Title page

Title: Immune modulation as a result of helminthes infestation in patients with idiopathic inflammatory disease, Crohn's disease and ulcerative colitis: cases control study.

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Abstract

The UC and Crohn's disease are chronic, idiopathic, inflammatory diseases of the GIT that share common symptoms such as diarrhea, abdominal pain, fever, and weight loss. Ulcerative colitis involves all or part of the colon, whereas, Crohn’s disease commonly involves the terminal ileum and proximal colon.The two major forms of IBD share many clinical and epidemiological characteristics, suggesting that underlying causation may be similar. Yet, UC & Crohn's disease are distinct syndromes with divergent treatment and prognosis.

Aim of the study:

1- Study a relationship between some parasitic infections such as helminthes infestation, and the development of IBD.
2- Understanding the correlation between parasitic infections and autoimmune disorders may be helpful in prediction, early identification and conceivably the prevention of these diseases
3- To detect the IL1B and IL10 related to disease pathophysiology.
4- To determine whether a patient may respond well to IBD disease medications.
5- To help differentiate Crohn's disease from ulcerative colitis patients.

Conclusion:

1. The current study also showed immunological evidence that helminthes can modulate host immune response to prevent and minimize the inflammatory response in cases of IBD through increasing the level of anti-inflammatory cytokines and decreasing the level of pro-inflammatory cytokines.
2. The combined infestation with multiple intestinal helminthes has a better protective role than single parasite in protection against inflammatory bowel disease.

Key words: Crohn's disease, ulcerative colitis, IL1B and IL10**,**IBD.

Introduction

Inflammatory Bowel Disease (IBD) comprises those conditions which tend to be chronic or relapsing immune activation and inflammation within gastrointestinal tract (GIT). Ulcerative Colitis (UC) and Crohn's disease are the two major forms of this disease with unidentified etiopathology (Sands, 2002). The incidence of IBD has been rising not only in Western countries, but also in Asia, including Korea (Yang *et al*., 2008). Thanks to the inventions of vaccines and anti-microbial agents that dramatically reduced the rate of infectious disorders. The big picture in the developed world can be summarized by two main trends. The first tread is that infectious disorders such as mumps, rubella, T.B, pneumonia, meningitis, etc., have reached very low incidence rates in these developed countries; the second trend however; on the other hand is that a number of disorders such type 1 diabetes mellitus, hay fever, celiac disease, asthma, Crohn's disease and ulcerative colitis have witnessed marked increase in incidence rate particularly when compared with their incidence rates in developing countries (Scudellari, 2017).

Epidemiologic studies have shown that the prevalence rates of idiopathic inflammatory bowel disease, Crohn's disease and ulcerative colitis, are higher in developed countries such as USA and Western Europe than in underdeveloped and developing countries. On the other hand, the prevalence rate of parasitic infestation with round worms, helminthes, is significantly lower in developed countries in comparison with developing countries. Based on these epidemiologic data, a number of authors has suggested a link between high incidence rate of Crohn's disease and ulcerative colitis and low incidence rate of helminthes infestation in developed countries and has hypothesized that under exposure to children in their

early lives to helminthes infestation resulted in maldevelopment of their immune system with subsequent predisposition to autoimmunity that may manifest itself in the form of either Crohn's disease of ulcerative colitis (Sýkora *et al*., 2018; M'Koma, 2013). However; because of the lack of clear consensus about this suggestion and because of the high prevalence of helminthes infestation in our community in Iraq (Saheb *et al*., 2017), the present study was planned and conducted to investigate the possible association among the immune system function, the prevalence of helminth infestation and the prevalence and pathogenesis of idiopathic inflammatory bowel diseases.

Patients and methods

After sterilizing the area with alcohol (70%), aspiration blood sample (5ml) was collected from cubital fossa vein from GIT patients and control groups.

Collected sample was transferred immediately in to two tubes as follows:

A. Two milliliter of blood in 5 ml tube (EDTA tube) used for PCR technique to detect NOD/CARD15 gene polymorphism

B. Three milliliter of blood in a gel tube (serum tube),then the blood samples were centrifuged at (4700 RPM for 5 min) to obtain blood serum then frozen at -20 C for screening of IL1B,IL10 cytokines levels .

Two groups were included in this study;

A- Patients Group : A total of fifty patients from Al-Diwaniyah province (males and females) with inflammatory bowel disease; 31 patients with Ulcerative Colitis and 19with Crohn's Diseases patients, who have been diagnosed by specialist physicians in Al-Diwaniyah Teaching Hospital for Gastrointestinal Tract and Hepatic diseases unit, depending on clinical features, biopsy for

histopathology, and endoscopy. All were regularly attending the consultant clinic for treatment and follow-up during the period from January 2018 to august 2018.

B- Control Group; A total number of thirty individuals, who were apparently healthy, were involved as a control group. They matched the patients group regarding sex, and age and had no history of / or clinical features of IBD, no obvious abnormalities, none of them had an acute or chronic diseases.

Study Protocol and Sampling Members of the two groups were subjected all were subjected to the following assays;

1- GSE.

Stool examination for parasites

1. Saline wet mount: It is used to detect worms, bile stained eggs, larvae, protozoan trophozoites and cysts. In addition, it can reveal the presence of RBCs and WBCs.

2. Iodine wet mount: It is used to stain the glycogen and nuclei of the cysts. A cyst is appreciated better in an iodine preparation, but the motility of the trophozoite is inhibited in the iodine preparation.

Procedure:

• Place a drop of saline on the left half of the slide and one drop of iodine on the right half.

• With an applicator stick, pick up a small portion of the specimen (equivalent to the size of a match head) and mix it with a saline drop.

• Similarly, pick up a similar amount and mix with a drop of iodine.

• Put the cover slip separately on both and examine under the microscope.

• The ova, cysts, trophozoites and adult worms can be identified as per their characteristic features.

2- Screening the bellow:

. EDTA tube for blood extraction and then RFLP PCR.

.Gel tube for investigate IL1B, IL10.

.Tube for stool extraction and PCR.

Human IL-1β (Interleukin 1 Beta) ELISA Kit was used in this study for quantitative determination of IL-1β concentrations in serum of human blood samples and done according to company instruction.

Human IL-10 (Interleukin 1 0) ELISA Kit was used in this study for quantitative determination of IL-1β concentrations in serum of human blood samples and done according to company instruction

Result

Serum levels of the anti-inflammatory cytokine IL-10 and of the pro-inflammatory cytokine IL-1 β were measured for all participants and results are shown in table 1. Median serum level of IL-10 in control group was 16 pg/ml, while that of patients with UC was 18 pg/ml and those with CD was 11 pg/ml. Hence serum level of IL-10 was significantly lower in patients with CD than both control group and UC group ($P < 0.05$), table 1 and figure 1. Moreover, there was no significant difference in the serum level of IL-10 between patients with UC and control group ($P > 0.05$), table 1and figure 1.

Serum IL-1 β was significantly ($P < 0.05$) highest in patients with CD followed by patients with UC and then by control group, 232 pg/ml, 65 pg/ml and

59.5 pg/ml, respectively, as shown in table 1 and figure 1; in addition there was no significant difference in serum Il-1 β level between patients with UC and control group ($P > 0.05$), table 1 and figure 2.

Table 1: Serum interleukin levels in patients and control subjects

Serum level	Statistic	Control *n* = 50	UC *n* = 31	CD *n* = 19
IL-10	Median (IQR)	16.00 (20.00) A	18.00 (23.00) A	11.00 (12.00) B
	Range	4.00 -56.00	3.00 -54.00	2.00 -65.00
IL-1β	Median (IQR)	59.50 (116.25) B	65.00 (222.00) B	232.00 (278.00) A
	Range	23.00 -653.00	3.00 -876.00	4.00 -866.00

UC: ulcerative colitis; CD: Crohn's disease; *n*: number of cases; IQR: inter-quartile range; Capital letters (A and B) where used to indicate significance level following Mann Whitney U test; similar letters indicate no significant difference at $P \leq 0.05$; different letters indicate significant difference at $P \leq 0.05$

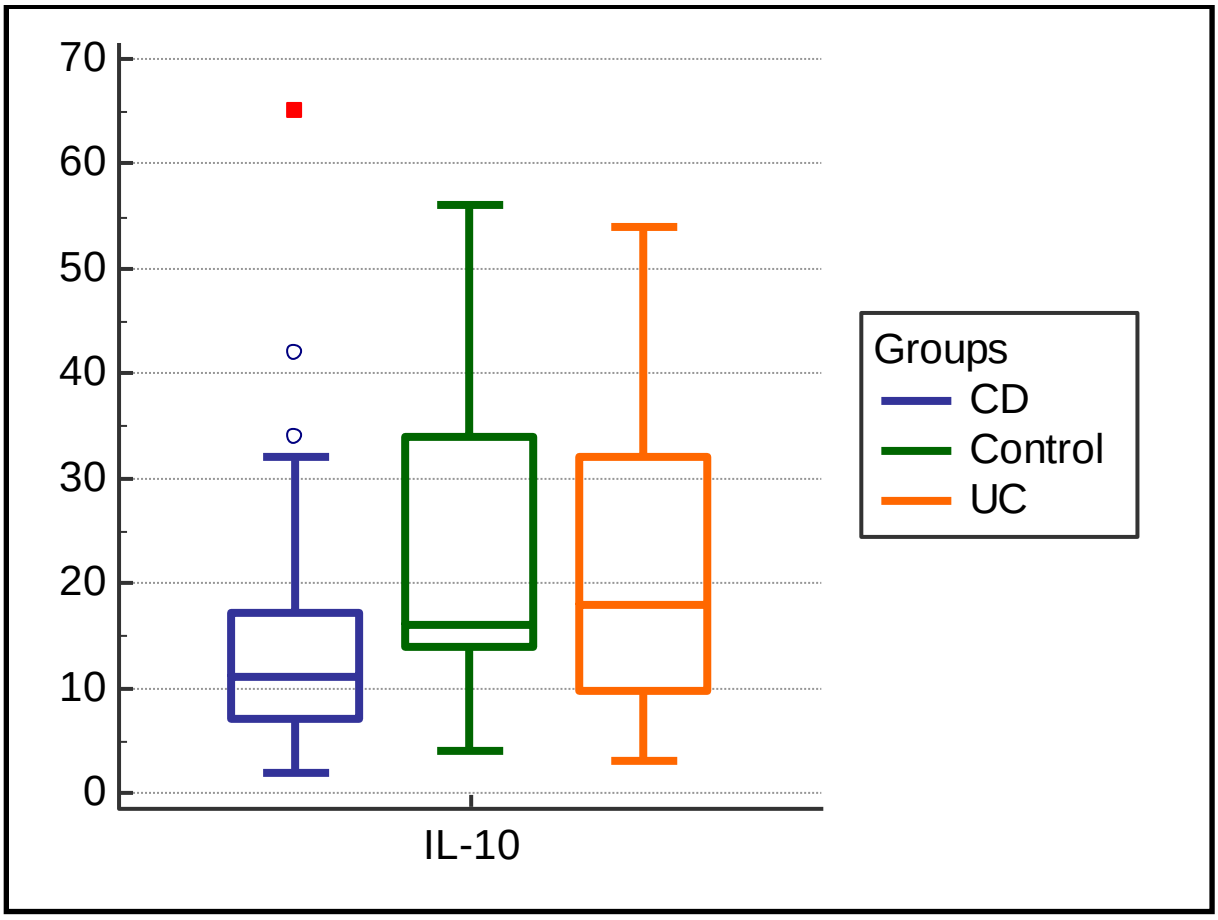

Figure 1: Box plot showing comparison of median serum IL-10 level among patients with Ulcerative colitis (UC) and Crohn's disease and control subjects

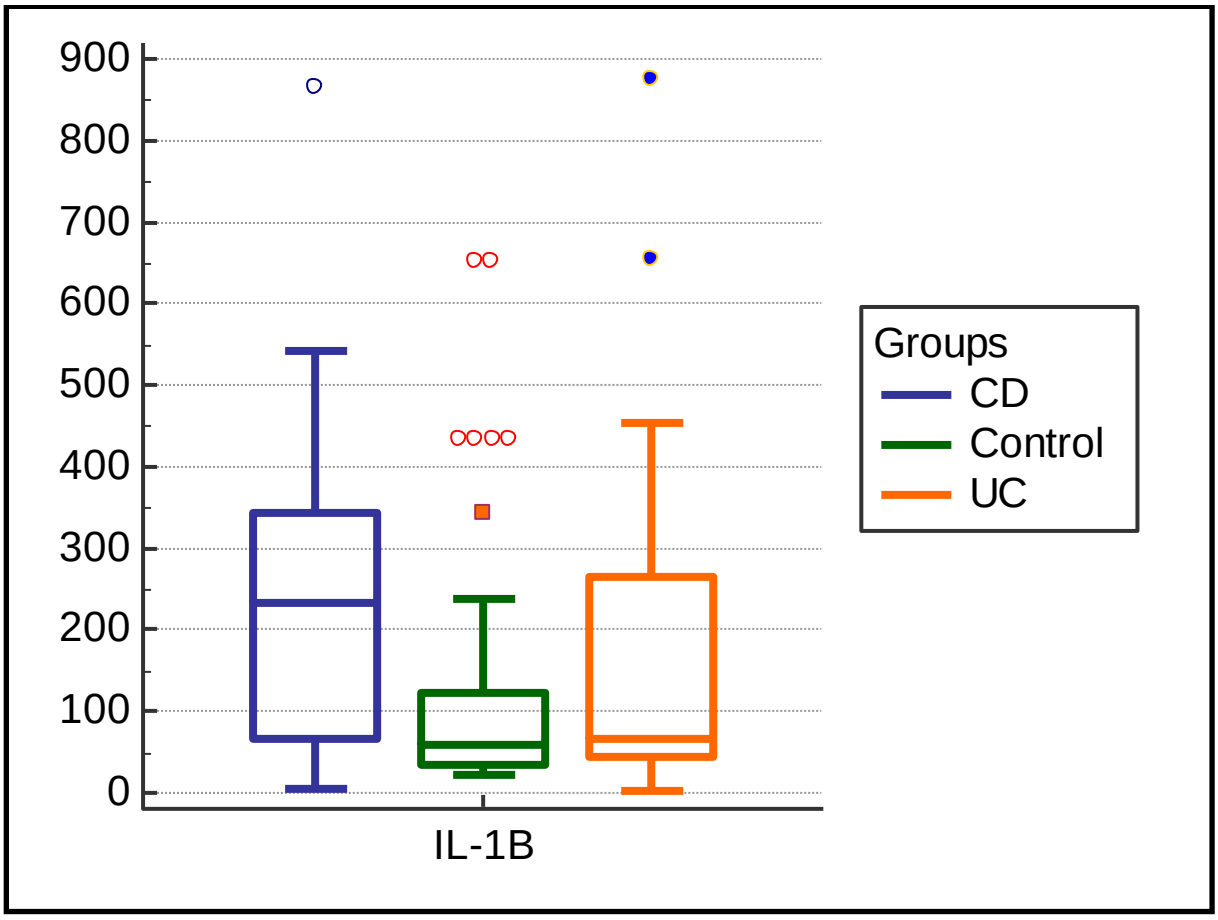

Figure 2: Box plot showing comparison of median serum IL-1β level among patients with Ulcerative colitis (UC) and Crohn's disease and control subjects

In control group, median serum IL-10 level was significantly higher in those having helminth infestation than those who are free of parasite, 17 versus 14 pg/ml, respectively (P = 0.002), as shown in table 2 and figure 3. In addition, there was no significant difference in median serum IL-1 β those having helminth infestation and those who are free of parasite, 59.5 versus 59.5 pg/ml, respectively (P = 0.633), as shown in table 3 and figure 3.

In patients with UC, median serum IL-10 level was significantly higher in those having helminth infestation than those who are free of parasite, 31 versus 8 pg/ml, respectively (P < 0.001), as shown in table 4 and figure 3. In addition,

median serum IL-1 β was higher in those who are free of parasite in comparison with those having helminth infestation, 222 versus 54 pg/ml; however, the difference did not reach statistical significance (P = 0.172), as shown in table 4 and figure 3.

In patients with CD, median serum IL-10 level was significantly higher in those having helminth infestation than those who are free of parasite, 32 versus 7.5 pg/ml, respectively (P = 0.001), as shown in table 4.10 and figure 4.3. In addition, median serum IL-1 β was lower in those who are free of parasite in comparison with those having helminth infestation, 132 versus 321 pg/ml; however, the difference did not reach statistical significance (P = 0.290), as shown in table 4 and figure 3.

Table 2: Serum IL-10 and IL-1β according to presence or absence of parasitic infestation in control

Serum IL	Statistic	Parasite infestation		*P* €
		Positive ***n*** **= 36**	**Negative** ***n*** **= 14**	
IL-10	Median (IQR)	17.00 (26.50)	14.00 (5.50)	0.002 HS
	Range	10.00 -56.00	4.00 -18.00	
IL1B	Median (IQR)	59.50 (168.50)	59.50 (127.25)	0.633 NS
	Range	23.00-653.00	23.00 -653.00	

n: number of cases; IQR: inter-quartile range; €: Mann Whitney U test; NS: not significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$

Table 3: Serum IL-10 and IL-1β according to presence or absence of parasitic infestation in UC

Serum IL	Statistic	Parasite infestation		*P* €
		Positive ***n*** **= 19**	**Negative** ***n*** **= 12**	
IL-10	Median (IQR)	31.00 (16.00)	8.00 (9.75)	<0.001 HS
	Range	12.00 -54.00	3.00 -21.00	
IL1B	Median (IQR)	54.00 (217.00)	222.00 (257.75)	0.172 NS
	Range	3.00 -655.00	8.00 -876.00	

n: number of cases; IQR: inter-quartile range; €: Mann Whitney U test; NS: not significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$

Table 4: Serum IL-10 and IL-1β according to presence or absence of parasitic infestation in CD

Serum IL	Statistic	Parasite infestation		*P* €
		Positive ***n*** **= 7**	**Negative** ***n*** **= 12**	
IL-10	Median (IQR)	32.00 (31.00)	7.50 (7.75)	0.001 HS
	Range	11.00 -65.00	2.00 -12.00	
IL1B	Median (IQR)	321.00 (445.00)	123.00 (269.50)	0.290 NS
	Range	4.00 -543.00	12.00 -866.00	

n: number of cases; IQR: inter-quartile range; €: Mann Whitney U test; NS: not significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$

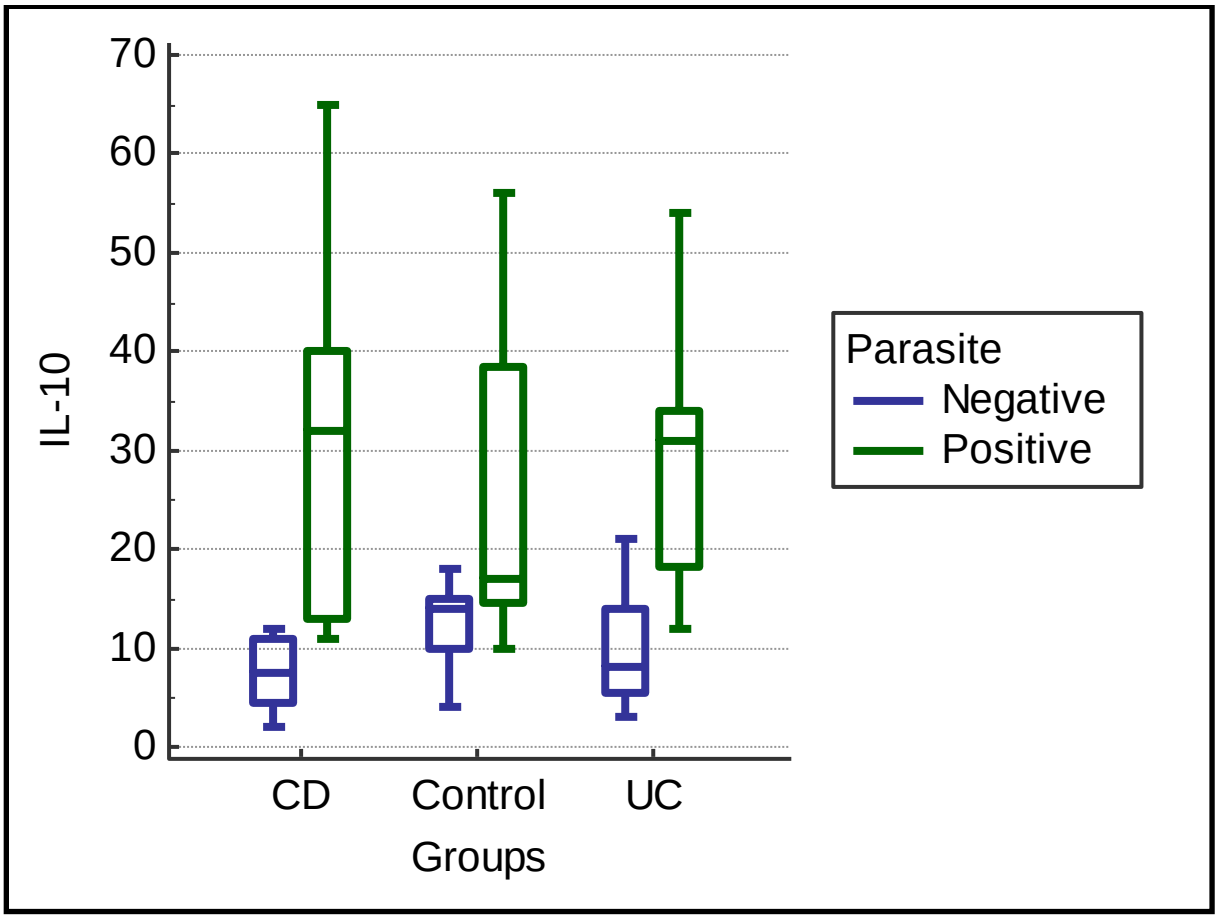

Figure 3: Box plot showing comparison of median serum IL-1β level among patients with Ulcerative colitis (UC) and Crohn's disease and control subjects according to presence or absence of parasitic infestation

Discussion:

In the present study, serum level of IL-10 was significantly lower in patients with CD than both control group and UC group ($P < 0.05$). Moreover, there was no significant difference in the serum level of IL-10 between patients with UC and control group ($P > 0.05$). The current study has shown that patients with concomitant inflammatory bowel disease and parasitic infestation have well defined immune modulation response when compared to those patients with inflammatory bowel disease and devoid of parasitic infestation. The immune modulation was that the level of anti-inflammatory interleukin 10 was higher and the level of the pro-inflammatory interleukin 1 beta was lower favoring immune suppression.

Immune regulation by parasites compromises immunity but also protects the host from damaging immunopathological reactions to the presence of parasites (McSorley and Maizels, 2012).In gastrointestinal nematode infections, particularly infections by the highly prevalent *Ascaris*, hookworm, and *Trichuris* species, the pathology is less intense, and long-term infestation is common. Infection is associated with a regulatory set of cells and cytokines, as IL-10 and TGF-β are significantly linked with hyporesponsiveness and susceptibility (Figueiredo *et al*., 2010; Turner JD, *et al*. 2008).

In support for the current study observation, Turnner *et al*. in 2008 have shown enhanced production of the anti-inflammatory cytokines IL-10 and TGF-β1. (Turnner et al. 2008) have demonstrated that constitutive levels of the regulatory cytokines IL-10 and TGF-β1 are enhanced in direct relation to the intestinal worm burden and provide evidence that this elevation in anti-inflammatory cytokine secretion in peripheral blood induces immunological hypo responsiveness. This observation is striking when considering the plethora of other microorganisms

encountered and the many other environmental, dietary, and lifestyle factors that are typically varied in the setting of a natural human infection, all of which could potentially induce or suppress IL-10 and/or TGF-β1 output. We infer from our data, therefore, that chronic gut-worm infection plays an important role in driving human immunoregulatory networks. Thus, our findings provide a mechanism for the observed lower incidence of autoimmune disease, allergies, and asthma in communities where gut worms are endemic (Stene and Nafstad, 2001; ISAAC, 1998).

It has been hypothesized that worm-driven immunoregulatory networks, exemplified by the induction of IL-10 and or TGF-β secreting T_{reg} cells, represent a parasite survival strategy that enables suppression of an effective immune response (Maizels *et al*., 2004). Gut helminths are generally considered to benefit from the modulation of Th2-like responses because data generated in model systems clearly demonstrate that Th2 cytokines drive effector mechanisms at the site of infection that lead to parasite expulsion (Cliffe *et al*., 2005). Furthermore, we and others have observed that Th2 cytokines and antibody responses linked to Th2 activity are inversely associated with human intestinal helminth infection (Turner et al., 2003) and with reinfection following chemotherapeutic intervention (Jackson *et al*., 2004). Our data are consistent with this hypothesis; accumulations of secreted IL-10 and TGF-β1 are inversely associated with Th2 (IL-4) recall responses to parasite antigens. However, in the case of TGF-β1, we have concluded that this suppressive cytokine is also associated with diminished Th1 responsiveness to bacterial antigen and a nonspecific stimulus. This may be a consequence of gut worm–driven regulatory activity spilling over onto unrelated adaptive cellular responses in heavily polyparasitized individuals. Alternatively, given that TGF-β1 expression increases during wound healing and has thus been implicated in the regulation of such responses (Kulkarni *et al*., 2002), the suppression of Th1

responses by TGF-β may be a consequence of tissue repair responses to damage of the gut mucosa, liver, or lungs by heavy worm infection and continuous larval reinvasion. A further possibility is that enteric bacterial infections triggered by worm-mediated disruption of the gut mucosa might also up-regulate counter inflammatory cytokine expression.

Dissection of the mechanism by which gut worms promote secretion of IL-10 and TGF-β1 is valuable in understanding why allergies and autoimmunity are increasing in the developed world and may present new avenues for treating or preventing such disorders. In fact, gut worms have already been demonstrated to have therapeutic value in the treatment of inflammatory bowel disease (Thompson and Weinstock, 2005). Furthermore, considering the frequency with which intestinal worm infections occur in humans (approximately 1 billion people are infected with at least 1 species) (Bethony *et al*., 2006) and their overlapping geographical distribution with devastating diseases (Borkow *et al*., 2000).

Since the etiology of IBD is still unknown and causative therapies aren't available, the patients exposure to helminths appeared to be a novel and promising approach in the treatment of colitis. During helminth infection, pronounced Th2 immune responses as well as an activation of B cells, basophils, mast cells, dendritic cells, and eosinophils are evoked in the host to control and expel the parasites. By the expansion of regulatory cells such as alternatively activated macrophages, $CD4^+$ and $CD8^+$ Tregs or regulatory B cells, and the consequent induction of anti-inflammatory cytokines, e.g. IL-10 or TGF-β, helminths constitute immunoregulatory conditions to ensure their survival (Allen and Maizels, 2011). This immunomodulatory state was suggested to limit intestinal inflammation in IBD. However, utilization of helminths in different human studies and animal experiments of colitis highlighted controversial results. First results of

clinical trials of *Trichuris suis* ova (TSO) therapy in UC and Crohn`s disease patients showed a reduction of the disease activity index (Summers et al., 2005).

In the present study it was shown that the level of the pro-inflammatory cytokine IL-1 beta was lower in patients with parasite infestation. The mechanism of this may be attributable to the increased level of the anti-inflammatory IL-10. The exact mechanism of the interaction between these two cytokines has been extensively studies by a number of authors and the down regulation of IL-1 beta mediated by TL-10 has been shown in a number of experimental studies (Jenkins *et al*., 1994).

The current study also contributed to some extent in the explanation of the varying severity of inflammation between ulcerative colitis and Crohn's diseases. It is well documented that Ulcerative colitis causes an inflammation that is mainly seen in the mucosa and sub-mucosa whereas, Crohn's disease causes more sever inflammation that involves all intestinal layers starting form mucosa reaching the serosa, termed by pathologist as transmural inflammation. In the current study, the serum level of IL-10, anti-inflammatory cytokine, was higher, and the level of IL-1 beta, pro-inflammatory cytokine, was lower, in ulcerative colitis than in Crohn's disease. This may provide clues to the different pathogenic pathways involved in the two inflammatory bowel disorders, as well as provide some explanation for less sever, and limited inflammatory response in ulcerative colitis in comparison with Crohn's disease. To the best of our knowledge, this is the first study that has brought insight toward the role of variation in cytokine level expression in relation to severity of intestinal inflammation.

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