

Cell phenotypic change due to *Cryptosporidium parvum* infection in immunocompetent mice

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Abstract

Cryptosporidium parvum is an intracellular parasite causing enteritis which can become life-threatening in immunocompromised host. Immunoregulatory T cells play a central role in the regulatory network of the host. Here, we proposed to characterize the populations of immune cells during infection and reinfection with *C. parvum*. Four-week-old BALB/C mice were inoculated with oocysts of *C. parvum* at days 0 and 22. Fecal and blood samples, spleens, and small intestines were collected for analysis. Peripheral blood and spleen cell populations were characterized by flow cytometry. After infection (days 0 to 21), mice presented higher values of neutrophils, eosinophils, NK cells and CD4⁺CD25^{high} T cells in peripheral blood. After reinfection, this upward trend continued in the following days for all four populations in infected mice. At day 35, infected mice presented similar values to the control group, except for CD4⁺CD25^{high} T cells, which remained higher in infected mice. A possible correlation between alterations in blood and spleen cell populations was also studied, but no consistent association could be established. Small intestine sections were screened for intracellular stages of the parasite but no evidence of pathology was observed. Here, we report information which may be important for the understanding of the specific cell-mediated response in immunocompetent mice to *C. parvum* infection. Although some questions remain unanswered and complementary studies are needed, our results are expected to contribute to a better understanding of innate and Treg cells role in the clearance process of this parasite.

Keywords

Cryptosporidium, immunophenotype, flow cytometry, B-lymphocytes, T-lymphocytes

Introduction

Cryptosporidium spp. are apicomplexan protozoan parasites with a worldwide distribution, causing gastrointestinal disease in humans and animals. *Cryptosporidium parvum* is the most prevalent species and causes short-term self-limiting diarrheal illness in immunocompetent hosts. Recovery is complete, and the vast majority of recovered individuals are resistant to reinfection. Immunocompromised individuals such as AIDS patients experience prolonged diarrhea associated with persistent infection of intestinal epithelial cells, which can be life-threatening (Fayer and Ungar 1986, Blanshard *et al.* 1992, Fayer *et al.* 2000, Matos *et al.* 2004, Xiao and Fayer 2008). Humans

can acquire *Cryptosporidium* infections through several routes, such as anthroponotic, zoonotic, food and waterborne transmission. Oocysts are immediately infectious when excreted in feces and highly resistant to environmental stress, disinfectants, and some treatment technologies. These characteristics facilitate their dissemination in water, and have given rise to several waterborne outbreaks (Mac Kenzie *et al.* 1994, Fayer *et al.* 2000, Xiao *et al.* 2006, Xiao and Feng 2008, Xiao and Ryan 2008).

Furthermore, *C. parvum* has a considerable economic importance in veterinary medicine, particularly in newborn ruminants where it causes weight loss, dehydration and death (Fayer and Ungar 1986).

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A complex sequence of events involving various components of the host response has shown to be important in the control of *C. parvum* infection and critically dependent on the immune status of the host. It means that the study of the immune response to the parasite would improve the knowledge of the host defense mechanisms and the choice of better targets for therapy. To date, specific therapeutic or preventive interventions are not yet available. Few studies on the immune response in humans have been done, some of which mainly dealing with systemic antibody responses and others addressing their attention on the humoral and cell-mediated immune response. Generally, these studies conclude that despite the many advances made in understanding immune responses to *C. parvum*, some infection mechanisms not clear and a lot remains unknown (Theodos 1998, Riggs 2002, Gomez Morales *et al.* 2004, Borad and Ward 2010, Petry *et al.* 2010). Improvements in this area have been hampered by the lack of studies in immunocompetent animal models. Most experiments have been performed in neonatal or immunocompromised mice, with lacking functional T and/or B cells, and targeted mutations on major histocompatibility complex (MHC) class-II, alpha-beta-T-cell receptor ($\alpha\beta$ -TCR) and interferon-gamma (INF- γ) (McDonald *et al.* 1992, Aguirre *et al.* 1994, McDonald and Bancroft 1994, Perryman *et al.* 1994, McDonald 2000).

Several studies suggest that innate immune response initially limits the parasite number but the clearance of the infection ultimately requires a T-cell-mediated response, emphasizing the importance of CD4⁺ cells in the protective immunity towards *C. parvum*.

Nevertheless, limited information is available on the specific subset and function of these cells (Aguirre *et al.* 1994, Waters and Harp 1996). More recently, it has become clear that avoidance of damage to the host by foreign microorganisms is also possible to achieve by active suppression mediated by regulatory T cells (Treg) populations (Maloy and Powrie 2001, Belkaid and Tarbell 2009). However, we should keep in mind that, although Treg cells have an important role in the regulation of immune responses, T-helper type 1 (Th₁) and type 2 (Th₂) cells can also regulate immune responses in this context through the production of specific cytokines (Lean *et al.* 2002). CD4⁺CD25⁺ Treg cells are "naturally occurring" subpopulation of Treg and their role in the initiation and development of immune response has been widely studied (O'Garra and Vieira 2004, Belkaid and Tarbell 2009, Borrego *et al.* 2009). Some authors showed, in mice, that these cells are the hematopoietic components that reverse, or at least, prevent the disease (Fontenot *et al.* 2003). Due to the inherent difficulties in using CD25 as a marker for the purification of Treg cells, the finding that selection of the CD25^{high} subset of CD4⁺CD25⁺ T cells minimizes the co-isolation of contaminating activated CD4 T cells is important for future studies on the role of these cells in human disease (Baecher-Allan *et al.* 2005). Although not tested in this study, there are other molecules that can also be used for the identification and isolation

of Treg cells (transcription factor Foxp3 and IL7R - CD127) (Fontenot *et al.* 2003, Baecher-Allan *et al.* 2005, Banham 2006, Liu *et al.* 2006).

In this study, four-week-old BALB/C ByJ strain mice under specific-pathogen-free (SPF) conditions were used as animal model. *Cryptosporidium* infection was performed with purified oocysts. Evaluation of *C. parvum* cell-mediated response was done by flow cytometry (FC) through the peripheral blood and spleen cell population analysis. Histological analysis of small intestine and spleen has also been done. It is expected to analyze and characterize (immunophenotyping) changes in peripheral blood and spleen cell populations due to *C. parvum* infection, which could help clarify some aspects of the immune response to this infection.

Materials and Methods

Mice: Four-week-old female BALB/C ByJ mice were obtained from Charles River Laboratories (France). Mice were acclimated and housed in separate sterilized plastic cages with filter top, and fed with sterilized food and water (pH 3.5) *ad libitum* under SPF conditions.

Parasites and infection studies: Purified oocysts of *C. parvum* (Moredun strain) from cervine origin were purchased at Creative Science Company/Moredun Institute (Edinburgh, Scotland). According to the supplier, oocysts were resuspended in phosphate buffered saline (PBS) and stored at 4°C in the presence of antibiotics (penicillin and streptomycin). The viability of the oocysts preparation, checked by *in vitro* excystation, was 94.7%.

Seven groups of three animals each were inoculated with 1×10^6 *C. parvum* oocysts in 80 μ l of PBS per mouse by oral gavage. Four groups of three mice each were gavaged with PBS and used as control. One group of infected mice was euthanized on each of days 3, 7, 14 and 21 post-infection (PI) and one group of control mice was euthanized on each of days 3 and 14. In order to analyze the mechanisms conferring resistance towards infection with *C. parvum*, the remaining groups of infected mice were rechallenged with 1×10^6 oocysts in 80 μ l of PBS per mouse on day 22 PI. On each of days 25, 28 and 35 PI (days 3, 6 and 13 post-reinfection [PRI], respectively) a group of infected mice was also euthanized and on days 28 and 35 a group of control mice was also euthanized.

At each time point the feces, blood, spleens and small intestines were collected for analysis. Care was taken to keep the same level of animal handling for all groups.

The presence of *C. parvum* DNA in feces was confirmed by a nested-PCR of the 60-kDa glycoprotein (GP60) gene (Glaberman *et al.* 2002, Alves *et al.* 2003) after extraction of DNA by a Mini-BeadBeater/silica method (Alves *et al.* 2001). The presence of oocysts in feces was determined in fecal smears stained by a modified Ziehl-Neelsen method (Casemore *et al.* 1985) after oocyst concentration using a modified water-

ether sedimentation method (Alves *et al.* 2000) and then observed under an optical microscope with 400x magnification.

Simultaneously, the active infection in small intestines of mice was confirmed by histological methods. Morphological changes in the spleen were also evaluated. For this purpose, tissues samples were removed from euthanized mice and fixed in 10% buffered formalin solution, processed for paraffin embedding and sectioned. Small intestine sections were stained with hematoxylin-eosin and with FITC-labeled monoclonal antibody reagent for direct immunofluorescence detection of *Cryptosporidium* oocysts (Crypt-a-Glo™, A400FLR-1X, Waterborne, Inc, New Orleans, Los Angeles) in accordance with the laboratory procedures and examined at a magnification of x400. Spleen sections were stained only with hematoxylin-eosin and examined at a magnification of x200.

All protocols and experiments involving animals were performed in compliance with the relevant laws and institutional guidelines with specific approval by the animal care committee.

Flow cytometric analysis of spleen and peripheral blood cell populations: Peripheral blood and spleen were aseptically collected from euthanized mice. Peripheral blood was collected from the vena cava. Splenocytes were obtained by gentle removal of spleen's contents, followed by a mechanical disassociation protocol, using BD Medimachine system/BD Medicon and BD Filcon Filters (BD Biosciences, San Jose, California). The protocol was adjusted according to the cytometer manufacturer's instructions. Red blood cells from mouse peripheral blood and spleen cell suspension were lysed using BD Biosciences Pharmingen's PharM Lyse™ solution. Five milliliters of 1X Lysing Solution per spleen cell suspension or per 250 µl of mouse peripheral blood were added. Samples were gently vortexed and incubated at room temperature, in the dark, for 5 min. After centrifugation at 200 x g for 5 min, supernatants were carefully aspirated and discarded. Each pellet was resuspended in 500 µl of cold wash buffer (Facs Flow BD Biosciences). After centrifugation at 350 x g for 5 min, the supernatants were discarded and the pellet resuspended to a concentration of 2×10^7 cells/ml (i.e., 10^6 cells per 50 µl). The used mAbs (obtained from BioLegend, San Diego, California) were: anti-mouse CD4 APC (clone GK1.5), anti-mouse CD8a PE (clone 53-6.7), anti-mouse CD19 APC (clone 6D5), anti-mouse CD3ε PerCP (clone 145-2C11), anti-mouse CD45 FITC (clone 30-F11), anti-mouse CD49b PE (clone DX5), anti-mouse CD25 PE (clone PC61), anti-mouse TCRγδ FITC (clone GL3) and anti-mouse TCRβ FITC (clone H57-597). Each mAb was diluted to predetermined optimal concentration in wash buffer and placed in several tubes in a volume of 50 µl. About 100 µl of resuspended pellet (10^6 cells) were added to each tube already containing 50 µl of mAb (or 50 µl wash buffer for negative controls). After mixing by gently vortexing, all tubes were incubated at 4°C for 30 min in the dark. Two washes were made with 200 µl of wash buffer followed by centrifugation at 350 x g for 5 min and supernatant removed by aspiration. Labeled cell pellets were re-

suspended in 200 µl of wash buffer and analyzed on a BD FACS Calibur™ Flow Cytometer (BD Biosciences, San Jose, California) equipped with an air-cooled argon laser, with an excitation wavelength of 488 nm, and a red diode laser, with an excitation wavelength of 685 nm. Instrument settings were previously adjusted and controlled following the standard procedures.

Cell Quest 3.3™ (BD Biosciences) software was used for both acquisition and analysis. In each tube, 40,000 leukocyte events (CD45⁺) were acquired. Results were presented as a percentage of total leukocytes for major populations, identified according to their forward angle light scatter (FSC) and side angle light scatter (SSC), and also the expression of CD45 (neutrophils, monocytes, lymphocytes and eosinophils). Within the lymphocytes, T, B and natural killer (NK) populations were identified according to the expression of CD3, CD4, CD8, CD19 and CD49b, and presented as a percentage of total lymphocytes. TCRαβ⁺ T cells (assessed as the population of cells expressing the TCRβ chain) were also evaluated according to their expression of CD4 and CD8, and classified in CD4⁺, CD8⁺, double negative (DN) and double positive (DP) cells. Finally, a combined Boolean Gating Strategy was used for the evaluation of CD25 expression within CD3⁺CD4⁺ T cells. For this purpose, 10,000 to 15,000 CD3⁺CD4⁺ T cells were acquired in each tube, and a fluorescence minus one (FMO) control tube was also used. CD3⁺CD4⁺CD25⁺ T cells were identified as the cells with higher fluorescence intensity than the FMO tube. Within this population, CD3⁺CD4⁺CD25^{high} T cells were identified as the small cluster presenting higher CD25 expression (above the second decade in the fluorescence logarithmic scale).

Statistical analysis: Statistical analysis was performed using the nonparametric Mann-Whitney test, since the data did not meet the criteria of normality and homogeneity of variance necessary to apply the t-test. Statistical significance was defined by a *p*-value of less than 0.05. With this test the authors analyzed if there were statistical significant differences among the values of the populations in the spleen and peripheral blood, determined by FC for each mouse, after infection and reinfection. Tests were performed using IBM SPSS Statistics version 19 software (SPSS Inc., Chicago, U.S.A.).

Results

Infectivity assay: Fecal samples were collected from each group of mice on days 3, 7, 14, 21, 25, 28 and 35 PI. The occurrence of oocysts in feces was determined in fecal smears of concentrated oocysts stained by a modified Ziehl-Neelsen method, followed by observation under an optical microscope with 400x magnification.

Although the load of oocysts was low (8–12 oocysts), they could be observed on days 3 and 7 PI. The presence of gDNA of *C. parvum* in feces was determined by a nested-PCR of 60-kDa glycoprotein gene and a band of DNA (400 bp) was ob-



Fig. 1. Histological examination of small intestine. Light photomicrograph of small intestine from a *C. parvum* infected mouse, at day 25 PI (day 3 PRI). Arrows indicate intracellular stages of the parasite lining the luminal surface of enterocytes (hematoxylin-eosin stain, $\times 400$)

served only on days 3, 7 and 14 PI. Feces from control mice were also analyzed in all time points, by microscopy and nested-PCR, in duplicate, and were all negative. The active infection in small intestines of mice was confirmed by histological examination. Small intestine sections from euthanized mice in all time points only showed a low number of intracellular parasite stages (5–10 parasites) in the superficial border of the villi (Fig. 1) on days 21, 25 and 35 PI. In sections ana-

lyzed by immunofluorescence in all time points, a low number of oocysts (5–10 oocysts) were also observed. Particular attention was also given to the possibility of an influx of eosinophils to the site of infection that could be correlated with the increased number of systemic eosinophils. No noticeable changes consistent with infection were observed in any of those days. In order to check any possible correlation between alterations in blood and spleen cell populations, histological changes in sections of spleen of infected mice were also evaluated by light microscopy. On days 25 and 28 PI (3 and 6 days PRI, respectively), all spleens of infected mice have shown signs of splenomegaly. Microscopically, the major feature of these spleens was a mild congestion of the red pulp, with no noticeable changes in the appearance of the white pulp.

Flow cytometry analysis: Analysis of peripheral blood – leukocyte subpopulations: Considering the major leukocyte subpopulations, neutrophils mean percentages varied between 31.42%–52.49% in infected mice, and 29.04%–45.33% in the control group (as shown in Table I). Analyzing the evolution of this population after infection and reinfection (days 0 and 22), neutrophil percentages appeared to rapidly increase (days 3 and 25), and then start to recover, approaching the values observed in the control group.

For eosinophils, mean percentage values of this population were higher in infected mice, varying between 3.29%–5.61%, compared to 1.00%–4.78% for the control group. Although eosinophil values in infected mice were significantly higher than those in control mice until day 28, this difference decreased and reached similar values at day 35.

The lymphocyte population presented values between 36.36%–57.01% in infected mice and values between 39.86%–56.25% in control mice. Lymphocytes and neutrophils are normally the two major leukocyte populations in mice peripheral blood, although they present inverted tendencies. Lymphocytes percentage increased after infection, until day 21, and then rose again after reinfection. Nevertheless, after both exposure periods, the lymphocyte population seemed to recover, presenting values closer to those observed in the control group.

Table I. Flow cytometry analysis for leukocyte subpopulations. Mean percentage values for leukocyte populations (neutrophils, eosinophils, lymphocytes and monocytes) in peripheral blood, along the infection and reinfection processes (35 days), in both infected and non-infected control mice

Day	Neutrophils (%)		Eosinophils (%)		Lymphocytes (%)		Monocytes (%)	
	IM	CM	IM	CM	IM	CM	IM	CM
3	49.44	45.33	4.25	1.99	40.23	39.86	5.20	10.05
7	43.12	–	3.29	–	45.95	–	7.36	–
14	37.72	34.80	3.69	1.30	50.39	52.03	8.19	6.36
21	35.76	–	5.61	–	51.46	–	6.60	–
22	Reinfection							
25	52.49	–	4.93	–	36.36	–	6.22	–
28	31.42	29.04	3.75	1.00	57.01	56.25	7.38	10.00
35	31.96	40.60	4.81	4.78	55.01	46.53	8.22	7.95

IM - infected mice; CM - control mice.

Table II. Flow cytometry analysis for lymphocyte subpopulations. Mean percentage values for lymphocyte subpopulations in peripheral blood, along the infection and reinfection processes (35 days), in both infected and non-infected control mice. The discrepancy observed between the summary of CD4⁺ and CD8⁺ T cells and the total T cells matches to the DP and DN cells (data not shown)

Day	B (%)		NK (%)		T (%)		CD4 ⁺ T (%)		CD8 ⁺ T (%)	
	IM	CM	IM	CM	IM	CM	IM	CM	IM	CM
3	33.27	26.16	10.15	6.87	53.72	64.47	42.20	44.81	7.78	7.57
7	23.79	–	6.84	–	65.72	–	48.65	–	8.92	–
14	31.69	36.18	12.01	6.34	52.89	55.07	42.59	41.02	7.58	7.10
21	40.29	–	11.39	–	44.30	–	28.92	–	8.56	–
22	Reinfection									
25	42.64	–	11.72	–	43.35	–	26.74	–	4.99	–
28	38.65	35.74	11.67	8.22	48.41	54.01	33.15	38.59	8.97	8.94
35	32.73	44.55	14.91	20.14	51.60	30.94	37.80	11.07	6.36	5.51

B – B cells (CD19+ lymphocytes); NK – Natural Killer cells (CD49b+ lymphocytes); T – T cells (CD3+ lymphocytes); DP – Double positive cells; DN – Double negative cells; IM – infected mice; CM – control mice.

Table III. Flow cytometry analysis of lymphocytes in spleen cells. Mean percentage values for lymphocytes and lymphocyte subpopulations in spleen samples, along the infection and reinfection processes (35 days), in both infected and non-infected control mice

Day	L (%)		B (%)		NK (%)		T (%)		CD4 ⁺ T (%)		CD8 ⁺ T (%)	
	IM	CM	IM	CM	IM	CM	IM	CM	IM	CM	IM	CM
3	84.36	80.12	57.84	50.70	2.55	2.26	36.65	44.92	20.30	16.71	7.48	7.76
7	83.53	–	47.41	–	3.67	–	47.75	–	25.51	–	9.82	–
14	89.05	–	44.07	–	3.97	–	50.73	–	21.22	–	9.04	–
21	82.21	–	53.07	–	4.46	–	41.29	–	18.87	–	7.47	–
22	Reinfection											
25	78.21	–	51.50	–	5.31	–	42.85	–	19.34	–	7.45	–
28	85.02	–	50.99	–	6	–	42.07	–	25.97	–	8.16	–
35	87.22	–	53.16	–	4.12	–	41.81	–	23.53	–	8.68	–

L – Lymphocytes (evaluated within total leukocyte events); B – B cells (CD19+ lymphocytes); NK – Natural Killer cells (CD49b+ lymphocytes); T – T cells (CD3+ lymphocytes); IM – infected mice; CM – control mice.

Monocytes showed mean percentage values between 5.2%–8.22% in infected mice, and between 6.36%–10.05% in control mice, which are not significantly different. Considering the values obtained in both groups, we can only point out minor decreases in percentage populations values in infected mice after infection and reinfection (days 3 and 25).

The significance of the results was limited due to the small number of mice. In most leukocyte populations (neutrophils, eosinophils, lymphocytes, monocytes) no statistically significant differences were observed among the mean values of infected and reinfected mice.

Analysis of peripheral blood – lymphocyte subpopulations: Lymphocytes, as previously mentioned, showed a tendency to decrease on days 3 and 25, after both infection and reinfection (days 0 and 22). In the control groups (Table II), within the lymphocyte subpopulations, B cells (CD19⁺) and NK cells (CD49b⁺) seemed to gain relevance through time. B cells and NK cells percentage ranged between 26.16%–44.55% and 6.34%–20.14%, respectively, while T cells

(CD3⁺) showed a decreasing tendency from 64.47% to 30.94%, as well as the CD4/CD8 ratio (data not shown).

In infected mice, mean values of B cells oscillated between 23.79% and 42.64%, but with the initial percentage of cells (33.27%) similar to the final one (32.73%). From day 25 to day 35, however, data show that B cells in infected animals did not follow the increasing tendency observed in the control group.

In infected animals, NK cells presented generally higher values than those observed in the control group. The only exception was day 35, with infected animals presenting a decrease in percentage values when compared to the control group (infected mice: 14.91%; control mice: 20.14%).

The data also showed that the percentage of T cells in infected mice, at days 3 and 25 diminished when compared with the control group, and then started to recover in value, achieving normal levels one week after the infection challenge. At day 35, however, both T cells and CD4⁺ T cells appeared to maintain higher values in infected animals.

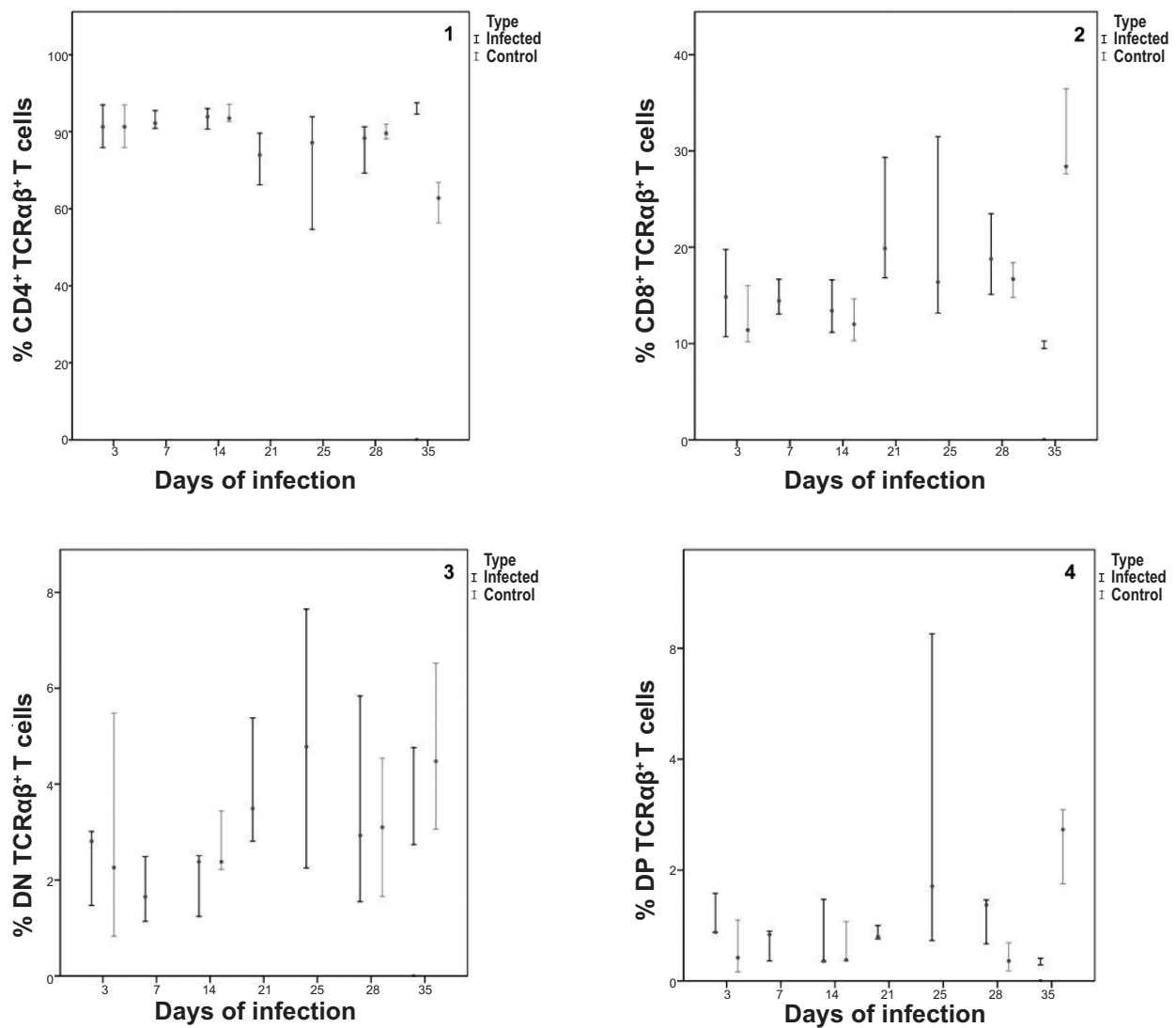


Fig. 2. Variability of TCRαβ⁺ T cell subpopulations. Graphics showing the variability in the data obtained from the analysis of peripheral blood, along the infection period (35 days), of TCRαβ⁺ T cells subpopulations divided according to their expression of CD4 and CD8: CD4⁺ TCRαβ⁺ T cells (graphic 1), CD8⁺ TCRαβ⁺ T cells (graphic 2), DN TCRαβ⁺ T cells (graphic 3) and DP TCRαβ⁺ T cells (graphic 4). Each graphic represents the results obtained for a group of three mice [first (-), second (dot), and third (-) values], infected and control, except for day 35, in which a mouse died in the infected group

To evaluate whether there were differences in the TCRαβ and TCRγδ T cell populations or not, CD4 and CD8 expression was assessed in TCRαβ⁺ T cells (identified by the expression of the TCRβ chain). Figure 2 represents the three values obtained for each group, except for day 35, which had only two mice because one mouse died. No significant differences were observed between infected and non-infected mice. Due to the small sample size, no statistical tests could be applied. At day 35, only DN TCRαβ⁺ T cells (3) followed the control tendencies. Compared to control values, CD4⁺ TCRαβ⁺ T cells (1) percentage values showed consistently higher values in infected mice, and DP (4) and CD8⁺ TCRαβ⁺ T cells (2) showed consistently lower values in infected mice. Generally, variability appeared to be larger for the infected group.

The significance of the results was limited due to the small number of mice. In lymphocyte subpopulations (B cells, NK cells, T cells, and within T cells, CD4⁺ and CD8⁺ cells) no statistically significant differences were observed among the mean values of both infected and reinfected mice. Among all populations in this study, CD8⁺ and DN cells presented lower *p*-values than all the other cells (CD8⁺, *p* = 0.054 and DN, *p* = 0.105), which, although not statistically significant, is an interesting result that deserves attention in future research studies.

Finally, CD25 expression was also evaluated in the CD3⁺CD4⁺ T cell population. CD25 is an activation marker in T cells, and within a special population of T cells with regulatory properties (Treg), that express high levels of this mole-

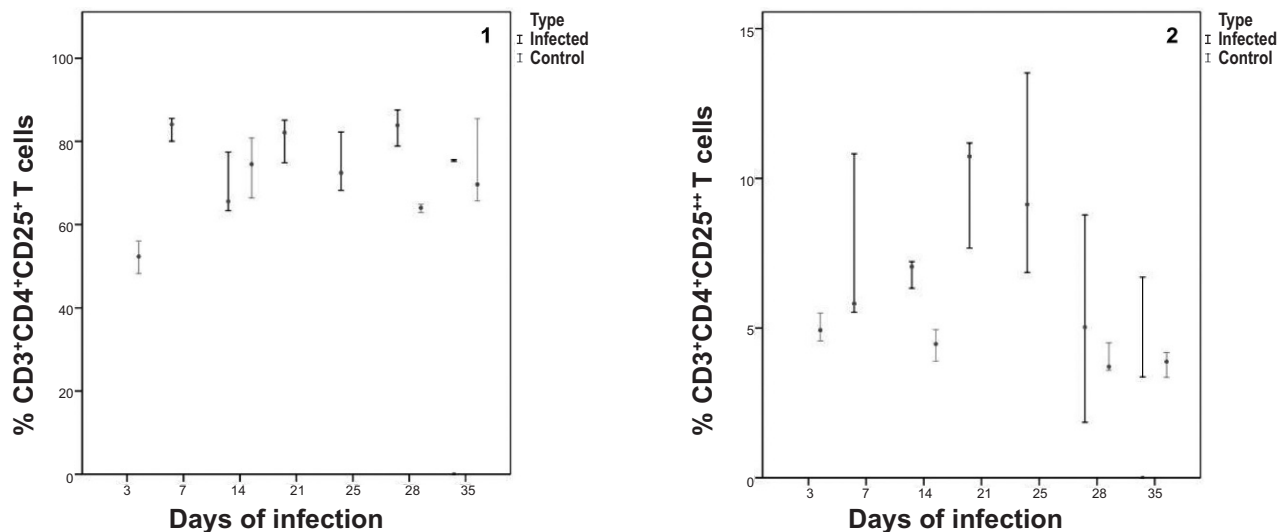


Fig. 3. Variability of CD25 expression in CD3⁺CD4⁺ T cells. Graphics showing the variability in the data obtained from the analysis of peripheral blood, along the infection period (35 days), of CD25 expression in CD3⁺CD4⁺ T cells: CD3⁺CD4⁺CD25⁺ T cells (graphic 1) and CD3⁺CD4⁺CD25⁺⁺ T cells (graphic 2). Each illustration represents the results obtained for a group of three mice [first (-), second (dot), and third (-) values], infected and control, except for day 35, in which a mouse from infected group died

cule. Thus, the expression of CD25 was assessed assuming two populations: CD3⁺CD4⁺CD25⁺ and CD3⁺CD4⁺CD25^{high} T cells. The analysis of the graphic 1 in Fig. 3 suggests that CD3⁺CD4⁺CD25⁺ T cells were generally present in higher values in infected animals, 68.80%–83.42%, versus 52.21%–73.94% in control mice. No significant differences between both groups of mice were observed in each set point and, at day 35, both groups presented similar values. Furthermore, the population of CD4⁺ T cells showing higher CD25 expression (CD3⁺CD4⁺CD25^{high} T cells), presented significantly higher percentage values in infected mice throughout the study (Fig. 3, graphic 2). These cells, a small percentage within CD3⁺CD4⁺ T cells (5.04%–9.86% in infected mice versus 3.8%–5.0% in control mice), presented even higher values in the infected group at days 21 and 25, a period of time during which the reinfection process occurred (day 22).

Analysis of spleen cell populations: Spleen samples were collected at each time point in infected mice, while in control mice, spleen samples were analyzed only for the first time point (day 3). Since lymphocytes are the major population in the spleen, only they were evaluated (Table III). For all types of lymphocytes, a pattern can be observed across the two infection challenges, where after the first week the lymphocyte percentages tended to increase (days 14, 28 and 35), while B cells showed an increase immediately after infection, and then they oscillated, near the mean value found in the spleen of the control group (50.7%). NK cells started with a 2.55% mean value then increased constantly to 6% until day 35, in which they started to decrease to 4.12%.

CD3⁺, CD4⁺ and CD8⁺ T lymphocytes were also analyzed. In CD3⁺ T cells, a peak was observed at days 7 (47.75%) and 14 (50.73%) PI, reaching higher values than those observed in the spleen of the control group (44.92%). However, after this

period, the percentage values within this population plateaued at a slightly lower level than the peak value. As for CD4⁺ and CD8⁺ T cells, both populations increased one week after the infection challenge (day 7). Nevertheless, after this period, both populations percentages started to decrease (days 21 and 25), closer to those observed in control mice. The reinfection episode, in day 22, somewhat mimics what was observed after the first infection point, as both populations achieved higher values a week after (day 28).

Discussion

The more we know about immune effector mechanisms that protect vertebrate hosts against opportunistic pathogens, the better the chances are to develop immunotherapeutic strategies for controlling diseases such as cryptosporidiosis. *C. parvum* is predominantly an intestinal pathogen. However, we decided to analyze immunophenotypic changes and potential differences in cell population from peripheral blood and spleen in orally infected mice because modifications induced in the immune system to overcome the infection, may be reflected as changes in peripheral blood and spleen cell populations.

Although the majority of the studies have been done on neonatal and immunosuppressed mice (Certad *et al.* 2007), we consider it important to study what occurs in the immunocompetent animal because this type of infection also takes place in immunocompetent persons.

In previous studies using the same animal model (Kapel *et al.* 1996, Marriotte *et al.* 2004), infective doses ranged from 10⁴ to 10⁶ oocysts and infection was not monitored beyond 27 days PI. So, in this work, we decided to monitor the infection for a longer period of time (35 days), with seven time points

and a higher inoculum of oocysts (1×10^6), instead of increasing the number of mice. With this first approach, we attempted to understand the tendencies of the cell population's evolution.

Due to the high amount of administered inoculum by oral gavage to mice, it would be expected to find a large amount of oocysts in feces, which did not occur. Our data showed that the onset of parasite shedding was low and only detectable by microscopy on days 3 and 7 PI. The presence of DNA of *C. parvum* in feces was detected on days 3, 7 and 14 PI. Those events might be due to the fact that oocysts have undergone excystation or have been destroyed during the normal gut transit. Furthermore, mice did not show any signs of disease such as weight loss, diarrhea or inactivity. In contrast to humans, who are susceptible to infection throughout life, animals including mice develop resistance to infection after the neonatal period.

Small intestinal sections from infected mice euthanized on days 3, 7, 14, 21, 25, 28 and 35 PI were screened for intracellular stages of the parasites. Histological examination with hematoxylin-eosin stain showed 5–10 intracellular parasite stages in the superficial border of villi of intestine. However, there was no evidence of histopathological changes due to the infection when compared with the intestinal sections of the uninfected mice, in agreement with previous studies (Current and Garcia 1991; Wyatt *et al.* 1997; Del Coco *et al.* 2012). Particular attention was given to the possibility of an influx of eosinophils to the site of infection that could be correlated with the increased number of systemic eosinophils, but we did not observe any changes consistent with infection.

Spleen sections were observed with hematoxylin-eosin for the same time points and the possible correlation between alterations in the blood and spleen cell populations was also studied. On days 25 and 28 PI (days 3 and 6 PRI, respectively), all of the euthanized mice presented with enlarged spleens (splenomegaly) with a size three times higher than the control mice. In the enlarged spleens we could observe that the proportion of both red and white pulps were similar. A mild congestion was observed in the red pulp of the enlarged spleens due to the increase in the cellular density; while the cellular density of the white pulp was similar in the spleens of both infected and control mice. Enlarged spleens may be attributable, in part, to the observed red pulp congestion. Thus, splenomegaly may be consistent with reinfection and related to the reinoculation event, may be due to stimulation of the immune response in the spleen, in agreement with what was reported by other authors (Miller and Schaefer 2007).

In this work, we expected to see an inflammatory response initiated by *C. parvum* attachment and development in the small intestine, characterized by an increase of circulating neutrophils followed by a gradual increase in circulating lymphocytes immediately after infection. Effectively, we observed an increase of neutrophils at day 3 PI, followed by decrease of their percentage until day 21 PI, and a new increase on day 25 PI (3 days PRI) followed by decrease until day 35 PI reaching

similar values to the control group. These data suggest an initial inflammatory response to inoculation events (primary and secondary infection), with an increase of circulating neutrophils number, perhaps followed by the potential recruitment of these cells towards the affected tissues. However, the percentage of neutrophils in control mice also showed fluctuation, though not as significant when compared with the values in infected mice.

Relative to eosinophils after infection and reinfection there was always an initial increase in their percentage (two to four times the control mice), followed by a decrease to values observed in control group at day 35 in a similar way to what was observed for neutrophils. This may be the characteristic profile exhibited by eosinophils during an infection by *C. parvum*, since increased eosinophilia has also been identified as a hallmark of infection with parasites like *Toxoplasma gondii* and *Plasmodium* spp. (Kasper and Buzoni-Gatel 2001, Male *et al.* 2006).

Relative to other cell populations, no significant variation was observed for monocytes among infected and control mice. In this context, it would be of interest to study if the monocytes express an activation marker (e.g. Gr-1⁺), which can be found in the mesenteric lymph nodes (MLNs) or lamina propria.

For B cells an increase was observed throughout the infection. However, between days 25 and 35, these values did not increase as observed in the values of the control group, probably related to T cell increase at these time points, which is more evident than the increase of B cells percentage.

NK cells values were higher in infected mice than in the control group. However, on day 35, we observed a percentage decrease in infected animals compared to the control group. Again, this difference might be related to T cell compartment within lymphocytes, which did not decrease in infected animals (51.7%) as occurred in control animals (30.94%). More conclusive results were not possible due to the lack of absolute counting values for these cell populations.

In this study, we decided to analyze the spleen of the infected mice since it is the organ of the lymphatic system that has been less studied in the context of an infection by *C. parvum* (mostly studied organs are Peyer's patches or mesenteric lymph nodes). In the spleen, the increase of CD4⁺ and CD8⁺ T cells one week after infection (day 7) and six days after reinfection (day 28), followed by its decrease, may be related to the establishment of the adaptive response upon which the protection against the infection depends. The low input presented by B cells may be related to the less important role of antibodies in this type of intracellular infection. Splenomegaly observed in mice challenged with *C. parvum* oocysts but not harboring pathogen developmental stages in the intestine is intriguing but probably the result of the increased cell proliferation. Considering the association of IgE with other extracellular parasites and the relevance of IgA in the mucosa of respiratory and digestive systems (Male *et al.* 2006), it would be important to study this type of humoral response in future studies.

The population of CD3⁺CD4⁺CD25^{high}, which is related to Treg cells, became progressively higher with time, and the values in the infected group were two times higher than the control group. In parasitic infectious diseases, as portrayed in an infection by *Schistosoma mansoni*, Treg cells may be induced in antigen-specific manner and may suppress tissue destruction resulting from immune responses (Watanabe *et al.* 2007). These authors showed that the percentage of circulating Treg cells was increased in some people infected with *S. mansoni*, and effective treatment decreased the levels of these cells. In the context of the present study, we hypothesized that the behavior of Treg cells may be similar, which needs to be proven in future studies and we would recommend using Foxp3 staining and/or IL-7R staining for a better discrimination between activated and Treg cells (Levings *et al.* 2006, Belkaid and Tarbell 2009).

There were no statistically significant differences among the mean values of infected and reinfected mice in most peripheral blood cell populations. However, it is interesting to notice that there was a greater variability after reinfection. In spite of the small sample size, and consequent *p*-values obtained (always greater than the chosen significance level), these findings allowed us to consider the hypothesis that this variability may be related to the fact that mice were older at PRI, and as such, the immune system was more mature. Although the age-related changes may be rather subtle, they may become very relevant during the course of the disease. This may explain the greater variability in the collected data after reinfection with oocysts of *C. parvum*. Additionally, some authors indicate that age should be considered in the selection of appropriate mouse models for immunological research (Pinchuk and Filipov 2008). This was one of the strongest reasons for choosing four-week-old animal model to understand how the immune system responds in the context of an infection by *C. parvum* since they are not yet in adulthood and the immune system is almost mature.

In spite of the numerous advances made in understanding the immune response to *C. parvum*, a significant amount of facts remains unknown. Limited information is available on the specific subset and function of the CD4⁺ cells that are required for protection against infection.

In conclusion, our data provides the first information about the evaluation of *C. parvum* specific cell-mediated response trends in immunocompetent mice. These results should be taken as a starting point for more deep studies to be performed with the main objective of understanding the role of innate immune response in the context of this infection in immunocompetent animals.

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