Immunobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

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

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ARTICLE INFO

Keywords:
Tuberculosis
Immune response
Clinical outcomes
Immunophenotyping

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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http://dx.doi.org/10.1016/j.imbio.2017.05.016
Received 22 December 2016; Received in revised form 5 April 2017; Accepted 23 May 2017

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