

RESEARCH ARTICLE

TNF- α blockade impairs *in vitro* tuberculous granuloma formation and down modulate Th1, Th17 and Treg cytokines

Djalma A. Alves da Silva¹*, Marcos V. da Silva¹*, Cleyson C. Oliveira Barros¹, Patrícia B. Dias Alexandre², Rodolfo P. Timóteo¹, Jonatas S. Catarino¹, Helioswilton Sales-Campos¹, Juliana R. Machado^{1,3}, Denise B. R. Rodrigues^{1,4}, Carlo J. Oliveira¹, Virmondes Rodrigues¹*

1 Laboratory of Immunology, Department of Biological Sciences, Triângulo Mineiro Federal University, Uberaba, Minas Gerais, Brazil, **2** Uberaba Municipal Department of Health, Uberaba, Minas Gerais, Brazil, **3** Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiania, Goiás, Brazil, **4** Laboratory of Biopathology and Molecular Biology, University of Uberaba, Uberaba, Minas Gerais, Brazil

* These authors contributed equally to this work.

* virmondes.rodrigues@uftm.edu.br



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Abstract

Tuberculosis (TB) is a granulomatous disease that has affected humanity for thousands of years. The production of cytokines, such as IFN- γ and TNF- α , is fundamental in the formation and maintenance of granulomas and in the control of the disease. Recently, the introduction of TNF- α -blocking monoclonal antibodies, such as Infliximab, has brought improvements in the treatment of patients with chronic inflammatory diseases, but this treatment also increases the risk of reactivation of latent tuberculosis. Our objective was to analyze, in an *in vitro* model, the influence of Infliximab on the granulomatous reactions and on the production of antigen-specific cytokines (TNF- α , IFN- γ , IL-12p40, IL-10 and IL-17) from beads sensitized with soluble Bacillus Calmette-Guérin (BCG) antigens cultured in the presence of peripheral blood mononuclear cells (PBMC) from TB patients. We evaluated 76 individuals, with tuberculosis active, treated and subjects with positive PPD. Granuloma formation was induced in the presence or absence of Infliximab for up to 10 days. The use of Infliximab in cultures significantly blocked TNF- α production ($p < 0.05$), and led to significant changes in granuloma structure, *in vitro*, only in the treated TB group. On the other hand, there was a significant reduction in the levels of IFN- γ , IL-12p40, IL-10 and IL-17 after TNF- α blockade in the three experimental groups ($p < 0.05$). Taken together, our results demonstrate that TNF- α blockade by Infliximab directly influenced the structure of granuloma only in the treated TB group, but negatively modulated the production of Th1, Th17 and regulatory T cytokines in the three groups analyzed.

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Introduction

Tuberculosis (TB) is an infectious and contagious disease that has affected the world society for thousands of years [1]. Currently it is estimated that one-third of the world population is infected with *Mycobacterium tuberculosis* [2] of these, 90–95% of cases remain symptomatic and non-communicable in a state called latent tuberculosis [3,4]. Since *M. tuberculosis* resides essentially in vacuoles within macrophages, the protective immune response against the *Mycobacterium* is dependent on the interaction between infected cells and CD4⁺ T cells [5,6]. This interaction occurs after the dissemination of *M. tuberculosis* to regional lymph nodes, where bacterial antigens are presented by dendritic cells, promoting the expansion of antigen-specific T cells [7].

M. tuberculosis infect macrophages and induce the formation of granulomas, which are composed of differentiated macrophages, lymphocytes and other cells of the immune system [8]. Granuloma is the structure that restricts the growth and spread of *M. tuberculosis*, and Th1 profile cytokines are classically associated with this structure [7]. TNF- α acts in synergy with IFN- γ in the stimulation of nitric oxide (NO) production by macrophages, besides being a key cytokine in the formation and maintenance of granulomas [9]. Changes in the immunological status of the individual, such as treatment with immunosuppressant drugs and HIV infection, potentiate the risk of reactivation of the disease [10,11].

Recently, the increase in the therapeutic indication of immunobiologicals agents as TNF- α inhibitors has brought a new risk factor for reactivation of latent tuberculosis in the last two decades. The use of Immunobiologicals drugs has revolutionized the treatment of chronic inflammatory diseases, especially in rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA) [11], as well in Inflammatory Bowel Diseases [12,13]. Rheumatic disease alone increases by 2 to 4 times the risk of TB [14] and by 30 times with the use of Infliximab, [15] thus characterizing the use of immunobiologicals as a risk factor for reactivation of TB [16]. Factors that disturb this delicate balance between mycobacteria and the maintenance of granuloma inevitably increase the risk of reactivation of the disease [11]. The understanding of microorganism-host interaction in granulomas is of paramount importance for the understanding of mycobacterial infections, but access to human granulomas through biopsies is very limited. The use of *in vitro* models that simulate the granulomatous reaction is an important strategy for the identification of mediators that influence its formation and maintenance [17,18]. In the view that the anti-tuberculosis immune response is distinct in patients with the active infection compared to post-treatment subjects or those with positive PPD (Purified Protein Derivative) intradermal test, we evaluated, *in vitro*, in these 3 groups of patients the effects of the formation of granuloma and the levels of cytokines involved in the control of *M. tuberculosis* in the presence of Infliximab, through peripheral blood mononuclear cells of patients with active tuberculosis, after treatment and individuals with positive PPD.

Methods

Patients

Blood samples were collected from 32 patients diagnosed with Active Tuberculosis (Active TB), 27 patients with previous TB episode who completed the treatment successfully (Treated TB) and 17 positive PPD individuals (PPD+ Control) with negative diagnosis for active TB and without previous history of tuberculosis. Patients with pulmonary or extra pulmonary forms of active and/or treated TB were included. Patients with active and treated disease were selected from Basic Health Units and UFTM General Hospital (Uberaba, State of Minas Gerais, Brazil). PPD+ subjects were volunteers with no previous history of tuberculosis, with

induration > 10 mm in intradermal test (Statens Serum Institut, Copenhagen, Denmark). Blood of patients with active TB was collected until the 21st day after the start of treatment, in order to minimize its interference. In all cases, the diagnosis of TB was defined through clinical, radiographic and laboratory criteria, according to guidelines of the World Health Organization (WHO) [19,20]. As criteria for inclusion in this study were considered: subjects aging over than 18 years that have not received organ transplantation or are in use of immunosuppressant's, HIV infection, have clinical disease or not. All individuals accepted to participate in this study, and after clarification, they signed a consent form. This study was approved by the ethics committee of the Federal University of the Triângulo Mineiro (UFTM) under the protocols number: 852 and 1475.

Obtaining peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated by density gradient in Ficoll-Hypaque (GE Health Care, Uppsala, Sweden), centrifuged at 400XG for 30 minutes at 21°C. They were resuspended in RPMI 1640 (GE) medium containing 50mM Hepes (GIBCO, Grand Island, NY, USA), 5% inactivated fetal bovine serum (GIBCO), 2mM L-glutamine (GIBCO), 40 μ g/mL gentamicin (Neoquímica, Anápolis, State of Goiás, Brazil), 1 mL 2 β -mercaptoethanol (Merck, Darmstadt, Germany), in a final concentration of 1x10⁶/mL. They were then cultured in 96-well plates (FALCON, San Jose, CA, USA) in the presence of conjugated and non-conjugated BCG Beads.

Obtaining BCG antigens

Mycobacterium bovis samples (Bacillus Calmette-Guérin—BCG), Moreau strain (Fundação Aatualpho de Paiva, Rio de Janeiro, State of Rio de Janeiro, Brazil) were used for the extraction of *Mycobacterium* antigens. Mycobacteria were resuspended in 0.85% physiological solution, incubated in a water bath at 90°C for 30 minutes, according to the manufacturer's protocol, and then autoclaved for 30 minutes. Soon after, they were centrifuged at 10,000xG at 4°C, for 30 minutes. The protein portion of the supernatant was collected, filtered through a 0.22 μ m filter (MILLIPORE, Molsheim, France), aliquoted and stored at -20°C. An aliquot was collected for dosing the protein concentration by the Bradford method (Pierce, Rockford, IL), according to the manufacturers protocol.

Conjugation of BCG antigen to polyacrylamide beads

The above antigenic preparation was conjugated to the polyacrylamide beads, (BIO-GEL[®] P-4 GEL BIO-RAD cat# 150–450 Hercules, CA, USA). 10 grams of beads were sterilized by gamma radiation at the dose of 100 cGy (Centigrays) (LINEAR ACCELERATOR VARIAN CLINAC 600 C, SN:310 Palo Alto, CA, USA), and hydrated in 1000 mL distilled water. After washing in 0.5M carbonate/bicarbonate buffer, 200 mg beads were incubated in this buffer for four hours in a water bath at 63°C with slow and continuous stirring. Next, 40 mg BCG antigens were added in the presence of 100 mg EDAC {N-ethyl-N'-(3-dimethylaminopropyl) Carbodiimide, Hydrochloride} SC 219152,(Santa Cruz Biotechnology, Dallas, Texas, USA) in 100 mL of sterile distilled water pH 6.5 at 4°C for 18 hours under slow and continuous stirring. Subsequently, the beads were washed and stored in sterile phosphate-buffered saline (PBS). Concomitantly, negative control beads were subjected to the same processes in the absence of BCG antigens, thus obtaining naked beads. At the time of use, beads were washed three times with incomplete RPMI medium. The whole procedure was carried out under axenic conditions, with sterile solutions and materials.

Granuloma *in vitro* model and treatment with anti-TNF- α monoclonal antibody

The *in vitro* granuloma formation assay was adapted according to the procedure described by Silva-Teixeira 1993, as described below [21].

Cultures for granuloma formation assay were performed on 96-well flat bottom culture plate (FALCON, San Jose, CA, USA) at the concentration of 2.5×10^5 cells/well for 10 days, kept at 37°C, in humid air containing 5% CO₂. The cultures were performed in the presence of medium, soluble antigen (BCG), non-conjugated beads, BCG-conjugated beads in the presence and absence of 40 μ g/mL of anti-TNF- α monoclonal antibody (Remicade[®] 100mg Infliximab Janssen Biologics BV, Leiden—Nederland and Schering-Plow (Brinny) Company, County Cork—Ireland.).

The granuloma index was assessed by quantification of the level of cellular reactivity in around 50 beads/well, as described [22]. According to the formation of the granuloma, scores were assigned as shown in Fig 1: A) score 1 (No bead-bound cell); B) score 2 (up to 5 bead-bound cells), C) score 3 (5 or more bead-bound cells without cell migration or blastic transformation), D) score 4 (5 or more bead-bound cells with cell migration and blastic transformation); E) score 5 (monolayer of cells covering the whole bead, cell migration and presence of blastic cells); F) score 6 (multiple layers of bead-bound cells, cell migration and blast transformation). The results were expressed as the granuloma index calculated by the mean in each well.

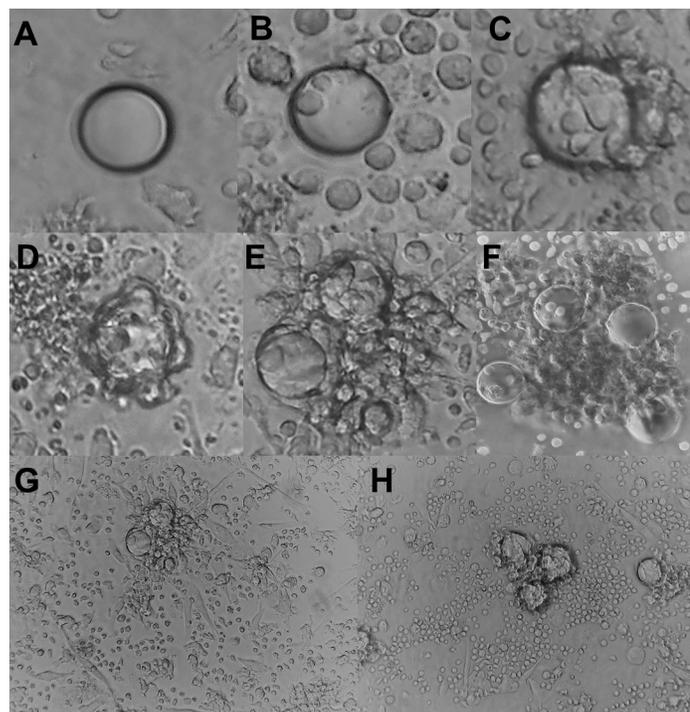


Fig 1. Classification of cellular reactivity around the beads, granuloma index. Representative image of cell reactivity index, in which A (No bead-bound cell), B (up to 5 bead-bound cells), C (5 or more bead-bound cells without cell migration or blast transformation), D (5 or more bead-bound cells, with cell migration and blast transformation), E (monolayer of cells covering the whole bead, cell migration and presence of blastic cells), and F (Multiple layers of bead-bound cells, cell migration and blast transformation). Representative image of an Treated TB patient at 10th day without (G) or with Infliximab.

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Quantification of cytokines in culture supernatants

The concentrations of TNF- α , IFN- γ , IL-10, IL-12p40 in the culture supernatants collected on the 5th and 10th day were measured by ELISA using monoclonal antibody pairs according to the manufacturer's specifications (BD Pharmingen, Franklin Lakes, NJ, USA) and IL-17 (R & D Systems, Minneapolis, MN, USA). Cytokine concentration was calculated using linear regression analysis of absorbance values obtained for recombinant cytokines, and was expressed in pg/mL. The sensitivity of the tests ranged from 2 to 20 pg/mL.

Statistical analysis

Statistical analysis was performed using EXCEL 2007 for WINDOWS (MICROSOFT—USA), STATVIEW (ABACCUS-USA) and GRAPHPAD PRISM 5.0 (GRAPHPAD SOFTWARE—USA). The normal distribution of the quantitative variables was checked by the D-Agostino & Pearson test. Continuous variables that presented normal distribution were expressed as mean \pm standard deviation and those with non-normal distribution were expressed in medians and percentiles. The variables that did not present normal distribution or did not have homogeneous variance were analyzed by the Mann-Whitney test for comparison of two independent groups or Kruskal-Wallis for three or more groups, with Dunn's post-test when necessary. The analysis of the data corresponding to the repeated measurements was evaluated by the Wilcoxon test. Differences were considered statistically significant when $p < 0.05$.

Results

Modulation of granuloma by TNF- α blockade

Here we evaluated the impact of TNF- α blockade by Infliximab on the ability of PBMCs derived from active and clinically cured tuberculosis patients and PPD+ healthy donors to form a granulomatous reaction through an *in vitro* model using mycobacterial antigens-coated polyacrylamide beads. In addition, we used this *in vitro* model of antigen-specific granulomatous reaction to evaluate the effects of TNF- α blockade on the ability of these PBMCs to produce cytokines related to the Th1, Th17 and T regulatory subsets.

After the blockade of TNF- α by Infliximab, we sought to check for differences between the groups, and whether this blockade influenced or not the formation of the granuloma. On the 5th and 10th days, in order to evaluate the *in vitro* granuloma formation shown in Fig 1, the counts of 50 beads/well were expressed as means. On the 5th day, in the absence of TNF- α blockade, the Treated TB group presented a significantly higher granuloma index than the PPD+ Control and Active TB groups ($p = 0.01$ and $p = 0.005$) (Kruskal-Wallis). On the 10th, no statistical differences were detected. TNF α blockade significantly reduced the granuloma index in the group with treated tuberculosis on the 5th and 10th day in the BCG-bead condition ($p = 0.015$ and $p = 0.039$) (Wilcoxon). In the other conditions, no significant difference was observed (Fig 2).

Modulation of cytokine production by TNF- α blockade

After determining the capacity for granuloma formation, *in vitro*, we sought to evaluate the effect of TNF- α blockade in the levels of cytokines with potential role in promoting this reaction. Cytokines were evaluated on the 5th day of culture. The TNF- α blockade, by using infliximab, was effective and significantly decreased cytokine levels in the four conditions tested and in all groups of patients studied (Figs 3–5). In individuals in the PPD+ control group, TNF- α blockade significantly reduced IFN- γ levels only in the BCG-bead condition ($p = 0.034$) (Wilcoxon), (Fig 3B). IL-10 levels, following TNF- α blockade, decreased significantly in cultures

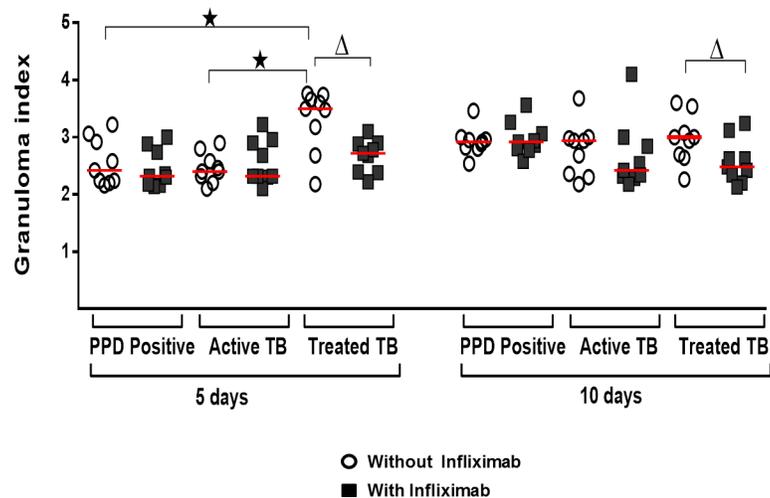


Fig 2. Effects of TNF- α blockade on *in vitro* granuloma formation. Granuloma index under different culture conditions—bead or BCG-bead—on days 5 and 10, in the presence or absence of infliximab. *significant difference between groups on the same day and in the same culture condition. Δ significant difference in the same group and same day of culture, after addition of Infiximab. Wilcoxon or Kruskal-Wallis tests followed by Dunn’s post-test. Significant difference was p-value <0.05. The horizontal lines represent the medians, the bars represent the 25–75% percentiles and the vertical lines represent the 10–90% percentiles.

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with Medium, Antigen and non-conjugated bead (p = 0.031, p = 0.015, p = 0.025) (Wilcoxon), (Fig 3C). IL-12p40 levels decreased significantly at all culture conditions after TNF- α blockade (p = 0.015, 0.015, 0.006, 0.001), (Wilcoxon), (Fig 3D). IL-17 levels significantly decreased in the Medium, bead and BCG-bead conditions after TNF- α blockade (p = 0.015, p = 0.001, p = 0.003), (Wilcoxon), (Fig 3E).

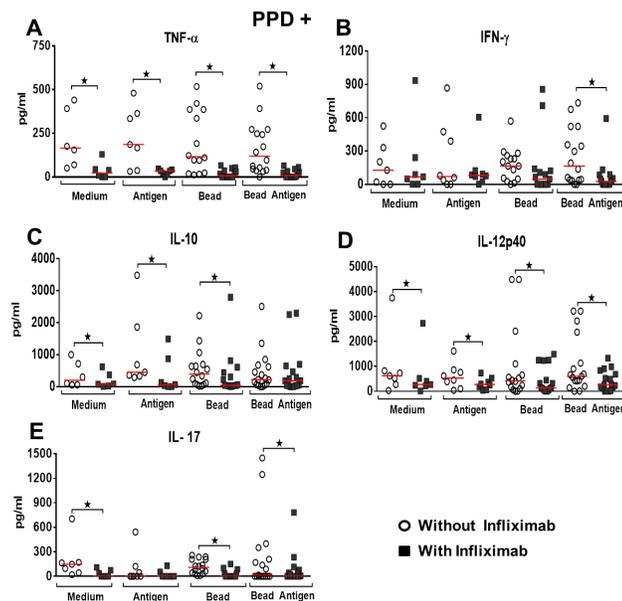


Fig 3. Effect of TNF- α blockade on cytokines production in PPD+ group. Levels of cytokines after granuloma formation, *in vitro*, PPD+ Control group (A-F) under different culture conditions—Medium or Antigen Medium and Bead or BCG-Bead, in the presence or absence of Infiximab. *Statistical differences between the conditions tested after the addition of Infiximab, in the different cytokines. Wilcoxon and Kruskal-Wallis tests, p-value <0.05. The horizontal lines represent the medians, the bars represent the 25–75% percentiles and the vertical lines represent the 10–90% percentiles.

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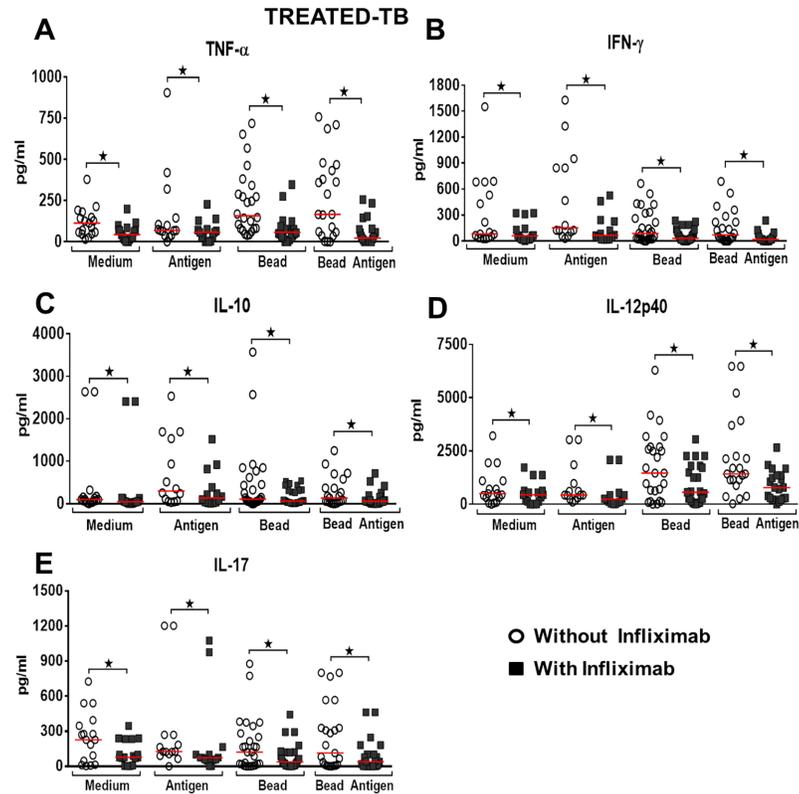


Fig 4. Effect of TNF- α blockade on cytokines production in treated Tb group. Level of cytokines after granuloma formation, *in vitro*, Treated Tb group (A-F) under different culture conditions—Medium or Antigen Medium and Bead or BCG-Bead, in the presence or absence of Infliximab. *Statistical differences between the conditions tested after the addition of Infliximab, in the different cytokines. Wilcoxon and Kruskal-Wallis tests, p-value <0.05. The horizontal lines represent the medians, the bars represent the 25–75% percentiles and the vertical lines represent the 10–90% percentiles.

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In patients in the treated TB group, TNF- α blockade significantly reduced IFN- γ levels in the four conditions tested ($p = 0.001$, $p = 0.002$, $p = 0.001$, $p = 0.001$), (Wilcoxon), (Fig 4B). IL-10 levels after TNF- α blockade were significantly decreased in the four conditions ($p = 0.004$, $p = 0.002$, $p = 0.005$, $p = 0.001$), (Wilcoxon), (Fig 4C). IL-12p40 levels decreased significantly at all culture conditions after TNF- α blockade ($p = 0.004$, $p = 0.004$, $p = 0.001$, $p = 0.001$), (Wilcoxon), (Fig 4D). IL-17 levels significantly decreased at all culture conditions after TNF- α blockade ($p = 0.004$, $p = 0.005$, $p = 0.005$, $p = 0.001$), (Wilcoxon), (Fig 4E).

In patients in the active TB group, TNF- α blockade significantly reduced IFN- γ levels in the four conditions tested ($p = 0.001$, $p = 0.001$, $p = 0.001$, $p = 0.001$), (Wilcoxon), (Fig 5B). IL-10 levels after TNF- α blockade significantly decreased in the four conditions ($p = 0.001$, $p = 0.001$, $p = 0.001$), (Wilcoxon), (Fig 5C). IL-12p40 levels decreased significantly at all culture conditions after TNF- α blockade ($p = 0.001$, $p = 0.001$, $p = 0.001$, $p = 0.001$), (Wilcoxon), (Fig 5D). IL-17 levels significantly decreased at all culture conditions after TNF- α blockade ($p = 0.001$, $p = 0.001$, $p = 0.001$, $p = 0.003$), (Wilcoxon), (Fig 5E).

Discussion

In the present study, in an *in vitro* model of granulomatous reaction, we evaluated the effects of TNF- α inhibition on the organization of granuloma and the production of prototypical

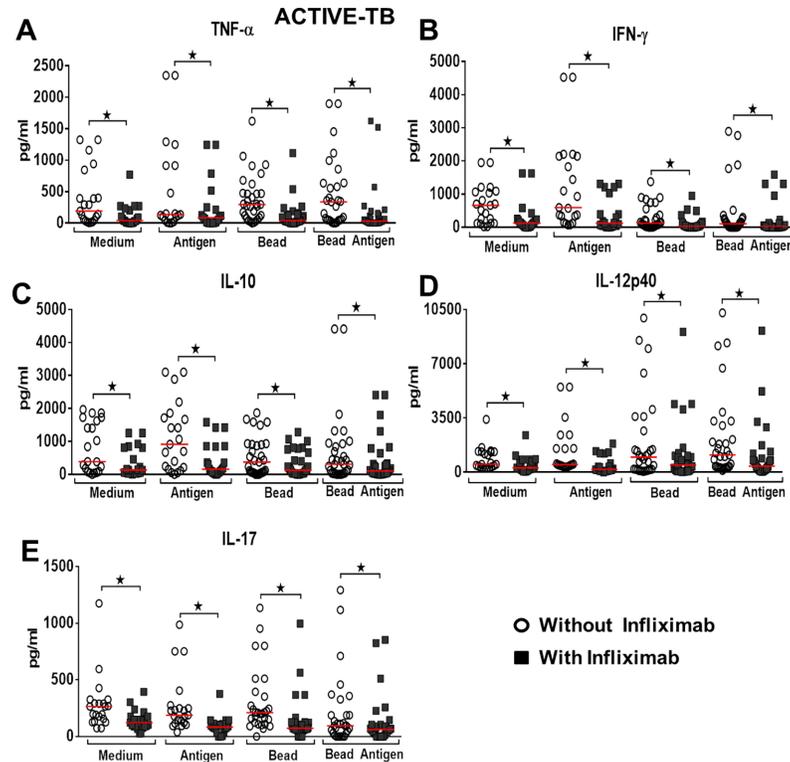


Fig 5. Effect of TNF- α blockade on cytokines production in active Tb group. Level of cytokines after granuloma formation, *in vitro*, Active Tb group (A-F) under different culture conditions—Medium or Antigen Medium and Bead or BCG-Bead, in the presence or absence of Infliximab. *Statistical differences between the conditions tested after the addition of Infliximab, in the different cytokines. Wilcoxon and Kruskal-Wallis tests, p-value <0.05. The horizontal lines represent the medians, the bars represent the 25–75% percentiles and the vertical lines represent the 10–90% percentiles.

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response cytokines Th1, Th17 and regulatory T in three groups of patients. The *in vitro* model of granuloma allows us to study the initial steps in the formation and maintenance of granulomas, facilitating the approach of translational aspects of human *M.tuberculosis* infection. However, they do not have the pulmonary structure and the conditions of the complete tissue microenvironment, being one of the limitations of the study [23]. Our data point to a significant inhibitory effect of TNF- α blockade in the group of patients with treated tuberculosis, both in the formation of granuloma and in the production of the cytokines tested. TNF- α blockade in patients with active tuberculosis and in the PPD positive control group did not interfere with the formation of granuloma, but modulated some of the cytokines analyzed. The chronic character and maintenance of the latency state of tuberculosis depend on several immune mechanisms involved in this process, such as the activation of macrophages and the formation and maintenance of granulomas that have a key role in the containment of the infection [24–26]. Although situations of evident immunosuppression are directly linked to the reactivation of tuberculosis, it is not always possible to determine which immune changes lead to the reactivation of the disease. Several situations cause the individual to develop immunosuppression, and in recent years one of them is highlighted: the use of immunobiologicals drugs for the treatment of chronic inflammatory diseases, such as TNF- α blockers [11].

We have shown that patients clinically cured of tuberculosis have granuloma indices significantly higher than the others. In these patients, TNF- α blockade significantly reduced granuloma formation *in vitro*, making it with similar rates to the other groups. In experimental

models with zebrafish, TNF- α deficiency leads to the formation of a disorganized granuloma with increased mycobacteria growth and macrophages necrosis [27]. In humans, therapeutic blockade of TNF- α leads to an increased risk of reactivation of some diseases, especially tuberculosis [28].

In this study, we also analyzed the effect of TNF- α blockade on the modulation of Th1, Th17, and regulatory T cytokines both in the granuloma model and after the stimulation with soluble antigens. We also evaluated IL-4 and LT- α , but with undetectable dosages in the culture supernatants, and IL-9 cytokine was not evaluated in this study. The dose of Infliximab used significantly reduced TNF- α levels in the supernatants at all culture conditions, indicating the efficacy of the concentration used to reduce the bioavailability of this cytokine. TNF- α is a proinflammatory cytokine, predominantly produced and secreted by monocytes, macrophages and T lymphocytes and its predominant effects on mycobacterial infection are the maintenance of granuloma structure, increase in adhesion molecule expression, production of reactive oxygen and nitrogen intermediates and increased expression of chemokines such as CCL-2, CCL5, CCL9 and CXCL10 [9,29–32].

Although in individuals PPD positive control, TNF- α blockade did not significantly alter the granuloma index, the blockade was shown to significantly reduce IL-12p40 levels in all conditions of antigenic stimulation, solubilized and immobilized in bead. IFN- γ and IL-17 were modulated negatively only in the condition of antigen immobilized in bead and IL-10 only when culture-soluble antigen was used. The granuloma index was not affected by TNF- α blockade in the active disease group, but significantly reduced the levels of IFN- γ , IL-12p40, IL-17 and IL-10 in all antigenic stimulus conditions tested. In the group of patients with treated tuberculosis, a reduction in all cytokines tested was verified. There was also a significant reduction in the granuloma index.

Interestingly, although cytokine levels were similar between the active disease and after treatment groups, the granuloma formation in the latter was significantly greater. The ability to form granuloma in the treated TB group was not exclusively associated with the cytokine pattern analyzed in this study. This suggests the involvement of other cytokines or chemokines, as well as adhesion molecules that may be directly responsible for intercellular adhesion in the formation of granuloma [33–35] or downstream pathways in the action of these and other cytokines, which may reflect different immune responses triggered in environments with similar concentrations of cytokines, especially TNF- α [36].

In vitro blockade of TNF- α with Infliximab led to a negative modulation of important cytokines in *M. tuberculosis* infection. Experimental studies in primate models and in human disease make clear the importance of the IL12/IFN- γ axis and the IL-10/TNF- α balance in the control of *M. tuberculosis* infection [37,38]. Carriers of innate errors in the IL-12/23-IFN- γ axis are more susceptible to *M. tuberculosis* infections and also by atypical mycobacteria [39]. IFN- γ and TNF- α are crucial for the complete activation of bactericidal functions of the macrophage producing NO and restricting the growth of mycobacteria [31,40–42].

The presence of IL-17 producing T cells during a secondary immune response in the lung environment has been shown to be important for protection against active tuberculosis [43]. Negative modulation of IL-17 promoted by the *in vitro* blockade of TNF- α may also be implicated in the risk of reactivation of *M. tuberculosis* infection in treated patients [44]. Similarly, a negative modulation of IL-10 was observed, which despite being described as a regulatory cytokine has been associated with the clinical cure of patients with tuberculosis [38] and experimental models have associated its production with IFN- γ /IL-10—double producers T cells [45]. The introduction of immunobiologics in the therapy of autoimmune diseases such as: rheumatoid arthritis, juvenile idiopathic arthritis as well as in Inflammatory Bowel Diseases, has brought about an improvement in the therapeutic response and quality of life of these

patients [11]. Nevertheless, modulation of the immune response may impair the fragile balance of the microorganism/host relationship in some latent or subclinical infectious diseases, increasing in these patients the risk of reactivation of latent tuberculosis [15].

In this study using *in vitro* granuloma model, we observed that TNF- α blockade not only blocks its bioavailability, with significant modulation of granuloma, but also significantly reduces the levels of IL-12p40, IFN- γ , IL-17 and IL-10. The participation of IL-10 in the formation of granuloma in tuberculosis requires further studies, since this cytokine is associated with the clinical cure of the disease and in the repertoire of antigen-specific T cells against *M. tuberculosis* [38]. The negative modulation of TNF- α , IFN- γ , IL-10, IL-12p40, IL-17, may be associated with the reactivation of the infection observed in patients taking Infliximab or other TNF- α blockers, which increases the importance of screening for latent tuberculosis in patients with chronic inflammatory diseases candidates for the use of immunobiological TNF blockers.

Supporting information

S1 Table. Demographic data.

(PDF)

S2 Table. Granuloma index.

(PDF)

S3 Table. Cytokine levels.

(PDF)

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Author Contributions

Conceptualization: Virmondés Rodrigues.

Data curation: Helioswilton Sales-Campos, Virmondés Rodrigues.

Formal analysis: Djalma A. Alves da Silva, Marcos V. da Silva, Cleyson C. Oliveira Barros, Rodolfo P. Timóteo, Jonatas S. Catarino, Helioswilton Sales-Campos, Juliana R. Machado, Carlo J. Oliveira, Virmondés Rodrigues.

Funding acquisition: Virmondés Rodrigues.

Investigation: Djalma A. Alves da Silva, Marcos V. da Silva, Cleyson C. Oliveira Barros, Patrícia B. Dias Alexandre, Rodolfo P. Timóteo, Jonatas S. Catarino, Helioswilton Sales-Campos, Juliana R. Machado, Denise B. R. Rodrigues, Carlo J. Oliveira, Virmondés Rodrigues.

Methodology: Djalma A. Alves da Silva, Marcos V. da Silva, Cleyson C. Oliveira Barros, Patrícia B. Dias Alexandre, Jonatas S. Catarino, Virmondés Rodrigues.

Project administration: Virmondés Rodrigues.

Supervision: Denise B. R. Rodrigues, Carlo J. Oliveira, Virmondés Rodrigues.

Validation: Marcos V. da Silva, Denise B. R. Rodrigues, Carlo J. Oliveira, Virmondés Rodrigues.

Visualization: Djalma A. Alves da Silva, Marcos V. da Silva, Cleyson C. Oliveira Barros, Patrícia B. Dias Alexandre, Rodolfo P. Timóteo, Jonatas S. Catarino, Helioswilton Sales-Campos, Juliana R. Machado, Denise B. R. Rodrigues, Carlo J. Oliveira, Virmondés Rodrigues.

Writing – original draft: Djalma A. Alves da Silva, Marcos V. da Silva, Cleyson C. Oliveira Barros, Jonatas S. Catarino, Helioswilton Sales-Campos, Carlo J. Oliveira, Virmondés Rodrigues.

Writing – review & editing: Djalma A. Alves da Silva, Marcos V. da Silva, Cleyson C. Oliveira Barros, Helioswilton Sales-Campos, Denise B. R. Rodrigues, Virmondés Rodrigues.

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