ORIGINAL ARTICLE

CYTOKINE RESPONSE IN MONO AND MALARIA-*S.MANSONI* CO-INFECTED INDIVIDUALS IN FINCHA SUGAR ESTATE, WESTERN ETHIOPIA

Mebrate Dufera [1] Hans C.Aass [2], Nega Berhe [3] Beyene Petros [4], Berhanu Erko [5]*

ABSTRACT

***Background**: In Ethiopia, where malaria parasite and Schistosoma mansoni infections are coendemic, the general public is quite vulnerable to both malaria and S. mansoni infections singly and concomitantly. However, data about immunological effects are lacking. The aim of this study was to assess the reciprocal effects of malaria- S. mansoni co-infections with a particular emphasis on immunological interactions.*
***Methods:** A community-based cross sectional study was conducted in Finchaa Sugar Estate, western Ethiopia. Plasma was collected for cytokine assay and measurements of selected cytokines were performed using luminex IS 100 instrument. SPSS statistical software version 20 was used, and P-value <0.05 was reported as statistically significant.*
***Results:** Of the three groups of infections (malaria, S. mansoni and malaria- Schistosoma mansoni co-infections), the levels of IL-10 and IL-4 were higher in co-infected individuals than in mono-infected ones.*
***Conclusion:** This study showed that Plasmodium falciparum and S. mansoni co-infection reciprocally increase anti-inflammatory cytokine (IL-10). However, further studies are required to investigate how malaria and S.mansoni co-infections could reciprocally contribute to increased levels of anti-inflammatory cytokines IL-10 and IL-4.*

***Key words:** co-infection, cytokine, Finchaa Sugar Estate, Plasmodium falciparum, Plasmodium. vivax, Schistosoma mansoni, Ethiopia*

INTRODUCTION

Cytokines are vital intercellular communication molecules. These proteins carry signals or messages when they are released from one cell and subsequently sensed by another. According to (1), there is increasing evidence that immune mechanisms are involved in the pathogenesis of many parasitic infections. In most cases, a state of immunosuppression can be evidenced in chronic parasitic infections. This hyporesponsiveness to antigen-specific could be related to immunosuppressive cytokines (IL-10 and TGF-β) secreted by antigen presenting cells and regulatory T cells. For this study, systemic cytokines indicative of CD4+Tcell-mediated T helper (Th)1 (IFN-γ and TNF-α) and Th2-type (IL-4 and IL-10) responses were selected for investigation, as these are amongst the main immunological correlates of pathology and protective immunity to malaria parasite and schistosome infections (2,1,3,4).

The immune response to malaria parasite is complex and partially understood. Immunity to malaria develops slowly, and protection against the parasite occurs later than the protection against disease symptoms (5).

[1]Department of Biology, Wollega University, Post Box No: 395, Nekemte, Ethiopia, [2]Department of Medical Biochemistry, Oslo University Hospital, Ullevål, Norway, E-mail: H.C.Aass@medisin.uio.no, [3]Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Post Box No: 1176, Addis Ababa, Ethiopia, Centre for Imported and Tropical Diseases, Oslo University Hospital-Ulleval. E-mail: nega.berhe.belay@gmail.com, [4]Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Post Bo No: 1176, Addis Ababa, Ethiopia. E-mail: abule2002@gmail.com, [5]Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Post Box No: 1176, Addis Ababa, Ethiopia. E-mail: berhanue@yahoo.com
***Correspondence author:** Dr. Mebrate Dufera, Department of Biology, Wollega University, P.O.Box: 395, Nekemte, Ethiopia, E-mail: mebratedufera@gmail.com*

In endemic malaria areas, the prolonged carriage of *P.falciparum* triggers the development of acquired immunity that controls blood-stage parasitaemia, thereby reducing clinical symptoms and life-threatening complications in older children and adults (6).

The development of pathology in *Plasmodium* infection is associated with the imbalance of cytokines involved in the regulation of inflammatory responses (7). Although pro-inflammatory responses are associated with protective immunity to malaria during the early phases of infection, overproduction of Interferon-γ (IFN-γ) or tumor necrosis factor alpha (TNF-α) predisposes a subject to severe malarial pathology (8).

The regulatory responses indicated above seem to suppress immune responses and thereby allow parasite growth. They might also contribute to the control of inflammatory responses and prevent the onset of severe malaria. Indeed, the anti-inflammatory cytokines Interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) and the ratio of IL-10/TNF- α could have a protective effect against pathology, as suggested by (9). Acute *P. falciparum* infection is usually associated with an increase of INFγ and TNF, regarded as the markers of the T helper 1 (Th1) and pro-inflammatory response (10). This pro-inflammatory response is thought to be needed to impede the multiplication of the parasite and favor its clearance, both in human and animal models (11).

Helminth infections, common in developing countries, can result in a chronic state of immune activation that is characterized by a dominant Th2 type of cytokine profile, high IgE levels, and eosinophilia (12). In the murine model, *Schistosoma* egg deposition induces a type-2 immune response, which is characterized by the production of IL- 4, IL-5 and IL -13 cytokines that, in addition to IL-10, has been associated with the down-modulation of the initial type-1 immune response and granuloma formation (13,2).

Studies conducted by (14) suggested that IL-10 was an important cytokine in regulating the immune response and possibly controlling morbidity in human schistosomiasis mansoni and that the production of IFN- γ might be associated with resistance to infection. Egg-positive people had significantly higher levels of specific antibodies, IL-2, IFN-γ and IL-23. In contrast, egg-negative individuals had significantly higher circulating IL-10, IL-4, IL-13 and IL-21 that were detected with high frequency in all participants. When analyzed by age, IL-4 and IL-10 increased significantly as did schistosome-specific antibodies (15). Also, (16) additionally indicated that systemic cytokine levels rose with age as well as with schistosome infection and exposure.

Regarding host immunity, co-infection by malaria and schistosomiasis may have an important influence on the regulation of immune response associated with the development of infections and their respective morbidity (17). Most studies that examined naturally occurring co-infection in humans indicated that co-infection with malaria and schistosomiasis has an effect on the host, both in terms of pathology and immunological response (18). A study conducted by (19) showed that co-infections with schistosome and malaria parasites aggravated malaria severity as shown by increased parasitemia and severe gross pathology of the liver and the spleen in co-infected mice.

Cytokines contribute to both infection-related pathological processes and the development of protective immunity to malaria and schistosome parasites(3). IL -10 and IFN-γ are involved in isotype switching to protective IgG sub-classes in *Plasmodium* parasite infections (20), while IL-4, IL-5 and IL-10 appear to

be important for the development of resistance to schistosome infection (2). There is a growing body of evidence suggesting that there is a significant interaction in the development of protective immunity and pathology in individuals co-infected with these parasites (21,22,23,24) also further explained that during the early phase of *Plasmodium* parasite infection, Th1 and inflammatory cytokines, such as IFN-γ, IL-12 and TNF-α are important to control the first cycles of parasitaemia, and overproduction of IFN-γ or TNF-α predisposes a subject to severe malarial pathology, whereas the anti-inflammatory cytokines IL 10 and TGF-β could have a protective effect against pathology.

For example, acute infection with schistosomes increases the levels of Th1 cytokines and may help to control parasitaemia, while it may enhance the risk for severe malaria. In contrast, chronic infection with schistosomes will induce Th2 as well as regulatory cytokines, and may decrease the risk for severe malaria but increase the risk for early clinical malaria. The co-endemicity of these two tropical diseases has prompted investigation into the mechanisms of co-infection, particularly the competing immunological responses associated with each disease (25).

Higher levels of regulatory modulators are available amongst *S. mansoni* positive children compared to *S. mansoni* and malarial negative individuals, but if both cases are exposed to malaria, *S. mansoni* infection may augment the underlying anti-inflammatory reaction (26). However, data about malaria-*S. mansoni* co-infection and their reciprocal immunological effects that could serve as a guide in designing, developing and implementing intervention strategies to mitigate co-morbidity due to co-infection among the high risk groups are lacking. Therefore, the aim of this study was to assess the reciprocal effects of malaria-*S. mansoni* co-infections with emphasis on immunological interactions and provid an additional immunological data on the reciprocal effects of *P. falciparum* and *S. mansoni* co-infection.

SUBJECTS, MATERIALS AND METHODS

Study area and population

Study area: This study was done in Finchaa Sugar Estate, located in Finchaa valley, Oromia regional state, western Ethiopia (Figure 1).The area is about 325 km west of Addis Ababa and is situated between 9^0 30'N to 9^0 60' N latitudes and 37^0 10' to 37^0 30' E longitudes and at an altitude of about 1,350-1,600 m above sea level with the average annual rainfall of 1,300 mm. The Sugar Estate was cultivating more than 18,000 hectares of irrigated land using the sprinkle irrigation system and a producing of 10,000 kg of sugar per day. The Estate had about seven camps each with one elementary school and one community health agent and one health center in one of the villages (Agemsa).

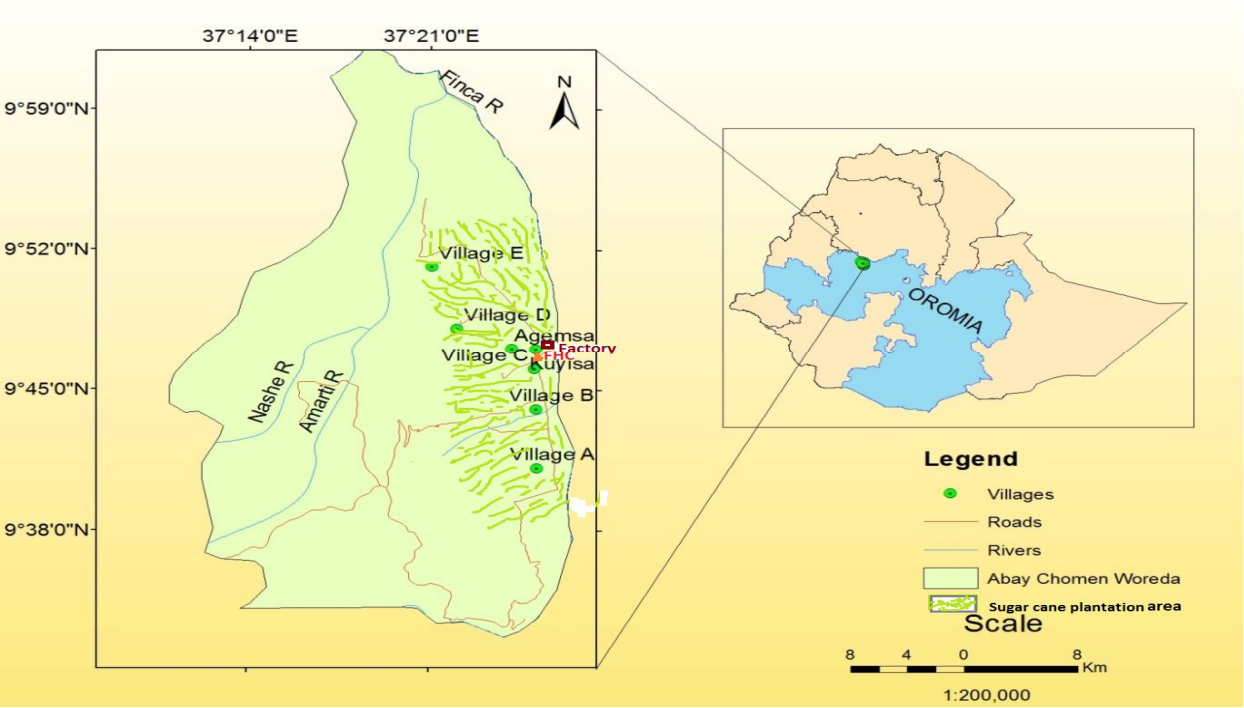

Fig 1 *Sketch-map of the study area -Finchaa Sugar Estate, western Ethiopia (Source: Garmin 72 GPS)*

Study population: The study populations were residents of the three purposively selected villages, Camp 7, Kuyissa and Agemsa. A study participant who fulfilled the inclusion criteria were selected from community lists, using the stratified random sampling methods from the three villages after informed consent/assent. In addition, about 50 individuals (non-endemic healthy controls) were selected randomly from Holleta town, 25km from Addis Ababa to the west.

Diagnostic techniques

Blood collection and plasma preparation for Cytokine measurement: For cytokine assays, three to four ml of venous blood was collected in vacuum K3 -EDTA tubes following the standard procedures. Plasma was separated from the peripheral blood mononuclear cells by Ficoll-Paque Plus density gradient centrifugation and transferred into two crovial tubes and kept at -20^{o} ^{C} in Finchaa Sugar health center for further cytokine assay. At the end of the day, every sample was packed in liquid nitrogen storage container and transported from the field to the Biomedical Sciences Research Laboratory in the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, and stored frozen at -70^{o}$_{C.}$

Four cytokines, namely, IFN -g , TNF-α, IL-10 and IL-4 were selected to be analyzed for all five arms of the study groups. A total of 250 plasma samples (malaria positive (n=50), *S. mansoni* positive (n=50), malaria-*S. mansoni* co-infection (n=50), endemic non -infected controls (n=50) and non-endemic non-infected controls (n=50) were packed in an ice box and sent to Oslo University hospital, Ulleval, Norway, through a research collaboration arrangement.

Determination of cytokine concentration: Plasma levels of the cytokines IFN-g, TNF-a, IL-10 and IL-4 were measured using a Luminex IS 100 instrument (Bio-Rad, Hercules, CA, USA) powered by the Bio-Plex Manager (version 6.0.1) software.

The plasma samples were thawed on ice, vortexed and spun at 10, 000xg for 10 min at 4^{O} C and the supernatant subsequently diluted three fold of the Bio -plex sample diluent). Fifty µL of the gently vortexed

diluted samples were mixed in the well together with IFN-g, TNF-a, IL-10 and IL-4 conjugated beads and incubated for 1 hour. The magnetic beads were then washed three times using a Bio-Plex Pro Wash Station (Bio Rad, Hercules, CA, USA) and 25 µL Biotin labelled reporter antibody was added and incubated on a IKA micro titre shaker (MTS) (IKA -Werke, GMBH and Co, Staufen, Germany) at 1,400 rpm for 30 sec and then 900 rpm for 30 min. The plate was washed, and the beads were incubated for 10 min with streptavidin bound phycoetrhythrin. The beads were then washed again prior to plate reading on the Luminex IS 100.

Treatment: All participants positive for malaria were treated with anti-malarial drugs. Also, individuals found positive for *S. mansoni* and STH infections were treated with a single dose of praziquantel 40 mg/kg and mebendazole 500 mg, respectively. All treatments were given free of charge under the supervision of a physicians at the health center according to the national treatment protocols (27).

Data analysis: Data were entered into a computer and validation was performed in Microsoft Excel 2007 spreadsheets and transferred to SPSS version 20.0 software for statistical analysis. Descriptive statistics were used to provide a clear picture of background variables. The frequency distribution of both dependent and independent variables was determined. Cytokine distributions were log transformed for analysis, and the geometric means were used for comparisons among the five study groups. All graphs were drawn using MS-Excel and all box-plots were drawn using SPSS version 20.0. To determine sample cytokine concentrations of a total of 250 plasma samples, (50 samples from each group) were analyzed for the respective cytokines: IFN-g, TNF-a IL-10 and IL-4. P-value <0.05 was reported as statistically significant.

Ethical Considerations: The study was approved by the Research Ethics Review Committee of the College of Natural Sciences, Addis Ababa University, and by the National Research Ethics Review Committee. To participate in the research project, written consent/assent was obtained from the participants.

RESULTS

Socio-demographic characteristics: A total of 810 participants, 415 (51.23%) male and 395 (48.77%) female, were included in the study. Of the total of 810 participants, 452 (55.81 %) harbored at least one parasitic infection each and 358 (44.20%) had none of the investigated parasitic infections. Among mono -infections, the most prevalent parasitic infection was *S. mansoni* 117 (14.44%), followed by malaria 104 (12.84%), malaria-*S. mansoni* co-infection 98 (12.10%) and other intestinal helminth parasites, such as Hookworm, *T.trichiura, A.lumbricoides, S.stercoralis* andTaenia spp. 96 (11.85%).Males were more infected (32.72%) than females (23.09%). As age increased, infection prevalence also decreased, and individuals within 5-9 and 10-14 age ranges were more affected than other age groups (Table 1).

Table 1: Prevalence of investigated parasitic diseases stratified by sex and age among study participants (n=810) at Finchaa Sugar Estate, western Ethiopia, 2012-2014

Sex	Uninfected	Parasite							Over all total n (%)
		Malaria n (%)	mal +Sm n (%)	Mal+OIHP n (%)	Sm n (%)	Sm+ OIHP n (%)	OIHP* n (%)	Total infected n (%)	
Male	164(20.25)	55 (6.79)	71(8.77)	172.10)	73(9.01)	5(0.62)	44(5.43)	265(32.72)	415(51.23)
Female	194(23.95)	49(6.05)	27(3.33)	9(1.11)	44(5.43)	6(0.74)	52(6.42)	187 (23.09)	395(48.77)
Total	358(44.20)	104(12.84)	98(12.10)	26(3.21)	117(14.44)	11(1.36)	96(11.85)	452(55.81)	810(100)
Age (Years)									
5-9	6(0.74)	3(0.37)	14(1.73)	5(0.62)	48(5.93)	8(0.99)	49(6.05)	127(15.68)	133(16.42)
10-14	31(3.83)	7(0.86)	19(2.35)	9(1.11)	35(4.32)	3(0.37)	37(4.57)	110(12.35)	141(17.41)
15-19	22(2.72)	18(2.22)	14(1.73)	8(0.99)	5(0.62)	0(0)	2(0.25)	47(5.80)	69(8.52)
20-24	56(6.91)	25(3.09)	23(2.84)	2(0.25)	12(1.48)	0(0)	4(0.49)	66(8.15)	122(15.06)
25-29	86(10.62)	15(1.85)	10(1.23)	2(0.25)	5(0.62)	0(0)	2(0.25)	34(4.20)	120(14.81)
³30	157(19.38)	36(4.44)	18(2.22)	0(0)	12(1.48)	0(0)	2(0.25)	68(8.40)	225(27.78)

***OIHP-**Other Intestinal Helminth Parasites (Taniea spp, T.trichiura, E.vermicularies, S.stercoralis)*

Cytokine levels in malaria positive and negative individuals: Elevated levels of both IFN-γ and TNF -α and low IL-10 and IL-4 cytokine levels were observed in malaria positive study participants (Table 2).

Table 2: Mean cytokine expression in malaria positive and negative individuals among study participants Finchaa Sugar Estate, western Ethiopia, 2012-2014

Cytokines (pg/ml)*	Malaria status	n	Mean	P
logIFN_γ	Positive	96	0.7034	0.000
	Negative	56	0.0813	
logTNF_α	Positive	99	1.2029	0.000
	negative	60	0.9448	
logIL_10	Positive	100	1.1997	0.000
	negative	60	1.9206	
logIL_4	Positive	93	0.8893	0.024
	negative	55	1.0151	

**Log transformed*

Cytokine levels in *Schistosoma mansoni* positive and negative individuals: The expressions of the cytokines, INF_γ and TNF-α were significantly (P<0.005) higher among *Schistosoma mansoni* positives compared to ***Schistosoma mansoni*** negatives, whereas IL-10 expression was higher among *Schistosoma mansoni* negatives compared to *Schistosoma mansoni* positives (Table 3).

Table 3: Expression of selected cytokines in *Schistosoma mansoni* positive and negative individuals among study participants Finchaa Sugar Estate, western Ethiopia, 2012-2014

Cytokines (pg/ml)*	*Schistosoma mansoni* Infection status	n	Mean	P
logIFN_γ	positive	83	0.744	0.001
	negative	69	0.250	
logTNF_α	positive	88	1.208	0.003
	negative	71	1.023	
logIL_10	positive	89	1.420	0.000
	negative	71	1.939	
logIL_4	positive	82	0.926	0.664
	negative	66	0.949	

**Log transformed*

Cytokine levels in malaria-*Schistosoma mansoni* co-infected individuals and non-infected controls: Malaria-*Schistosoma mansoni* co-infected individuals had relatively higher Th2 anti-inflammatory cytokines (IL-10 and IL-4) and lower Th1 inflammatory cytokines (IFN-γ and TNF-a) compared to mono-infected groups (Figure 2).

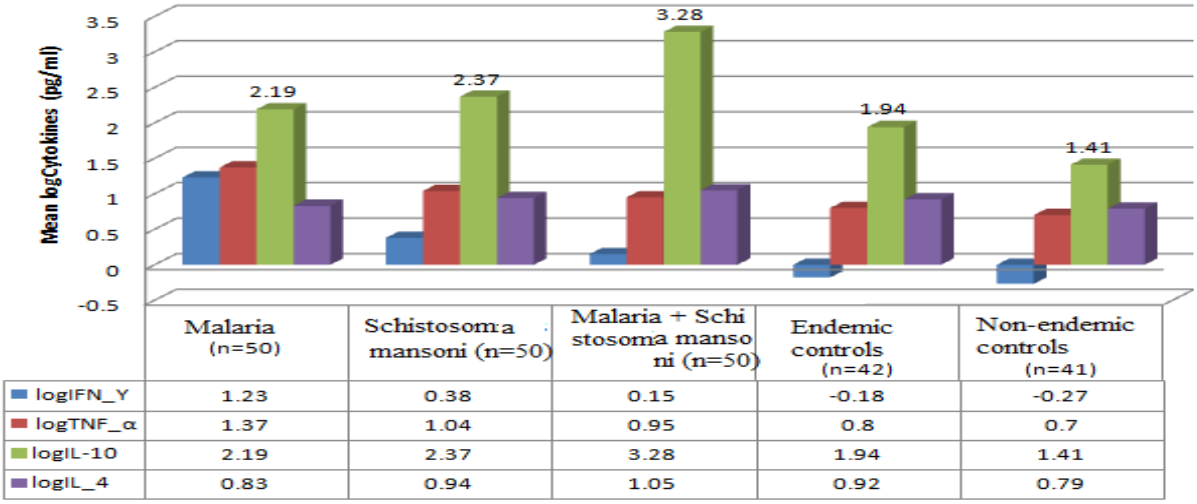

Figure 2: *Expression of cytokines among the five arms of the study groups, malaria-Schistosoma mansoni co-infection study participants at Finchaa Sugar Estate, western Ethiopia, 2012-2014*

DISCUSSION

This study showed that malaria and *S. mansoni* co-infection and co-morbidity reciprocally increased IL-10.

The study also showed that malaria positive patients had significantly higher mean levels of INF-γ and TNF-α than the uninfected, indicating that parasite induced inflammatory response results in the production of higher IFN-γ and TNF-α during acute malaria infections as shown by (28) that the low IL-10 plas-

ma levels associated with acute malaria could be attributed to the modulatory role of IL-10 during early infection of malaria parasites. This is in agreement with the findings by (4) who reported that in human malaria infections elevated levels of both IFN -γ and TNF-α were associated with acute malaria.

The high levels of IL-10 and IL-4 in *S.mansoni* egg-negative individuals and higher mean levels of IFN-γ and TNF-α in *S.mansoni* egg-positive individuals detected in the present study is similar to the report by (29) a study in northern Ethiopia. In this regard, (30) reported that low levels of inflammatory mediators were associated predominantly with uninfected individuals.

Regarding immune consequences of malaria and *S. mansoni* co-infection, it has been hypothesized that helminth infections interfere with immune responses to *P. falciparum* infection by inducing the production of Th2, characterized by the production of cytokines, such as IL-4, IL-5, IL-10, IL-13 as well as IgE (31,32,33) and cytokines contribute to the development of protective immunity to both malaria and *S. mansoni (3).*

In the present study, comparisons of cytokine profiles among the five arms of the study categories (malaria only, *S. mansoni* only, malaria- *S. mansoni* co-infection, endemic and non endemic controls) showed that malaria- *S. mansoni* co-infected individuals had higher Th2 anti-inflammatory cytokines (IL-10 and IL-4) and lower Th1 inflammatory cytokines (IFN-γ and TNF-a) when compared to mono-infected groups. This is supported by the findings of (24) who reported that chronic infection with *S.mansoni* and *S. mansoni* dominated malaria co-infection induces Th2 cytokines.

Cytokine quantification and analysis was made in collaboration with the University of Oslo, Faculty of Medicine, Oslo, Norway, using appropriate technologies which could be considered as the strengths of the paper, while, co-infected individuals were smaller in number compared to mono-infected ones which could be considered as the weaknesses of the attempt.

The study has provided an additional immunological data on the reciprocal effects of *P. falciparum* and *S. mansoni* co-infection. However, the immunological implications of this observation require further exploration and investigation, especially on how malaria and *S.mansoni* co-infections could reciprocally contribute to increased levels of anti-inflammatory cytokines.

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