Natural regulatory T cells in children with *Entamoeba histolytica* infection

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Declaration of interest

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Abstract

Background: Parasites exemplified by *Entamoeba histolytica* can manipulate natural regulatory T cells in order to lessen the infection burden and maintain successful infection in the host for a longer time frame. The aim of this work is to investigate natural regulatory cells level in *Entamoeba histolytica* infection.
Methods: This study comprised: 53 pediatric patients with *E.histolytica*, 47 pediatric patients with other intestinal parasites, 40 healthy children as control group. Each patient was subjected to coprological study to determine different parasites and their parasitic load, differentiation of *E. histolytica* from *E. dispar* trophozoites in stool samples will be demonstrated by ELISA, and determination of CD4, CD25 and Foxp3 by Flow Cytometric Analysis
Results: The most common parasitic infection in the studied group was *Entamoeba histolytica* followed by *Giardia lamblia.* There was highly significant difference between *Entamoebia* infection and Control and Other parasite and Control as regard CD4+CD25+Foxp3 level. CD4+CD25+Foxp3level was significantly higher in severe *Entamoeba* infection than mild and moderate infection**.**

Conclusion: CD4+CD25+Foxp3 Tcells increased in parasitic infection including *Entamoeba histolytica* infection and their increase proportionally related to severity of *Entamoeba histolytica* infection so they may be implicated in silencing immunity to *Entamoeba* infection and persistence of infection.

Key words: Regulatory T cell, CD4+CD25+FOXp3+, parasitic diseases, Entamoeba histolytica.

Running title: T cells in children with *Entamoeba histolytica*

1. Introduction

In 1970, an originalfinding had been made by Gershon, and Kondoby finding T cells restrain immune responses different from helper T cells, named them suppressor (regulatory) T cells [1]. T regulatory cells are divided into 2 major groupsbased on their inception, their specific antigen and used effector mechanisms into natural TR (nTR) which maturate in thymus, and display $CD4^+CD25^+$ phenotype and are "naturally" addressed to cause immunosuppression. Induced TR (iTR), are also derived from thymus, however, they developafter antigen exposure from naïve, $CD4^+CD25^-$T cells in the periphery [2,3,4]. Recently, Foxp3 was identified as a signature transcription factor for development and function of nTR [5]. Natural regulatory T cells have been shown to play an important role in prevention of several autoimmune diseases, and can also modify immunity to infectious agents and transplantation [6,7].

Entamoeba histolytica is a scarcely investigated intestinal parasite, although it is considered the second killer from parasitic diseases worldwide [8]. Moreover, there is no current vaccine for this ruinous disease until now. So, understanding human intestinal immune response toward *Entamoeba histolytica* could pave the way to develop effective immunotherapy for this devastating parasite. The main role of immune system is not only protection against infection, but also control of infection-induced immunopathology. The latter could be achieved by generation of antigen specific T regulatory cells. Parasites have the ably to lessen the infection burden by manipulating natural Tregs, in order to prevail in the host for a longer time frame [9]. So, the reciprocal action between the host and the arms of immunity including: Th1, Th2 and Tregs are important in defending against parasitic infections [10].

Hypothesis: natural regulatory Tcells levels increase in *Entamoeba histolytica* infection thus silencing immune response to its infection
Null hypothesis: natural regulatory Tcells levels decrease or normal in *Entamoeba histolytica* infection thus has no effect in immune response to its infection.

Aim of the work: To investigate natural regulatory cells level in *Entamoeba histolytica* infection.

2. Material & methods

Fifty three pediatric patients with *Entamoeba histolytica* infections and forty seven pediatric patients with other intestinal parasitic infections, from Mansoura University Children Hospital, were included in this study. Exclusion criteria were 1) any form of malnutrition, 2) AIDS or any other immune deficiency diseases, 3) ongoing inflammatory diseases, 4) diabetes mellitus, 5) leishmanianiasis *or* malaria or 6) antiparasitic therapy in the last three months before. Forty healthy children, parasitologicaly free, age and sex matched was included as control.

The stool examination was done by direct smear, Formol-Ether concentration method [11], acid fast stain were used for Coccidea [12], Gomori's trichrome stain [13], Weber's trichrome stain for Microsporidia [14] and agar plate culture for *Strongyloides stercoralis*

[15]. Parasites cyst load were determined by examining stool sediment by haemocytometer.The differentiation of *E. histolytica* from *E. dispar* trophozoites in stool samples ELISA kit was used (E histolytica Test TechLab, Blacksburg, VA, USA) [16].

CD4, CD25 and Foxp3 expression in peripheral blood mononuclear cells (PBMCs) were analyzed by using a FACS calibur flow cytometer (Becton Dickinson: BD FACS CantoTM II flow cytometer, company BD Biosciences, San Jose, CA95131, USA). Flow cytometric analysis of cell surface CD4 and CD25 expression on PBMCs was performed by combining of a direct standard two-color immunofluorescence labeling antibodies strategy includes phycoerythrin (PE)-conjugated anti-CD4 monoclonal antibodies (mAbs) with fluorescein isothiocyanate (FITC)–labeled-anti-CD25mAbs(BD eBioscience Inc, San Diego, CA, USA). For intracellular staining, cells were subsequently treated with cell fixation and permeabilization reagents following the manufacturer's instructions, and then incubated with a phycoerythrin-cyanine 5 (RPE-CY5)–labeled anti-Foxp3, (eBioscience, San Diego, USA) and their appropriate isotype controls (Dakocytoformation, Denmark) for 30 min at 4 °C. Appropriate isotypic controls were used to determine specific binding for each fluorescent channel. T-regulatory cells were defined as the percentage of Foxp3+cells in CD4+CD25+ T cells.

2.1. Ethical consideration

Approval of Institutional Research Board (IRB) at faculty of Medicine, Mansoura University, was obtained with code number R/16.10.45. Parents of each patient made an informed verbal consent to participate in the study with a full right to withdraw at any time of research with assurance of confidentiality and anonymity of the data.

2.2. Statistical analysis

SPSS version 21was used to analyze the data. Testing of normality of data was done by one-sample Kolmogorov-Smirnov test. Qualitative data were presented as percentage and quantitative data as mean ± SD and range. ANOVA test was used to compare between two groups. p is significant if < 0.05 at confidence interval 95%.

3. Results

The most common parasitic infection (Figure 1) is *Entamoeba* (32.5%) followed by *Giardia* (19.2%). Table 1 show that there is a high significant difference between *Entamoeba* infection and control and between other parasite and control as regard CD4+CD25+Foxp3 level ($p \leq 0.001$). On the other hand, there is no significant difference between *Entamoeba* infection and other parasite. CD4+CD25+Foxp3 level higher in *Entamoeba* infection and other parasite than control.

Figure [1] Percentage of parasites in the studied group

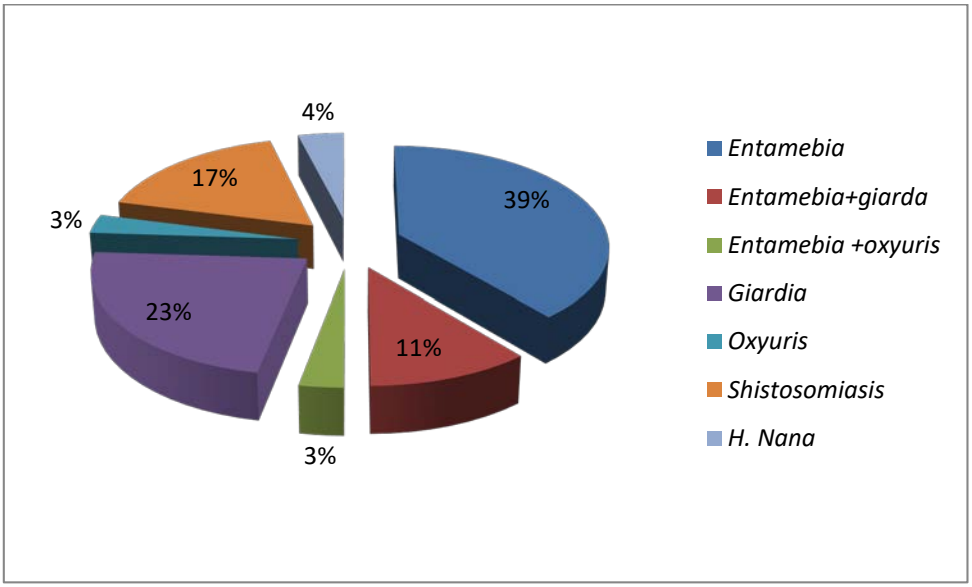

Table (1): Comparison between studied groups regarding CD4+CD25+Foxp3

CD4+CD25+Foxp3	***Entamoeba infection*(n=53)**	**Other parasite(n=47)**	**Control(n=40)**	**ANOVA p-value**
Mean±SD	7.79±3.91	7.62±2.59	2.43±0.53	F=24.513 p=≤.001**
Min-Max	3.10-14.90	4.20-15.10	1.70-3.30	
p-value	P_1= 0.782	p_2=≤.001**	p_3=≤.001**	

Data expressed asMean ± SD *F: ANOVA test* **high significant p ≤0.001

P1 comparison between *Entamoeba* infection and Other parasite
p2 comparison between *Entamoeba* infection and Control
p3 comparison between Other parasite and Control

Table 2 shows that there is high significant difference between severity of *Entamoeba* infection and CD4+CD25+Foxp3 level (p ≤0.001).
CD4+CD25+Foxp3 level is higher in severe *Entamoeba* infection than mild and moderate infection while there is no significant difference between moderate and severe *Entamoeba* infection (p=0.182).

Table (2): Relation between Entamoeba infection load andlevels ofCD4+CD25+Foxp3

CD4+CD25+Foxp3	Mild *Entamoeba* infection (n=22)	Moderate *Entamoeba* infection (n=9)	Severe *Entamoeba* infection (n=22)	ANOVA p-value
Mean±SD	5.35±0.84	7.03±4.11	10.54±4.04	F=15.36 p=≤.001**
Min-Max	4.20 -6.30	3.10 -12.30	5.30 -14.90	
p-value	P_1= 0.007 p_2=≤.001**		p_3= 0.182	

P1 comparison between Mild and Moderate infection
P2 comparison between Mild and sever infection
P3 comparison between Moderate and severe infection

4. Discussion

Intestinal amoebiasis can manifest as amoebic colitis in a small percentage of symptomatic patients however, mortality can reach 50% worldwide 8. The role of intraepithelial and lamina propria T lymphocytes in mediation of pathology and infection resolution are poorly understood [17].

In this study, CD4+CD25+Foxp3 levels were higher in *Entamoeba* infection and other parasite than control. This could be explained by fact that CD4+CD25+FoxP3+ Tcells secret IL-10 cytokine in a murine model of *E. histolytica* infection which can lead to colonic inflammatory Resolution [18,19].
Under inflammatory processes, Treg cells migrate into the intestinal mucosa under influence of CCR4 and CCR6, CCR9 chemokine receptors [20,21].
CCR9 chemokine receptor is induced by retinoic acid on CD4+T cells, enabling lymphocyte migration to the small intestine. Under inflammatory conditions, CD4+CD25+FoxP3+ Treg cells can be induced under influence of retinoic acid [22].

The interplay between natural regulatory T cells and intestinal epithelial cells (IEC) could stimulate IEC to mucin secretion and suppressing the release of proinflammatory cytokines and, thus leading to preserving epithelial barrier integrity and aborting *E. histolytica* infection [23].

This is also the scenario in schistosomasis *mansoni*, natural regulatory T cells produce IL-10 protecting the mice from the egg induced hepatocyte damage and prevent the resulting pathology induced mortality [24].

In this study, CD4+CD25+Foxp3 level higher in severe *Entamoeba* infection than mild and moderate infection while there is no significant difference between moderate and severe *Entamoeba* infection table 2.

Higher parasitic load were also reported in other parasites as *Plasmodium falciparum* with higher T regulatory cells by Minigo and his colleagues [25]; and Todryk and his colleagues[26] and concluded that there is associations between severe disease and exacerbated immune pathology determining the outcome of malaria infection.
T regulatory cells may be advantageous to the host in late infection (where parasitemia is low) by reducing the inflammatory reaction and thus lowering immune induced pathology [27]. On the contrary, if T regulatory cells mediate early suppression, could lead touncontrolled parasite growth, which may also lead to higher parasitic burdern and severe disease [28].

Effective antibilharizal therapy could decrease T regulatory cells owing to decreasing antigen shedding from egg and adult in human vasculatures [29]. In experimental mice infection, treatment with *S. mansoni* egg antigen (SEA) was shown to expansion of Foxp3 by upregulating TGF-ß on T cells and Th2 cells which remain important in egg mediated pathology [30].However, T regulatory cells can accumulate in targeted chronic infected tissues and so their number could be reduced in the peripheral blood. However being most accessible compartment, peripheral blood is the extensively used in human studies that evaluate Treg cell functions or numbers [31].

In conclusion, CD4+CD25+Foxp3 Tcells increased in parasitic infection including *Entamoeba histolytica* infection and their increase proportionally related to severity of *Entamoeba histolytica* infection so they may be implicated in silencing immunity to Entamoeba infection and persistence of infection.

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