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Evaluation of profile and functionality of memory T cells in pulmonary tuberculosis

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ABSTRACT

The cells T CD4+ T and CD8+ can be subdivided into phenotypes naïve, T of central memory, T of effector memory and effector, according to the expression of surface molecules CD45RO and CD27. The T lymphocytes are cells of long life with capacity of rapid expansion and function, after a new antigenic exposure. In tuberculosis, it was found that specific memory T cells are present, however, gaps remain about the role of such cells in the disease immunology. In this study, the phenotypic profile was analyzed and characterized the functionality of CD4+ T lymphocytes and CD8+ T cells of memory and effector, in response to specific stimuli *in vitro*, in patients with active pulmonary TB, compared to individuals with latent infection with *Mycobacterium tuberculosis* the ones treated with pulmonary TB. It was observed that the group of patients with active pulmonary tuberculosis was the one which presented the highest proportion of cells T CD4+ of central memory IFN-γ+ e TNF-α+, suggesting that in TB, these T of central memory cells would have a profile of protective response, being an important target of study for the development of more effective vaccines; this group also developed lower proportion of CD8+ T effector lymphocytes than the others, a probable cause of specific and less effective response against the bacillus in these individuals; the ones treated for pulmonary tuberculosis were those who developed higher proportion of T CD4+ of memory central IL-17+ cells, indicating that the stimulation of long duration, with high antigenic load, followed by elimination of the pathogen, contribute to more significant generation of such cells; individuals with latent infection by *M. tuberculosis* and treated for pulmonary tuberculosis, showed greater response of CD8+ T effector lymphocytes IFN-γ+ than the controls, suggesting that these cells, as well as CD4+ T lymphocytes, have crucial role of protection against *M. tuberculosis*. These findings have contributed to a better understanding of the immunologic changes in *M. tuberculosis* infection and the development of new strategies for diagnosis and prevention of tuberculosis.

1. Introduction

The lymphocytes memory T cells comprise a heterogeneous group of cells of long life, which are developed at the immune response antigen-specific and exhibit ability to expand quickly after a second exposure to the antigen, although in low concentrations [1–3]. Various surface markers can be analyzed to subdivide phenotypically between the populations of T cells, being more used those involved in phenomena of activation (CD45Ra and CD45Ro), co-stimulation (CD27 and CD28) and cell migration (CCR7) [2,4]. According to the expression of molecules CD45Ro and CD27, CD4+ T cells and CD8+ can be subdivided into four phenotypes: T naïve (CD45Ro-CD27+), T central memory − T_{MC} (CD45Ro + CD27+) T effector memory − T_{ME} (CD45Ro + CD27-) and T effector (CD45Ro-CD27-) [2,5,6,7–9]. The T_{ME} cells are present in the blood and non-lymphoid tissue and are able to respond quickly against antigens presented previously; the T_M cells are mainly found in lymphoid organs and would be able to differentiate themselves and assume a phenotype of effector memory [3,10].

Abbreviations: PBMC, peripheral blood mononuclear cells; IL-17, interleukin-17; IL-2, interleukin-2; IFN- γ, Interferon-gamma; TNF-α, tumor necrosis factor-alpha; Mtb, *Mycobacterium tuberculosis*; Mtb-Ags, antigens of the strain H37Rv of *Mycobacterium tuberculosis*; TB, Tuberculosis; T_{MC}, T of central memory; T_{ME}, T of effector memory; LTBI, individuals with latent infection by *Mycobacterium tuberculosis*; TBPT, patients after treatment of pulmonary TB; ATBP, patients with active pulmonary tuberculosis

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The knowledge about the memory T cells was initiated from studies of acute and chronic viral infections, being that chronic bacterial infections, such as tuberculosis (TB), have been little studied [11]. The importance of subpopulations of memory T lymphocytes in infection caused by *Mycobacterium tuberculosis* (*Mtb*) has been demonstrated in studies performed with animals [12–14]. In some of the studies that evaluated new vaccine candidates against TB [15] the effectiveness of new alternatives of immunization was analyzed according to the immunological memory developed by analysis of subpopulations of memory cells [14,16]. These populations have also been the subject of research in comparative studies of the immune response anti-*Mtb* in groups of individuals infected by the Koch bacillus, among them, those who developed active pulmonary TB, those who were treated and cured of the disease and those who maintained the infection in the state of latency [7,17,18].

Although memory T cells-Mtb are induced in individuals infected by bacillus [19], gaps remain about the similarities and/or differences of this specific cellular response among individuals with latent infection by bacillus, patients with active pulmonary TB and treated for pulmonary TB, both at the phenotypic level and functionality of response. The role of these cells in the human being is still little understood: the single vaccine available against TB is not able to generate a response of memory effectively, since it promotes variable protection in the human population and appears to be effective in protecting the population against only the most severe forms of the disease. Furthermore, the fact those individuals treated for TB could be infected again and, develop again the disease, raises the question of how protective the memory T cells are, specific to *Mtb* [7].

Through this study, the phenotypic profile was analyzed and the functionality of CD4+ T lymphocytes and CD8+ T cells were characterized in response to stimulation in vitro with antigens of the strain H37Rv *Mtb*, in patients with active pulmonary TB (ATBP), compared to individuals with latent tuberculosis infection (LTBI), to patients after treatment of pulmonary TB (TBPT) and to healthy non-infected individuals (controls).

2. Materials and methods

2.1. Case analysis and samples collection

Four groups of participants were selected for the study: I − healthy volunteers were asymptomatic with negative tuberculin test (first test = 0 mm and booster effect negative, not infected by *M. tuberculosis* − controls); II − asymptomatic volunteers with positive tuberculin test (first test = 10 mm or first test < 10 mm and second test, after a week, > 10 mm with an increment of 6 mm from the first reading − LTBI); III − patients with active pulmonary TB (diagnosed by sputum smear microscopy and/or culture of identification for mycobacteria − ATBP); IV − Patients treated for active pulmonary TB and cured (finalized treatment, presented two sputum smear microscopy or, by absence of expectoration, were discharged on the basis of the data clinical and radiological findings − TBPT). Individuals aged between 18 and 60 years were included, of both genders, all with negative serology for HIV infection (assessed by immunochromatographic method fast, HIV-EIC, Gold analisa, Brasil) and no history of pathology or use of a substance that affects the immune status (for example, pregnant women, diabetics, patients with autoimmune disease, in use of corticosteroids). Participants were recruited of the Federal University of Minas Gerais (HC/UFMG) and the Pharmacy Faculty of UFMG. All individuals who agreed to participate in the study signed an Free and Informed Consent Form, were interviewed by health professionals and responded to a clinical and socioeconomic questionnaire, standardized by the network-TB of research. 20 mL of peripheral blood of each participant eligible for the study was collected, being 16 mL collected in anticoagulant heparin (for completion of *in vitro* assays) and 4 mL collected in EDTA anticoagulant (for research of HIV infection). The collection of blood samples from participants of the group ATBP was performed before or up to two weeks after the beginning of the anti-TB treatment [6,7,20]. This study was approved by the Research Ethics Committee at the UFMG − COEP, No 28/13, registration Nº. 13864313.3.0000.5149.

2.2. Antigens of M. tuberculosis

Antigens of the strain H37Rv Mtb (Mtb-Ags) were recovered in the Mycobacteria Laboratory (Hospital das Clínicas/UFMG, Brazil). The strain was cultivated in the medium Loweinstein-Jensen and incubated at 37 °C until evidence of bacterial growth. The 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 sterilized by gamma radiation (dose of 5000 Gy for 2 h and 15 min). The proteins concentration was measured by the method of Lowry [21].

2.3. Culture of peripheral blood mononuclear cells and stimulation

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation in a gradient of Ficoll Hypaque (density 1.077g/mL, Sigma Chemical Co. St. Louis, MO). The PBMC were cultivated on plates of cultivation of 24 holes (Corning Costar Corporation, Cambridge, MA) for 144 h at 37 °C, in an atmosphere of 5% CO_2 and 95% humidity. 1.5×10^6 cells were grown per well, containing 1.0 mL of RPMI-1640 (Gibco − Grand Island, NY) supplemented with 5% of human AB serum (Sigma-Aldrich, St. Louis, MO), 50IU penicillin/0.05 mg streptomycin/mL (Sigma-Aldrich, St. Louis, MO) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO); in the presence of Mtb-Ags (10 μg/mL − cell culture stimulated − SC), in the absence of Mtb-Ags (supplemented RPMI-1640 medium − cell culture without stimulation or control − CC). One aliquot of cells serving as positive control was stimulated with phytohemagglutinin (PHA − 5 μg/mL).

2.4. Immunophenotyping of lymphocytes and analysis of intracytoplasmic cytokines

Brefeldina A (1.0 μL/mL, BD Golgi PlugTM, BD Biosciences) was added to cultures, leaving 4 h to the end of cultivation. Finalizing the incubation period, the cells were transferred to tubes of expanded polystyrene of 5 mL and washed in PBS-Wash (phosphate buffered saline [PBS] 0.015 M pH 7.4, supplemented with 0.5% bovine serum albumin [BSA, Sigma Chemical Co.] and 0.1% sodium azide [VETEC]). Then the cell suspension was incubated with a combination of cocktail of monoclonal antibodies anti CD4-FITC (PAR-T4, BD Biosciences), anti CD8-APC (PAR-T8, BD Biosciences), anti-CD45RO-PerCp Cy5.5 (UCHL1, BD Biosciences) and anti-CD27-efluor 450 (The323, eBiosciences). The cells were incubated for 30 min at room temperature and protected from light. After incubation, the cells were lysate and the leukocytes were fixed, with 2 mL of aqueous solution of pH 7.85, containing sodium citrate hydrogen orthophosphate; 0.25% w/v, formaldehyde PA 5.4% v/v, diethylene glycol PA 3.0% v/v and commercial heparin 4,0UI/mL for 10 min at room temperature and protected from light. The cells were washed with 1 mL of PBS-Wash, permeabilized by incubation with PBS-Perm (phosphate buffered saline 0.015 M, pH 7.4, supplemented with 0.5% BSA, 0.1% of sodium azide and 0.5% saponin), for 10 min at 4 °C, under the light. After the permeabilization, the cells were incubated with monoclonal antibodies anti IFN-ɣ-V500 (B27, BD Biosciences), anti-IL-2-PECy7 (MAb11, BD Biosciences) and anti TNF-α-PE (Qm1-17H12, BD Biosciences) for 30 min at 4 °C in the dark. Finally, the cells were washed with 2 mL of PBS-Perm and PBS-Wash, using the centrifugation (400*g* for 7 min at 4 °C), fixed with MFF (10 g/L of paraformaldehyde, 1% of cacodylate buffer of sodium, 6.67 g/L sodium chloride, pH 7.2) and stored at 4 °C in the dark until the moment of reading on flow cytometry LSR fortessa™ (BD Biosciences).