Systemic Immunological changes in patients with distinct clinical outcomes during *Mycobacterium tuberculosis* infection

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ARTICLE INFO

ABSTRACT

Background: The lung lesions in an individual infected with tuberculosis (TB) are surprisingly variable and independent of each other. However, there is no circulating biomarker yet able to segregate patients according to the extent of lung lesions.

Materials and methods: In this study, the phenotypic and functional profile of leukocytes of patients with active pulmonary tuberculosis (TB) and controls (CO) were fully scrutinized by immunophenotyping assays and *in vitro* short-term whole blood culture. The TB group was subdivided according to the extent of lung lesions as unilateral (UNI) and bilateral (BI).

Results: The results show that TB group display an altered leukocyte profile in the peripheral blood with significant lower counts of NK-cells, CD3$^+$CD56$^+$CD16$^{+/-}$ NKT-cells, CD4$^+$T-cells, CD19$^+$B-cells when compared to CO. Increased CD4$^+$T-cells and CD8$^+$T-cell activation was observed by the upregulation of activation markers (HLA-DR) as well as of chemokine receptors (CCR2, CCR3, and CXCR4). In addition, TB group presented a significant decrease proportion of CD14^{Low}CD16$^+$ monocytes despite the increase in HLA-DR expression. Regarding the severity of the disease, in the BI group a reduction in frequency of CD19$^+$CD5$^+$ B-cells and expression of HLA-DR in CD14^{Low}CD16$^+$ monocytes was observed. Furthermore, the extent of lung lesions influences the production of molecules as observed by significantly larger production of IL-4 by neutrophils, total T-cells, CD4$^+$T-cells, CD8$^+$T-cells and CD19$^+$B-cells in UNI as compared to BI. By contrast, in BI group the frequency of high producers of both IL-17$^+$CD4$^+$T-cells and IL-17$^+$CD8$^+$T-cells were significantly increased than UNI, suggesting the deleterious role of these subsets during active pulmonary *Mtb* infection.

Conclusion: The immunophenotypic characterization of unilateral and bilateral active TB performed in the present study indicates that the extent of lung lesion could be associated with a fine-tuning between immunological responses during untreated *Mtb* infection.

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*Mtb*) and remains as a highly prevalent infectious disease worldwide (Lee et al., 2015). In 2015, 10.4 million new active cases of TB were notified and 1.5 million people died from TB, including 400,000 HIV-positive individuals (WHO, 2016).

Heightened morbidity and mortality associated with this disease are consequences of complex processes triggered by *Mtb*. The vaccine composed of the bacillus Calmette Guérin (BCG) has contributed for

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decreasing lethality. However, BCG has been proven to be of inadequate efficacy against the most frequent outcome of pulmonary tuberculosis in adults (Fine, 2005). Regarding the current therapy for the disease, the number of multi-drug resistant (MDR) isolates is on the rise in many areas of the world (Korbel et al., 2008).

Variations in the host immune response against the pathogen, as the type and intensity, may influence the status of *Mtb* infection in active or latent tuberculosis, which differ in the type of treatment needed and the risk of its transmission (Lyadova and Panteleev, 2015).

Mtb is an intracellular pathogen that infects preferentially myeloid cells such as macrophages (Russell et al., 2009). Therefore, it is suggested that development of a cellular immune response, with special participation of Th1-lymphocytes, is critical for disease control (Dorhoi et al., 2011) by decreasing the growth and spread of *Mtb* in the host cells (O'Garra et al., 2013). Other immune cells, such as B-lymphocytes, appear to exert protective role in tuberculosis (Maglione et al., 2007) through the production of cytokines such as IL-12 and IL-4, which directs the response to proinflammatory or regulatory profiles, respectively (Maglione et al., 2007). In addition, NK-cells seem to be able to limit pathogen growth by lysis of *Mtb*-infected cells mediated by the perforin and granzyme pathway (Sia et al., 2015).

Studies that proposed to phenotypically and functionally characterize the innate and the adaptive immune cells in tuberculosis are complex and not completely conclusive (da Silva et al., 2015). Therefore, the present study aimed at analyzing innate and adaptive immune cell subsets by *ex vivo* immunophenotyping and short-term culture with *Mtb* (H37Rv) antigen. Our findings suggest that unilateral and bilateral TB are composed of a different immunophenotypic profile, which is associated with a fine-tuning of immune responses during untreated *Mtb* infection.

2. Population, material and methods

2.1. Study population

This study was conducted in Belo Horizonte and surrounding cities, at Minas Gerais state, Brazil. Twenty-one patients with active tuberculosis (TB) were included in the study and had confirmed diagnosis by examination of sputum smear and culture. The severity of illness of patients with TB, directly related to the extension of lung involvement, was assessed through the analysis of chest radiographs and patients were segregated as bearing lesions classified as unilateral (UNI) with involvement of only one pulmonary lobe, and bilateral (BI) with involvement of both pulmonary lobes (Kobashi et al., 2007). A control group (CO) of twenty-eight co-inhabitants of the same endemic area with no evidence of disease by clinical examination and negative culture for *Mtb* were enrolled in this study. For the immunophenotyping assays, the study groups were constituted by: CO (n = 21), TB (n = 15), UNI (n = 05) and BI (n = 08). Two patients from the TB group had no chest radiograph and thus were excluded from the analysis. For the *in vitro* short-term whole blood culture, the groups were: CO (n = 09), TB (n = 08), UNI (n = 03) and BI (n = 05). Hematological parameters, smoking, alcohol, body mass index (BMI), blood glucose and C-reactive protein were described in Table 1. All subjects included in this evaluation were HIV-negative. This study was carried out in full accordance with all International and Brazilian accepted guidelines and was approved by the Ethics Committee for Research at Federal University of Minas Gerais (UFMG protocol # 228/03).

2.2. Blood sample

Venous peripheral blood sample of 5 mL was collected from each subject using heparin (whole blood culture) or EDTA (*ex vivo*) as anticoagulant. Blood samples from TB patients were collected immediately prior to the chemotherapy treatment.

Table 1
Demographic, clinical and laboratorial features of the study population

Parameters	Groups	
	Control (CO)	Pumonary tuberculosis (TB)
Genre		
Male	16 (57.1%)	14 (66.7%)
Female	12 (42.9%)	7 (33.3%)
Age		
	42 (19–62)	44 (21–70)
Pulmonay disease Severity		
Unilateral	–	08
Bilateral	–	11
Hematological Profile		
RBC (mm^3)[1]	5,200 (4,900 − 5,600)	5,000 (4,000 − 5,000)
Platelets (mm^3)[2]	233,000 (190,000 − 321,000)	377,000 (288,000 − 488,500)
Hemoglobin (g/dL)[3]	15.0 (13.9–16.6)	12.3 (11.5–13.2)
Leukocytes (mm^3)[4]	6,620 (4,965 − 7,900)	8,540 (6,540 − 10,190)
Neutrophils (mm^3)[5]	3,970 (2,560 − 5,227)	6,170 (4,260 − 8,085)
Eosinophils (mm^3)	170 (95–245)	140 (80–405)
Monocytes (mm^3)[6]	520 (415–700)	680 (520–940)
Lymphocytes (mm^3)[7]	1,910 (1,515 − 2,155)	1,360 (830 − 1,665)
Alcoholism		
Yes	16 (57.1%)	09 (42.9%)
No	12 (42.9%)	12 (57.1%)
Smoking		
Yes	13 (46.4%)	14 (66.7%)
No	15 (53.6%)	07 (33.3%)
BMI (weight/height^2)[8]	26.2 (24.2–30.6)	20.0 (17.0 − 21.0)
C-reactive protein (mg/L)[9]	5.0 (3.0 − 10.1)	54.0 (21.5–76.0)
glycemia (mg/L)	82.0 (76.0 − 90.0)	80.0 (74.0 − 92.5)

[1](p = 0,005); [2](p = 0,001); [3](p = 0,0001); [4](p = 0,005); [5](p = 0,0004); [6](p = 0,03); [7](p = 0,003); [8](p = 0,0001); [9](p = 0,0001); BMI (body mass index).

2.3. Mycobacterium tuberculosis antigen

Mtb antigen (H37Rv *Mtb*-Ag) was provided by the Microbiology Laboratory (Departamento de Bioquímica/USP/Brazil). The *Mtb* was cultured in tubes with Loweinstein Jensen medium and incubated at 37°C until evidence of bacterial growth. Colonies were inactivated at 80°C for 1 h and sonicated in 2 cycles of 20 s at 40 Hz in an ice bath. The suspension was then sterilized using gamma radiation (dose of 5000 Gray for 2:15 h). The protein concentration was measured by the Lowry method.

2.4. Immunophenotyping assays

For the realization of *ex vivo* immunophenotyping assays, peripheral blood leukocytes from TB patients (TB) and controls (CO) were collected. EDTA blood aliquot of 50 μL was transferred to 5 mL polystyrene tubes (Falcon – BD, USA) containing aliquots of monoclonal antibodies labeled with fluorochromes: FITC (anti-CD4, anti-CD14, anti-CD5), PE (anti-HLA-DR, anti-CD54, anti-CD25, anti-CD18, anti-CD62, anti-CD69, anti-CD23) and TC (anti-CD8, anti-CD19, anti-CD16). The samples were homogenized and incubated for 30 min at room temperature, protected from light. Red blood cell lysis was performed by adding 2 mL of lysis solution to the cell suspension (FACS Lysing Solution- Becton Dickinson) and incubated for 10 min at room temperature. Subsequently, cell suspension was washed twice with 1 mL of phosphate buffered saline-PBS (0.015 M, pH 7.4) and centrifuged for 10 min, 18°C at 400 × g (Beckman, Model J-6B, USA). The samples were fixed with 200 μL of FACS fixative solution (10 g/L

paraformaldehyde, 1% sodium cacodylate, 6.67 g/L sodium chloride, pH 7.2). After 15 min at 4°C, samples were acquired using FACScan flow cytometer (BD, San Jose, CA, USA).

2.5. In vitro short-term whole blood culture

The short-term whole blood cultures were performed as described by Luiza-Silva et al., 2011 modified as follows: 1 mL aliquots of heparinized peripheral blood were dispensed into duplicates of 14 mL polypropylene tubes (Falcon®, B.D. Pharmingen, San Diego, CA) and incubated for 24 h at 37°C in a 5% CO_2 humidified atmosphere in the presence of 1 mL of RPMI 1640 (GIBCO – Grand Island, NY) (non-stimulated culture) or in the presence of *Mtb-Ag* diluted in RPMI at a final concentration of 15 μg/mL (stimulated culture). After priming, 10 μg/mL of Brefeldin A (BFA) (Sigma – Chemical Company – St Louis, MO) were added and the samples were re-incubated for an additional 4 h at 37°C in a 5% CO_2 humidified atmosphere. Following incubation, the cultures were treated with 2 mM EDTA, (Sigma – Chemical Company – St Louis, MO) and maintained at room temperature for 15 min. The duplicates were pooled into a 15 mL polypropylene tubes and then washed with 6 mL of FACS buffer (0.015 M PBS, supplemented with 0.5% bovine serum albumin—BSA and 0.1% sodium azide) by centrifugation at 600*g* for 7 min at room temperature. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1 mL of FACS buffer. The cell suspension was immediately submitted to immunophenotyping and intracytoplasmic cytokine analysis by flow cytometry. Positive control cultures were performed using Phorbol 12-Myristate 13-Acetate-PMA, ionomycin and BFA at final concentration of 25 ng/mL, 1 μg/mL and 10 μg/mL, respectively. These cultures were characterized by high levels of activation and cytokine production were used in all immunophenotyping and intracytoplasmic cytokine analysis to confirm cell viability of all blood samples (data not shown).

2.6. Intracytoplasmic cytokine staining

Aliquots of whole blood cultures were stained with anti-human cell surface TC-labeled monoclonal antibodies (anti-CD4, anti-CD8, anti-CD14, anti-CD16 and anti-CD19) for 30 min at room temperature. After membrane staining, erythrocyte lysis and leukocytes fixation, cell suspension was permeabilized with FACS perm-buffer (FACS buffer supplemented with 0.5% saponin) and aliquots incubated for 30 min at room temperature, in the dark, with fluorescent anti-cytokine monoclonal antibodies, including PE-labeled anti-IL-6, anti-IFN-γ, anti-TNFα, anti-IL-4, anti-IL-17, anti-IL-8 and anti-IL-10. After intracytoplasmic cytokine staining, the leucocytes were washed with FACS perm-buffer and FACS buffer and fixed in FACS fixative solution. Immunostained samples were acquired using a FACScan flow cytometer and analyzed using Flowjo software (Tree Star, San Jose, CA, USA). A total of 20,000 events/tube were acquired and stored for further analysis. Distinct gating strategies were used to analyze the cytokine-expressing leukocytes subsets, including neutrophils, monocytes and lymphocytes (B-cells, CD4$^+$T-cell and CD8$^+$T-cell subsets). The results were first expressed as percentages of cytokine-positive cells for different gated leukocyte subpopulations analyzed in this study. These values were used to obtain the cytokine index, calculated as the ratio between the percentage of cytokine-positive cells observed in the stimulated culture (*Mtb*-Ag) by the percentage of cytokine positive cells observed in the unstimulated culture (control).

2.7. Flow cytometric acquisition and analysis

Flow cytometry acquisition was performed in a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). After acquiring 30,000 events/tube, distinct gating strategies were used to analyze the frequency of different leucocyte subsets according to surface molecules defined by specific clusters of differentiation (CD). Samples were first

gated on lymphocytes using the morphometric parameters side-scatter (SSC low) and forward-scatter (FSC low). Subsequently, T and B lymphocytes were identified. CD4$^+$-T and CD8$^+$-T lymphocytes subsets were defined within the lymphocyte gate and the expression of chemokine (CC and CXC) receptors (CCR2, CCR3, CCR5, CXCR3 and CXCR4), cell adhesion markers (CD62L, CD18, CD54) and cell activation markers (CD25 and HLADR) were evaluated. Thus, the phenotype of CD4$^+$T and CD8$^+$T-lymphocytes were defined as: CCR2$^+$CD4$^+$/CD8$^+$, CCR3$^+$CD4$^+$/CD8$^+$, CCR5$^+$CD4$^+$/CD8$^+$, CXCR3$^+$CD4$^+$/CD8$^+$, CXCR4$^+$CD4$^+$/CD8$^+$; CD62^{neg}CD4$^+$/CD8$^+$, CD18$^+$CD4$^+$/CD8$^+$, CD54$^+$CD4$^+$/CD8$^+$; CD4$^+$CD25$^+$ and HLADR$^+$CD4$^+$/CD8$^+$. The reason for testing chemokine receptors on CD4$^+$T-cells and CD8$^+$T-cells is that these surface molecules can shape the migration potential of T-lymphocytes and induce polarization towards protective or pathogenic responses (Kim et al., 2001).

B-lymphocyte subsets were defined by expression of CD19$^+$. Within this subset, cell activation markers CD23, CD5 and CD69 were assessed. Gates were created from CD19 vs. CD23 or CD5 and B- lymphocyte subsets were defined as: CD23$^+$CD19$^+$, CD23^{Low}CD19$^+$, CD23^{High}CD19$^+$ and CD5$^+$CD19$^+$. In addition, the density of expression of CD69 on CD5$^+$CD19$^+$B-lymphocytes was determined by the geometric mean fluorescence intensity (MFI). NK and NKT subsets were defined by the absence or presence of CD3, CD56 and CD16 expression as follows: gates were created from CD3 versus SSC for selection of populations CD3$^-$ and CD3$^+$. Subsequently, gates were created from CD56 vs. CD16 and NK-cells were defined as: NK (CD56$^+$CD16$^{+/-}$CD3$^-$) and (CD56$^+$CD16$^{+/-}$CD3$^+$), respectively. NK-cells were evaluated according to the expression intensity of CD56, which were classified as: CD56^{neg}CD16$^+$, CD56^{dim}CD16$^+$ and CD56^{bright}CD16$^-$ (Fig. S1). The analysis of the subpopulations of circulating monocytes were defined by expression of HLA-DR, CD14 (receptor for binding of lipopolysaccharide complex and protein) and CD16 (low affinity receptor for IgG). For this purpose, a gate was created in HLA-DR$^+$ and intermediate SSC and, subsequently, gates were created from CD14 versus CD16. The circulating monocytes were defined as: classical monocytes (CD14$^+$CD16$^-$), proinflammatory monocytes (CD14$^+$CD16$^+$) and patrol monocytes (CD14^{Low}CD16$^+$). Activation each subset was assessed by the expression intensity of HLA-DR, determined by the geometric mean intensity of fluorescence (MFI). The software FlowJo version 7.0 (TreeStar, San Diego, CA, USA) was utilized for flow cytometric data analysis.

2.8. Phenotypic and functional signature analysis

The Phenotypic and functional signatures of patients with TB (UNI and BI) as well as controls (CO) were first calculated by assessing the percentages of low and high cytokine producers, as previously suggested by Luiza-Silva et al. (2011), using the global median as the cut off values to segregate low and high producers. The percentage of TB patients and CO volunteers showing high frequency was calculated for each leukocyte subset. For the *in vitro* study, *Mtb*-Ag/control indices were calculated. The ascendant curve of high frequency subjects on control group was then used as the reference curve to identify changes in the overall immune patterns from the other groups. Radar charts (Campi-Azevedo et al., 2016; Coelho-dos-Reis et al., 2013) were further used to summarize the cytokine signatures in a range of leukocyte subsets of innate [neutrophils (orange), monocytes (red)] and adaptive immunity [lymphocytes T (green), lymphocytes CD4 (blue), lymphocytes CD8 (salmon) e lymphocytes B (lilac)]. Each axis represents the frequency (%) of volunteers showing high cytokine-producing cell indices or high frequency of a specific cell subset.

2.9. Statistical analysis

Statistical analyses between groups were performed by parametric analysis by Student *t* test or nonparametric (Mann-Whitney) test. In all
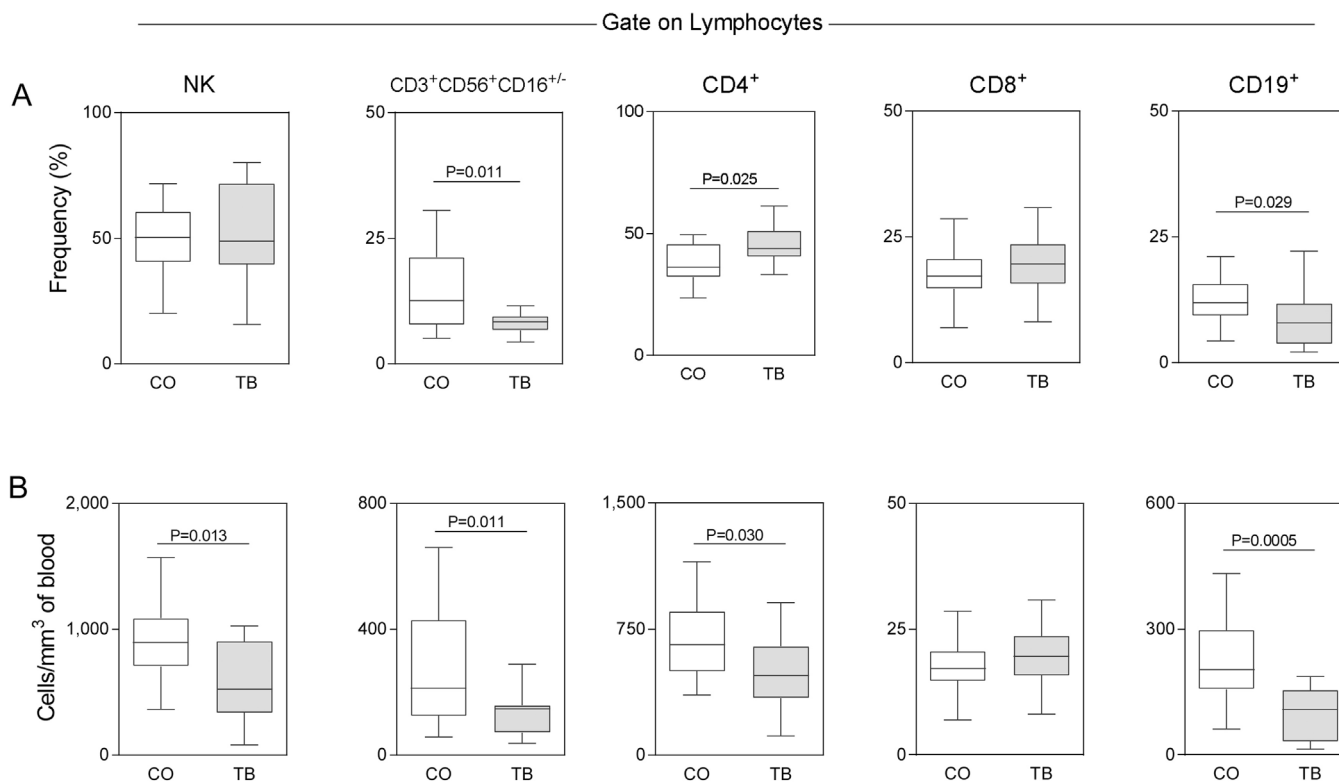
Fig. 1. Patients with pulmonary tuberculosis display altered leukocyte profile in the peripheral blood. (A) Percentage and total number (B) of circulating NK-cells (CD3$^+$CD56$^+$CD16$^{+/-}$), CD4$^+$T-cells, CD8$^+$T-cells and CD19$^+$B-cells in Control-CO (n = 20), Tuberculosis-TB (n = 15) groups, obtained by flow cytometry. The respective subsets of lymphocytes were determined within the gate of total lymphocytes. Results were plotted in box plot graphics, with median and interquartile range. Data were analyzed using the Mann-Whitney test. Differences were considered significant when p ≤ 0.05.

cases, significance was considered at p ≤ 0.05. All tests were provided by GraphPad Prism version 5.0 (San Diego, CA). The analysis of cytokine signatures was performed using the cytokine signature from control group as the reference curve, and significant differences were considered when the indices were superior to the 50th percentile as compared to the one in the reference signature (CO).

3. Results

3.1. Patients with pulmonary tuberculosis display altered leukocyte profile in the peripheral blood

The profile of circulating leukocytes in peripheral blood of patients with pulmonary tuberculosis was evaluated. Fig. 1 shows the *ex vivo* phenotypic results as percentages (Fig. 1A), as well as absolute numbers (Fig. 1B) of NK-cells, NKT-cells, CD4$^+$T-cells, CD8$^+$T-cells and B-cells from peripheral blood of CO and TB groups. Regarding NK-cells and CD8$^+$T-cells, no differences were observed between the groups (Fig. 1A). Data analysis demonstrated that there is significant decrease in both proportions and absolute numbers of the CD3$^+$CD56$^+$CD16$^{+/-}$ NKT- cell and CD19$^+$B-cell subsets in TB as compared to CO. A statistically significant increase was observed in the CD4$^+$T-cells in the TB group as compared to CO group. On the other hand, the opposite was observed when the absolute of CD4+T-cell numbers were assessed. Despite no differences were observed in the frequency of NK-cells (Fig. 1A), TB patients displayed decreased absolute number compared to CO (Fig. 1B). These data suggest that the TB group has selective alterations in CD3$^+$CD56$^+$CD16$^{+/-}$ cells, CD4$^+$-T as well as CD19$^+$-B lymphocytes.

3.2. Monocyte subsets display distinct activation profile during tuberculosis

In order to verify if subsets of monocytes and their HLA-DR

expression could be related to *Mtb* infection, the phenotypic profile of three monocyte subsets was evaluated. For that, classic (CD14$^+$CD16$^-$), proinflammatory (CD14$^+$CD16$^+$) and patrolling (CD14^{Low}CD16$^+$) monocyte subsets were analyzed (Fig. 2A). TB presented a significant decrease in the frequency of patrolling monocytes when compared to CO (Fig. 2B). No differences were observed in classic and proinflammatory monocytes. Analysis of the HLA-DR on each subset indicated that the proinflammatory monocytes displayed the highest levels of expression in both CO (classic and patrolling) and TB (Classic) (Fig. 2C). Higher HLA-DR expression was observed in patrolling monocytes from TB when compared to CO. No differences in HLA-DR expression were observed between classic and proinflammatory monocytes.

Mtb-infected patients were segregated according to disease severity as unilateral (UNI) and bilateral (BI) based on the extent of pulmonary lesions observed by the analysis of chest radiographs. The results show that despite similar frequencies of distinct monocyte subsets, patrolling monocytes from BI display a significant decrease in HLA-DR expression compared to UNI. These findings indicate that the activation profile of patrolling monocytes is not only altered in TB patients but also decreased significantly in patients with bilateral TB.

3.3. Phenotypic analysis of circulating CD4$^+$T-cell and CD8$^+$T-cell subsets of patients with pulmonary tuberculosis

We next investigated the percentage of circulating CD4$^+$T-cells and CD8$^+$T-cells expressing the chemokine receptors CCR2, CCR3, CCR5, CXCR3 and CXCR4 as well as the cellular activation markers CD62L, CD54, CD18, CD25 and HLA-DR in CO and TB groups. The results displayed in Fig. 3A showed that there is a statistically significant increase in the percentage of CCR2$^+$CD4$^+$T-cells, CCR3$^+$CD4$^+$T-cells, CD18$^+$ CD4$^+$T-cells and HLA-DR$^+$CD4$^+$T-cells in TB when compared to CO. In regards to the CD8$^+$T-cells (Fig. 3B), there is statistically
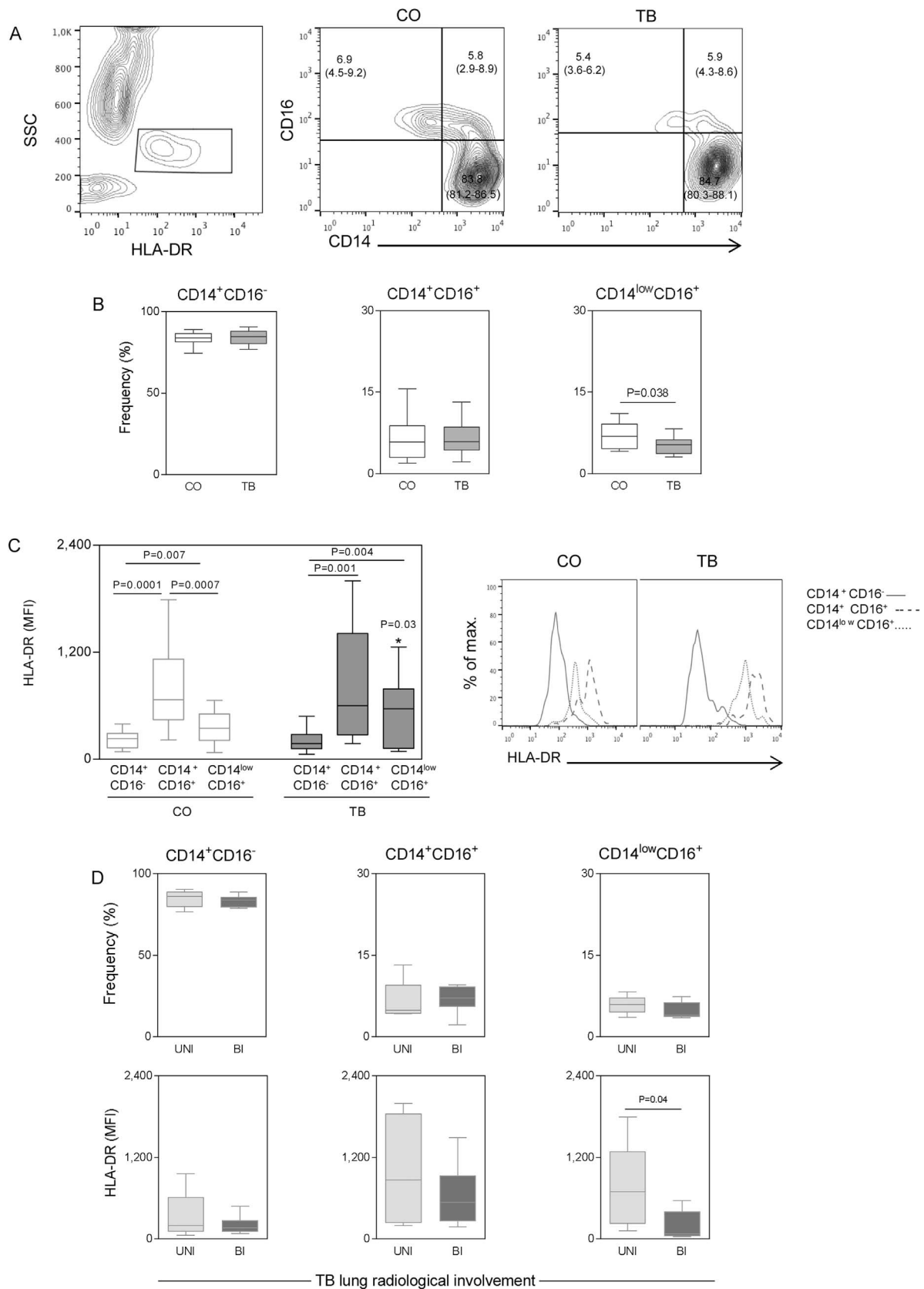
Fig. 2. Circulating monocytes profile in patients with pulmonary tuberculosis. (A) FACS plots showing the distribution of circulating subtypes of monocytes defined by markers HLA-DR, CD14 and CD16 in Control-CO (n = 20) and Tuberculosis-TB (n = 15) groups, with the respective values of the median and Q2 and Q3 quartiles. (B) Monocyte subsets $CD14^+CD16^-$, $CD14^+CD16^+$ and $CD14^{low}CD16^+$ are shown in box plot graphics, with median and interquartile range. Data were analyzed using the Mann-Whitney test. (C) On the left, expression of HLA-DR (Median Fluorescence Intensity − MFI) in subsets of monocytes in the group CO (white bars) and TB (black bars). Data were analyzed using the t-test. On the right, FACS histogram graphs showing HLA-DR expression in $CD14^+CD16^-$ (continuous line), $CD14^+CD16^+$ (dashed line) and $CD14^{low}CD16^+$ (dotted line) subsets of monocytes. (D) Left, percentage of subsets of monocytes $CD14^+CD16^-$, $CD14^+CD16^+$ and $CD14^{low}CD16^+$ in TB group patients with unilateral radiological abnormalities-UNI (n = 05) and bilateral-BI (n = 08). Below, median intensity of HLA-DR fluorescence in subsets of monocytes in the group UNI and BI, using the Mann-Whitney test. Differences were considered significant when
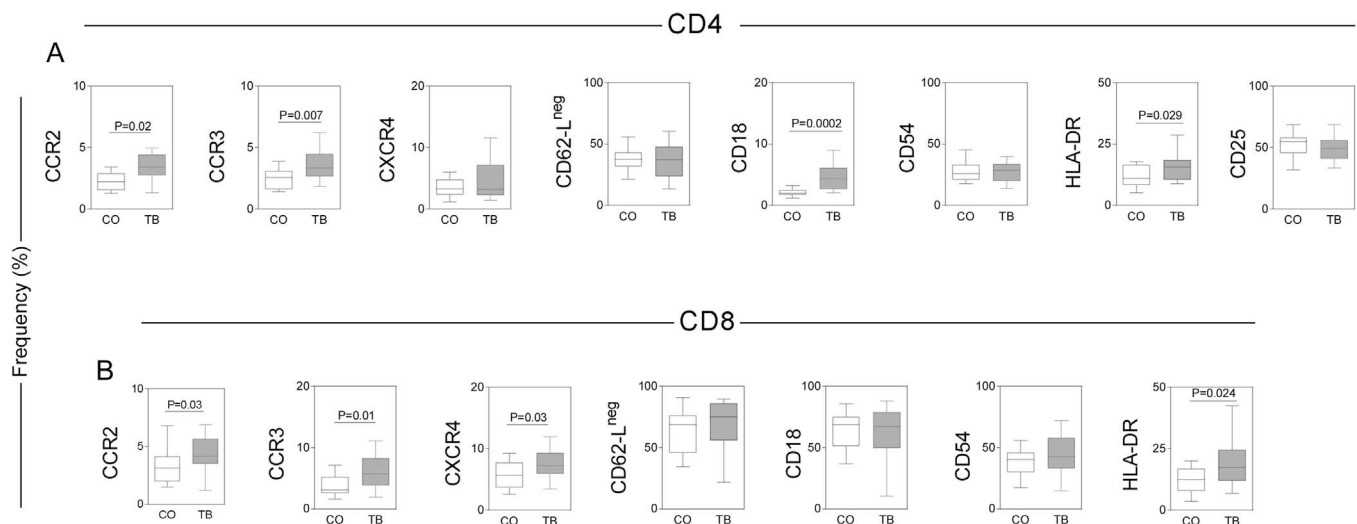
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Fig. 3. Phenotypic analysis of circulating CD4$^+$T-cell and CD8$^+$T-cell subsets of patients with pulmonary tuberculosis. Percentage of circulating CD4$^+$T-cells and CD8$^+$T-cells expressing the chemokine receptors CCR2, CCR3, CCR5, CXCR3 and CXCR4 as well as the cellular activation markers CD62L, CD54, CD18, CD25 and HLA-DR in Control-CO (n = 20) and Tuberculosis-TB (n = 15) groups, obtained by flow cytometry. The CD4$^+$T-cell and CD8$^+$T-cell subsets were determined within the gate of total lymphocytes. Results were plotted in Box plot graphics, with median and interquartile range. Data were analyzed using the Mann-Whitney or t-test. Differences were considered significant when p ≤ 0.05.

significant increase in the percentage of CCR2$^+$CD8$^+$T-cells, CCR3$^+$CD8$^+$T-cells, CXCR4$^+$CD8$^+$T-cells and HLA-DR$^+$CD8$^+$T-cells in TB versus CO. On the whole, the analysis of circulating CD4$^+$T-cells and CD8$^+$T-cells suggest that TB patients displayed higher activation and migration pattern in these lymphocyte subsets.

3.4. TB patients with bilateral pulmonary lesion display lower percentages of CD19$^+$CD5$^+$ B-lymphocytes

Fig. 4 shows the percentage of CD19$^+$B-cells and subsets according CD23 expression (CD19$^+$CD23$^+$, CD19$^+$CD23^{Low} and CD19$^+$CD23^{High}) in patients with pulmonary tuberculosis (Fig. 4A). The data allowed the identification of distinct frequency of B subsets in TB. There is a significantly reduced percentage of CD19$^+$CD23$^+$ and percentage of CD19$^+$CD23^{High} cells in the TB group compared to CO. The percentage of CD19$^+$CD23^{Low} cell subset did not differ significantly between groups. No significant differences were observed in the CD19$^+$B-cell subsets when UNI and BI were compared.

In addition, we evaluated the percentage of CD19$^+$CD5$^+$B-cells as well as the activation of these cells by CD69 expression. The data demonstrated that TB patients displayed significantly lower percentages of CD19$^+$CD5$^+$ cells when compared to CO. The activation of these cells did not differ significantly between groups. The percentage of CD19$^+$CD5$^+$ cells were higher in UNI when compared to BI. The results reveal that the innate B-cell subsets such as CD19$^+$CD5$^+$-cells may be important for the protective mechanisms triggered during the lung lesion formation.

3.5. Ex vivo immunophenotypic signature analysis reveal increase and decrease in innate and adaptive immune components in TB patients with distinct radiological involvement

The phenotypic leukocyte signatures in the peripheral blood of CO and TB were determined (Fig. 5). For that, continuous values were transformed in categorical data and trend curves for the CO group (reference curve) were plotted and overlaid on the histograms of TB, UNI and BI groups. It is possible to observe that both the innate and adaptive immunity curves of TB show a peak above the reference curve, indicating that there is an important role of acquired immunity with emphasis on CD4$^+$T-cell and CD8$^+$T-cell subsets during TB. On the other hand, there are clear gaps at the end of both the innate and adaptive immunity curves, composed mainly by CD4$^+$T-cells and B-

cells. However, when comparing UNI and BI groups, there was a mixed profile in relation to the innate and adaptive immunity; the first of which resembles the profile of the individuals in the group CO, and the second, the TB group. These findings reveal that the overall innate and adaptive immune profiles of TB patients with different lung lesion commitment are diversified, which may be relevant for the foundation of such clinical patterns.

3.6. Production of proinflammatory and regulatory cytokines of the innate and adaptive immunity are distinct in tuberculosis patients with different extent of lung lesions

The production of intracytoplasmic chemokine/cytokines by circulating leukocytes of patients with pulmonary tuberculosis was analyzed after short-term whole blood culture in the presence or absence of Mtb-Ag (Fig. 6). Categorical data for chemokine/cytokine-producing neutrophils, monocytes, total T-cells, CD4$^+$T-cells, CD8$^+$T-cells and CD19$^+$B-cells are displayed as radar graphs built with the frequency of high producers (%) from each group (CO, TB, UNI and BI). Left wing indicates molecules (black) associated with inflammation and right wing indicates molecules (gray) associated with regulation of the immune response. It is possible to observe that the chemokine/cytokine profile of CO delineates a graph area, which is confined in the 50% threshold and left and right wings show balanced percentages.

Expansion of the area graphs is observed in TB. Involvement of innate an adaptive immunity in the production of cytokines is observed in TB group as compared to CO. Interestingly, TB patients present lower frequency of high IL-4$^+$ and IL-10$^+$ neutrophils when compared to controls. In general, a mixed profile in the innate and adaptive immunity was observed in patients with UNI or BI pulmonary lesions. However, the expansion of area graph on the left wing of UNI was evident when compared to BI, which was contrasted by significantly larger production of IL-4 by neutrophils, total T-cells, CD4$^+$T-cells, CD8$^+$T-cells and CD19$^+$B-cells. The frequency of high producers of IL-17$^+$CD4$^+$T-cells and CD8$^+$T-cells were significantly increased in BI as compared to UNI. These results suggest that IL-17 could play a deleterious role, while IL-4 may be associated with a possible protective role during active pulmonary Mtb infection.

4. Discussion

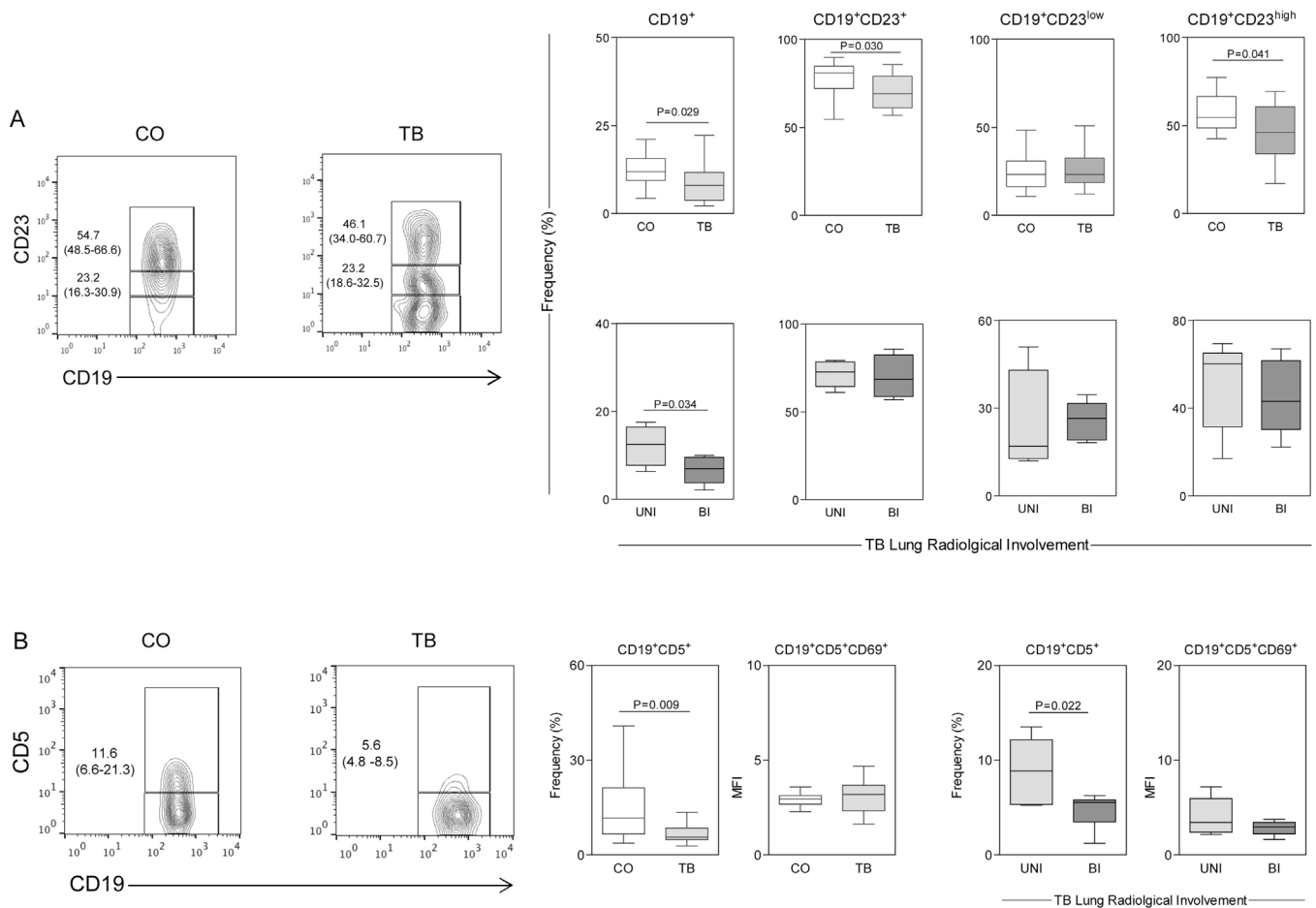Active tuberculosis is a spectrum disease, which assumes influence

Fig. 4. Circulating B-cell profile in patients with pulmonary tuberculosis. (A) On the left, FACS plots showing the distribution of circulating subtypes of B-cells defined by markers CD19 and CD23 in Control-CO (n = 20) and Tuberculosis-TB (n = 15) groups, with the respective values of the median and Q2 and Q3 quartiles. Right, percentage of CD19^{+}, CD19^{+}CD23^{low} and CD19^{+}CD23^{high} subsets of B-cells in CO (n = 20), TB (n = 15), unilateral radiological abnormalities-UNI (n = 5) and bilateral-BI (n = 8) groups are shown in box plot graphics, with median and interquartile range. The CD19^{+}CD23^{+} subsets of B-cells were determined by the sum of CD19^{+}CD23^{low} and CD19^{+}CD23^{high}. Data were analyzed using the Mann-Whitney test. (B) On the left, FACS plots showing the distribution of circulating subtypes of B-cells defined by markers CD19 and CD5 in CO (n = 20) and TB (n = 15) groups, with the respective values of the median and Q2 and Q3 quartiles. On the right, percentage of CD19^{+}CD5^{+} subsets of B-cells in CO (n = 20), TB (n = 15), UNI (n = 05) and BI (n = 08) groups shown in boxplot graphs, with median and interquartile range. Activation of CD19^{+}CD5^{+} subsets of B-cells was determined by the median fluorescence intensity (MFI) of the CD69 marker. Data were analyzed using the Mann-Whitney test. Differences were considered significant when p ≤ 0.05.

of several factors such as bacterial load, the host immune response and the extent of lung commitment. The activation of the immune system and inflammation are, with no doubt, key players in the immunopathogenesis of disease. However, the complexity of the interactions between *Mtb* and host factors hinders the establishment of biomarkers related to activation of the disease and protection (Lyadova and Panteleev, 2015).

In this context, the present study aimed at evaluating the profile of circulating leukocytes in TB patients as compared to the profile observed in co-inhabitants without *Mtb* infection (CO group) from an urbanized region of Minas Gerais state, in Brazil. It is important to consider that these patients are living in an endemic area and were analyzed before treatment. The results of this study showed significant reduction in the absolute numbers of NK-cells, NKT-cells, CD4^{+}T-cells and CD19^{+}B-cells in the TB group when compared to the controls. Regarding the percentage, there was reduction in CD19^{+}B-cells and NKT-cells, but an increase in CD4^{+}T-cells. Wu et al. (2009) observed similar results in relation to the percentage of NK cells and CD4^{+}T-cells, however, increased in CD19^{+}B-cells and CD8^{+}T-cells. Moreover, Guglielmetti et al. (2013) observed reduction in the absolute numbers of CD4^{+}T-cells and no difference in the percentage of these cells. They also showed an increase in the percentage of CD8^{+}T-cells and variation in the absolute numbers of CD19^{+}B-cells. So far, it has not yet been possible to establish the profile of circulating lymphocytes in active

pulmonary tuberculosis, as published studies showed controversial results. However, it is suggested that patients with pulmonary tuberculosis have changes in circulating lymphocyte homeostasis, which can be associated with disease progression (Wu et al., 2009). The contrast on the results of percentage versus absolute numbers of CD4^{+}T-cells may reflect a general leukopenia with a more severe damage to B- lymphocytes and compared to CD4^{+}T-cells. The general leukopenia explains the lower absolute numbers of CD4^{+}T-cells in TB patients. On the other hand, considering that CD4^{+}T-cell percentages were measured within the lymphocyte gate which includes B-cells and NKT-cells, an alteration in latter could reflect on the percentage of the former, in such a way that the more severe decrease in B cell and NKT-cell subsets among the total lymphocyte population may account for the increased percentage of CD4^{+}T-cells among lymphocytes.

In this regard, monocytes have been described as essential players not only in disseminating the pathogen in the host, but also in priming innate events as well as in triggering acquired immunity against *Mtb* (Tang et al., 2015). In *Mtb* infection, little is known about the relationship between the phenotype of monocytes and their function against the pathogen. Previous studies have shown distinct profiles in the percentage of circulating monocytes in patients simultaneously infected with tuberculosis and other pathogens. Andrade et al. (2014) found that patients co-infected TB-HIV, who developed IRIS (Immune Reconstitution Inflammatory Syndrome), displayed decreased
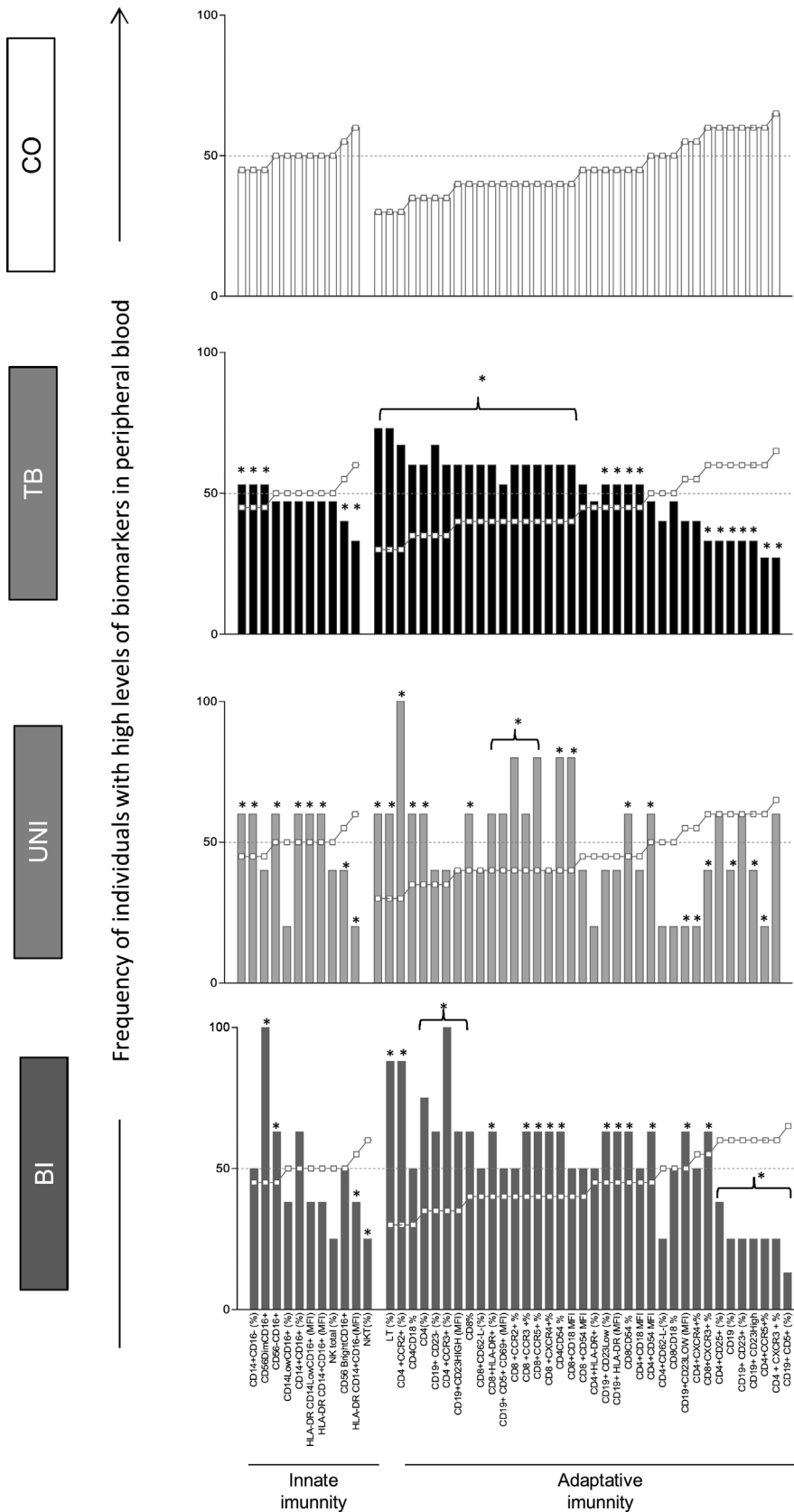
Fig. 5. Phenotypic cellular signature of TB. Continuous values were transformed in categorical data and trend curves for the Control-CO group (reference curve) were plotted and overlaid on the histograms of Tuberculosis-TB (Black), unilateral radiological abnormalities-UNI (light gray) and bilateral-BI (dark gray) groups.

percentage of patrolling monocytes (CD14^{Dim}CD16^{+}), and increased frequencies of classic and inflammatory monocyte subsets compared to patients who did not developed the syndrome. Castaño et al. (2011) observed an increase in inflammatory and patrolling monocytes during TB. The present data partially agree with these previous findings, showing a conspicuous reduction in circulating patrolling monocytes in the TB group and, at the same time, increased activation of this subset assessed by the up-regulation of the HLA-DR expression. It is suggested
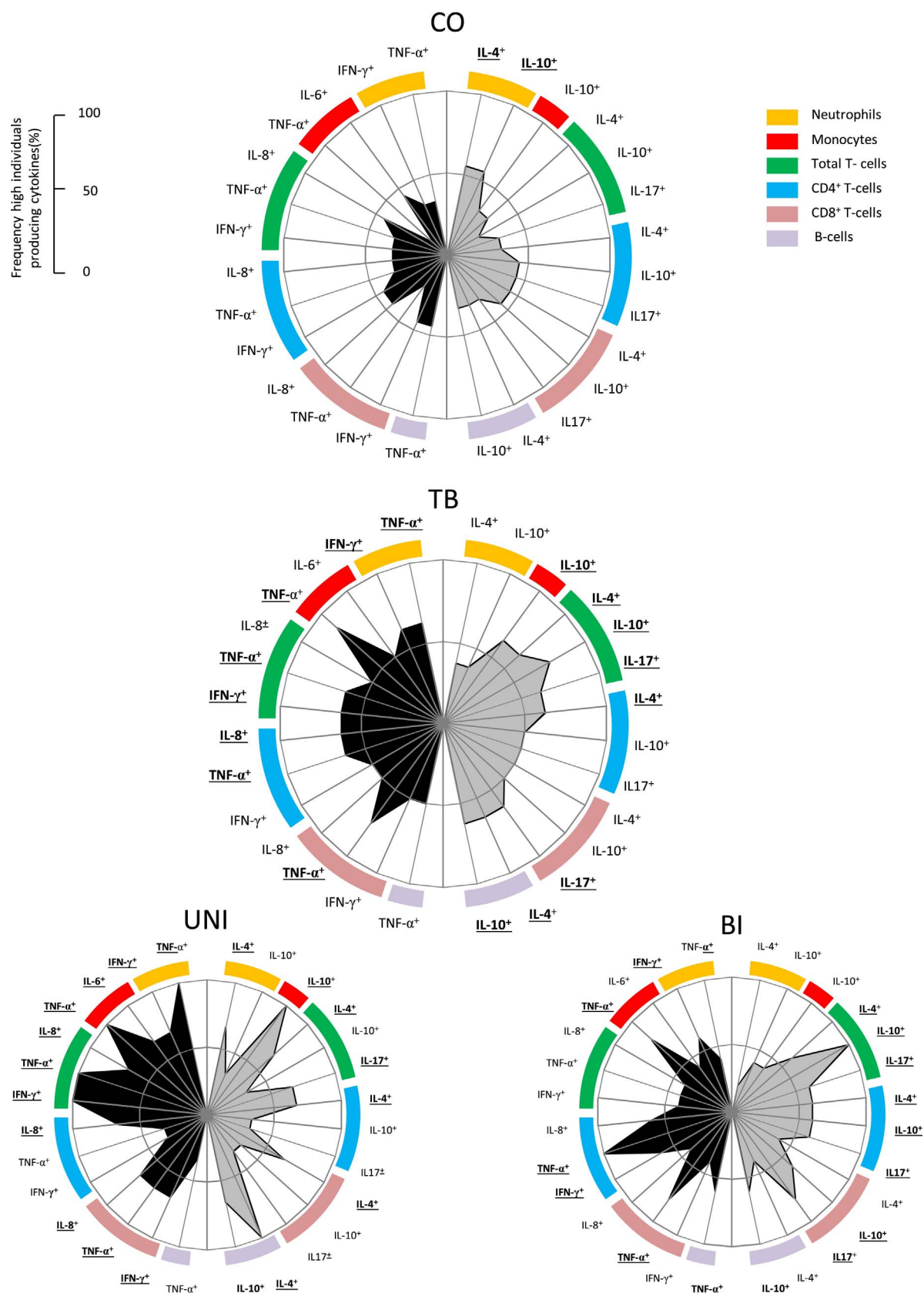
Fig. 6. Overall pattern of high cytokine producers triggered by *Mtb*-Ag. Categorical data for chemokine/cytokine-producing subsets neutrophils (orange), monocytes (red), total T-cells (green), CD4^{+} T-cells (blue), CD8^{+} T-cells (salmon) and B-cells (lilac) are displayed as radar graphs built with the frequency of high producers (%) by Control-CO (n = 09), Tuberculosis-TB (n = 08), unilateral radiological abnormalities-UNI (n = 03) and bilateral-BI (n = 05). Left wing indicates proinflammatory molecules (black) and right wing indicates regulatory molecules (gray). Cytokines/chemokines were above 50% were placed in bold and underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that the decrease in the percentage of circulating patrolling monocytes in TB patients is due to the recruitment of these cells to the site of infection (Belge et al., 2002), which is highly influenced by the cell composition and chemokine/cytokine milieu (Stríz et al., 1993).

Furthermore, increased expression of HLA-DR is directly related to cell activation and improves antigen presentation to T lymphocytes (Lapa e Silva et al., 1996). A hallmark of *Mtb* is its ablility to efficiently evade the immune system. Thus, despite activated in the circulation we cannot rule out the possibility of these monocytes become suppressed after recruited to the lung. Balboa et al. (2015) have shown that CD16$^+$ monocyte subpopulations can contribute to the infection, as they are somewhat resistant to microbial growth and present decreased nitric oxide production capacity compared to CD16$^-$ monocytes. Strengthening this hypothesis, data from the present study show that TB patients with bilateral pulmonary involvement have reduced HLA-DR expressing patrolling (CD14^{Low}CD16$^+$) monocytes as compared to unilateral involvement. Previous studies have shown that the reduction of HLA-DR expression on monocytes is related to the severity of infectious diseases (Ditschkowski et al., 1999) and could be employed as a reliable prognostic marker for the surveillance of severe sepsis (Lekkou et al., 2004).

Contrary to what it is known about the importance of the proinflammatory immune response in the host defense against *Mtb*, much remains to be clarified on the role of B-lymphocytes during tuberculosis (Kozakiewicz et al., 2013). In this study, there was significantly reduced percentage and absolute numbers of CD19$^+$B-cell and CD19$^+$CD5$^+$B-cell subsets in the TB when compared CO. A similar profile was observed in BI group when compared to the UNI. Patients with severe tuberculosis have lower percentage of circulating B-cells. These data corroborate previous studies that described decreased numbers of circulating CD19$^+$B-cells in patients with pulmonary tuberculosis (Abreu et al., 2014; Hernandez et al., 2010). In addition, the reduction of CD19$^+$CD5$^+$B-cell subsets could be related to the severity of the disease (Guglielmetti et al., 2013). However, other studies showed an increase in the percentage of circulating B-cells in pulmonary tuberculosis patients (Zhang et al., 2012; Wu et al., 2009).

Reports using mouse models suggest that anti-TB B-cell response in the lung has clear pathological and doubtful protective role (Linge et al., 2017). Linge and collaborators demonstrate that B-lymphocytes forming follicle-like structures in the lung tissue of tuberculosis-infected mice presented a regulatory profile with absence of surface molecules CD5 and CD23 (LINGE et al., 2017). CD5 is a cell surface receptor that negatively regulates B-cell function by inhibiting BCR activation (Gary-Gouy et al., 2002). CD5 is expressed by B-1 cells which are regarded as regulatory/innate-like B-cells. The significant decrease of CD5 + CD19 + -B cells found in BI as compared to UNI may indicate that regulation by this subset may be protective. In parallel, CD23 is a receptor for IgE of low-affinity, which is involved in allergy and resistance to parasites, as well as in the regulation of IgE levels (Payet et al., 1999). Mossalayi et al. (2009) reported that surface CD23 mediates antimicrobial activity of human macrophages against *Mycobacterium*, in part, through the induction of effector responses including TNF-α production. These results are in line with the present findings that show lower percentages of CD19$^+$CD23$^+$B-cells and CD19$^+$CD23^{high}B-cells in TB as compared to CO.

In addition to antibody production, B-cells regulate the immune response though the production of modulatory cytokines and the interaction with effector T-lymphocyte subsets (du Plessis et al., 2015). Our results are in line with this hypothesis considering that BI group displayed a clear mixed inflammatory/regulatory profile, when compared to UNI, with evident IL-4, IL-10 and IL-17 production by both CD4$^+$T-cells and CD8$^+$T-cells. Zhang et al. (2012) demonstrated that, in patients with pulmonary tuberculosis, circulating CD19$^+$CD5$^+$B-cells are able to inhibit the production of IL-17 by CD4$^+$T-cells. These data suggest that the production of IL-17, associated with a reduction in the production of IFN-γ by cells from the innate and the adaptive

immunity in the BI group may contribute to the pathogenesis of tuberculosis, considering the inhibitory properties of IFN-γ in IL-17 production (Lyadova and Panteleev et al., 2015). It has been suggested that one of the possible mechanisms involved in the deleterious action of IL-17 during *Mtb* infection is related to the increase in the neutrophil migration into the lung (Kozakiewicz et al., 2013), which contributes to the pathogenesis of tuberculosis (Eruslanov et al., 2005).

In addition, considering that these patients come from endemic areas affected with helmiths and environmental mycobacteria, infections with such pathogens may influence the response towards a Th2 profile, in detriment to the IFN-γ response, which could affect the phenotype observed. It is important to mention, however, that the patients evaluated herein underwent close and deep clinical evaluation prior to enrollment in the study and no evidence of such infection were detected. Eosinophil counts were considered normal during the clinical evaluation, also indicating that no helminth infection was occurring at the time of blood collection.

5. Conclusion

All in all, the immunophenotypic characterization of unilateral and bilateral active TB performed in the present study indicates that the extent of lung lesion could be associated with a fine-tuning between immunological responses during untreated *Mtb* infection.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2017.05.016.

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