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Ascaris lumbricoides Co-Infection does not Alter Clinical Evolution of Pulmonary Tuberculosis nor Th1/Th2/Th17 Cytokine Profile but May Reduce Tissue Damage by Decreasing Il-6 Levels

João Hugo Abdalla Santos[1,2], Samira Bührer-Sékula[2,3], Gisely Cardoso Melo[1,2], Marcelo Cordeiro-Santos[1,2], João Paulo Diniz Pimentel[4,6], Adriano Gomes-Silva[8], Allyson Guimarães Costa[1,2,4,7], Valeria Saraceni[8], Alda Maria Da-Cruz[5] and Marcus Vinícius Guimarães Lacerda[1,2,9*]

[1]*Programa de Pós-Graduação em Medicina Tropical, Universidade do Estado do Amazonas (UEA), Manaus, AM, Brazil*

[2]*Instituto de Pesquisa Clínica Carlos Borborema, Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), Manaus, AM, Brazil*

[3]*Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás (UFG), Goiânia, GO, Brazil*

[4]*Diretora de Ensino e Pesquisa, Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM), Manaus, AM, Brazil*

[5]*Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (Fiocruz-IOC); Rio de Janeiro, RJ, Brazil*

[6]*Laboratório de Pesquisa Clínica em Micobacterioses, Instituto Nacional de Infectologia Evandro Chagas, Fundação Oswaldo Cruz (Fiocruz-INI), Rio de Janeiro, RJ, Brazil*

[7]*Programa de Pós-Graduação em Imunologia Básica e Aplicada, Instituto de Ciências Biológicas, Universidade Federal do Amazonas (UFAM), Manaus, AM, Brazil*

[8]*Secretaria Municipal de Saúde, Prefeitura da Cidade do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

[9]*Instituto de Pesquisas Leônidas & Maria Deane, FIOCRUZ-Amazônia, Manaus, AM, Brazil*

[*]**Corresponding author:** Marcus Vinícius Guimarães Lacerda, Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), Av. Pedro Teixeira, 25, Dom Pedro, Manaus, AM, CEP 69040-000, Brazil, Tel: +55 92 99114-7633; E-mail: marcuslacerda.br@gmail.com

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Abstract

Immunological control of Mycobacterium tuberculosis (MTB) infection is dependent on the cellular immune response, mediated predominantly by Th1 type $CD4^+$ T cells. Polarization of the immune response to Th2 can inhibit the host immune protection against pathogens. Patients with tuberculosis (TB) co-infected with helminths show more severe pulmonary manifestations, a deficiency in the immune response against TB and an impaired response to anti-TB therapy. We evaluated the cellular immune response and the impact of the presence of *Ascaris lumbricoides* (Al) on the immune and clinical response in pulmonary TB patients. Ninety-one total individuals were included: 38 TB patients, 11 TB patients coinfected with Al and other helminths, 10 Al patients, and 34 non-infected control individuals. Clinical evolution of pulmonary tuberculosis was studied on 0, 30, 60, and 90 days post-diagnosed by MTB and Al. Furthermore, immune cells and plasmatic cytokine profiles in mono/co-infection MTB and Al by Flow Cytometry. There were no statistical differences for any of the evaluated parameters and the results indicated that Al infection does not appear to lead to significant clinical repercussions in the presentation and evolution of pulmonary tuberculosis. Unexpectedly, the association with Al also did not influence the Th1, Th2 and Th17 type responses, as well as the percentage of the T lymphocyte subpopulations. However, higher serum levels of IL-6 in TB patients may explain lung parenchyma damage.

Keywords: *Mycobacterium tuberculosis*; Helminths; Co-infection; Cells imunes; Cytokines

Introduction

World Health Organization (WHO) estimates that 10.4 million new cases of tuberculosis worldwide occur annually, with a total of 1.4 million deaths [1]. According to data from the Brazilian Ministry of Health (MS), Amazonas is the state with the highest incidence in Brazil, accounting for 67.2/100.000 inhabitants in 2016 [2].

The *Mycobacterium tuberculosis* (MTB) is a facultative intracellular organism, obligate aerobe, infecting primarily lungs via the aerogenic route [3]. MTB is a classic example of a pathogen for which an appropriate protective response is dependent on an effector immune response. The immune control of this infection is dependent on the cellular immune response, mediated predominantly by Th1-type CD4 (+) T cells [4].

The main Th1 inducing cytokines are IL-12 and IFN-. IL-12 is an important proinflammatory cytokine partially responsible for triggering a Th1 response, whereas IFN- activates macrophages, stimulates phagocytosis, phagosome maturation, production of reactive oxygen intermediates (ROS) and antigen presentation [5,6].

IFN-γ is considered the main cytokine in the control of infection and MTB elimination. It acts by activating the infected macrophage to produce reactive oxygen and nitrogen species, exerting their microbicidal role. In addition, IFN-γ stimulates macrophages to release TNF, a cytokine important for granuloma formation and for controlling the spread of infection [7].

An estimated 820 million people are infected with *Ascaris lumbricoides* (Al) around the world, especially in tropical and subtropical areas, representing a source of high morbidity and mortality in sub-Saharan Africa, America, China, and East Asia [8]. The typical immune response induced by intestinal helminths is dependent on Th2 cells, a response involving production of IL-4, IL-5, IL-10 and IL-13, as well as IgE production and mobilization and

expansion of non-specific, such as mast cells, eosinophils and basophils. Patients with chronic helminthic infections maintain persistent activation of the immune system by parasitic antigens associated with a decrease in the transduction of signals in the T cell, with consequent decrease of the immune response and energy [9].

Polarization of the immune response to a Th2 response can negatively influence a Th1 response by inhibiting the immune protection of the host against pathogens whose protective response is Th1 dependent. Th2 cells produce IL-10, which acts on macrophages by inhibiting activation of Th1 response and blocking the IL-12 synthesis. They also lead to an increase in CTLA-4 expression, decreased chemokine secretion and reduced late-type immune response.

Patients chronically infected with intestinal helminths showed a decrease in the number of CD4+ T cells and an increase in circulating CD8+ T cells with a high level of activation [10]. In addition, there is evidence that helminth infection can alter expression levels of TLRs and modulate downstream signaling following Toll-like receptor (TLR) stimulation [11]. Interestingly, some studies have reported that helminth infections can modulate the immune response by influencing the development of some co-infections such as malaria, tuberculosis (TB) and HIV [12–14]. It has been demonstrated that patients with TB who have helminthic infections show more severe pulmonary manifestations, a deficiency in the immune response against tuberculosis and an impaired response to anti-MTB therapy [13]. However, as the induction of the immune response can be differentiated according to the type of interaction that each species of helminth establishes with the host, it is difficult to determine the real influence of each helminth in the clinical manifestation of TB.

This study evaluated the cellular immune response in TB patients coinfected with intestinal helminths, focusing on ascaridosis, the most prevalent helminthic infection in urban areas of the Amazon, and the impact of this parasite on the immune and clinical response in pulmonary TB. The data presented in this study suggests MTB and Al co-infection did not influence the immune responses, as well significant clinical repercussions in the presentation and evolution of pulmonary tuberculosis.

Materials and Methods

Study population

93 patients were included in the study. Of these, 38 patients were diagnosed with pulmonary Tuberculosis (TB) by M. tuberculosis (MTB), 11 patients with MTB coinfected with *A. lumbricoides* (MTBAl), 10 patients with Al only, and 34 negative for both infections (control). All participants included were recruited at the same health unit and represented new cases of TB with positive baciloscopy result.

Sample collection and laboratory testing

Three fecal samples were collected on consecutive days using the Lutz, Kato-Katz and Baerman-Moraes techniques for parasitological diagnosis and cure control. All patients with positive serology for HIV were excluded. Blood, stool and sputum samples from MTB and MTBAl patients were studied on 0, 30, 60, and 90 days. Blood from Al patients and controls were collected only at the time of inclusion in the study. Ten mL of peripheral blood were used for hematology analyses, mononuclear cell phenotype, and cytokines plasma quantification.

Ethics approval and consent to participate

This project was approved by Ethical Committee from Fundação de Medicina Tropical Dr Heitor Vieira Dourado (#process 2030, CAAE: 0020.0.114.000-10), according to Declaration of Helsinki and Resolution 466/12 of the Brazilian National Health Council for research involving human subjects. All participants gave consent and signed the form. Patients with MTB were treated with the standard four-drug regimen (rifampicin, isoniazid, pyrazinamide and ethambutol) according to the recommendations of the Brazilian Ministry of Health. For therapeutic monitoring, we used quantification of bacillary load in sputum and semi quantification (images of parenchymatous changes visualized on the chest radiograph). Two independent observers analyzed the radiographs. Patients with helminthiasis were treated with mebendazole (500 mg/day for three days) or thiabendazole (500 mg/day for three days) [15,16].

Blood cells phenotypic analysis

EDTA whole blood samples (100 µL) was incubated with 5 µL monoclonal antibodies (mAbs). The cell populations were defined according to panels of the specific mAbs labeled with fluorochromes (BD Biosciences, San Diego, CA): anti-CD14-FITC/anti-CD80-APC (Monocytes); anti-CD3-PerCP/anti-CD16-FITC/anti-CD56-PE (NK and NKT Cells); anti-CD3-PerCP/anti-CD4-PE/anti-CD8-FITC/anti-CD69-APC (CD4+ and CD8+ T Cells); anti-CD19-PE (B cells); and anti-CD4-PE/anti-CD25-PerCP/anti-CD152-FITC/anti-FoxP3-APC (Regulatory T Cells - Treg). The cells were incubated for 30 min at room temperature followed erythrocyte lysis using BD FACS™ lysing solution (BD Biosciences, San Diego, CA) for 10 min. Leukocytes were washed in 0.01% sodium azide in PBS and ressuspended in 200 µL of FACS - FIX solution (10 g/L paraformaldehyde, 1% sodium cacodylate, 6,65 g/L sodium chlorate, 0,01% sodium azide). FACSCanto II® flow cytometer (Becton-Dickinson Company, San Jose, CA, USA) was used for data acquisition and storage as FCS files. 10,000/100,000 events were acquired to quantify whole blood cells. The FlowJo software (v. 9.4.1, TreeStar Inc., Ashland, OR, USA) was used for data analysis. The results were expressed as percentage of positive cells within the leukocyte or lymphocyte gate.

Cytokine measurements

Cytometric Bead Array (CBA) Human Th1, Th2, Th17 kit (BD® Biosciences, San Jose, CA, USA) used to measured plasmatic cytokine levels. The Processed was performed according to the manufacturer's protocol. The following cytokines were quantified: IL-6, TNF, IL-2, IL-10, IFN-γ IL-4 and IL-17A. The sample were acquired using a FACSCanto II® flow cytometer (Becton-Dickinson Company, San Jose, CA, USA. The mean fluorescence intensity (MFI) of each plasmatic cytokine was calculate used with FCAP-Array software (v.3.0.1, Soft Flow Inc., USA).

Statistical analyses

The comparison groups were described in terms of frequencies, proportions and measures of central tendency and dispersion. The categorical variables were compared using homogeneity of proportions, by means of the Pearson's chi-square or Fischer's exact test. The bacillary load was split into 3 categories, according to the acid-fast bacilli results (1+/3+, 2+/3+ and 3+/3+) and compared by McNemar's test. This part of the analysis was performed with the statistical package Stata (v.13, College Station, TX, USA). Comparative

analysis between groups were performed to evaluate the hemoglobin levels using the nonparametric Mann-Whitney test. Kruskal-Wallis test followed by Dunn's multiple comparison post-test were used to evaluate the quantitative analysis of non-parametric variables regarding phenotypes and cytokines. The values were expressed in medians and interquartile range. Spearman correlation test was performed to assess the association between the frequencies and levels of each cells and cytokine tested. Software GraphPad Prism (v.5, San Diego, CA, USA) was used for comparative analyzes. The correlation index (r) was used to categorize the correlation strength as weak ($r \leq 0.35$), moderate ($r \geq 0.36$ to $r \leq 0.67$), or strong ($r \geq 0.68$). Networks were assembled to assess the associations amongst the phenotypes and cytokines. Significant correlations were compiled using the open access software Cytoscape v3.3 (Cytoscape Consortium, San Diego, CA), as previously reported [17]. The significance level was established at 5% for all tests.

Results

Demographic, clinical and laboratorial compendium of the study population

Co-infection with *Ascaris lumbricoides* does not alter the clinical evolution of pulmonary tuberculosis, but may influence the severity of pulmonary lesions. There was homogeneity of the patients studied with no statistically significant differences regarding demographic data and clinical/laboratory characteristics. However, 28% (7/25) of the MTB patients presented radiological evidence of an advanced degree of pulmonary parenchyma involvement, while none of the 11 MTBAl patients had more severe disease. In addition, more than half of MTBAl patients were paucibacillary (54.6%, 6/11), in contrast to a lower frequency observed among MTB cases (34.2%, 13/38) (Table 1).

	MTB (n[a]=38)	MTBAl (n=11)	p-value
Demographic data			
Males (%)	71.1	63.6	0.638
Age (median)	41	45	0.231
Symptoms			
Cough (%)	89.5	100	0.261
Chest pain (%)	86.8	90.9	0.717
Weight loss (%)	86.8	90.9	0.717
Fever (%)	84.2	81.8	0.85
Dyspnea (%)	60.5	72.7	0.46
Sweating (%)	52.6	72.7	0.236
Asthenia (%)	47.4	63.6	0.342
Abdominal pain (%)	29	27.3	0.914
Diarrhea (%)	18.4	18.2	0.986
Lung bleeding (%)	13.2	9.1	0.717
Chest radiograph			
Non-advanced degree[b] (n)	18	11	0.076
Advanced degree (n)	7	0	_
Bacillary load			
Paucibacillary (1+/3+++)	34.2	54.6	<0.001[c]
Intermediate (2++/3+++)	39.5	18.2	
Multibacillary (3+++/3+++)	26.3	27.2	

Note: a: n=number; b: images "non-advanced degree" include the minimum and moderate change criteria; c: McNemar test.

Table 1: Baseline demographic and clinical characteristics, laboratory tests and chest radiograph of patients with *Mycobacterium tuberculosis* (MTB) or co-infected with *Ascaris lumbricoides* (MTBAl).

Hemoglobin post-diagnosis MTB and Al

Simultaneous remission of the main MTB-associated symptoms such as cough, chest pain, weight loss and fever, occurred similarly from the second month of follow-up in both groups (MTB and MTBAl). Furthermore, there was no difference in the percentage of blood cells, in the four evaluations of hematological alterations. Hemoglobin recovery (mean) were faster in the group with MTB, although the difference was not significant (Figure 1).

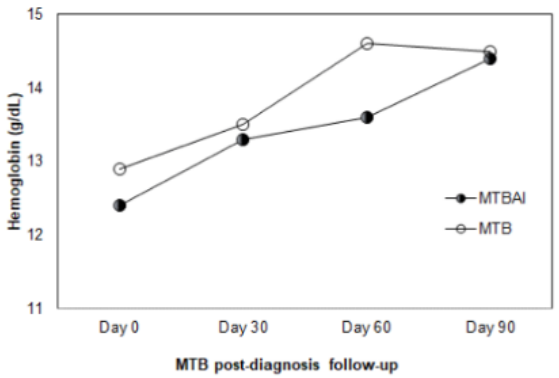

Figure 1: Hemoglobin levels among the MTB post-diagnosis follow-up. Data is expressed as median in g/dL. Statistical analyses were performed by the Mann-Whitney test.

Phenotypic features of immune cells

The global analysis of the cellular immunophenotyping data of the studied groups are summarized in Figure 2. The results showed a significant increase in the proportion of monocyte, activated $CD4^+CD69^+$ T and Treg cells in MTB patients when compared to the control group (Figures 2A, 2G and 2I). There was no statistical difference for any of the parameters evaluated when co-infected patients (MTBAl) were compared to cases of MTB and Al mono-infection or control.

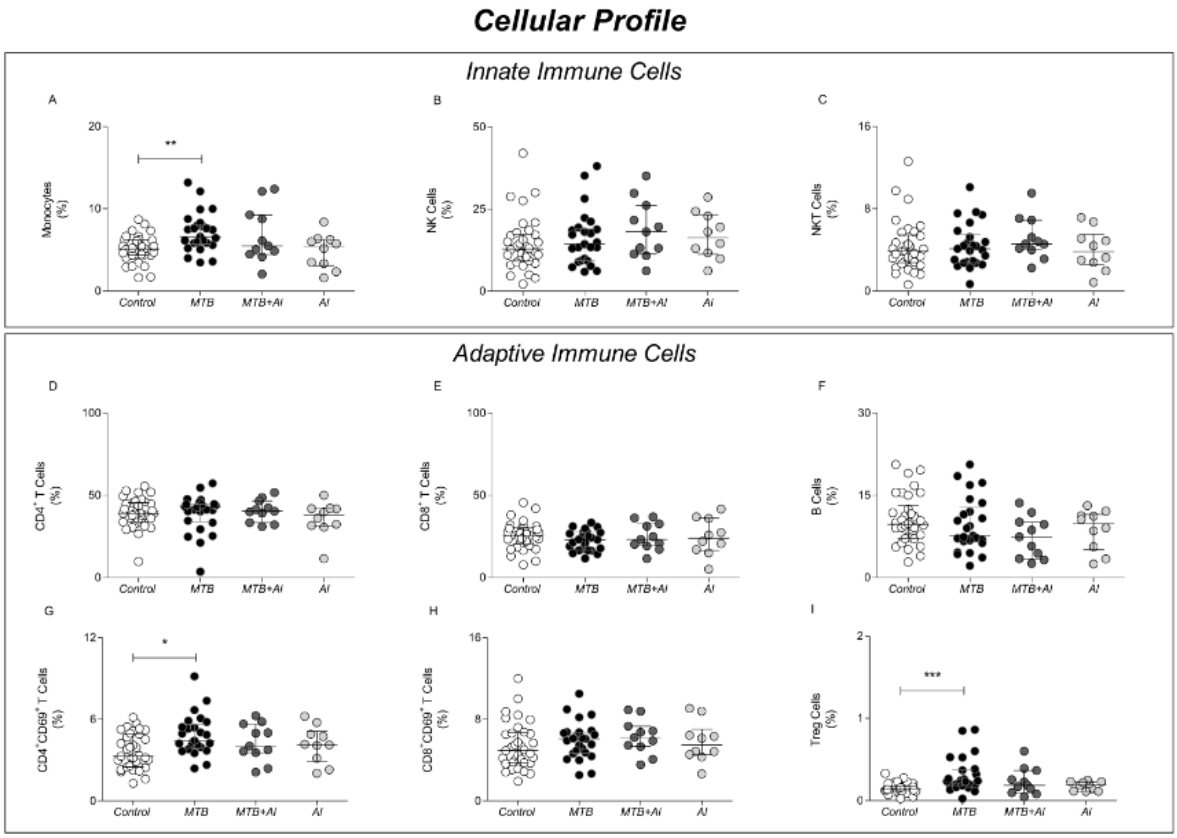

Figure 2: Immune cellular profile in Control, MTB, MTBAl and Al patients enrolled in the study. Frequency of circulating innate (monocytes, NK and NKT cells) and adaptive cells (CD4+, CD8+, Treg, CD4+CD69+ and CD8+CD69+ T-cell subsets and B cells) categorized into groups referred to as Control (○), MTB (●), MTBAl (●) and Al (○). Data is expressed as median ± IQR in percentile. Statistical analyses were performed by the Kruskal-Wallis test, followed by Dunn´s test to compare pairs. "*", "**"and "***" denote p<0.05, p<0.001 and p<0.0001 respectively.

Plasmatic cytokine profiles in mono/co-infection MTB and Al

Analysis of cytokine profiles in MTB, MTBAl and Al patients are shown in Figure 3. Elevated levels of IL-6 and IFN-γ were observed in MTBAl co-infection cases compared to control subjects (Figures 3A and 3E). In addition, a significant increase in IL-6 was observed in MTB patients compared to Al and control groups (Figure 3A). Furthermore, low concentrations of IL-17A were observed in cases of monoinfection MTB in relation to the Al group (Figure 3G).

Interactions of immune cells and plasmatic cytokines in co-infection MTBAl

Data analysis demonstrate that MTBAl patients presented a cell and cytokine network similar to those exhibited by MTB monoinfection (Figure 4). Although this similarity was noted between the interaction networks in both groups, MTBAl patients presented a response profile with greater interactions between the regulatory profile cells (Treg and NKT). In addition, proinflammatory and Th1 profile cytokines (TNF and IFN-γ) have less interactions with the other components evaluated.

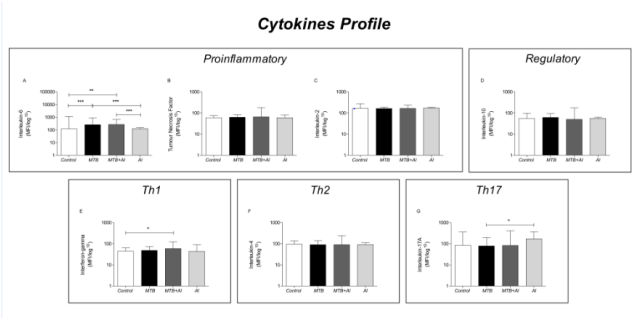

Figure 3: Cytokine profiles in Control, MTB, MTBAl and Al patients enrolled in the study. Levels of plasmatic cytokines of IL-6, TNF, IL-2 (Proinflammatory), IL-10 (Regulatory), IFN-γ (Th1), IL-4 (Th2) and IL-17A (Th17) categorized into groups referred to as Control (□), MTB (■), MTBAl (■) and Al (□). Data is expressed as median ± IQR in MFI. Statistical analyses were performed by the Kruskal-Wallis test, followed by Dunn´s test to compare pairs. "*", "**"and "***" denote p<0.05, p<0.001 and p<0.0001 respectively.

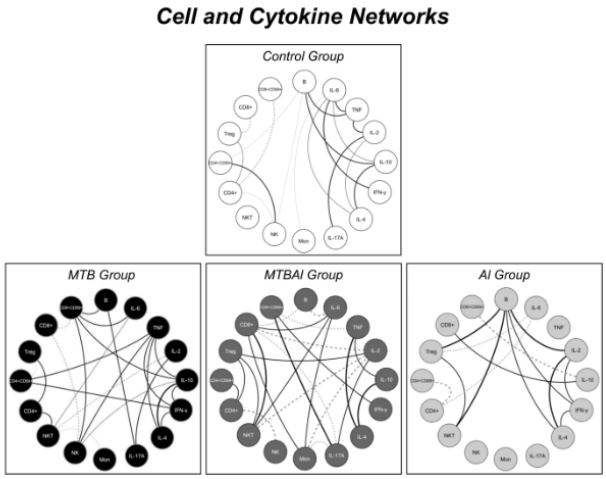

Figure 4: Cell and Cytokine networks in control, monoinfection and coinfection categorized into groups referred as Control (nodes white circles), MTB (nodes black circles), MTBAl (nodes dark gray circles) and Al (nodes light gray circles). Significant Spearman's correlations at $p<0.05$ were represented by connecting edges to highlight positive (thick continuous line) or negative (thick dashed line). The correlation index (r) was used to categorize the correlation strength as weak ($r \leq 0.35$), moderate ($r \geq 0.36$ to $r \leq 0.67$), and strong ($r \geq 0.68$).

Discussion

This study characterized the co-infection of pulmonary MTB with *A. lumbricoides*, including clinical aspects and immune response.

Surprisingly, the association of MTB with Al did not influence the Th1, Th2 and Th17 responses or the percentage of innate and adaptive cells subpopulations. *A. lumbricoides* in patients co-infected with MTB and the degree of involvement of the pulmonary parenchyma of TB patients differed from a previous study in a cohort where *S. stercoralis* was the most frequent helminth, in which advanced-grade lung injury was more frequent in co-infected patients [18]. However, a more recent study also with frequent *S. stercoralis* co-infection found no association between TB and infection with any of the prevalent helminths, however *Schistosoma mansoni* infection was associated with low bacillary load and a tendency for fewer pulmonary cavitations [14]. Similarly, in our study co-infected patients did not present advanced degree of lung injury. This may indicate that, depending on the helminth species, a more or less favorable clinical expression of pulmonary TB may occur.

After treatment for TB, mean hemoglobin levels showed a slower recovery in coinfected patients, but the difference was not statistically significant. Helminth co-infection in malaria patients has been reported to lead to erythropoiesis [12]. These results support the idea that helminths aggravate chronic anemia associated with infectious diseases.

A significant increase in the percentage of monocyte, activated $CD4^+CD69^+$ T and Treg cells was shown in MTB patients compared to healthy individuals controls. Several studies have demonstrated an increase of Tregs in TB which are believed to be related to the role of the adaptive immune system to restrict MTB. Tregs are able to delay the initiation of effector T cells, probably prolonging the phase of bacterial expansion. Thus, T-cells recognizing MTB-derived antigens specifically and potently restrict protective immune responses during TB [19–21].

Some studies have demonstrated an increase in monocyte subpopulations in individuals with MTB, especially in the neutrophils subpopulations, however, the role of these subpopulations in MTB is not yet clear [22,23]. The elevation of activation levels in $TCD4^+$ observed in cases with TB occurred in coinfected patients (MTBAl), although not significant.

IL-6 may be related to lung parenchyma lesion processes [24]. The data from the present study supports this hypothesis since the highest serum levels of IL-6 were observed in cases of TB, together with a higher frequency of involvement of the lung parenchyma with advanced degree when compared to the MTBAl group. Co-infected patients (MTBAl) had lower IL-6 levels when compared to MTB cases, although the difference was not significant. These results may indicate a possible immunomodulation at IL-6 levels due to the presence of Al [25–27]. IL-6 is crucial for resistance against MTB, and is essential for the generation of Th1 and Th17 [24]. This result corroborates the fact that 100% of MTBAl coinfected patients did not present an advanced degree of pulmonary parenchymal lesion. The reduction of IL-17A was significant in cases of MTB with and without Al, indicating that the absence of this cytokine should favor the infectious process.

The present study demonstrates the interactions of immune cells and cytokines in MTB and Al monoinfection and co-infection (MTBAl). The observed similarity between the interactions present in the networks of the MTB and MTBAl groups demonstrate that the pathophysiological events of the MTB infection prevail in the coinfection. In addition, our data suggest that Al co-infection produces a less inflammatory response profile, with greater participation of regulatory cells (Treg and NKT), which could explain the lower involvement of the pulmonary parenchyma in MTBAl patients. A possible beneficial effect of Al in MTB co-infections is further supported by the higher frequency of paucibacillary in these patients. The absence of significant changes in serum cytokines and blood cells could be related to the fact that these MTB and Al infections did not cause an expressive systemic repercussion in individuals.

The results may have been influenced by some limitations of the study, such as the number of patients studied; the non-assessment of polyparasitism in patients with TB for comparison purposes; the indication of treatment for helminths at the time of diagnosis, not allowing us to evaluate their immunomodulation along the clinical follow-up, and the non-genetic evaluation of the groups for immune factors. On the other hand, due to the challenges in recruiting these cases, especially to form a prospective cohort of three months with many parameters evaluated, the design of this study was quite complex and it is the first report of this kind. In addition, the strategy to study co-infection with only one helminth of great epidemiological importance due to its high prevalence as is the case of Al, allows to infer that only the immunopathogenic consequences of this parasite should be responsible for possible observed differences between groups.

Conclusion

In summary, our results suggest that Al infection does not lead to significant clinical repercussions in the presentation and evolution of

pulmonary TB. Interestingly, it may have a decreasing effect on lung parenchymal injury as well as bacillary load, and IL-6 may be involved in this mechanism. Unexpectedly, the association with Al did not influence the Th1, Th2 and Th17 response as well as the percentage of T lymphocyte subpopulations. Therefore, the evaluation of the specific immune response to ascaris and mycobacteria antigens should be a new approach to understand this association. Thus, a future perspective could be to study the specific antigen response and regulatory cells *in situ* lung tissue.

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Authors' Contributions

JHAS collected the clinical data and followed the patients. JPP and AGC carried out the immunologic studies and participated in the acquisition of data. VS, AMC, AGS, AGC and SBS participated in the analysis and interpretation of data and drafting and revising the manuscript. MVGL, GCM and MCS conceived the study and participated in its design, coordination and drafting the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This project was approved by Ethical Committee from Fundação de Medicina Tropical Dr Heitor Vieira Dourado (#process 2030, CAAE: 0020.0.114.000-10), according to Declaration of Helsinki and Resolution 466/12 of the Brazilian National Health Council for research involving human subjects. All participants gave consent and signed the form.

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