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Modulation of the immune response by infection with *Cryptosporidium* spp. in children with allergic diseases

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Running title. The allergic immune response and *Cryptosporidium* infection.

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Abstract

It has been demonstrated that the allergic response can be ameliorated by the administration of pathogen derivatives that activate Toll-like receptors and induce a Th1-type immune response (IR). *Cryptosporidium* is a parasite that promotes an IR via Toll-like receptors and elicits the production of Th1-type cytokines, which limit cryptosporidiosis. The aim of this study was to investigate allergy-related immune markers in children naturally infected with *Cryptosporidium*. In a cross sectional study, 49 children with or without clinical diagnosis of allergies, oocysts of *Cryptosporidium* spp. in the faeces were screened microscopically. We microscopically screened for leukocytes, examined T and B cells for allergy-related activation markers using flow cytometry and evaluated serum for total IgE using chemiluminescence. Children with allergies and *Cryptosporidium* in the faeces had significantly lower levels of total IgE, B cells, CD19⁺CD23⁺ and CD19⁺CD124⁺ cells as well as a greater percentage of IFN- γ ⁺ and IL-4⁺ CD4⁺ cells than children with allergies without *Cryptosporidium*. This is the first description of the modulation of the IR in children with allergic diseases in the setting of natural *Cryptosporidium* infection. Our findings suggest the involvement of CD4⁺ cells producing IL-4 and IFN- γ in the IR to *Cryptosporidium* in naturally infected children.

Key words: *Cryptosporidium*; Children; Allergy; IgE; IFN- γ ; IL-4.

1. Introduction

Allergic disease is the result of an immunological reaction regulated by T helper (Th) cells with a pattern of Th2 activation. Th2 cells release interleukin (IL)-4 and IL-13¹, which are required for the production of IgE by B cells². Additionally, IL-4 inhibits the generation of Th1 cells by decreasing the production of cytokines such as IL-2, interferon-gamma (IFN- γ), and IL-12. In sensitized individuals, exposure to an allergen results in a cross-linking of allergen to IgE-bound Fc ϵ RI on the surface of mast cells. As a consequence of allergen-IgE cross-linking, mast cells release inflammatory mediators responsible for the allergic symptomatology³. In the last phase, the Th2 inflammatory response is characterized by the recruitment of eosinophils, neutrophils, basophils, and activated CD4⁺ T cells, which are associated with the severity of a reaction and a reduction in the quality of life in allergic individuals⁴⁻⁷.

The role of exposure to non-pathogens, pathogens or their derivatives in the control of the allergic response has been previously studied. Studies on the efficacy of live bacteria and bacterial extracts for the treatment of allergic diseases suggest the participation of IL-10-producing B cells and Treg cells^{8,9}. In some experimental models and in children with allergies, suppression of the allergic response has been observed through the administration of whole cell inactivated mycobacterial vaccines, including administration of *M. vaccae*, the live attenuated strain of *M. bovis* (bacillus Calmette-Guérin, BCG)¹⁰⁻¹² or lipopolysaccharide, via the activation of Toll-like receptors capable of inducing Th1 responses¹³. In some allergies (including allergic asthma), the role of treatment with parasites or molecules derived from parasites in modulating the immune response (IR) has been analysed¹⁴. Some authors have shown that infection by intestinal helminths such as *Schistosoma mansoni*, *Ascaris lumbricoides*, and *Trichuris trichiura* may cause a decrease in the reactivity of skin tests and asthmatic symptomatology¹⁵⁻¹⁷. However, some of these pathogens and their derivatives have had controversial effects on allergen-mediated immune responses^{18,19}.

Cryptosporidium is a coccidian protozoan that infects a wide range of hosts, such as reptiles, birds, and mammals, including humans^{20,21}. It is an opportunistic parasite with worldwide distribution and causes a self-limiting diarrhoea in immunocompetent individuals. However, in immunocompromised subjects (patients with AIDS, children with malnutrition, and the elderly), the disease can be severe, prolonged, and lethal²²⁻²⁴. The defence mechanisms responsible for limitation of disease in humans have not been fully characterized, due to the self-limiting nature of the disease in healthy individuals and general underdiagnosis of *Cryptosporidium* infection. This underdiagnosis is due to the asymptomatic disease course or unspecific clinical conditions, and only in rare cases is the true aetiology suspected^{25,26}. Evidence in murine and bovine models and in some human studies suggests an important role of the innate and adaptive IR during cryptosporidiosis with a balance between the production of Th1 cytokines, needed to control parasite growth, and Th2 cytokines, needed for disease resolution²⁷⁻³¹. It is known that *Cryptosporidium* is capable of inducing Th1-type responses through the activation of Toll-like receptors 2 and 4^{32,33}. During the early and late phases of the infection, the activation of CD4⁺ T cells has been demonstrated^{34,35}, as well as an increase in the levels of IFN- γ and IL-4³⁶⁻³⁸, and infection has been associated with the expression of anti-inflammatory cytokines, including IL-10 and transforming growth factor-beta (TGF- β)³⁹⁻⁴¹. Taken together, these data suggest that *Cryptosporidium* modulates the IR in allergic children. The aim of this study was to investigate allergy-related immune markers in children naturally infected with *Cryptosporidium*. To achieve this, we

analysed the following characteristics in a population of children with and without allergies, in a setting of natural *Cryptosporidium* infection: the levels of total IgE; the total counts of eosinophils, segmented neutrophils and total lymphocytes; the percentage of T (CD3⁺), Th (CD4⁺), and B (CD19⁺) lymphocytes; the proportion of Th1 and Th2 CD4⁺ lymphocytes (IFN- γ - and IL-4-producing cells, respectively); the expression of activation markers such as CD69, one of the earliest markers induced upon activation in T and B lymphocytes ⁴²; the low-affinity receptor for IgE (CD23), with an important role in the regulation of IgE synthesis in allergic diseases ⁴³; and the interleukin-4 receptor- α on B cells (CD124), whose expression is increased in nasal and bronchial mucosal biopsies in allergic patients ⁴⁴.

2. Materials and Methods

2.1. Subjects

The protocol was approved by the Ethics Committee of the Faculty of Medicine, Juárez University of the State of Durango, and a signed letter of consent from each child's parents or guardians and an assent letter from each child were obtained. The sample included children from a region in the Comarca Lagunera, which includes two cities in the state of Durango (Gómez Palacio and Lerdo) and one in the state of Coahuila (Torreón), Mexico, the latter which is known for environmental lead contamination problems ⁴⁵. From the Dr. Roberto Garcia Sosa Urban Health Center in Lerdo, Durango, and the Heavy Metals Care Center (CAMP) in Torreon, Coahuila, Mexico, we enrolled 65 children, of both genders, aged six to nine years during the period from April 2012 to February 2013. Thirty-four non-allergic children (52%) attending for healthy child control and 31 children (48%) with suspected allergies were referred to the CAMP; all of children were evaluated by allergy specialists.

2.2. Diagnosis of Allergy

An allergy diagnosis was established by any total IgE level in serum and the two following criteria: 1) clinical data of allergy without active allergic disease symptoms, assessed using an adapted questionnaire from the International Study of Asthma and Allergies in Childhood (ISAAC), and clinical evaluation that included the evaluation of allergic rhinitis and associated comorbidities, such as conjunctivitis, asthma, and dermatitis, carried out by a

certified physician, taking into account the criteria set by the international guidance for respiratory allergies ⁴⁶, the Global Initiative for Asthma ⁴⁷, and Hanifin and Rajka's diagnostic criteria for atopic dermatitis ⁴⁸. Historic rhinitis, conjunctivitis and asthma severity were determined according to the ARIA Guidelines, based on the impact of the allergic diseases upon the patient's quality of life ⁴⁶ while dermatitis severity was determined using the Eczema Area and Severity Index (EASI)⁴⁸. To perform rhinitis severity analysis, mild intermittent and mild persistent rhinitis were grouped together as mild rhinitis, while moderate-severe intermittent and moderate-severe persistent rhinitis were grouped together as moderate-severe rhinitis. Additionally, in the detailed medical history used for the diagnosis of allergies, questions about contact with risk factors related with allergies, such as past or current contact with animals (including cats, dogs, horses, chicken and cattle) and about living near farms were asked. 2) Presence of at least one allergic sensitization to common allergens (ALK, Allergologist; Laboratorium A/S, Copenhagen, Denmark). The skin tests included 32 common aeroallergens, such as cat dander, dog dander, cockroach, two house dust mites, five fungi, seven trees, five types of grass and 10 thickets. Histamine hydrochloride (10 mg/mL) and phenol glycerol saline served as positive and negative controls. Only children with a clinical history of allergies received a sensitization test. Inclusion criteria for the non-allergic group were negative allergy clinical history and serum total IgE levels <300 IU/mL. Exclusion criteria: 1) helminth eggs in faeces and 2) insufficient sample.

2.3. Detection of *Cryptosporidium* spp.

The detection of parasite eggs, cysts, and oocysts was performed in three consecutive stool samples collected from each subject. The samples were placed in a 10% formaldehyde solution and stored appropriately until analysis. The samples were processed by the modified Ritchie method ⁴⁹ with iodine smears analysed by optical microscopy (40x magnification, Carl-Zeiss, México) for detection of parasitic helminth eggs and protozoan cysts, and stained with the modified Ziehl Neelsen stain, specific for direct examination of oocysts of *Cryptosporidium* spp and other coccidia using optical microscopy (100× magnification, Carl-Zeiss, México) ⁵⁰. A faecal sample was considered positive if at least four *Cryptosporidium* oocysts were observed in at least one of the collected samples; an average of four to five oocysts per field was considered scarce, six to nine was moderate, and >nine was abundant. Once subjects with helminths were excluded from the sample, subjects with and without *Cryptosporidium* were identified within the allergic and non-allergic groups.

2.4. Quantification of Total IgE

Total IgE levels were quantified in serum by chemiluminescence (Immulite Total IgE, DPC Diagnostic Products Corp., Los Angeles, CA, USA), and the concentration of total IgE in each sample was calculated from a standard curve. We utilized mouse anti-IgE monoclonal antibodies, including two internal controls for each assay, one high (1200 IU/mL) and one low (49 IU/mL). The detection limit was 1 IU/mL.

2.5. Cellular Percentages in Blood

We carried out a haemogram with total and differential counts of leukocytes using optical microscopy (Carl-Zeiss, México).

2.6. Phenotyping

To evaluate the expression of surface markers on T and B cells, we utilized the following monoclonal antibodies: anti-CD3 FITC, anti-CD4 PerCP, anti-CD19 PerCP, anti-CD69 PE, anti-CD124 PE, and anti-CD23 PE (BD Biosciences). We employed anti-IgG1 as an isotype control (BD Biosciences). The cells were incubated with the antibodies for 15 min at room temperature in the dark. After labelling, we lysed red blood cells by incubation in FACS lysing solution (BD Biosciences) for 10 min. Finally, the cells were fixed with 0.5% formaldehyde prior to analysis by flow cytometry.

2.7. Cellular Activation

The activation and labelling of cells for flow cytometry analysis was carried out according to the method described by BD Immunocytometry Systems (BD Biosciences, Becton Dickinson and Company, San José, CA, USA). The blood samples were collected in sodium heparin tubes (BD Biosciences) and processed within 8 h of collection. The blood was diluted 1:1 with culture medium (RPMI-1640; GIBCO, Grand Island, NY, USA) and incubated with phorbol myristate acetate (PMA, 25 ng/mL; Sigma-Aldrich, St. Louis, MO, USA), ionomycin (1 µg/mL, Sigma) and

Brefeldin A (GolgiPlug, 1 µg/mL, BD Biosciences) for 3.5 h at 37°C in an atmosphere of 5% CO₂ to facilitate cellular activation.

2.8. Identification of Cytokine Production by Intracellular Staining

After the activation process and labelling with anti-CD4 PerCP, the cells were incubated with fixation and permeabilization solution (BD Cytofix/Cytoperm; BD Biosciences) for 10 min at 4°C, washed with Perm/Wash buffer, and then incubated with anti-IL-4 PE and anti-IFN-γ FITC monoclonal antibodies (BD Biosciences) at 4°C for 30 min in the dark. After labelling, the cells were washed with Perm/Wash buffer and resuspended in Staining Buffer prior to flow cytometric analysis. The samples were analysed using a FACSCalibur flow cytometer (BD Biosciences) equipped with a 488-nm argon laser. Lymphocytes were identified based on size and complexity, and 30,000 events were acquired per sample. Sample analysis was performed using CellQuest software (BD Biosciences), and quadrants statistics were expressed by percent of the total lymphocytes. The cut off value of the immune markers studied after *in vitro* PMA and ionomycin stimulation was established using the value average + 1 standard deviation of each variable analysed.

Due to the relationship of blood lead levels with Th2 and allergy responses⁵¹, we included an analysis of blood lead levels for all subjects. Blood was collected in 5 mL EDTA-vacutainer trace-metal free tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were transported in a cooler to be stored at 4°C until analysis. The samples were analysed in duplicate using Atomic Absorption Spectrophotometry (Zeeman-Graphite Furnace-Analyst 800, Perkin Elmer Norwalk, CT), according to Bischoff et al.⁵²

2.9. Statistical Analysis

The normality of the variables was determined using a Kolmogorov-Smirnov test. For the mean comparison between numerical variables and according to their parametric or non-parametric distribution an independent t-test or a U-Mann-Whitney test were used, and for median comparison, Mann-Whitney Wilcoxon test was employed. A one-way analysis of variance with Bonferroni correction or Kruskal-Wallis test with Dunn correction were used for

multiple comparisons between groups, allergic children with and without *Cryptosporidium* and non-allergic children with and without *Cryptosporidium* for parametric or non-parametric distribution, respectively. Two-way analysis of variance was used for multiple comparisons between groups. In categorical variables a Chi-square test was used for comparing the proportions of children in the allergic and non-allergic groups and in with and without *Cryptosporidium* in faeces groups of children. The comparison of previous history of allergic disease severity in children with or without *Cryptosporidium* was performed using a Chi-square test. We considered the results statistically significant when the p value was <0.05. The data were analysed using SPSS version 15 (SPSS, Chicago, IL, USA).

3. Results

3.1. Frequency of Allergic Diseases and of *Cryptosporidium* spp. in Faeces

All of the children included in this study lived in Comarca Lagunera, and no one of the children reported past or current contact with farms or cattle. From 65 children enrolled in this study, 12 children (8 with allergy and 4 without allergy) who provided insufficient samples and four children without allergy with helminths in their faeces were excluded, leaving 49 children. The mean age of the children ($n = 49$) was 7.38 ± 1.09 years and 59% ($n = 29$) of the children were males. In 57% ($n = 28$), a clinical diagnosis of allergy was established. All had a positive diagnosis for allergic rhinitis, 21% ($n = 6$) presented only rhinitis, and 39% ($n = 11$) had rhinitis associated with at least one other allergic disease [(conjunctivitis, $n = 5$), (asthma, $n = 3$), (dermatitis, $n = 1$), (insect bite, $n = 1$) and (drug reaction, $n = 1$)]. In 25% ($n = 7$) allergic rhinitis was associated with two different allergic diseases [(asthma and mosquito bite, $n = 3$), (conjunctivitis and drug reaction, $n = 2$), (conjunctivitis and dermatitis, $n = 1$), and (conjunctivitis and asthma, $n = 1$)]. In 7% ($n = 1$), rhinitis was associated with three allergic diseases (conjunctivitis, mosquito bites and asthma) and in 7% ($n = 1$), rhinitis was associated with other four allergies (conjunctivitis, mosquito bites, asthma, and drug reaction). The allergic sensitization in the allergic group was the following: 20 children (71%) were sensitized to thickets, 19 children (68%) to grass, 14 children (50%) to trees, six children (21%) to animal dander, and two children (7%) to mites.

Protozoans were recovered from 34 (69%) of the 49 individuals examined. The prevalence of *Cryptosporidium* spp. oocysts in the faeces for the total sample was 51% ($n = 25$), without a statistically significant difference in the frequency of the parasite in subjects with allergies vs. subjects without allergies [43% ($n = 12$) vs. 62% ($n = 13$) ($p = 0.18$)] (Table 1). The next most common parasite was *Blastocystis* spp with 31% ($n = 15$). Samples from two individuals (5%) harboured *G. lamblia* and *E. histolytica*; 2% ($n = 1$) harboured *C. cayetanensis*, *E. coli* and *I. bütschlii*. The most frequent combination of parasites was *Cryptosporidium* spp.- *B. spp* with 21% ($n = 7$); *Cryptosporidium* spp.- *B. spp*-*E. coli* with 3% ($n = 1$) and *Cryptosporidium* spp.- *B. spp*-*E. histolytica*- *I. bütschlii* with 3% ($n = 1$). Finally, the groups of children with allergies ($n = 28$ children) and without allergies ($n = 21$ children) were divided into subgroups according to the absence or presence of *Cryptosporidium* spp. oocysts in the faeces. The *B. spp* distribution into the groups with or without oocysts was similar, while the frequency of other protozoan was minimal (approximately one per group, data not shown).

Because all of allergic children were diagnosed with allergic rhinitis and the proportion of other comorbidities was low, the severity of the disease was analysed according to the rhinitis severity. The previous history of moderate-severe rhinitis was documented in 81% ($n = 13$) of the allergic children without *Cryptosporidium* vs. 50% ($n = 6$) in children with *Cryptosporidium* ($p = 0.08$). In addition, we did not observe statistically significant differences in *Cryptosporidium* oocysts frequencies relative to age, gender, or body mass index (BMI) (data not shown). We observed moderate/abundant *Cryptosporidium* oocysts in 61% ($n = 8$) of non-allergic children ($n = 13$), and 58% ($n = 7$) of the allergic children ($n = 12$). No diarrheal samples were found, and the children did not show clinical manifestations of cryptosporidiosis (data not shown).

3.2. Immunological Characteristics in Children with or without Allergies

The immunological variables measured are shown in Table 2. Subjects with allergies showed significantly higher total cell counts of eosinophils, percentages of activated CD3⁺, CD4⁺, and CD19⁺ cells (e.g., those expressing CD69), and percentages CD19⁺CD23⁺ cells. In contrast, subjects without allergies had a significantly greater percentage of Th (CD4⁺) cells and IL-4-producing CD4⁺ cells. Between the groups, we did not observe differences in total counts of leukocytes, lymphocytes or segmented neutrophils and the percentage of T lymphocytes (CD3⁺), B

lymphocytes (CD19⁺), or B cells expressing CD124. We observed a greater percentage of IFN- γ -producing Th cells in the group without allergies, although significance was not achieved ($p = 0.06$).

3.3. Cellular Populations and *Cryptosporidium* in Faeces

Figure 1 shows cellular populations in allergic and non-allergic subjects with and without *Cryptosporidium* oocysts. The cellular subtypes detected in the blood from the group of subjects without allergies did not exhibit effects related to the presence of *Cryptosporidium* spp. in the faeces. The absence of the *Cryptosporidium* in subjects with allergies vs. those without allergies was associated with significantly greater total counts of eosinophils ($p = 0.01$, Fig. 1b) and significantly lower total counts of segmented neutrophils ($p = 0.04$, Fig. 1c), while the presence of *Cryptosporidium* in subjects with allergies vs. without allergies was associated with lower percentages of CD4⁺ T lymphocytes ($p = 0.03$, Fig. 1e). In allergic children with vs. without *Cryptosporidium*, lower percentage of B lymphocytes (CD19⁺; $p = 0.01$, Fig. 1f) were observed, with non-significant lower percentage of eosinophils ($p = 0.05$, Fig. 1b).

3.4. Activation Markers Related to Allergy and *Cryptosporidium* spp. in the Faeces of Children with Allergies

Figure 2 depicts the behaviour of allergy-related activation markers in subjects with allergies. In the group of subjects with allergies, the presence of *Cryptosporidium* spp. was significantly associated with lower serum levels of total IgE ($p = 0.00$; Fig. 2a), a lower percentage of activated Th cells (CD4⁺CD69⁺; $p = 0.03$, Fig. 2c), and fewer B cells expressing CD23 ($p = 0.04$) and CD124 ($p = 0.03$; Fig. 2e and f, respectively) compared with the levels in subjects with allergies but without *Cryptosporidium* oocysts. Furthermore, we did not observe differences in the percentages of activated (CD69⁺) CD3⁺ and CD19⁺ cells (Fig. 2, b and d). A significantly greater percentage of T cells producing IL-4 ($p = 0.03$) or IFN- γ ($p = 0.03$) after activation *in vitro* was detected in subjects with allergy when *Cryptosporidium* oocysts were detected in the faeces (Fig. 2g and h, respectively).

3.5. Allergy-Related Activation Markers and *Cryptosporidium* spp. in the Faeces of Children without Allergies

As observed in Fig. 3, in the group of subjects without allergies, the presence of *Cryptosporidium* spp. in the faeces was not associated with significant effects on any of the markers.

3.6 *Cryptosporidiosis*-related IRs in Children with or without Allergies

We evaluated the percentage of CD4⁺ lymphocytes, CD4⁺ lymphocytes producing IL-4 and INF- γ , and total counts of segmented neutrophils, as immunological parameters previously associated with immune response to *Cryptosporidium* (Table 3). In subjects without *Cryptosporidium*, no significant differences in allergic and non-allergic subjects were observed regarding the percentage of CD4⁺ lymphocytes ($p = 0.32$) or CD4⁺IFN- γ ⁺ cells, while significant differences were observed in the counts of segmented neutrophils ($p = 0.04$) and the percentage of CD4⁺ IL-4⁺ cells ($p = 0.00$) in children without allergies. In subjects with *Cryptosporidium*, no differences were observed in subjects without or with allergies regarding the counts of segmented neutrophils ($p = 0.36$), CD4⁺IFN- γ ⁺ ($p = 0.54$) and CD4⁺IL-4⁺ ($p = 0.33$), and significant differences were observed in CD4⁺ lymphocytes ($p = 0.03$) in children without allergies. Comparing subjects with and without *Cryptosporidium* and with or without allergies, we observed differences in the percentages of CD4⁺ lymphocytes ($p = 0.03$), CD4⁺IFN- γ ⁺ ($p = 0.01$) and CD4⁺IL-4⁺ ($p = 0.02$), without differences in segmented neutrophils counts ($p = 0.25$).

3.7 Lead in the Blood and *Cryptosporidium* spp. in the Faeces

Lead levels in the blood were under the values recommended by the U.S. Centers for Disease Control Guide and by the Official Mexican Norma (NOM-199-SSA1-2002), with a median of 1.5 $\mu\text{g/dL}$. There were no statistically significant differences between the groups with and without allergies or relating to the presence or absence of *Cryptosporidium* oocysts in the faeces (results not shown).

4. Discussion

The Lagunera region is a semi-desert area characterized by climatological conditions that favour extrinsic contact with airborne allergens such as thickets and grass, and a high prevalence of allergic rhinitis has been reported^{53,54}. In our study, the results obtained confirm higher total counts of eosinophils in the peripheral blood of children with allergies^{44,55}, associated with a higher percentage of Th and B cells that express CD69 and a greater percentage of B cells that express CD23 (the low-affinity receptor for IgE), without observed effects on the expression of the IL-4 receptor (CD124). As is discussed below, the highest percentages of CD4⁺ T lymphocytes and IL-4 producing CD4⁺ T lymphocytes observed in children without allergies suggests a previous history of exposure to *Cryptosporidium*, without disregarding the participation of other parasites.

The prevalence of *Cryptosporidium* in Mexico is not fully documented, although this disease is commonly reported in children less than five years of age⁵⁶. In Mexico City, the presence of *Cryptosporidium* oocysts has been associated with diarrhoea in children under one year in 41% of subjects⁵⁷. In northern Mexican children aged from six months to 13 years living on the border area between Mexico and US, anti-*Cryptosporidium* antibodies were found in 70% of the total samples, while for five to nine year- old children, the prevalence ranged from 24 to 37%⁵⁸. In the Laguna region, there are no previous studies documenting *Cryptosporidium* prevalence, and the prevalence of *Cryptosporidium* oocysts (51%) in this study is higher than reported by Leach⁵⁸. This work did not include an examination of associated risk factors for *Cryptosporidium* infection and the children did not report past or current contact with farms or cattle. However contact with cattle faeces cannot be excluded as a cause of the high prevalence of *Cryptosporidium* found because the Lagunera region is an important cattle-raising zone of Mexico. The Lagunera region experiences dust storms, which can mobilize faeces contaminated with *Cryptosporidium* and promote the contamination of food and water reservoirs⁵⁹.

Because of the difficulty to observe natural infection with *Cryptosporidium*, few studies have included immunocompetent individuals^{60, 61}, and the majority of the studies describing the IR to *Cryptosporidium* have therefore been carried out in animal models⁶²⁻⁶⁴, immunocompromised patients^{38, 60, 61}, or experimentally infected human volunteers without allergies^{40, 65}. To our knowledge, we have provided the first description of IR in children

with and children without allergies in the setting of natural *Cryptosporidium* infection. Previously, a reduced number of eosinophils were found in the mucosa of piglets infected with *C. parvum* compared to control non-infected mucosa⁶². Subjects with allergies infected with *Cryptosporidium* showed lower counts of eosinophils, lower percentages of CD4⁺CD69⁺ and CD19⁺ cells, CD19⁺CD23⁺ and CD19⁺CD124⁺ cells, and lower levels of serum total IgE compared with non-infected allergic children, supporting a low Th2-type IR and confirming the participation of a Th1-type IR previously associated with *Cryptosporidium*^{30, 31}. In our study, we found no relationship in the spectrum of allergic disease and the presence of *Cryptosporidium*, suggesting no relationship between the severity of allergic disease and *Cryptosporidium* infection. However more studies are needed to rule out this association.

Despite previously published reports describing a relationship between lead levels in the blood and the activation of Th2-type responses^{66, 67}, our data did not support this finding. However, the absence of significant differences in lead levels between children with and without allergies and those with and without *Cryptosporidium* spp. supports the observation that the findings in this study are directly related to the presence of *Cryptosporidium* spp. in faeces.

Our results showed significantly higher percentages of CD4⁺ cells producing IFN γ (Th1) and CD4⁺ cells producing IL-4 (Th2) in children with allergies and oocysts in faeces compared with children with allergies without *Cryptosporidium* (Table 3), supporting the participation of these cells in the IR of allergic children infected with *Cryptosporidium*. Closely related with this, studies that analyse the role of IFN- γ and IL-4 in the IR to *Cryptosporidium* consider them important mediators in the innate and acquired IRs related to the excretion of *Cryptosporidium* oocysts, resolution of the disease, and generation of immunological memory^{37, 41, 68}. Our results are consistent with findings from Kaushik⁶⁰, who found a greater Th1 (IFN γ and TNF α) and Th2 (IL-4 and IL-10) cytokines responses to *Cryptosporidium* antigens in infected and presumably immunocompetent subjects, immunocompromised HIV-seropositive subjects and patients receiving immunosuppressive therapy compared with apparently healthy subjects or HIV-seropositive subjects without *Cryptosporidium*, reinforcing the involvement of CD4⁺ cells producing IFN γ (Th1) and CD4⁺ cells producing IL-4 (Th2) in the IR to *Cryptosporidium*. With regard to segmented neutrophils, the presence of these cells is associated with the innate immune response to infection with *Cryptosporidium*. In our study population, we not observe a modulation in the total counts by the presence of the *Cryptosporidium* oocysts in faeces, however, these results should be treated with caution because the group sample

size did not allow us to evaluate possible interactions related to the presence of *Cryptosporidium* and the type of allergic diseases, which could explain the absence of significant differences observed after ANOVA analysis between groups.

We cannot determine whether the lower allergic response observed in children with oocysts of *Cryptosporidium* occurs only during infection, or if previous exposure might be sufficient to promote the changes in the IR for an extended period of time. In addition, it was not possible to prove if the included participants (allergic or non-allergic children) have ever been exposed to *Cryptosporidium* and other parasites. In our study, children without allergies have highest percentages of CD4⁺ T lymphocytes and IL-4 producing CD4⁺ T lymphocytes vs. children with allergy (Table 2), and when the groups were divided according to the presence or absence of *Cryptosporidium* in the faeces (Table 3), children without allergy and without oocysts showed higher counts of segmented neutrophils and IL-4 producing CD4⁺ cells than children with allergy without *Cryptosporidium*. In addition, the higher percentages of IFN γ producing T CD4⁺ cells vs. the percentage of IL-4 producing T CD4⁺ lymphocytes found in children with allergy without oocysts could suggest the presence of a predominant Th1 IR. All of the above findings with the background information on the participation of these cells in the IR by infection with *Cryptosporidium*, the high prevalence of *Cryptosporidium* oocysts found in our sample faeces and the self-resolving characteristic of the disease, suggest previous exposure to *Cryptosporidium* and possibly other parasites in the study population.

Additionally, in our study, the immunomodulatory effect of *Cryptosporidium* oocysts, observed in children with allergies (Fig. 2, Table 3) was not observed in children without allergy (Fig. 3, Table 3). Previously, Kaushik⁶⁰ showed that the proliferative response to *Cryptosporidium parvum* antigens in HIV-seronegative individuals, receiving or not receiving immunosuppressive therapy, or HIV-seropositive subjects infected with *Cryptosporidium*, was significantly higher compared to healthy subjects without history of exposure to *Cryptosporidium* and with HIV-seropositive subjects not infected with *Cryptosporidium*. Similar levels of IFN γ and IL-4 were found in healthy subjects without history of exposure to *Cryptosporidium* and immunocompromised subjects without cryptosporidiosis, but less compared to those found in HIV-seropositive immunocompromised subjects, subjects

receiving immunosuppressive therapy or immunocompetent subjects infected with *Cryptosporidium*, strengthening the idea of a previous exposure to *Cryptosporidium* in our study population.

Given the difficulty to establish, in non-experimental settings, a cohort with allergic and non-allergic subjects exposed and unexposed to *Cryptosporidium*, and to observe changes in the IR before and after the infection, the best way to show the relationship between IR and the presence of *Cryptosporidium* would be a case-control study. In a case-control study, the cases could be defined as allergic subjects with presence of *Cryptosporidium* when compared to control groups of: 1) allergic subjects without oocysts, 2) non-allergic subjects with oocysts, and 3) non-allergic subjects without oocysts. The nature of this study was exploratory, and in it, the findings in allergic subjects with *Cryptosporidium* compared with allergic uninfected and non-allergic infected and uninfected were reported. The major limitation of this study is the small group sample size, making it difficult to compare groups and identify differences. The main objective of this study was to compare immune markers related with allergy in allergic and non-allergic subjects with and without oocysts. In addition, we analysed whether the participation of segmented neutrophils and IL-4 and IFN γ producing CD4⁺ lymphocytes observed in other studies with animal models, normal volunteer subjects, and immunocompromised individuals (HIV⁺) infected with *Cryptosporidium* was present in allergic patients. Currently, the evidence supports the modulation of IR by *Cryptosporidium* infection, mainly with a type Th1 response, and our findings reveal the immunomodulatory effect of *Cryptosporidium* in allergic children, which presented lower levels of immunological markers associated with allergy (Th2), supporting the involvement of INF- γ and IL-4 producing CD4⁺ T cells in the IR of children infected with *Cryptosporidium*. Therefore, our results are expected to contribute to a better understanding of the immune response to *Cryptosporidium*.

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Authorship

Janeth O. Guangorena-Gómez and Rocío Meza-Velázquez performed the research.

Janeth O. Guangorena-Gómez, Maribel Cervantes-Flores, and Raquel C. Goytia-Acevedo designed the research study.

Mario Rivera-Guillén; Rocío Meza-Velázquez; Raquel C. Goytia-Acevedo; Guadalupe García-Arenas and Leonor C. Acosta-Saavedra, contributed essential reagents and tools.

Aurora Maravilla-Domínguez; Janeth O. Guangorena-Gómez and Raquel C. Goytia-Acevedo, analysed the data.

Aurora Maravilla-Domínguez; Raquel C. Goytia-Acevedo and Guadalupe García-Arenas wrote the paper.

Ethical approval: All procedures performed in these study involving human participants were in accordance with the ethical standards of Juárez University of the State of Durango Ethics Committee of the Faculty of Medicine and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure legends

Fig. 1 Analysis of cellular subtypes in the peripheral blood of children with or without *Cryptosporidium* oocysts in the faeces. Total cell counts of (a) lymphocytes, (b) eosinophils, and (c) segmented neutrophils. Cellular percentages of (d) CD3⁺ lymphocytes, (e) CD4⁺ lymphocytes and (f) CD19⁺ cells. The symbols denote children with allergies (Δ, ▲), without allergies (○, ●), without oocysts of *Cryptosporidium* spp. in the faeces (▲, ●), and with oocysts of *Cryptosporidium* in the faeces (Δ, ○). The results are represented as individual values with line graphs of the average and the standard deviation (SD). We performed a one-way analysis of variance with Bonferroni correction to compare the means of multiple groups (a, c, d, e, and f). The data that did not show a normal distribution were compared using the nonparametric Kruskal-Wallis test with Dunn correction to compare multiple groups' medians (b). Significant differences are indicated by $p \leq 0.05$.

Fig. 2 Allergy-related activation markers in children with allergies ($n = 28$), with ($n = 12$) and without ($n = 16$) *Cryptosporidium* spp. oocysts in the faeces. (a) Total serum IgE levels. (b, c, and d) Percentages of activated T or B cells obtained by double staining with anti-CD3 FITC, anti-CD4 PerCP, or anti-CD19 PerCP with anti-CD69 PE. (e and f) Double staining of cells with anti-CD19 PerCP and anti-CD23 PE or anti-CD124 PE to identify activation markers on B cells. (g and h) To detect intracellular cytokines, anti-CD4 PerCP and anti-IL-4 PE or IFN- γ FITC were used. The results are presented as individual values with line graphs of the average and the standard deviation (SD). A Student's t test or the U-Mann-Whitney tests were used to analyse differences between the means of the groups (with or without oocysts of *Cryptosporidium* spp.). The significant differences and the p values are shown with connector lines within the figure.

Fig. 3 Allergy-related activation markers in children without allergy ($n = 21$), with ($n = 13$) and without ($n = 8$) *Cryptosporidium* spp. oocysts in the faeces. (a) Total serum IgE levels. (b, c, and d) Percentages of activated T or B cells obtained by double staining with anti-CD3 FITC, anti-CD4 PerCP, or anti-CD19 PerCP with anti-CD69 PE. (e and f) Double staining of cells with anti-CD19 PerCP and anti-CD23 PE or anti-CD124 PE to identify activation markers on B cells. (g and h) To detect intracellular cytokines, anti-CD4 PerCP and anti-IL-4 PE or IFN- γ FITC were used. The results are presented as individual values with line graphs of the average and the standard deviation (SD). A Student's t test or the U-Mann-Whitney tests were used to analyse differences between the means of the groups (with or without oocysts of *Cryptosporidium* spp.). The significant differences and the p values are shown with connector lines within the figure.

Table 1. Characteristics of the population

Characteristic	Without allergies $n = 21$	With allergies $n = 28$	p value
Age (mean $\pm$ SD), years	7.62 $\pm$ 1.07	7.21 $\pm$ 1.01	0.20
Male, n (%)	13 (62)	16 (57)	0.73
Female, n (%)	8 (38)	12 (43)	
BMI (mean $\pm$ SD), kg/m ²	17.61 $\pm$ 3.25	18.05 $\pm$ 3.63	0.75
<i>Cryptosporidium</i> spp., faecal oocysts, n (%)	13 (62)	12 (43)	0.18

SD: Standard deviation; BMI: Body mass index.

Table 2. Values of immunological variables in groups with and without allergies

Variable	Without allergies n = 21	With allergies n = 28	<i>p</i> value
Total leukocytes, cells/ μ l ^a	7000 (4500-9100)	6050 (3400-10800)	0.19
Total lymphocytes, cells/ μ l ^a	1360 (611-2520)	1650 (680-2376)	0.62
Eosinophils, cells/ μ l ^a	222 (0-720)	416 (0-1296)	0.03
Segmented neutrophils, cells/ μ l ^a	5016 (2745-6699)	4158 (2108-7384)	0.08
CD3 ⁺ cells, %	61.28 $\pm$ 7.82	61.83 $\pm$ 8.54	0.82
CD3 ⁺ CD69 ⁺ cells, % ^a	1.43 (0.18-9.38)	3.45 (0.09-25.25)	0.00
CD4 ⁺ lymphocytes, %	41.23 $\pm$ 13.52	32.77 $\pm$ 8.79	0.01
CD4 ⁺ CD69 ⁺ cells, % ^a	2.42 (0.59-17.5)	7.13 (0.75-42.20)	0.00
CD19 ⁺ lymphocytes, %	15.92 $\pm$ 5.49	17.67 $\pm$ 5.08	0.25
CD19 ⁺ CD69 ⁺ cells, % ^a	0.55 (0.1-5.9)	1.43 (0.09-11.16)	0.00
CD19 ⁺ CD23 ⁺ cells, % ^a	6.03 (1.30-15.91)	10.45 (4.15-25.68)	0.01
CD19 ⁺ CD124 ⁺ cells, % ^a	4.90 (0.68-13.96)	6.99 (1.03-19.13)	0.26
CD4 ⁺ IL-4 ⁺ cells, % ^a	7.28 (0.21-28.32)	3.65 (0.21-26.08)	0.02
CD4 ⁺ IFN- γ ⁺ cells, % ^a	7.44 (0.16-15.28)	3.83 (0.36-29.70)	0.06

^a Expressed as median (range). All other percentages are shown as the mean $\pm$ standard deviation (SD).

Table 3. Percent of segmented neutrophils, CD4⁺ lymphocytes, CD4⁺INF- γ ⁺ and CD4⁺IL-4⁺ cells, in subject with and without allergic disease and with and without *Cryptosporidium* oocysts in faeces.

Variable	<i>Cryptosporidium</i> Oocysts (-)			<i>Cryptosporidium</i> Oocysts (+)			Anova <i>p</i> -value
	n = 24			n = 25			
	Without allergies n = 8	With allergies n = 16	<i>p</i> value	Without allergies n = 13	With allergies n =12	<i>p</i> -value	
Segmented neutrophils, cells/μl	5269.8 ± 903.4	4010.5 ± 1708.8	0.04	4631.7 ± 1172.3	4436.4 ± 1332.5	0.36	0.25
CD4 ⁺ T lymphocytes, %	36.9±12.9	32.2±9.8	0.32	43.9±13.7	33.6±7.4	0.03	0.03
CD4 ⁺ INF-γ ⁺ cells, %	7.9±5.0	4.5±7.0	0.09	6.5±3.7	6.6±4.9	0.54	0.01
CD4 ⁺ IL-4 ⁺ cells, %	10.3±9.6	2.8±2.4	0.00	8.4±6.1	7.4±5.7	0.33	0.02

All values are shown as mean $\pm$ standard deviation (SD).



