

## Major Article

# *Ascaris lumbricoides* coinfection reduces tissue damage by decreasing IL-6 levels without altering clinical evolution of pulmonary tuberculosis or Th1/Th2/Th17 cytokine profile

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### Abstract

**Introduction:** Immunological control of *Mycobacterium tuberculosis* infection is dependent on the cellular immune response, mediated predominantly by Th1 type CD4<sup>+</sup> T cells. Polarization of the immune response to Th2 can inhibit the host immune protection against pathogens. Patients with tuberculosis coinfecting with helminths demonstrate more severe pulmonary symptoms, a deficiency in the immune response against tuberculosis, and an impaired response to anti-tuberculosis therapy. **Methods:** We evaluated the cellular immune response and the impact of the presence of *Ascaris lumbricoides* on the immune and clinical response in pulmonary tuberculosis patients. Ninety-one individuals were included in the study: 38 tuberculosis patients, 11 tuberculosis patients coinfecting with *Ascaris lumbricoides* and other helminths, 10 *Ascaris lumbricoides* patients, and 34 non-infected control individuals. Clinical evolution of pulmonary tuberculosis was studied on 0, 30, 60, and 90 days post-diagnosis of *Mycobacterium tuberculosis* and *Ascaris lumbricoides*. Furthermore, immune cells and plasma cytokine profiles were examined in mono/coinfection by *Mycobacterium tuberculosis* and *Ascaris lumbricoides* using flow cytometry. **Results:** There were no statistical differences in any of the evaluated parameters and the results indicated that *Ascaris lumbricoides* infection does not lead to significant clinical repercussions in the presentation and evolution of pulmonary tuberculosis. **Conclusions:** The association with *Ascaris lumbricoides* did not influence the Th1, Th2, and Th17 type responses, or the proportions of T lymphocyte subpopulations. However, higher serum levels of IL-6 in tuberculosis patients may explain the pulmonary parenchymal damage.

**Keywords:** *Mycobacterium tuberculosis*. Helminths. Coinfection. Cellular immunity. Cytokines.

### INTRODUCTION

World Health Organization (WHO) estimates 10.4 million new annual cases of tuberculosis (TB) globally, with a total of 1.4 million deaths<sup>1</sup>. According to data from the Brazilian

Ministry of Health (MS), Amazonas is the state with the highest incidence of TB in Brazil, accounting for 67.2/100,000 inhabitants in 2016<sup>2</sup>.

*Mycobacterium tuberculosis* (MTB) is a facultative intracellular organism, an obligate aerobe that primarily infects the lungs via the aerogenic route<sup>3</sup>. MTB is a prime 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<sup>+</sup> T cells<sup>4</sup>.

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The major Th1-inducing cytokines are IL-12 and IFN- $\gamma$ . IL-12 is an important pro-inflammatory cytokine partially responsible for triggering a Th1 response, whereas IFN- $\gamma$  activates macrophages, and stimulates phagocytosis, phagosome maturation, production of reactive oxygen intermediates (ROS), and antigen presentation<sup>5,6</sup>.

IFN- $\gamma$  is considered the primary cytokine controlling infection and MTB elimination. It acts by activating the infected macrophage, resulting in production of reactive oxygen and nitrogen species, which exert microbicidal role. In addition, IFN- $\gamma$  stimulates macrophages to release TNF, a cytokine which significantly affects granuloma formation and spread of infection<sup>7</sup>.

An estimated 820 million people are infected by *Ascaris lumbricoides* (Al) globally, especially in tropical and subtropical areas, and it represents a cause of high morbidity and mortality in sub-Saharan Africa, America, China, and East Asia<sup>8</sup>. The typical immune response induced by intestinal helminths is dependent on Th2 cells, involving the production of IL-4, IL-5, IL-10, and IL-13, as well as IgE production, mobilization, and expansion of non-specific immune cells, such as mast cells, eosinophils, and basophils. Patients with chronic helminthic infections demonstrate persistent activation of the immune system by parasitic antigens associated with a decrease in the transduction of signals in the T cell, and consequent decrease in immune response and anergy<sup>9</sup>.

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 IL-12 synthesis. Additionally, they increase CTLA-4 expression, decrease chemokine secretion, and reduce delayed immune response.

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

This study evaluates the cellular immune response in TB patients coinfecting with intestinal helminths, focusing on ascariidiosis, the most prevalent helminthic infection in the

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 that MTB and Al coinfection did not influence the immune responses. However, it highlights significant clinical repercussions in the presentation and evolution of pulmonary tuberculosis.

## METHODS

### Study Population

Ninety-three patients were included in the study. Of these, 38 patients were diagnosed with pulmonary tuberculosis (TB) by *M. tuberculosis* (MTB); 11 patients with MTB were coinfecting with *A. lumbricoides* (MTBAI), 10 patients with Al only, and 34 were negative for both infections (control group). All participants included were recruited at the same health unit and represented new cases of TB with positive bacilloscopy results. Furthermore, posterior-anterior chest radiographs were taken at the radiology department of Fundação de Medicina Tropical Dr Heitor Vieira Dourado (FMT-HVD) as a part of the clinical evaluation of patients diagnosed with TB.

### 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 MTBAI patients were studied on 0, 30, 60, and 90 days post-diagnosis. Blood from Al patients and controls were collected only at the time of inclusion in the study. Ten milliliters of peripheral blood were used for hematology analyses, mononuclear cell phenotyping, and cytokine plasma quantification.

### Ethics Approval and Consent to Participate

This project was approved by Ethical Committee from FMT-HVD (#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 provided consent through a signed 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 alterations 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)<sup>15,16</sup>.

### Blood Cell Phenotypic Analysis

EDTA whole blood samples (100  $\mu$ L) were incubated with 5  $\mu$ L monoclonal antibodies (mAbs). The cell populations were defined according to panels of the specific mAbs labeled with fluorochromes (BD Biosciences, San Diego, CA): Monocytes (anti-CD14-FITC [Clone: M5E2] and anti-CD80-APC [Clone: BB1]), NK and NKT cells (anti-CD3-PerCP [Clone: SK7],

anti-CD16-FITC [Clone: 3G8], and anti-CD56-PE [Clone: B159]), T cell subsets (anti-CD3-PerCP [Clone: SK7], anti-CD4-PE [Clone: RPA-T4], anti-CD8-FITC [Clone: RPA-T8], and anti-CD69-APC [Clone: FN50]), B cells (anti-CD19-PE [Clone: HIB19]), and regulatory T cells (anti-CD4-PE [Clone: RPA-T4], anti-CD25-PerCP [Clone: M-A251], and anti-FoxP3-AF647 [Clone: 259D/C7]). The cells were incubated for 30 min at room temperature, followed by erythrocyte lysis using BD FACSTM lysing solution (BD Biosciences, San Diego, CA) for 10 min. Leukocytes were washed in 0.01% sodium azide in PBS and resuspended in 200  $\mu$ L 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 as previously described<sup>17</sup>.

### Cytokine Measurements

Cytometric Bead Array (CBA) Human Th1, Th2, Th17 kit (BD® Biosciences, San Jose, CA, USA) was used to measure plasma cytokine levels according to the manufacturer's protocol. The following cytokines were quantified: IL-6, TNF, IL-2, IL-10, IFN- $\gamma$ , IL-4, and IL-17A. Briefly, several beads (microspheres) of known size, with capture antibody and distinct fluorescence intensity, were used to detect various soluble cytokines simultaneously. Beads were homogenized with plasma samples, and conjugate antibodies with fluorochrome PE (phycoerythrin) were used for detection of the cytokines. Captured bead + sample cytokine + detection antibody complexes were quantified by FACSCanto II® flow cytometer (Becton-Dickinson Company, San Jose, CA, USA) at Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM). The mean fluorescence intensity (MFI) of each plasmatic cytokine was calculated using 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 and bacillary load (split into 3 categories, according to the acid-fast bacilli results [1+/3+, 2+/3+, and 3+/3+]) were compared using homogeneity of proportions by Fischer's exact test. 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, was 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 of the cells and cytokines tested. Analyses were performed with the statistical packages Stata (v.13, College Station, TX, USA) and GraphPad Prism

(v.5, San Diego, CA, USA). 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<sup>18</sup>. The significance level was established at 5% for all tests.

## RESULTS

### Demographic, Clinical, and Laboratorial Compendium of the Study Population

Coinfection with *Ascaris lumbricoides* does not alter the clinical evolution of pulmonary tuberculosis, though it may influence the severity of pulmonary lesions. There was homogeneity in the patients studied with no statistically significant differences in demographic data or clinical/laboratory characteristics (**Table 1**). However, 28% (7/25) of the MTB patients presented radiological evidence of an advanced degree of pulmonary parenchymal involvement, while none of the 11 MTBAI patients presented greater severity of the disease. In addition, more than 50% of MTBAI patients were paucibacillary (54.6%, 6/11), in contrast to a lower frequency observed among MTB cases (34.2%, 13/38) (**Table 1**).

### Hemoglobin Post-Diagnosis MTB and AI

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

### Phenotypic Features of Immune Cells

The global analysis of the cellular immunophenotyping data of the studied groups is summarized in **Figure 2**. The results indicate a significant increase in the proportion of monocytes, activated CD4<sup>+</sup>CD69<sup>+</sup> T, and regulatory T cells (Treg) in MTB patients when compared to the control group (**Figure 2A**, **Figure 2G**, and **Figure 2I**). There was no statistical difference in any of the evaluated parameters when coinfecting patients (MTBAI) were compared to patients with MTB, AI mono-infection, or those belonging to the control group.

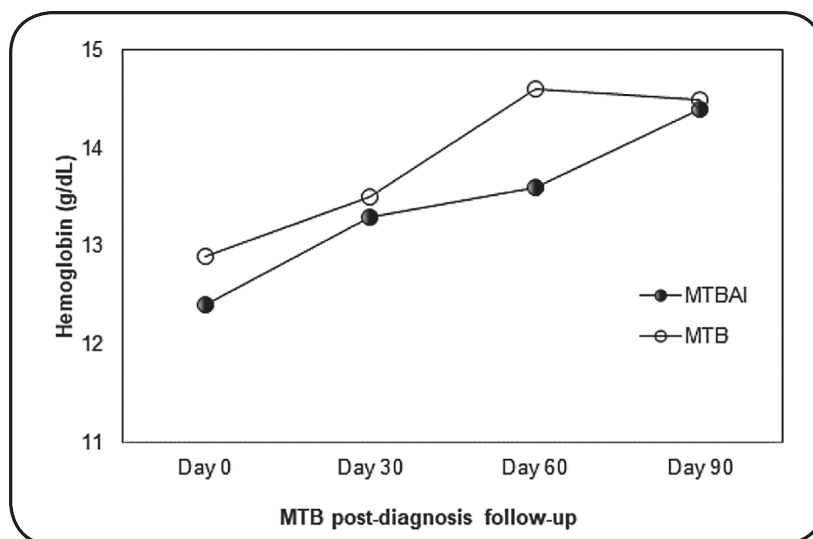
### Plasma Cytokine Profiles in mono/coinfection MTB and AI

Cytokine profile analysis in MTB, MTBAI, and AI patients are presented in **Figure 3**. Compared to control subjects, IL-6 and IFN- $\gamma$  levels were elevated in MTBAI coinfection cases (**Figure 3A** and **Figure 3E**). In addition, compared to AI and control groups, a significant increase in IL-6 was observed in MTB patients (**Figure 3A**). Furthermore, compared to the AI group, low concentrations of IL-6 were observed in MTBAI and IL-17A in cases of MTB mono-infection (**Figure 3G**).

**TABLE 1:** Baseline demographic, clinical and laboratorial characteristics of patients with MTB and MTBAI.

	MTB (n=38)	MTBAI (n=11)	<i>p-value</i> <sup>b</sup>
<b>Demographic data</b>			
Males (%)	71	64	0.638
Age (median [IQR])	41 [28-49]	45 [23-57]	0.146
<b>Symptoms</b>			
Cough (%)	90	100	0.261
Chest pain (%)	87	91	0.717
Weight loss (%)	87	91	0.717
Fever (%)	84	82	0.850
Dyspnea (%)	61	73	0.460
Sweating (%)	53	73	0.236
Asthenia (%)	47	64	0.342
Abdominal pain (%)	29	27	0.914
Diarrhea (%)	18	18	0.986
Lung bleeding (%)	13	9	0.717
<b>Chest radiograph</b>			
Non-degree <sup>a</sup> (%)	34	0	
Non-advanced degree (%)	47	100	<b>0.007</b>
Advanced degree (%)	19	0	
<b>Bacillary load</b>			
Paucibacillary (1+/3+++) <sup>a</sup> (%)	34	55	
Intermediate (2++/3+++) <sup>a</sup> (%)	40	18	0.362
Multibacillary (3+++/3+++) <sup>a</sup> (%)	26	27	

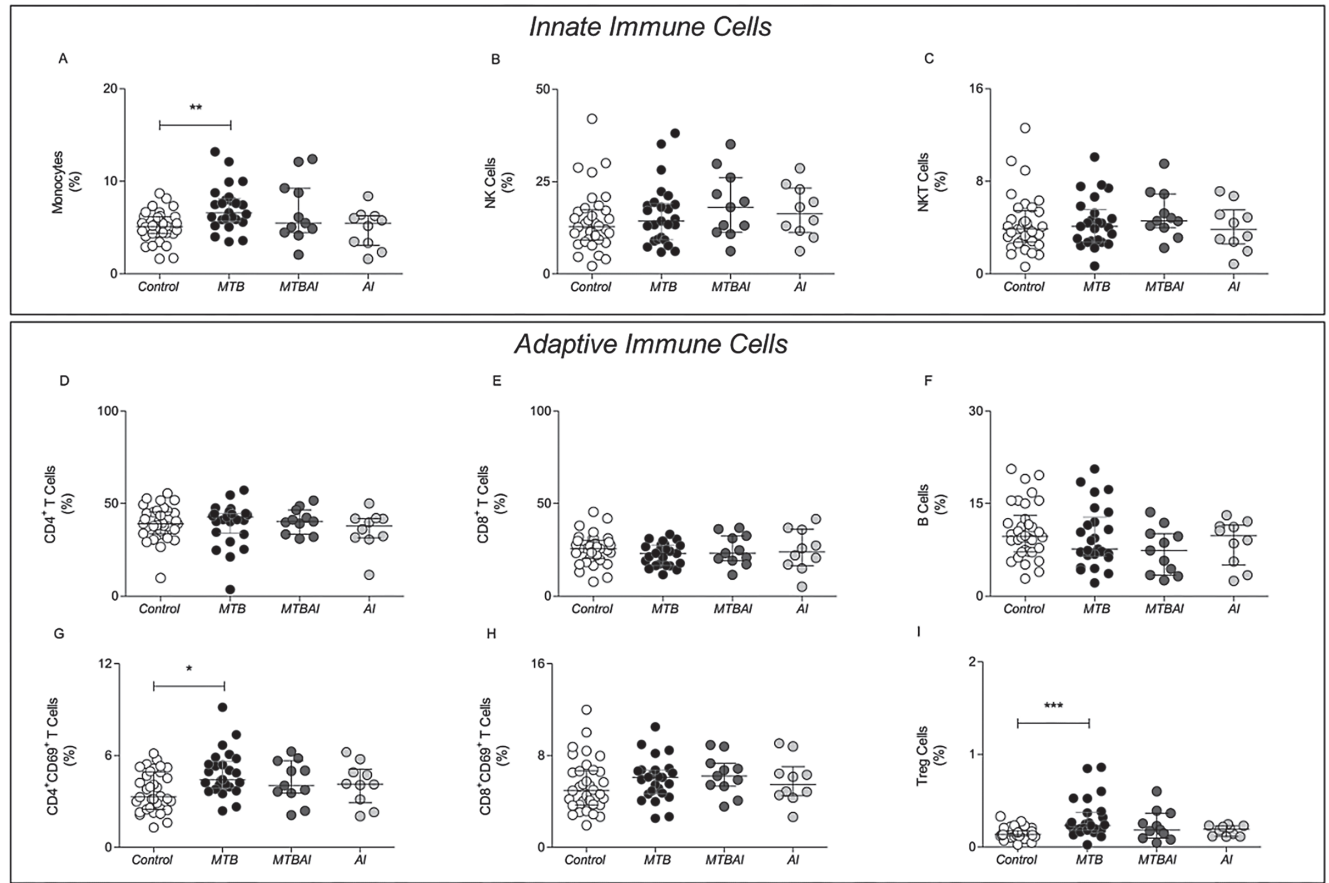
a: images "non-advanced degree" include the minimum and moderate change criteria; b: Fisher's exact or Mann-Whitney test.



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

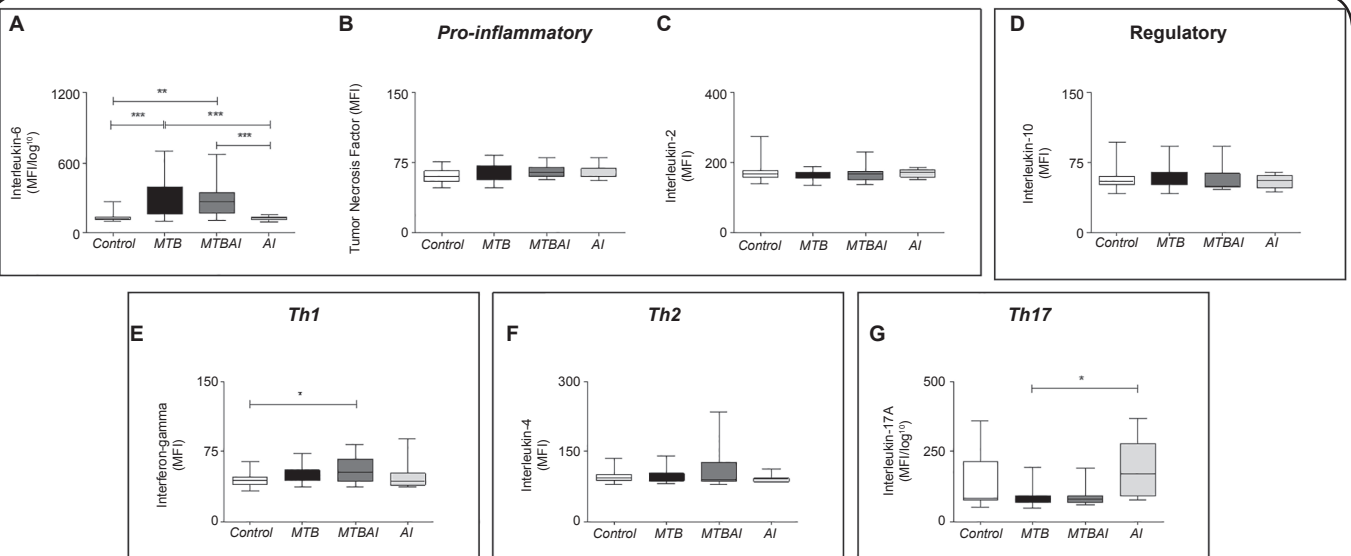


### Cellular Profile



Frequency of circulating innate (monocytes, NK, and NKT cells) and adaptive cells (CD4<sup>+</sup>, CD8<sup>+</sup>, Treg, CD4<sup>+</sup>CD69<sup>+</sup>, and CD8<sup>+</sup>CD69<sup>+</sup> T-cell subsets and B cells) categorized into groups referred to as Control (○), MTB (●), MTBAI (◐) and AI (◑). 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.

**FIGURE 2:** Immune cellular profile in Control, MTB, MTBAI, and AI patients enrolled in the study.



Levels of plasmatic cytokines IL-6, TNF, IL-2 (Pro-inflammatory), IL-10 (Regulatory), IFN- $\gamma$  (Th1), IL-4 (Th2), and IL-17A (Th17) are categorized into groups referred to as Control (□), MTB (■), MTBAI (◐) and AI (◑). Data is expressed as median±IQR in MFI. Statistical analyses were performed using 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 3:** Cytokine profiles in Control, MTB, MTBAI, and AI patients enrolled in the study.

## Interactions of Immune Cells and Plasma Cytokines in MTBAI coinfection

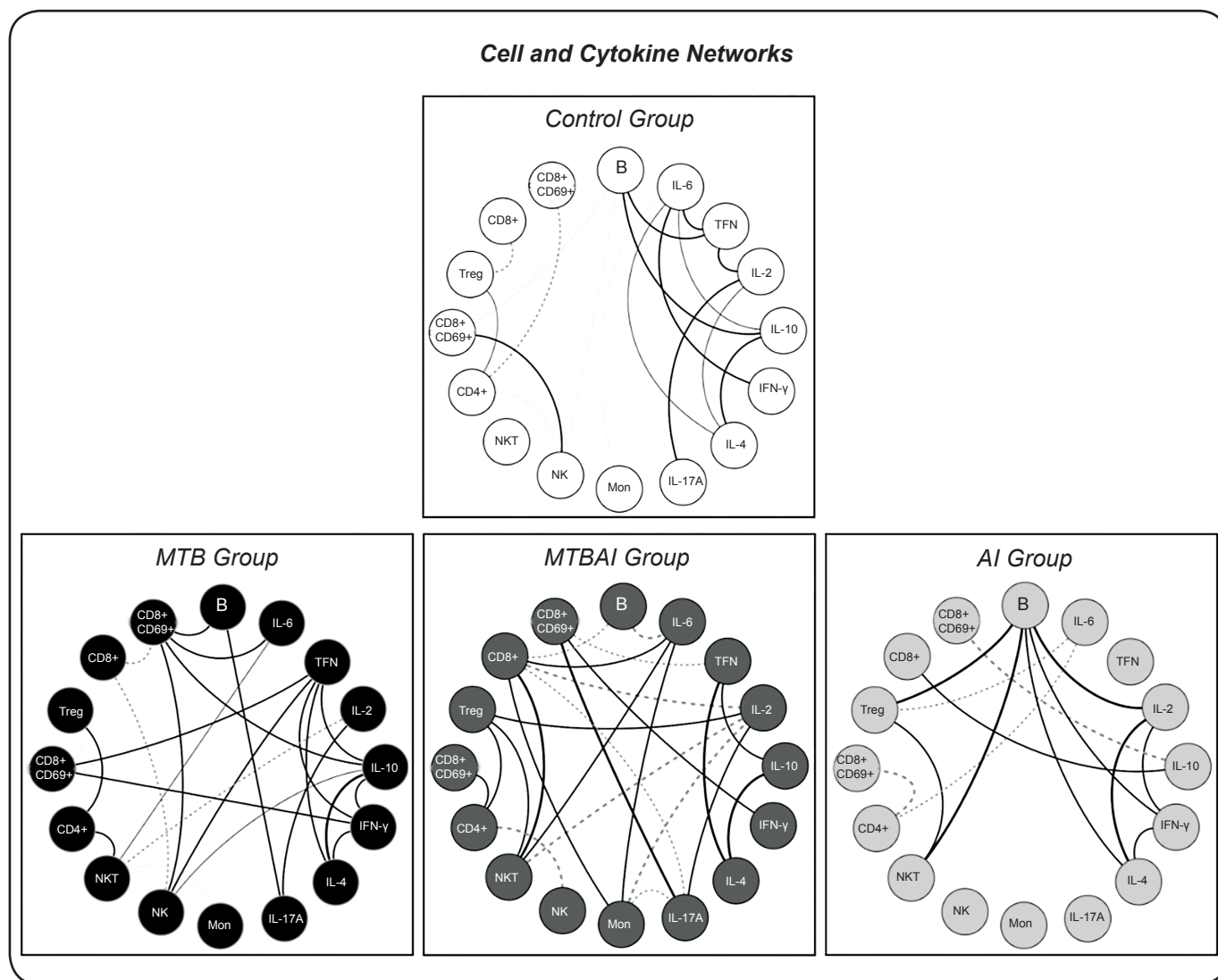
Data analyses indicated that MTBAI patients presented a cell and cytokine network similar to that exhibited by MTB monoinfection (Figure 4). Although this similarity was noted between the interaction networks in both groups, MTBAI patients presented a response profile with greater interactions between the regulatory profile cells (Treg and NKT). In addition, pro-inflammatory and Th1 profile cytokines (TNF and IFN- $\gamma$ ) have fewer interactions with the other components evaluated.

## DISCUSSION

This study characterized the coinfection of pulmonary MTB with *A. lumbricoides*, including clinical aspects and immune response. Contrarily, the association of MTB with AI did not influence the Th1, Th2, and Th17 responses or the percentage

of innate and adaptive cell subpopulations. *A. lumbricoides* in patients coinfecting with MTB and the degree of pulmonary parenchymal involvement in TB patients differed from a previous study in a cohort, where *Strongyloides stercoralis* was the most predominant helminth and advanced-grade lung injury was more frequent in coinfecting patients<sup>19</sup>. However, a more recent study with frequent *S. stercoralis* coinfection found no association between TB and infection with any of the prevalent helminths. However, *Schistosoma mansoni* infection was associated with a low bacillary load and a tendency of fewer pulmonary cavitations<sup>14</sup>. Similarly, in our study, coinfecting patients did not present advanced degree of lung injury. This may indicate that, depending on the helminth species, a favorable clinical expression of pulmonary TB may occur.

After treatment for TB, mean hemoglobin levels indicated a slower recovery in coinfecting patients, though the difference



**FIGURE 4:** Cell and Cytokine networks in control, monoinfection, and coinfection categorized into groups referred to as Control (nodes white circles), MTB (nodes black circles), MTBAI (nodes dark gray circles) and AI (nodes light gray circles). Significant Spearman's correlations at  $p < 0.05$  were represented by connecting edges to highlight positive correlation (thick continuous line) or negative correlation (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 ( $r \geq 0.68$ ).

was statistically insignificant. Helminth coinfection in malaria patients has been reported to lead to erythropoiesis<sup>12</sup>. These results support the idea that helminths aggravate chronic anemia associated with infectious diseases.

A significant increase in the percentage of monocytes and activated CD4<sup>+</sup>CD69<sup>+</sup> T and Treg cells was detected in MTB patients compared to healthy individuals from the control group. Several studies have indicated an increase of Tregs in TB, which are believed to be related to the role of the adaptive immune system in MTB restriction. Tregs can delay the initiation of effector T cells, prolonging the phase of bacterial expansion. Thus, T cells recognizing MTB-derived antigens specifically and potentially restrict protective immune responses during TB<sup>20-22</sup>.

Specific studies have demonstrated an increase in monocyte subpopulations in individuals with MTB, especially in the neutrophil subpopulations; however, the role of these subpopulations in MTB has not yet been clarified<sup>23,24</sup>. The elevation of TCD4<sup>+</sup> activation levels observed in TB patients occurred in coinfecting patients (MTBAI), although it was insignificant.

IL-6 may be related to pulmonary parenchymatous lesion processes, and its low concentration has been associated with lower incidence of organ damage<sup>25</sup>. Data from the present study support this hypothesis as the highest serum levels of IL-6 were observed in cases of TB, in conjunction with a higher frequency of advanced degree of pulmonary parenchymal involvement as compared to the MTBAI group. It was observed in murine models that diffused and poorly organized MTB lesions can develop from the onset of acute coinfection with immunosuppressive pathogens, such the helminths<sup>26</sup>.

The coinfecting patients (MTBAI) had lower IL-6 levels when compared to MTB patients, although the difference was statistically insignificant, since increased plasma IL-6 levels were detected in patients with severe pulmonary TB lesions<sup>27</sup>. These results may indicate a possible immunomodulation of IL-6 levels due to the presence of AI<sup>28-30</sup>. It is known that IL-6 is crucial for resistance against MTB, and is essential for the development of Th1 and Th17 profile<sup>25</sup>. In contrast, in vitro experiments with *Mycobacterium tuberculosis* demonstrated that IL-6 produced by macrophages inhibits cellular responses to IFN- $\gamma$ , contributing to the survival of MTB<sup>31</sup>. This result corroborates the fact that none of the MTBAI coinfecting patients presented an advanced degree of pulmonary parenchymal lesion. Furthermore, reduction of IL-17A was significant in cases of MTB with and without AI, indicating that the absence of this cytokine should favor the infection process.

The present study demonstrates the interactions of immune cells and cytokines in MTB and AI monoinfection and coinfection (MTBAI). The similarity observed between the interactions present in the networks of the MTB and MTBAI groups demonstrate that the pathophysiological events of the MTB infection prevail in the coinfection. In addition, our data suggest that AI coinfection produces a weaker inflammatory response profile, with greater participation of regulatory cells (Treg and NKT), which could explain the lower involvement

of the pulmonary parenchyma in MTBAI patients. A possible beneficial effect of AI in MTB coinfections is further supported by the higher paucibacillary frequency in these patients. The absence of significant changes in serum cytokines and blood cells could be related to the fact that MTB and AI infections did not induce an expressive systemic repercussion in individuals.

The results may have been influenced by limitations of this study, such as number of patients studied, non-assessment of polyparasitism in patients with TB for comparison purposes, and indication of treatment for helminths at the time of diagnosis. Furthermore, we did not evaluate the immunomodulation process along with clinical follow-up and the genetic background of the groups for immune factors. On the contrary, due to challenges in recruitment of patients, especially in formation of a prospective cohort of three months with multiple parameters evaluated, the design of this study was considerably complex, and it is the first report of its kind. In addition, the strategy to study coinfection with a single helminth of significant epidemiological importance due to its high prevalence, as is the case of AI, leads to the inference that only the immunopathogenic consequences of this parasite should be responsible for observed differences between groups.

In summary, our results suggest that AI infection does not significantly alter the percentages and/or concentrations of the immunological parameters evaluated; however, it may induce a significant clinical repercussion in the presentation and evolution of pulmonary TB. Interestingly, it may have a reducing effect on lung parenchymal injury as well as bacillary load, and IL-6 may be involved in this mechanism. Unexpectedly, the association with AI did not influence the Th1, Th2, and Th17 response or the percentage of T lymphocyte subpopulations. Therefore, the evaluation of specific immune response to *Ascaris* and *Mycobacteria* antigens should be adopted as a novel approach to evaluate this association. Thus, a future perspective could study the specific antigen response and regulatory cells in lung tissues.

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## Conflict of Interest

The authors declare no conflict of interests.

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