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NOTCH1 AND DLL4 ARE INVOLVED IN THE HUMAN TUBERCULOSIS PROGRESSION AND IMMUNE RESPONSE ACTIVATION

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Abstract

Tuberculosis (TB) is the leading cause of mortality among infectious diseases worldwide. The study of molecular targets for therapy and diagnosis suggested that Notch signaling is an important pathway for the maintenance of the immune response during *Mycobacterium tuberculosis* (Mtb) infection. We evaluated the participation of the Notch pathway in the modulation of immune response during Mtb infection, and observed that patients with active TB had increased DLL4 expression in intermediate and non-classic monocytes. Further, patients with moderate and advanced lung injury have higher Notch1 expression in $CD4^+$ T cells when compared to patients with a minimal lung injury. When we considered the severity of disease in active TB patients, the expression of the DLL4 in intermediate monocytes and the expression of Notch1 in $CD4^+$ T cells are positively correlated with the degree of lung injury. *In vitro,* PBMCs treated with the Notch pharmacological inhibitor reduced the production of IL-17A and IL-2, whereas anti-hDLL4 treatment promoted a significant increase in TNF-α and phagocytosis. We suggest that Notch1 and DLL4 are associated with immune response activation in human tuberculosis, and can be a novel target to be exploited in the future in the searching of biomarkers.

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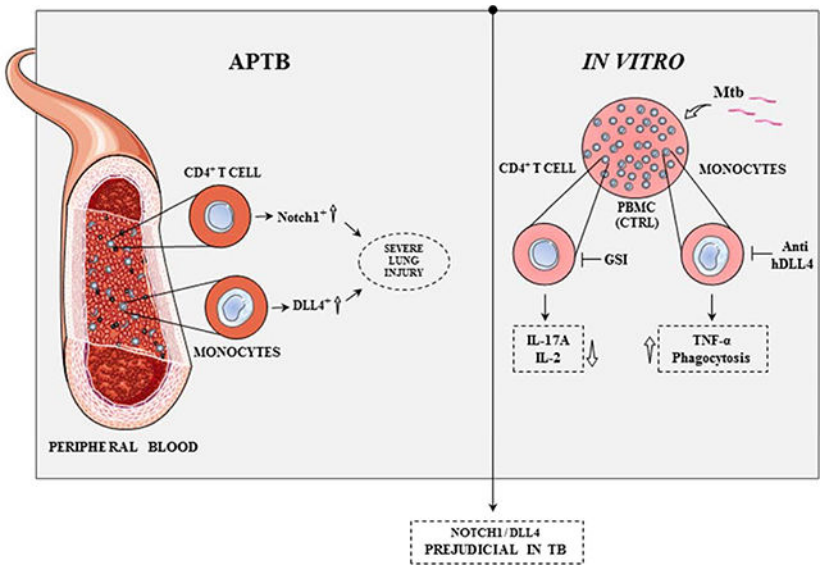
Potential conflicts of interest

All authors have declared that no reported conflicts of interest.

Declarations of interest: none

Graphical Abstract



Keywords

Mycobacterium tuberculosis ; progression biomarker; immune response; Notch signaling

1. Introduction

Tuberculosis (TB) is a bacterial infectious disease caused by *Mycobacterium tuberculosis* (Mtb) that has been affecting humans worldwide for thousands of years and is still considered a major challenge for science and medicine (1). Efforts from different clinical research groups around the world have supported the development of antibiotic therapies that led to inroads to TB cures in some instances. However, the disease is still a serious public health problem, since it is the main cause of morbidity and mortality from a single infectious agent worldwide (1). Thus, studies that seek new tools that aid in the prevention, diagnosis, identification of biomarkers and treatment of tuberculosis are still fundamental for the effective control of the disease.

In the search for diagnostic biomarkers, several molecules related to the host immune response have been selected as potential targets. One of these is the Notch signaling pathway, which influences the dynamics of the immune response during mycobacterial infection (2), (3), (4), (5), (6), (7). In mammals, there are four types of Notch receptors (Notch1–4) and five ligands: three Delta-like ligands (DLL1, DLL3, and DLL4) and two ligands of the Jagged family (Jag1 and Jag2) (8), (9). Once expressed on the cell surface, canonical signaling is initiated from the ligand/receptor interaction and is mediated by sequences of proteolytic events that leads to the transcription and upregulation of Notch target genes (10). Notch1 and Notch 2 receptors are constitutively expressed at different stages of the lymphocyte life cycle, and thus the Notch pathway seems to directly influence the development and effector function of these cells (11), (12). The expression of the Notch

ligands DLL1 and DLL4 in monocytes, macrophages, and dendritic cells influences several immunological mechanisms related to changes in pro-inflammatory responses as well as the activation and polarization of T cells and their cytokine profiles (6), (13), (14), (15).

The expression of different Notch pathway receptors and ligands during TB infection may result in different outcomes for the host, and consequently affect the progression and severity of tuberculosis (16), (17). In a model of *M. bovis* BCG infection, there was an increase in the expression of DLL4 in dendritic cells and progenitor cells of the bone marrow followed by an increase of IFN-γ and IL-17 cytokines released by T cells (6). Further, it was shown in PBMCs of patients with active TB that the expression of DLL4 ligand was increased in $CD14^+$ cells compared to those of healthy subjects or successfully treated patients (6). In addition, it has been shown that blocking Notch signaling with a pharmacological DAPT inhibitor in peripheral blood mononuclear cells (PBMC) samples from patients with latent and active TB can reduce the Th2 profile response and favor the Th1 response (7).

Here we characterize components of the Notch pathway in PBMC of patients with active TB compared to PBMC of healthy controls. We observed that patients with active TB had increased DLL4 expression in intermediate and nonclassic monocytes. In addition, patients with moderate and advanced lung injury have greater Notch1 expression. Expression of the DLL4 ligands in intermediate monocytes and expression of Notch1 in $CD4^+$ T cells in patients with active TB correlated positively with the degree of pulmonary injury. In addition, we suggest that our systemic analyses of these Notch pathway constituents in PBMCs of patients with active TB ranks them as potential biomarkers to assess disease progression.

2. Materials and methods

2.1 Ethical aspects

This research was approved by the ethics committees of the Faculty of Pharmaceutical Sciences of Ribeirão Preto (CEP/FCFRP-USP: n° 427-CAAE, n°62362216.8.0000.5403) and the Hospital das Clinícas de Ribeirão Preto-USP. All control subjects and patients with active tuberculosis who freely consented to participate in the study registered their authorization through the signing of the Informed Consent Form.

2.2 Study Groups

The blood samples from 13 healthy control subjects and 12 patients diagnosed with active TB, with less than 2 months of antibacterial treatment, were collected at the Infectious Diseases Unit of the General Hospital from Medical School of Ribeirão Preto. For the composition of the two groups, individuals of both genders, aged between 18 and 65 years and without co-infection with HIV, hepatitis and other chronic diseases were recruited. The control subjects were classified based on the IGRA (Interferon Gamma Release Assay) test. Patients with active tuberculosis were diagnosed through clinical signs, chest images combined with microbiological (acid-fast bacilli and MGIT-culture; BD BACTEC™ MGIT™, Brazil) and molecular assays (Xpert® MTB/RIF, Cepheid, EUA). In addition,

severity of disease was also categorized according to the pulmonary radiographic findings using a double-blind test and classified as the minimal, moderate and advanced disease as described (18).

The lesions were considered minimal (stage 1), when there was no evidence of the tissue density was classified as mild or moderate, and location was above the second chondrosternal junction. Moreover, these stage 1 lesions involved only a single segment of one or both lungs and the extent of combined lesions did not exceed the volume of a single lung. In moderate disease (stage 2), the lesions were densely confluent, but the area occupied by these lesions could not occupy more than a third of the volume of a lung. In addition, the overall diameter of cavitation did not exceed 4 cm in patients classified at this stage. Patients with advanced disease (stage 3), had lesions that were of greater extent than defined in moderate disease. Table 1 represents the general characteristics of the groups evaluated in the study.

2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

After venipuncture, the samples were centrifuged at room temperature for the collection of the plasma. Then, the cell portion was diluted in 1× PBS (Phosphate Buffered Saline) and PBMCs were isolated with the density separation technique using Ficoll-Paque ™ PLUS, d = 1.078 g / mL (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions.

2.4 Flow cytometry

For cellular characterization, cell surface markers were stained using CD14, CD16, DLL4 and DLL1 for populations of monocytes and CD3, CD4, CD8, CXCR3, CCR6, Notch 1 and 2 for T cell populations. For this, cells were re-suspended in 100 μL of PBS containing 2% FBS and 5 μL of each fluorescent antibody was added. The labeling was performed for 25 minutes at 4 °C in the dark. After the incubation time, 2 mL of PBS containing 2% FBS was added and the tubes were centrifuged for 10 minutes at 400 *g*, 4°C and the supernatants were discarded. Cells were fixed in 200 μL of PBS with 1% formaldehyde and stored at 4°C until analyses. 100,000 to 200,000 events from each sample were obtained using the Fortessa LRS II (BD Biosciences, San Diego, USA) and then analysed using BD FACSDiva 8.0.1. and FlowJo software, version 7.6.5.

2.5 *In vitro* culture experiments with *Mycobacterium tuberculosis.*

All procedures involving *in vitro* infection with *Mycobacterium tuberculosis* were developed in the FMRP -USP level 3 biological safety room. For the *in vitro* assays, *Mycobacterium tuberculosis* strain H37Rv (ATCC® 27294TM, Rockville, MD) was used. The 3rd generation strain, stored at −70 °C, was mixed in 7H9 liquid medium supplemented with Middlebrook ADC™ (BD Biosciences, Sparks, USA) and incubated at 37 °C for 10–11 days for bacterial growth. Then, the bacterial suspension was diluted with 1× PBS until reaching the same turbidity of the McFarland 1 scale, which corresponds to the concentration of 1×10^7 bacilli/mL.

The PBMC suspensions from control subjects were adjusted to the concentration of 2×10^6/well in RPMI 1640 culture medium (GibcoBRL), supplemented with 10% fetal bovine serum (RPMIc) and then distributed into 48-well plates. These cells were treated for 24 hours with modulators of the Notch signaling pathway, including: 10 μM Deshydroxy LY-411575 (GSI-I) (Sigma-Aldrich, Darmstadt, Germany), 5μg/mL anti-hDLL1 (R&D systems, R&D Systems, Minneapolis, USA) and 10 μM/mL anti-hDLL4 (as described by Robert Smith et al., 1994) which are all Notch signaling pathway blockers. After the treatment period, the cells were infected with Mtb (MOI 5) and maintained in culture for 72 hours. In the last 4 hours of infection, the cells were stimulated with phorbol-12-myristate 13-acetate (PMA) 50 ng/mL and ionomycin 500 ng/mL. Next the culture supernatants were harvested for cytokine quantification and the cell pellet was used for mRNA extraction.

2.6 Detection of cytokines

The quantification of cytokines and chemokines (IFN-γ, TNF-α, IL-17A, IL-4, IL-2, IL-12, IL-1β, IL-6, IL-8, IL-10, RANTES, and IP −10) was performed on plasma samples as well as on the culture supernatant of PBMCs treated and infected *in vitro* with Mtb. Detection assays were performed on the multiplex platform using the Magnetic Luminex Assay kit (R&D Systems a biotechne brand, Minneapolis, USA) analyzed on a plate reader (Luminex Multiplexing Instrument - EMD Millipore, Luminex Corporation, Austin, TX, USA) according to the manufacturer's instructions. All results were calculated by Milliplex Analyst 5.1® software using an individual standard curve for every cytokine/chemokine. Soluble CD163 and CD14 levels were quantified in plasma samples by enzyme-linked immunosorbent assay (ELISA - R&D Systems, Minneapolis, USA) according to the manufacturer's methodology.

2.7 Phagocytosis and microbicidal activity

PBMC isolated from control subjects were adjusted in RPMI culture medium with Fetal Bovine Serum (5%) and distributed in 96-well plates with 1×10^6 cells per well, in a volume of 100 μL/well. The cells were then treated with anti-hDLL1 (R&D Systems, Minneapolis, USA) at a concentration of 5μg/ml and anti-hDLL4 (as described by Robert Smith et al., 1994) at a concentration of 10μg/ml and incubated at 37 ° C for 24 hours. After the treatment period, infection was performed using Mtb H37Rv strains. For evaluation of phagocytic and microbicidal activity, the cells were distributed in two different plates, the phagocytosis plate was incubated for 2 hours, while the plate examining microbicidal activity was incubated for 24 hours. The colorimetric resazurin technique was used with the spectrofluorimeter (SpectraMax Gemini XPS, Molecular Devices) with excitation at 560 nm and emission at 590 nm, 24 hours after addition of resazurin (0.5 mg/mL).

2.8 Extraction of total RNA from PBMCs, cDNA synthesis and real-time PCR techniques

In order to evaluate gene expression, the extraction of messenger RNA (mRNA) was performed using the PureLink™ RNA Mini Kit (Ambion, Life TechnologiesTM, Carlsbad, USA) and the cDNA was generated using cDNA High Capacity Archive kit (Appied Biosystems, Foster City, USA) according to the manufacturer's methodology. The following genes were probed with TaqMan® for qPCR: IFN-γ, TNF-α, IL-17A, IL-17F, IL-6, IL-10, IL-21, TGF-β, DTX, HES1 and HEY1 (Applied Biosystems, Foster City, USA). The results

were analyzed using the $2^{-\Delta\Delta CT}$ method. GAPDH expression was used as constitutive control. The reaction was performed on StepOnePlus ™ equipment (Applied Biosystems, Foster City, USA).

2.9 Statistical analysis

The analyses were performed using GraphPad Prism 6.0. and IBM SPSS Statistics 21. Analysis used the Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lilliefor P value. For the parametric data we used the t-test and for the non-parametric data we used the Mann-Whitney test or Wilcoxon matched-pairs signed rank test. For all tests, p values less than or equal to 0.05 (Two-tailed) were considered significant. Correlations between the data were performed using the Kendall's tau-b and Spearman correlations tests.

Results from PBMCs related to the gene expression of cytokines and Notch-related genes were used to obtain a bioassinature analysis. For this purpose, the overall median of each target gene was calculated for all data, which was used as the cutoff point for the classification of individuals as "low" or "high" producers. Once the cutoff points were determined and the top producers of each indicator were identified, the results were plotted as diagrams and the frequencies were determined for each study group. Radar charts were built in Microsoft Excel (Microsoft Office 2013, Las Vegas, USA) to characterize the overall frequency of individuals with increased levels of each marker.

3. Results

3.1 Expression of Notch1 receptor in T cells of patients with active tuberculosis

To understand the participation of the Notch signaling pathway in TB, we evaluated Notch1 and Notch2 receptor expression in $CD4^+$ T cells isolated from PBMC of patients with active TB. Our results confirmed that patients with active TB have a significant decrease in the number of $CD4^+$ T cells when compared to the control individuals (Figure 1A-C). When the frequency of Notch receptors was measured on $CD4^+$ T cells, no significant differences were observed (Figures 1D and 1E). Then, we next evaluated the interaction between the Notch signaling pathway and the progression of TB. Based on the results obtained so far, the expression of Notch1 and 2 receptors on T $CD4^+$ cells from patients with active TB were associated with the severity of TB as determined by clinical data, including x-rays and computerized radiography. These parameters allowed the classification of patients into three categories, taking into account the extent of lung injury (Table 1).

Thus, we initially determined the correlation between the expression of Notch receptors and the degree of lung injury, and found that Notch1 receptor expression in $CD4^+$ T cells positively correlated with lung injury (Table 2). We saw that patients with moderate and advanced pulmonary lesions had a greater expression of the Notch1 receptor when compared to patients with a minimum degree of injury (Figure 1G-I).

We also evaluated the activation profiles of these cells through the expression of the CXCR3 and CCR6 receptors, activation markers of Th1 (T-helper 1) and Th17 (T-helper 17) cells, respectively. Our results showed a significant decrease in the frequency of CXCR3 receptor positive cells of patients with TB compared to controls (Supplementary 1A).

In contrast, *in vitro* infected PBMCs from healthy subjects demonstrated a significant increase in the frequency of CXCR3 receptor positive cells in $CD4^+$ 72 hours post infection (Supplementary 1B). We did not observe modulation in the expression of CCR6 (Supplementary 1A and 1B). Together our results suggest that in patients with active TB, Notch1 receptor expression in T $CD4^+$ cells is associated with progression of TB and that skewing to Th1 profile cells is decreased in $CD4^+$ cell populations.

3.2 Expression of DLL4 ligand in subpopulations of monocytes from patients with active tuberculosis.

We next evaluated the expression of Notch ligands, DLL1 and DLL4 in monocytes. We first characterized the different subpopulations of monocytes through the expression of CD14 and CD16. We observed that patients with active TB had a significant increase in the frequency of $CD14^+$ $CD16^{++}$ cells, regarded as non-classical monocytes, compared to control individuals (Figure 2A). The same results were observed during *in vitro* infection model (Supplementary 2A. In addition, the number of $CD14^{++}$ $CD16^+$ cells (intermediate monocytes) was significantly decreased in infected *in vitro* cells when compared to uninfected cells (Supplementary 2A).

Next we evaluated the expression of DLL1 and DLL4 ligands. We observed a significant increase in the number of $CD14^{++}$ $CD16^+$ cells and $CD14^+$ $CD16^{++}$ cells expressing the DLL4 ligand in patients with active TB compared to control individuals (Figures 2D and 2F). In the *in vitro* infection model, we also observed a significant increase in the number of $CD14^{++}$ $CD16^+$ cells that express DLL4 (Supplementary 2B). Thus, we suggest that the Notch pathway plays a role during Mtb infection, since expression of Notch receptors in T cells and ligands in mononuclear cells are modulated, as shown by the increase in DLL4 in subpopulations of monocytes.

3.3 Changes in inflammatory mediators in plasma of patients with active TB.

Considering the modulation in the expression of Notch receptors and ligands in patients with active TB and knowing the participation of this pathway in cell polarization, we explored the cytokine profile in plasma samples from patient.

We analyzed levels of circulating IFN-γ, IL-17A, IL-2, IL-12, TNF-α, IL-1β and IL-6 in the plasma of these patients. We detected a significant decrease in the levels of IFN-γ, IL-17A, IL-2 and IL-12 in active TB patients compared to control subjects, but we did not detect significant differences in relation of TNF-α (Figures 3A-E). In addition, we observed a significant increase in the levels of IL-6 in patients with active TB (Figures 3F). When we compare the levels of these cytokines between the groups of patients with minimal and moderate/advanced lung injury, we observed that all the cytokines mentioned above are significantly increased in patients with a higher degree severe lung injury (Figure 3I-N). These results suggest that patients with active TB present important alterations in their plasma cytokine profile, and these changes may be related to the changes in the Notch pathway observed in these individuals.

In addition, when we evaluated the gene expression of cytokines and Notch targets, we found that over 50% of patients with active TB were considered high producers of TNF-α,

IL-17A, IL-6, TGF-β, HES-1, and DTX, with frequencies of 54, 57, 50, 62, 58 and 53%, respectively (Figure 3H). In the control group, we observed high producers of IFN-γ, IL-17A and IL-6, with frequencies of 64, 50 and 54%, respectively (Figure 3G). Unlikely patients with active TB, control subjects were not considered high producers for genes related to the activation of the Notch pathway (HES-1 and DTX) (Figure 3G).

3.4 Association between via Notch, immunological mediators and the progression of tuberculosis.

Considering changes in receptor and ligand expression observed in patients with active TB, as well as in plasma cytokine profile and, consequently, in the pattern of immune response, we next evaluated the interaction between the Notch signaling pathway and the progression of TB. Based on the results obtained so far, the expression of Notch1 and 2 receptors on T cells and DLL1 and DLL4 in monocytes from patients with active TB were associated with the severity of TB as determined by clinical data, including x-rays and computerized radiography. These parameters allowed the classification of patients into three categories, taking into account the extent of lung injury (Table 1).

We also observed important correlations between the progression of TB and the expression of the ligands in the monocytes. Thus, we determined that, in a manner complementary to that observed with the Notch1 receptor, the expression of the DLL4 ligands in $CD14^{++}$ $CD16^{+}$ cells were also positively correlated with a higher degree of lung injury (Table 2). We infer that the increased expression of the constituents of the Notch pathway, including Notch1 and DLL4, is directly associated with severity in TB.

We next correlated the levels of plasma inflammatory mediators with expression of Notch receptors and ligands. The levels of IFN-γ, IL-17A and IL-2 showed a positive correlation with higher Notch1 receptor expression in $CD4^{+}$ T cells (Figures 4A-F). Furthermore, IL-17A levels were correlated to higher DLL4 expression in $CD14^{++}$ $CD16^{+}$ and $CD14^{++}$ $CD16^{+}$ cells (Figures 4G and 4H). After the systemic evaluation of our data, we suggest that the Notch pathway plays an important role during active TB.

3.5 Notch signaling alters cytokine production and blocking of DLL4 in monocytes favors phagocytosis during Mtb infection.

After characterizing the expression of receptors and ligands of the Notch pathway in PBMCs from patients with active TB and post *in vitro* infection with Mtb, we next investigated the mechanisms involved in the response to bacillus dependent on the activation of the Notch pathway. For this evaluation, we used Notch-pharmacological inhibitor (GSI), as well as blocking antibodies to the ligands, anti-hDLL1 and anti-hDLL4.

We observed that blocking Notch signaling, using GSI resulted in a significant decrease in the production of IL-17A and IL-2 (Figures 5A-C), while blockade DLL4 ligands with anti-hDLL4 resulted in increased levels of TNF-α (Figure 5D). These results suggest an important role of Notch pathway in modulate pro-inflammatory cytokine release during tuberculosis infection, and this modulation could be crucial for Mtb control

Thus, finally, as the modulation observed in cytokine production, we additionally evaluated the role of Notch signaling in the effector mechanisms of monocytes. The blockade of the Notch signaling pathway with anti-hDLL4 treatment significantly increased the phagocytic capacity of monocytes compared to the control group (Figure 5E-F). This suggested that the absence of DLL4 ligand favors phagocytosis of Mtb by monocytes at the site of infection, while blockage of DLL1 did not alter phagocytosis or killing in our model of infection.

4. Discussion

New molecular targets for therapy and diagnosis of TB, which take into account the host's immune response and mycobacteria type are sorely needed in order to improve TB prognosis and treatment. As we have shown here, the Notch signaling pathway plays an essential role in the immune response during mycobacterial infection

Monocytes, circulating mononuclear cells, play a central role in immunity, being indispensable during the process of inflammation and control of Mtb. The expression of Notch ligands, mainly DLL1 and DLL4, in monocytes, macrophages and dendritic cells influences several immunological mechanisms including changes in pro-inflammatory responses, as well as activation and polarization of T cells (6), (13), (19), (20). Thus, when we evaluated the expression of these ligands in monocyte subpopulations of patients with active TB, we observed a significant increase in DLL4 expression in $CD14^{++}$ $CD16^{+}$ and $CD14^{+}$ $CD16^{++}$ monocytes. In addition, when monocytes were infected *in vitro* with the Mtb H37Rv strain, we again demonstrated an increase in DLL4 expression in $CD14^{++}$ $CD16^{+}$ monocytes, suggesting that the monocyte interaction with Mtb induces the expression of this Notch ligand. Interestingly, the expression of DLL4 in $CD14^{++}$ $CD16^{+}$ monocytes correlates positively with the degree of lung injury in patients with active TB, which reinforces the importance of this molecule during the development of TB. Our collaborators, earlier, demonstrated that PBMCs from patients with active TB expressed higher levels of DLL4 in monocytes when compared to healthy individuals 6 months post-treatment (6). They also suggested that DLL4 is a potential biomarker to distinguish infected patients from uninfected individuals, since it is easy to measure this ligand samples of human peripheral blood (6). Our study complements and confirms their results and further defines the subtypes of monocytes that are most correlated both with the expression of these markers and with the severity of the disease.

The production and release of cytokines by cells of innate and adaptive immunity during Mtb infection are essential for disease control. In patients with active TB, these responses are not restricted to the site of infection but are also directed to the circulation. In addition, changes in the production of cytokines may directly reflect the progression of the disease. Thus, regarding T cells, especially Th1 profile, with a predominance of IFN-γ, is associated with host protection during Mtb infection and the dysregulation of this response may be deleterious in individuals affected by the disease (24), (25). In addition, studies have shown that Th17 cells play important protective response during TB (26), (27).

The Notch signaling pathway is also active in T cells, in which Notch1 and Notch2 receptors are constitutively expressed at different stages of the lymphocyte life cycle and therefore,

the Notch pathway seems to directly influence the development and effector function of these cells (11), (26). Studies have demonstrated only the positive regulation of Notch1 in murine peritoneal macrophages infected with *M. bovis* BCG (2), (28), but in our hands, we did not observe any modulation of this receptor during Mtb infection. In addition, we showed that Notch1 expression in $CD4^+$ T cells correlated positively with the degree of lung injury in patients with active TB. Although we did not observe significant differences in the expression of Notch1 in comparation of patients with active TB to healthy individuals, when we evaluated the expression of this receptor within the group of patients with active TB, we observed that cases with moderate and advanced lung injury are those that have the most Notch1 expression in $CD4^+$ T cells. This data reinforces the idea that this receptor is associated with the progression of the disease. Regarding the activation of the Notch pathway, we observed that more than 50% of the patients with active TB presented higher expression of of HES1 and DTX, compared to the control individuals, suggesting that the pathway is being more activated in TB patients. These observations suggest that the Notch pathway can be activated systemically during Mtb infection and that its activation may be detrimental to the outcome of the disease.

The expression of specific ligands influences the differentiation and function of $CD4^+$ T cells and the involvement of DLL ligands is associated with the development of Th1 and Th17 cells (6), (14), (21), (29), (30). Our results showed that IL-17A levels positively correlated with DLL4 expression in $CD14^{++}$ $CD16^-$ and $CD14^{++}$ $CD16^+$ monocytes. In addition, plasma levels of IFN-γ, IL-17A and IL-2 are positively correlated with Notchl expression in $CD4^+$ T cells. These data suggest an association between the immune response to TB developed by the host and the expression of the components of the Notch signaling pathway, and in addition supports the Notch pathway's relation to the progression of the disease. Together these data demonstrate the importance of the Notch pathway in human TB.

Besides systemic modulation of Notch pathway during tuberculosis, we also evaluated the effects of direct contact of mycobacteria with mononuclear cells *in vitro.* In this analysis, we observed that Notch receptors play a role in the modulation of proinflammatory responses. We demonstrated a reduction in IL-17 and IL-2 release in culture supernatant when PBMCs were infected with Mtb H37Rv and treated with GSI, confirming published studies. In 2009, Mukherjee and colleagues showed that in murine $CD4^+$ T cells, IL-17 production is dependent on Notch activation mediated by DLL4. In addition, Notch's active transcriptional complex has the ability to directly bind to promoters regions of the Rorc and IL-17 gene, and thereby modulate the activation of these genes (30). Increased expression of the intracellular portion of Notch can induce increased IL-2 production and IL-2 receptor a subunit expression in T cells (31).

Since we demonstrated the modulation of the Notch pathway during Mtb infection, we also sought to evaluate the role of signaling Notch in the effector mechanisms of monocytes. These cells, together with macrophages, neutrophils and dendritic cells, are known to be essential for the elimination of bacilli and for activation of the adaptive immune response. T cell activation directly influences the phagocytic capacity and microbicidal activity of innate immunity cells. By blocking DLL4 with the anti-hDLL4 antibody, we observed increased TNF-α production in mononuclear cells culture. From this perspective, we found that

DLL4 blockade also improves the phagocytic activity of monocytes during Mtb infection, corroborating the increase observed in innate pro-inflammatory cytokines. It is known that TNF-α, an important cytokine in Mtb infection, contributes to the activation of monocytes and macrophages, recruits new inflammatory cells to the site of infection and favors the formation of granulomas (33). Regarding other pathologies, recent studies presented results related to the DLL4 blockade. The treatment of non-obese diabetic mice with anti-DLL4 prevented the development of type 1 diabetes, promoting an increase in natural Treg cells (34). In another study, the blockade of DLL4 during the development of experimental autoimmune encephalomyelitis increased the numbers of Treg cells and decreased the severity of the disease (35). These results can suggest an uncoupled function of the Notch1 and DDL4, since the inhibition of Notch1 modulated the production of cytokines that are important for the activation of macrophages, while blocking DDL4 enhanced phagocytosis of Mtb by monocytes. These ligands are very promiscuous and this effect observed in *in vitro* experiments could be explained by the ligation into different receptors. One limitation of our study is the use of GSI as a blocker, since its effect is not specific for Notch1.

In summary, we showed here that Mtb infection activates the Notch signaling pathway, which can be detrimental to the host by modulating pro and anti-inflammatory responses. The Notch pathway also regulates the functional mechanisms of monocytes, such as phagocytosis and cytokine production, which are important for control of infection. In addition, the increase in Notch1 expression in T cells and DLL4 expression in intermediate monocytes is related to severe forms of the disease in the subjects evaluated in this study. Despite the fact that our number of samples is considered small and taking into account all results presented here, we were able to present important evidence of the relevance of Notch signaling, especially in patients with severe conditions. Thus, our data suggest that NOTCH1 and DLL4 could be assessed in different cohorts by researches that aim to search potential prognostic biomarker for tuberculosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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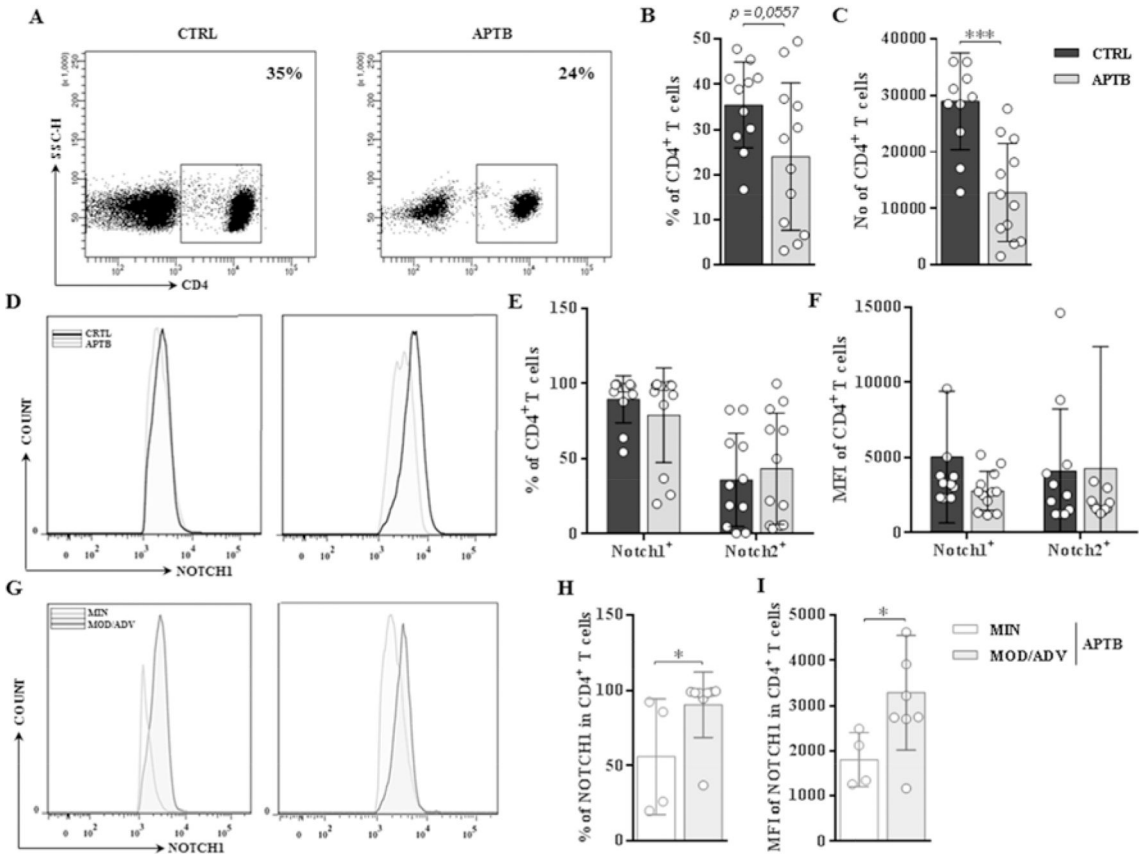

Figure 1. Expression of Notch1 receptor in CD4$^+$ T lymphocytes from patients with active TB.

The expression of Notchl and Notch2 receptors in PBMCs from control subjects and patients with active tuberculosis was evaluated by flow cytometry. A) Representative dotplot of distribution of CD4$^+$ lymphocyte populations. B and C) frequency (%) and number of the CD4$^+$ T cells. D) Representative histogram the frequency (%) of Notchl in CD4$^+$ T cells from control subjects and patients with active tuberculosis. E and F) frequency (%) and expression (Mean Fluorescence Intensity - MFI) of Notchland Notch2 receptors in CD4$^+$ T lymphocyte population. G) Representative histogram the frequency (%) of Notchl in CD4$^+$ T cells from patients with active tuberculosis. H and I) frequency (%) and expression (Mean Fluorescence Intensity - MFI) of Notchl receptor in the CD4$^+$ T cells from patients with minimum (MIN), moderate (MOD) and advanced (ADV) pulmonary lesions. The bars represent the mean ± standard deviation of each group. Control subjects (n=ll) and patients with active TB (n=l2) were used to evaluate of Notchland Notch2. Patients with minimum (n=4), moderate/advanced (n=8) pulmonary lesions were used to evaluate of Notchl. The results were analyzed with the t-test. ***P <0.0005, *P <0.05.

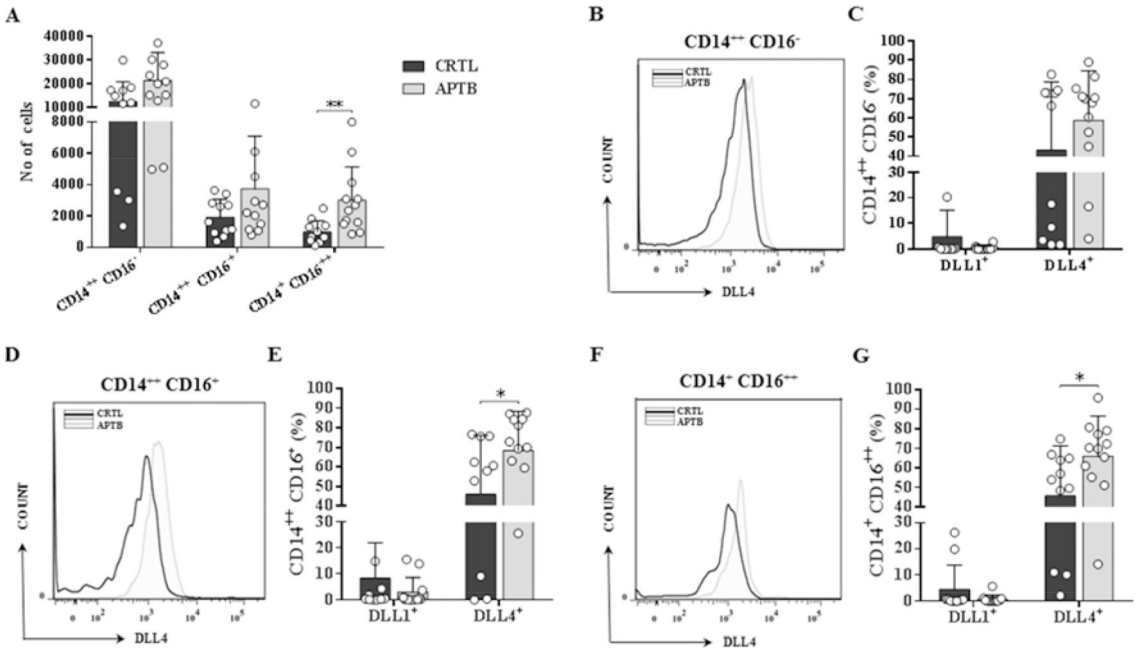

Figure 2. Expression of DLL4 in subpopulations of monocytes from patients with active TB.

Mononuclear cells from control subjects and active TB patients were labeled with anti-CD16 and anti-CD14 antibodies to divide monocyte subpopulations into classical ($CD14^{+}$ $CD16^{-}$), intermediate ($CD14^{++}$ $CD16^{+}$) and non-classical ($CD14^{+}$ $CD16^{++}$) monocytes. A) Distribution of subpopulations of monocytes (number of cells). B) Representative histogram the frequency (%) of DLL4 ligands in classical monocyte subpopulations from active tuberculosis patients and control subjects. C) Frequency (%) of DLL1 and DLL4 ligands in classical monocyte subpopulations. D) Representative histogram the frequency (%) of DLL4 ligands in intermediate monocyte subpopulations from patients with active tuberculosis and control subjects. E) Frequency (%) of DLL1 and DLL4 ligands in subpopulations of intermediate monocytes. F) Representative histogram the frequency (%) of DLL4 ligands in non-classical monocyte subpopulations from patients with active tuberculosis and control subjects. G) Frequency (%) of DLL1 and DLL4 ligands in non-classical monocyte subpopulations. The bars represent the mean ± standard deviation of each group. Control subjects (n=11) and patients with active TB (n=12). The results were analyzed using the t-test; * $P < 0.05$.

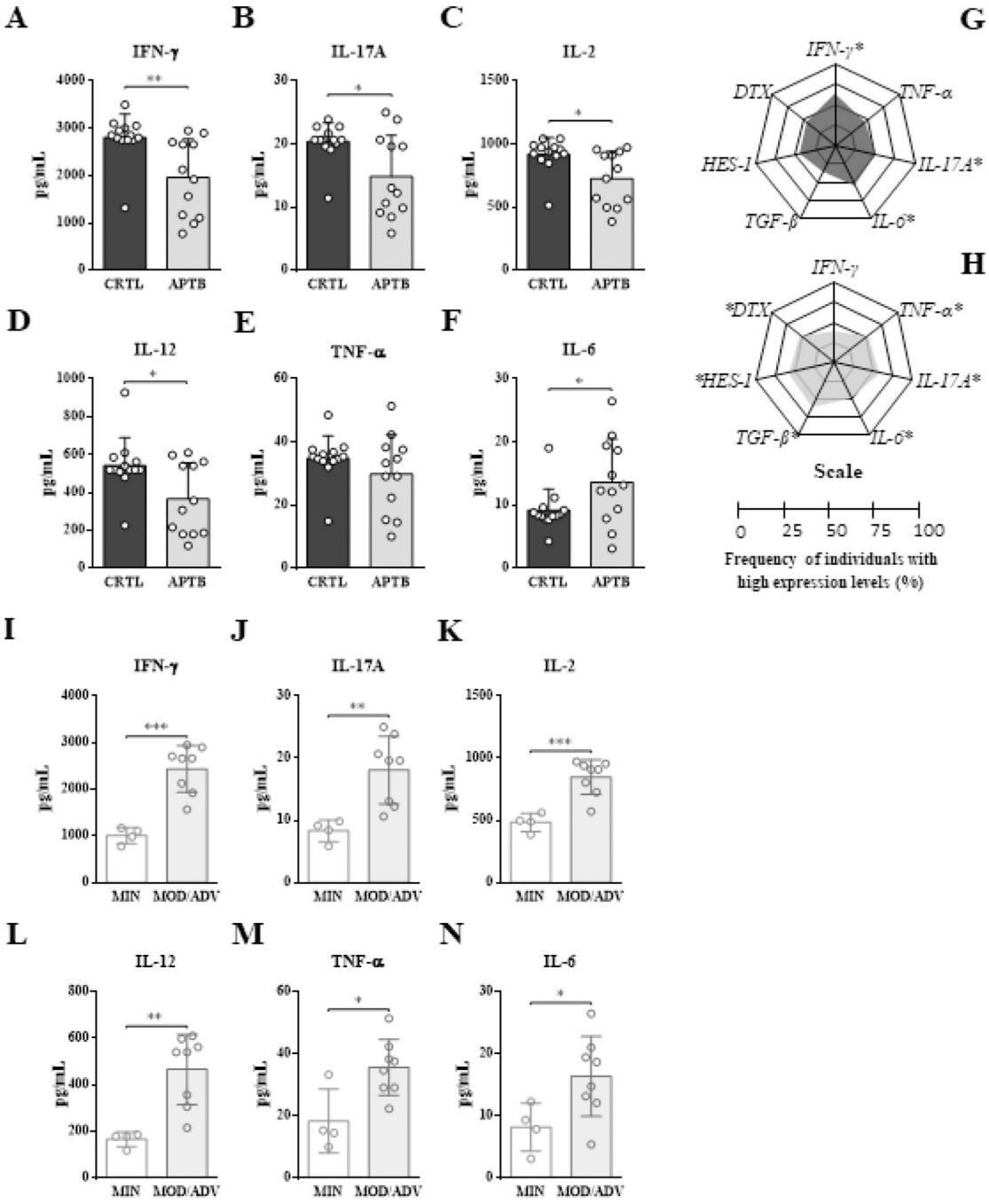

Figure 3. Quantification of cytokine levels in plasma and global signature of the gene expression of Notch targets and cytokines from patients with active TB.

A-N) Cytokines levels were evaluated in plasma samples from control subjects and patients with active TB through the Magnetic Luminex Assay Kit. The bars represent the mean ± standard deviation of each group and each spot is representative of an individual. Control group (n=13) and patients with active TB (n=12). Gene expression of Notch targets and cytokines were plotted as radar charts, in which the frequencies of higher producers (%) were determined based on the overall median value of each target gene as a cutoff point for individuals with “low” or “high” gene expression. Radar plots assume that each axis shows

the proportion of individuals with high levels of a given target gene. The relevant values (≥ 50%) are indicated (*). G) Representation of control individuals (black). H) Representation of patients with active TB (gray). The results were analyzed through the t-test. **** P <0.0005, ** P <0.005, * P <0.05.

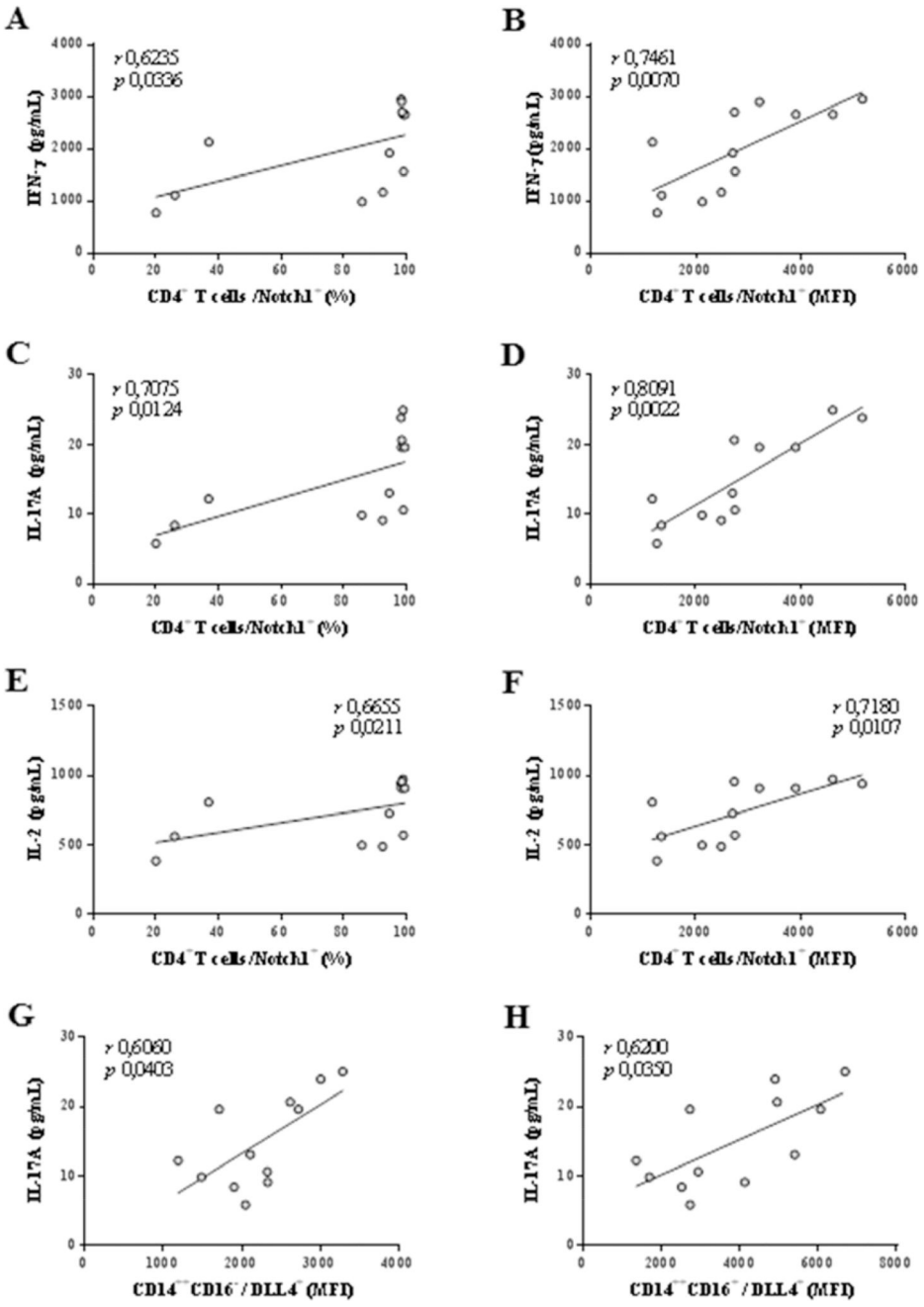

Figure 4. Correlation between expression of Notch1 and DLL4 with immunological mediators in patients with active TB.

The expression of Notch1 receptor on $CD4^+$ T cells was correlated with plasma levels of (A and B) IFN-γ, (C and D) IL-17A and (E and F) IL-2. Correlation studies between expression (MFI) of DLL4 in $CD14^{++}$ $CD16^-$ monocytes and plasma levels of IL-17A (G). Correlation studies between expression (MFI) of DLL4 in $CD14^{++}$ $CD16^+$ cells and plasma levels of IL- 17A (H). The analyses were performed using Spearman's statistical test. Each spot is representative of an individual and the values of *r* and *p* are specified in the graphs. Patients with active TB (n=12).

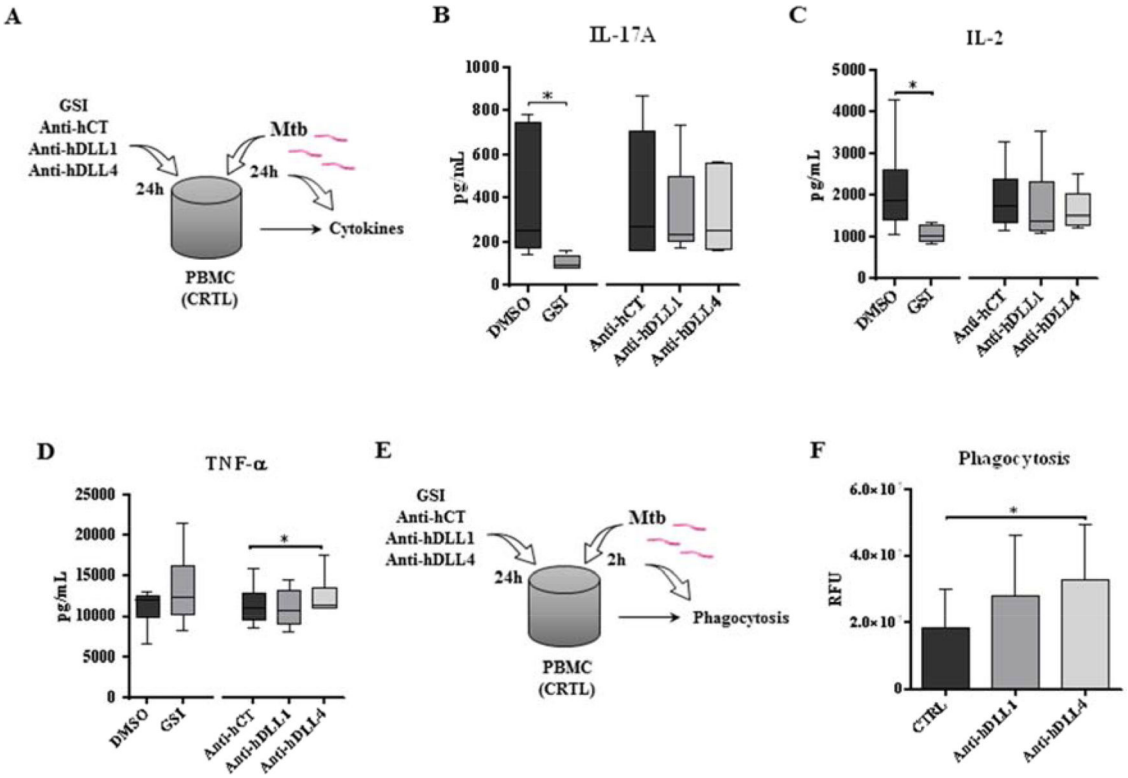

Figure 5. Effect of Notch pathway modulation in cytokine release and monocytes phagocytosis in response to Mtb *in vitro* infection.

IL-17A, IL-2 and TNF-α were evaluated in culture supernatant of PBMCs, previously treated with GSI, anti-hDLL1, or anti-hDLL4 for 24 h and infected with Mtb H37Rv for 72 h, using the Magnetic Luminex Assay Kit. (A) Experimental design, (B – D) Cytokine quantitation of IL-17, Il-2 and TNF-α, respectively. (E) Experimental design of phagocytic activity analysis - PBMCs from control subjects were pretreated with anti-hDLL1 and anti-hDLL4 for 24 hours and then infected with the Mtb H37Rv strain. (F) Phagocytosis analysis of this experiment was performed using the resazurin metabolism assay by viable internalized bacteria. The bars represent the mean ± standard deviation (n=6). The bars represent the mean ± standard deviation (n=6). The results were analyzed through the t-test. ** P <0.005, * P <0.05.

Table 1.

General characteristics of the groups included in the study

Groups	Control	Active TB patients
N	13	12
Gender (M/F)	10/3	9/3
Age in years (mean ± SD)	30±8.9	44±10
HIV+	0	0
Sputum smear +	NA	9
Culture +	NA	8
PCR +	NA	12
IGRA +	0	NA
Treatment time in days (mean ± SD)	NA	24±22
Radiological severity score		
1-Minimal	NA	4
2-Moderate	NA	1
3-Advanced	NA	7

Number of individuals per group (N); Male (M) / Female (F); SD (standard deviation); molecular test - Polymerase Chain Reaction (PCR), Interferon Gamma Release Assay (IGRA); treatment time (mean / days); Not applied (NA).

Table 2.

Correlation of Notch1, Notch2, DLL1 and DLL4 expression with radiological severity score in patients with tuberculosis

		RADIOLOGICAL SEVERITY SCORE	
		Correlation coefficient	***P***
CD4$^+$ T cells	Notch1$^+$ (%)	0,690	0,006 **
	Notch2$^+$ (%)	−0,217	0,384
	Notch1$^+$ (MFI)	0,611	0,014 *
	Notch2$^+$ (MFI)	−0,138	0,580
		RADIOLOGICAL SEVERITY SCORE	
		Correlation coefficient	***P***
CD14^{++}CD16$^+$	DLL1$^+$ (%)	−0,200	0,427
	DLL4$^+$ (%)	0,138	0,580
	DLL1$^+$ (MFI)	−0,020	0,937
	DLL4$^+$ (MFI)	0,493	0,048 *

Patients with active tuberculosis (n=12). Analyzes were performed using the Kendall's tau-b test.

** P <0.01

* P <0.05.