation of spleen cells and cytokine production of infected and non-infected BALB/c mice after stimulation with E/S-

Ags or soluble extract was evaluated (Fig. 6a). Basal proliferation in non-infected mice was observed in the presence of E/S-Ags or soluble extracts (Fig. 6a). High levels of proliferation were observed in infected mice after stimulation with E/S proteins on 7 days p.i., but low proliferation stimulated by soluble extract was observed. At this point (14 days p.i.), similar levels of proliferation of splenocytes were observed in the presence of soluble extract and E/S-Ags (Fig. 6a). After 21 days p.i., an increase of proliferation stimulated by E/S-Ags was detected with low levels of proliferation stimulated by soluble extract (Fig. 6a). Reduction of spleen proliferation was observed to the 28 days p.i. in response to E/S-Ags (Fig. 6a). The concentration of cytokines was evaluated by ELISA (Fig. 6b–e). For cytokines, 7 days p.i. was observed that E/S-Ags induced in spleen cells of infected BALB/c mice high secretion peak of IL-4, but a reduction of this cytokine after 14 days p.i. (Fig. 6b). For INF- γ , an increase of this cytokine 7 days p.i. was observed, with a peak of production at 14 days, but a reduction after stimulation by E/S-Ags (Fig. 6c). In Fig. 6d, the IL-5 production was detected initially at 14 days p.i. stimulated by E/S-Ags, but progressive reduction of this cytokines until 28 days p.i. Interestingly, increase of IL-5 after stimulation with soluble extracts was detected, with a peak at 21 days p.i., but a reduction after 28 days p.i. (Fig. 6d). Finally, gradual and progressive increase of IL-10 secretion stimulated in both preparations (E/S-Ags and soluble extract) was detected at 14, 21, and 28 days p.i., respectively (Fig. 6e).

Discussion

Humoral and cellular immune mechanisms are involved in the clearance of *G. intestinalis* infection in mouse and human (Djamiatun and Faubert 1998; Faubert 2000). However, despite of some progress in the knowledge of immune response against *Giardia* infection, only limited information is available on specific molecules able to induce specific immune response. Currently, it has been well established that orogastric inoculation of *G. intestinalis* or *G. muris* trophozoites into adult BALB/c mice provides a reproducible level of infection (Byrd et al. 1994; Larocque et al. 2003; Robert-Thompson et al. 1976). It has also been reported that proteins secreted by *G. lamblia* induce a Th2-specific response (Kaur et al. 1999; Shant et al. 2004). Other parasite infections have also well illustrated the central role played by the excretory/secretory proteins in host cell invasion, catabolism of host proteins, cell cycle progression, and stimulation and evasion of host immune response (Goodridge et al. 2001; Holland et al. 2000). For example, immune response against E/S-Ags of *Toxoplasma gondii* was associated with higher survival rate challenged experiments (Smith et al. 2000). In previous results, we have demonstrated that *Giardia* E/S-Ags elicited

Fig. 4 Total **a** and specific **b** IgE against E/S-Ags (black circle), SAEs (white circle) in infected or non-infected BALB/c mice (black inverted triangle), orally inoculated with 1×10^6 trophozoites per milliliter (four mice per group), were measured by ELISA. The total IgE was expressed as microgram per milliliter (geometric means $\pm$ SD), and specific IgE was measured in serum of uninfected and infected mice by isotype specific ELISA. Results of antigen-specific IgE were presented as antibody titers. * $p < 0.05$ by Student's *t* test



systemic and mucosal antibody response, as well as histological changes in the small intestine after oral administration (Jiménez et al. 2004). The present work aimed to determine whether experimental infection of BALB/c mice with *G. intestinalis* elicited specific response against excretory/secretory proteins of *G. intestinalis*. Infection of BALB/c mice with *G. intestinalis* trophozoites produce specific circulating IgG1-, IgG2a-, IgA-, and IgE-antibody responses against E/S-Ags, while for soluble extract low titers were observed, indicating the powerful of immunogenicity of excretory proteins. These results confirm our previous

observations in which mice orally immunized with E/S-Ags of *G. intestinalis* (without adjuvant) showed strong specific antibody responses (Jiménez et al. 2004). In addition, systemic immunization of excretory/secretory in Balb/c mice stimulated specific antibody production and cytokine profile of Th2-type, it which was reduced after immunization with the excretory/secretory proteins inhibited by cysteine–protease inhibitor (Jimenez et al. 2009). In the same way, oral immunization with cytosolic fractions of *G. intestinalis* trophozoites stimulated a specific immune response (Laroque et al. 2003). Results reported by Laroque et al. (2003) indicated that oral

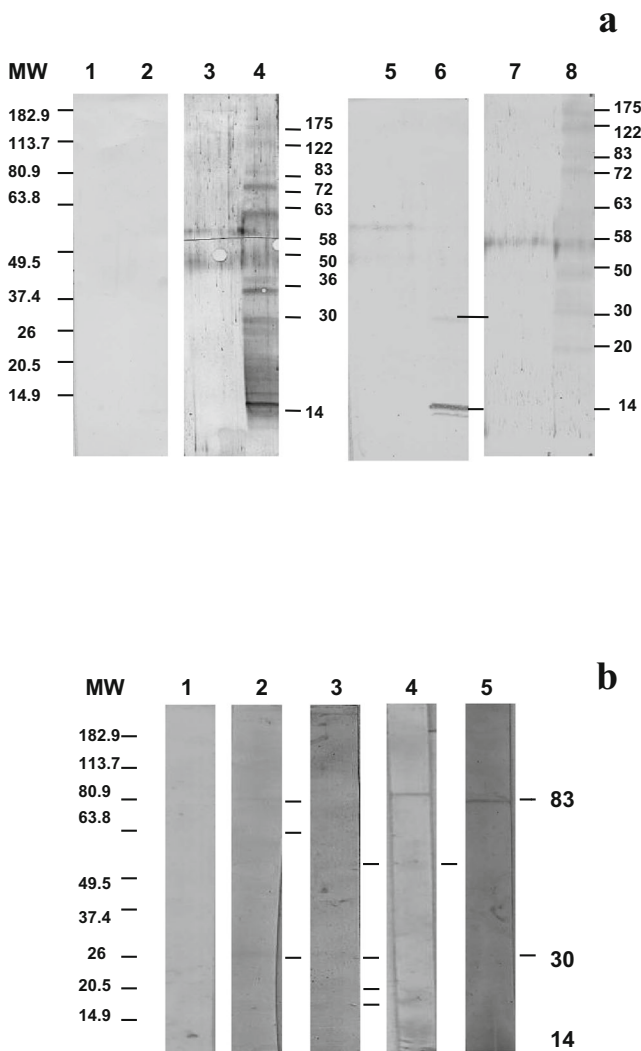
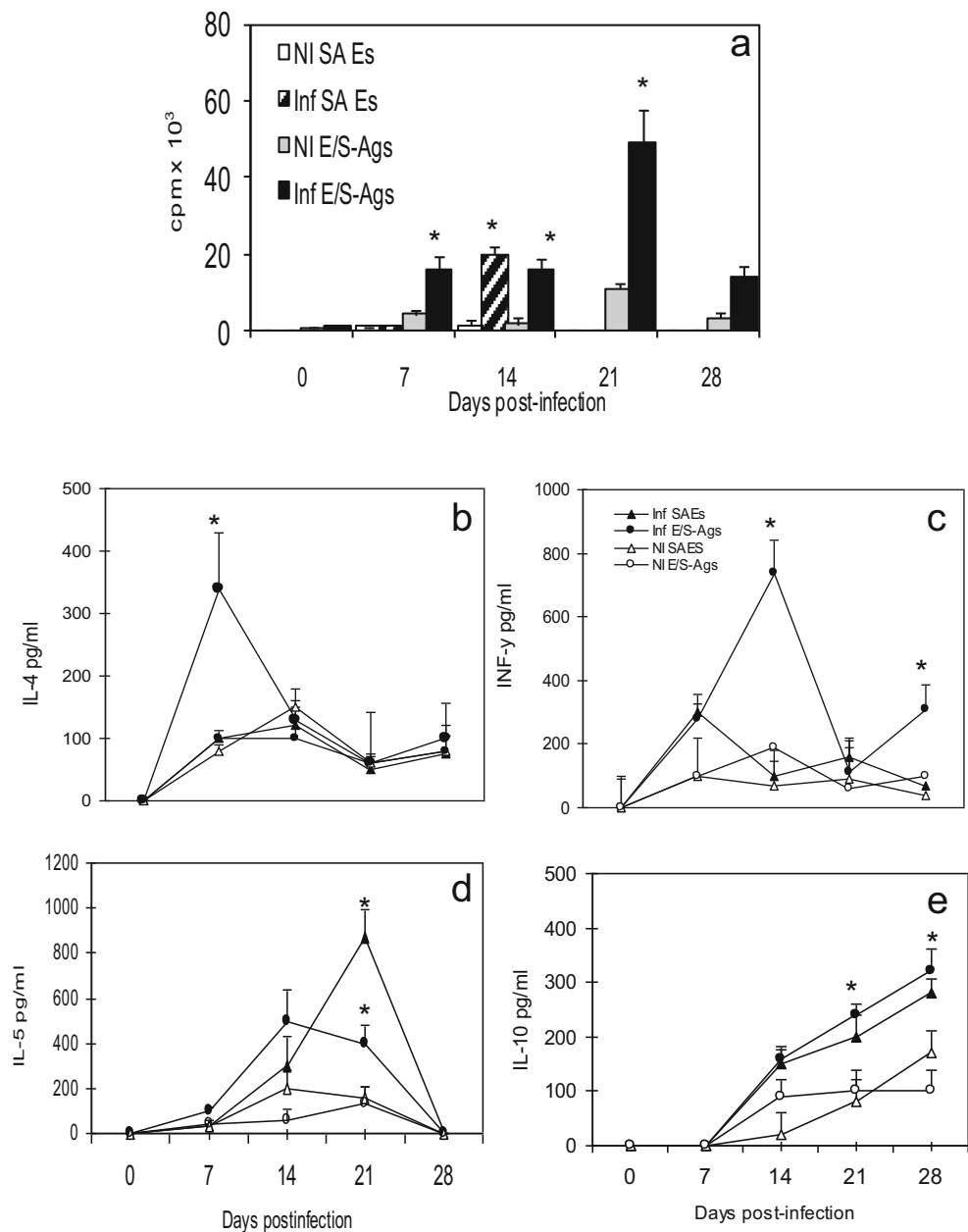


Fig. 5 Western blotting for E/S-Ags (lanes 1, 3, 5, and 7) and soluble extract (lanes 2, 4, 6, and 8) of pooled sera of infected mice **a**. Lanes 1 and 2 pooled sera of non infected mice; lanes 3 and 4, IgG1; lanes 5 and 6, IgA; and lanes 7 and 8 specific IgE. Specific secretory IgA antibody against soluble extract in non-infected and infected mice (7 and 21 days p.i.) from pooled intestinal washing of infected mice **b**. Intestinal washes of four mice of each group. Lane, 1 intestinal washing of non-infected mice; lanes 2 and 3, 7 days after infection; and lanes 3 and 4, 14 days p.i. The antigens bound to serum were detected using Liquid DAB system

immunization of BALB/c mice with a recombinant cyst wall-2 protein (rCWP2) elicited circulating IgG1 antibody able to interfere with cyst formation (Larocque et al. 2003). Therefore, the infection with *G. duodenalis* stimulates specific humoral and cellular responses. The specific IgA antibody plays a central role in the immune response involved in either trophozoites immobilization or detachment (from the epithelium) (Langford et al. 2002; Velázquez et al. 2005; Venkatesan et al. 1996, 1997). In previous work, we reported a significant reduction of growth rate in vitro of *G. intestinalis* trophozoites in the presence of heat-inactivated serum of orally immunized BALB/c mice with *Giardia* E/S-Ags, suggesting a possible

cytopathic effect in vitro of these antibodies on the trophozoites (Kaur et al. 1999). High production of specific IgA antibody was observed after infection in BALB/c mice. The parasite-specific secretory IgA antibody from intestinal fluid was also detected, but as early as day 7 p.i., with a reduction of secretory antibodies, 14 days after infection. The evaluation of E/S-Ags in serum and intestinal fluid reactivity by Western blotting revealed several antigens, recognized proteins between 83 and 30 kDa, respectively. Therefore, it would be important to identify the proteins associated to the development of secretory and systemic antibody response so better potential mucosal-vaccine candidates (Robert-Thompson et al. 1976). Previous studies in human giardiasis reported that *Giardia* infection induces the production of total and specific IgE antibodies (Di Prisco et al. 1998). Interestingly, infected BALB/c mice with *G. intestinalis* trophozoites showed high titers of total and specific IgE anti-E/S antibody responses, indicating the allergenic properties. In addition, several antigens from 175 to 17 kDa related to the specific IgE were identified, which to be related with allergic reactions observed in some individuals. The stimulation of spleen cells of infected mice with E/S proteins showed a proliferative activity and the secretion of cytokines, indicating that E/S-Ags have a strong capacity to induce a specific T cell response (Dunn et al. 2001). The production of cytokines stimulated by E/S-Ag showed a high but transient secretion of IL-4 at 7 days p. i.; after day 14 p.i., a reduction of this cytokine was accompanied of an increase in the production of IFN- γ in the infected mice until 14 days p. i. In addition, secretion of IL-5 by splenocytes of infected mice stimulated with E/S-Ags was also observed along with the production of IFN- γ at the same time. This profile of cytokines was also related with the reduction of cyst output 21 days after infection, suggesting that these cytokines could be involved in the control of *G. intestinalis* infection in BALB/c mice. In this regards, IFN- γ has been related with an increase of macrophage NO production in *Giardia* infection (Byrd et al. 1994; Holland et al. 2000), though it was reported that the parasite was able to inhibit NO production in vitro (Faubert 2000). A similar protective role of IFN- γ against *Cryptosporidium parvum* or *Eimeria vermiformis* infections has been documented (Smith et al. 2000; Wakelin et al. 1993). On the whole, our results suggest that the high production of IFN- γ may contribute to the early control of the infection, probably in synergy with the production of IL-5 more related with the elimination of the parasite. In the previous ex vivo experiments using lymph node cells from *G. muris*-infected BALB/c mice, only the IL-5 was detected followed of *G. muris*-SAE stimulation (Venkatesan et al. 1996). Therefore, our results are in contrast with the previous reports, in which *Giardia* infection could be controlled by experimentally infected Th1- or Th2-deficient mice, suggesting that neither IL-4 nor INF- γ would be needed to the control of the infection (Wakelin et al. 1993).

Fig. 6 Proliferative response of spleen cells of non-infected and infected BALB/c mice 72 h after stimulation with E/S-Ags or soluble extracts of *G. intestinalis* **a**. Antigen-specific proliferation was measured by Thymidine incorporation after stimulation in vitro with E/S proteins (10 μ g/ml) and SAEs (10 μ g/ml) expressed as counts per minute (mean $\pm$ SD). Antigen-specific cytokine production of IL-4 **b**, IFN- γ **c**, IL-5 **d**, and IL-10 **e** of spleen cells supernatants from infected and non-infected BALB/c mice (days 0, 7, 14, 21 and 28 p.i.). Supernatants were harvested 72 h after incubation and analyzed by sandwich ELISA. Results were expressed as (pg/ml) in three experiments are shown as means $\pm$ SE ($n=4$ mice per group), * $p<0.05$ was considered statistically significant compared to non-infected mice as control



Interestingly, gradual and progressive increase of the IL-10 secretion by spleen cells after *Giardia* E/S-Ag and soluble extract stimulation was recorded in concomitant with the reduction of INF- γ , suggesting a possible regulatory effect of *Giardia* antigens on the production of the INF- γ . Finally, the detection of specific antibody response against E/S-Ags indicates that these proteins are firstly secreted in vivo during infection and secondly can interact with the mucosal and systemic immune cells stimulating a mixed Th1/Th2 response. Although have been reported, other cells and cytokines able to the control of infection such as dendritic cell (Kamda and Singer 2009) and TNF- α (Zhou et al. 2007). However, in the present work, we demonstrate for the first

time that *Giardia* E/S-Ags participate directly in the development of the specific immune response by the secretion of antibodies and Th2-cytokines. However, whether some of these antigens mediate a protective immunity against *Giardia* infection is unknown.

Acknowledgement In memoriam of Jean-Marie Grzych. This work was developed in the framework of collaboration between Inserm U547 and BDEP (EA-4547–Lille-2 University) group at the Institute Pasteur of Lille (IFR-142). Juan C. Jiménez was supported by a fellowships from “Fondo Nacional para el Desarrollo de las Ciencias y Tecnología” (FONACIT) and “Consejo de Desarrollo Científico y Humanístico” (Universidad Central de Venezuela).

References

- Adam RD (2001) Biology of *Giardia lamblia*. Clin Microbiol Rev 14(3): 447–475. doi:[10.1128/CMR.14.3.447-475.2001](https://doi.org/10.1128/CMR.14.3.447-475.2001)
- Belosevic M, Daniels CW (1992) Phagocytosis of *Giardia lamblia* trophozoites by cytokine-activated macrophages. Clin Exp Immunol 87(2):304–309
- Byrd LG, Conrad JT, Nash TE (1994) *Giardia lamblia* infection in adult mice. Infect Imm 62(8):3583–3585
- Daryani A, Hosseini AZ, Dalimi A (2003) Immune response against excreted/secreted antigens of *Toxoplasma gondii* tachyzoites in the murine models. Vet Parasitol 113(2):123–134
- Di Prisco MC, Hagel I, Lynch NR, Jiménez JC, Rojas R, Gil M, Mata E (1998) Association between giardiasis and allergy. Ann Allergy Asthma Immunol 81(3):261–265
- Djamiatun K, Faubert GM (1998) Exogenous cytokines released by spleen and Peyer's patch cells removed from mice infected with *Giardia muris*. Parasite Immunol 20(1):27–36
- Dunn LA, Upcroft JA, Fowler EV, Matthews BS, Upcroft P (2001) Orally administered *Giardia duodenalis* extracts enhance an antigen-specific antibody response. Infect Immunol 69(10):6503–6510
- Eckmann L (2003) Mucosal defences against Giardia. Parasite Immunol 25(5):259–270
- Faubert G (2000) Immune response to *Giardia duodenalis*. Clin Microbiol Rev 13(1):35–54
- Goodridge HS, Wilson EH, Harnett W, Campbell CC, Harnett MM, Liew FY (2001) Modulation of macrophage cytokine production by ES-62 a secreted product of the filarial nematode *Acanthocheilonema vitae*. J Immunol 167(2):940–945
- Guy RA, Bertrand S, Faubert GM (1991) Modification of RPMI-390 1640 for use in vitro immunological studies of host-parasite interaction in giardiasis. J Clin Microbiol 29(3):627–629
- Heyworth MF, Vergara JA (1994) *Giardia muris* trophozoite antigenic targets for mouse intestinal IgA antibody. J Infect Dis 169(2):395–398. doi:[10.1093/infdis/169.2.395](https://doi.org/10.1093/infdis/169.2.395)
- Heyworth MF, Carlson JR, Ermak TH (1987) Clearance of *Giardia muris* infection requires helper/inducer T lymphocytes. J Exp Med 165(6):1743–1748
- Hill DR (1990) Lymphocyte proliferation in Peyer's patches of *Giardia muris*-infected mice. Infect Immun 58(8):2683–2685
- Holland MJ, Marcus YM, Riches PL, Maizels RM (2000) Proteins secreted by the parasitic nematode *Nippostrongylus brasiliensis* act as adjuvants for Th2 responses. Eur J Immunol 30(7):1977–1987. doi:[10.1002/1521-4141\(200007\)30:7<1977::AID-IMMU1977>3.0.CO;2-3](https://doi.org/10.1002/1521-4141(200007)30:7<1977::AID-IMMU1977>3.0.CO;2-3)
- Jiménez JC, Uzcanga G, Zambrano A, Di Prisco MC, Lynch NR (2000) Identification and partial characterization of excretory/secretory products with proteolytic activity in *Giardia intestinalis*. J Parasitol 86(4):859–862. doi:[10.1645/0022-3395\(2000\)086\[0859:IAPCOE\]2.0.CO;2](https://doi.org/10.1645/0022-3395(2000)086[0859:IAPCOE]2.0.CO;2)
- Jiménez JC, Fontaine J, Grzych JM, Dei-Cas E, Capron M (2004) Systemic and mucosal responses to oral administration of excretory/secretory (E/S) antigens from *Giardia intestinalis*. Clin Diagn Lab Immunol 11(1):152–160. doi:[10.1128/CDLI.11.1.152-160.2004](https://doi.org/10.1128/CDLI.11.1.152-160.2004)
- Jimenez JC, Fontaine J, Grzych JM, Capron M, Dei-Cas E (2009) Antibody and cytokine responses in BALB/c mice immunized with the excreted/secreted proteins of *Giardia intestinalis*: the role of cysteine proteases. Ann Trop Med Parasitol 103(8):693–703. doi:[10.1179/000349809X12502035776351](https://doi.org/10.1179/000349809X12502035776351)
- Kamda JD, Singer SM (2009) Phosphoinositide 3-kinase-dependent inhibition of dendritic cell interleukin-12 production by *Giardia lamblia*. Infect Immun 77(2):685–693. doi:[10.1128/IAI.00718-08](https://doi.org/10.1128/IAI.00718-08)
- Kaur H, Samra H, Ghosh S, Vinayak VK, Ganguly NK (1999) Immune effector responses to an excretory-secretory product of *Giardia lamblia*. FEMS Immunol Med Microbiol 23(2):93–105. doi:[10.1111/j.1574-695X.1999.tb01228.x](https://doi.org/10.1111/j.1574-695X.1999.tb01228.x)
- Kaur H, Ghosh S, Samra H, Vinayak VK, Ganguly NK (2001) Identification and characterization of an excretory-secretory product from *Giardia lamblia*. Parasitology 123(Pt4):347–356
- Keister DB (1983) Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans R Soc Trop Med Hyg 77(4):478–488
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685
- Langford TD, Housley MP, Boes M, Chen J, Kagnoff MF, Gillin FD, Eckmann L (2002) Central importance of immunoglobulin A in host defense against *Giardia* spp. Infect Immun 70(1):11–18. doi:[10.1128/IAI.70.1.11-18.2002](https://doi.org/10.1128/IAI.70.1.11-18.2002)
- Larocque RK, Nakagaki P, Lee A, Abdul-Wahid, Faubert GM (2003) Oral immunization of BALB/c mice with *Giardia duodenalis* recombinant cyst wall protein inhibits shedding of cysts. Infect Immun 71(10):5662–5669. doi:[10.1128/IAI.71.10.5662-5669.2003](https://doi.org/10.1128/IAI.71.10.5662-5669.2003)
- Nash TE, Gillin FD, Smith PD (1983) Excretory-secretory products of *Giardia lamblia*. J Immunol 131(4):2004–2010
- Palm JED, Weiland M, E-I G, Ljungström WJ, Svärd SG (2003) Identification of immunoreactive proteins during acute human giardiasis. J Infect Dis 187(12):1849–1859. doi:[10.1086/375356](https://doi.org/10.1086/375356)
- Robert-Thompson IC, Stevens DP, Mahmoud AA, Warren KS (1976) Giardiasis in the mouse: an animal model. Gastroenterology 71(1):57–61
- Savioli L, Smith H, Thompson A (2006) Giardia and Cryptosporidium join the “Neglected Diseases Initiative”. Trends Parasitol 22(5):203–208. doi:[10.1016/j.pt.2006.02.015](https://doi.org/10.1016/j.pt.2006.02.015)
- Scott KG, Logan MR, Klammer GM, Teoh DA, Buret AG (2000) Jejunal brush border microvillous alterations in *Giardia muris*-infected mice: role of T lymphocytes and interleukin-6. Infect. Immun 68(6):3412–3418
- Scott KG, Yu LC, Buret AG (2004) Role of CD8+ and CD4+ lymphocytes in jejuna mucosal injury during murine Giardiasis. Infect Immunol 72(6):3536–3542. doi:[10.1128/IAI.72.6.3536-3542.2004](https://doi.org/10.1128/IAI.72.6.3536-3542.2004)
- Shant J, Bhattacharyya S, Ghosh S, Ganguly NK, Majumdar S (2004) A potentially important excretory-secretory product of *Giardia lamblia*. Exp Parasitol 102(3–4):178–186
- Singer SM, Nash TE (2000) T-cell-dependent control of acute *Giardia lamblia* infections in mice. Infect Immun 68(1):170–175. doi:[10.1086/317659](https://doi.org/10.1086/317659)
- Smith LM, Bonafonte M-T, Mead JR et al (2000) Cytokine expression and specific lymphocyte proliferation in two strains of *Cryptosporidium parvum*-infected γ -interferon knockout mice. J Parasitol 86(2):300–307. doi:[10.1645/0022-3395\(2000\)086\[0300:CEASLP\]2.0.CO;2](https://doi.org/10.1645/0022-3395(2000)086[0300:CEASLP]2.0.CO;2)
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci U S A 76(9):4350–4354
- Velázquez C, Beltrán M, Ontiveros N, Rascón L, Figueroa DC, Granados AJ, Hernández-Martínez J, Hernández J, Astiazaran-García H (2005) *Giardia lamblia* infection induces different secretory and systemic antibody responses in mice. Parasite Immunol 27(9): 351–356. doi:[10.1111/j.1365-3024.2005.00793.x](https://doi.org/10.1111/j.1365-3024.2005.00793.x)
- Venkatesan P, Finch RG, Wakelin D (1996) Comparison of antibody and cytokine responses to primary *Giardia muris* infection in H-2 congenic strains of mice. Infect Immun 64(11):4525–4533
- Venkatesan P, Finch RG, Wakelin D (1997) A comparison of mucosal inflammatory responses to *Giardia muris* in resistant B10 and susceptible BALB/c mice. Parasite Immunol 19(3):137–143
- Wakelin D, Rose ME, Else P, Hesketh KJ, Grens RK (1993) Immunity to coccidiosis: genetic influences on lymphocyte and cytokine responses to infection with *Eimeria vermiformis* in inbred mice. Parasite Immunol 15(1):11–19
- WHO- The world Health Organization Report (1996) Geneva, World Health Organization, 1996
- Zhou P, Li E, Shea-Donohue T, Singer SM (2007) Tumor necrosis factor alpha contributes to protection against *Giardia lamblia* infection in mice. Parasite Immunol 29(7):367–374. doi:[10.1111/j.1365-3024.2007.00953.x](https://doi.org/10.1111/j.1365-3024.2007.00953.x)