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IMMUNOLOGICAL ASPECTS

Increased percentage of IFN-γ producing $CD56^+CD3^+$ cells in active tuberculosis patients upon CFP-10 stimulation of peripheral mononuclear cells

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SUMMARY

Aiming to identify a possible biomarker that distinguishes immune cellular response of active tuberculosis from latent infection. Peripheral blood mononuclear cells (PBMCs) of pulmonary tuberculosis patients (PTB), tuberculin positive household contacts (TST+ HHC), and tuberculin negative non-household contacts (TST− Non HHC) were stimulated with PPD or CFP-10 and the percentage of $CD69^+$ cells, proliferating precursor and IFN-γ producing $CD4^+$, $CD8^+$, $CD56^+CD3^-$ and $CD56^+CD3^+$ cells were compared. IL-2, IL-12p70, IL-15, IL-18 and IL-10 were measured in culture supernatants. PTB and TST+ HHC presented higher percentages of $CD69^+$ cells, IFN-γ^+ and proliferating precursors in all subpopulations studied and higher IL-12p70 levels than TST− Non HHC. The increased percentage of IFN-γ producing $CD56^+CD3^+$ cells in response to CFP-10 in PTB, compared with TST− Non HHC and the ratios between the percentage of $CD56^+CD3^+$ cells/$CD56^+CD3^-$ and $CD8^+$ cells producing IFN-γ suggest that these parameters may distinguish active TB from latently infected individuals.

1. Introduction

Tuberculosis continues to be a serious public health problem worldwide. According to World Health Organization (WHO), there were 8.7 million new cases and 1.5 million deaths in 2011 [1]. Traditionally, infection by *Mycobacterium tuberculosis* has been diagnosed by tuberculin skin test (TST); however, TST has limitations related to false positive reactions due to previous vaccination with Bacille Calmette-Guérin (BCG) or exposure to environmental mycobacteria, as well as false negatives associated to viral infections or immunosuppression [2]. Techniques based on Interferon-γ (IFN-γ) release assays have been developed for detection of *M. tuberculosis*. The IFN-γ quantification from cell culture supernatants stimulated with specific *M. tuberculosis* antigens, such as early secretory antigenic target-6 (ESAT-6) [3] and culture filtrate protein-10 (CFP-10) [3], which are absent in *Mycobacterium bovis* BCG vaccine, allowing to identify *M. tuberculosis* infection and to distinguish it from response to BCG vaccine [4,5]. However, in some endemic areas up to 80% of TST− individuals, with no exposure to tuberculosis, produced IFN-γ in response to ESAT-6 [6] and 30% of community controls, not exposed to tuberculosis patients, also responded to ESAT-6 [7]. These results had been explained by cross-reactivity with ESAT-6 produced by some non-tuberculosis mycobacteria [8,9]. These findings support the need to carry out further studies in search of other immunological markers that could be applied toward identification of infected and sick individuals in endemic populations with ample BCG vaccination.

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The CD69 is an early of T cells marker activation [10]. Its expression on $CD4^+$ T cells after PPD stimulus is higher in patients and TST+ individuals than in TST− individuals, indicating that CD69 expression may be a TB infection marker [11], however it did not differentiate TB disease from latent infection.

Few reports have shown that cellular immune parameter in response to mycobacterial antigen would differentiate active from latent TB. Mueller et al. found that the percentage of $CD4^+$ $CD45RO^{high}$-producing IFN-γ, TNFα, and IL-2 in response to mycobacteria and ESAT6-CFP-10 was significantly higher in children with tuberculosis than children with latent tuberculosis [12]. Also Harari et al. found a significant increase in the percentage of TNFα-producing $CD4^+$ T cells in response to CFP-10 and ESAT-6 in individuals with active tuberculosis, compared to latent TB [13].

The studies on NK and NKT cells responses to *M. tuberculosis* in patients and individuals with latent infection are scarce in comparison to studies of the response of $CD4^+$ and $CD8^+$ T lymphocytes. NK cells are activated by cytokines such as IFNα/β, IL-2, IL-12, IL-15 and IL-18, which induce their proliferation and IFN-γ production [14]. Patients with active and treated TB presented a higher percentage of mature NK cells than healthy TST+ [15]. Studies that evaluated NK cells effector functions have reported a decrease in cytotoxic capacity, activation, and IFN-γ production in TB patients [16,17].

NKT cells differ from NK cells by expression of CD3 molecule, these cells are activated by cytokines such as IL-12, IL-18, IL-15 and IL-2, as well as by mycobacterial glycolipids through CD1d [18]. In PBMCs cultures stimulated with mycobacteria, TB patients showed a higher percentage of IFN-γ producing NKT cells [19] and in ex vivo analysis of peripheral blood cells, TB patients presented an increased percentage of NKT cells compared to TST+ individuals [20].

With aim of contributing to understanding of anti-TB immune response and in search of parameters that may be evaluated as biomarkers of TB infection, this study compared CD69 expression, the percentage of proliferating precursor cells and the percentage of IFN-γ producing cells in $CD4^+$, $CD8^+$, $CD56^+CD3^-$ and $CD56^+CD3^+$ cells and levels of IL-2, IL-12p70, IL-15, IIL-18 and IL-10 in supernatants of cultures stimulated with PPD and CFP-10, in PTB, TST+ HHC and TST− Non HHC.

2. Materials and methods

2.1. Participants

Fifteen PTB were recruited at health centers in Popayán (Colombia) and 3 nearby villages (Piendamó, Timbío, and Caldono). PTB had negative anti-HIV antibody test. Home visits were carried-out to assign each patient one household contact, who was TST+ with an induration $\geq$ 10 mm, without any disease symptoms and negative for anti-HIV antibody test. Fifteen individuals without antecedents of cohabitation with TB patients, no disease symptoms, TST with an induration $\leq$ 10 mm and negative for anti-HIV antibodies were recruited from the upper socio-economic strata of Popayán. All participants were negative for diabetes, cancer or asthma; they were not taken immunosuppressive drugs, and none of women was pregnant. All participants signed an informed consent form for anti-HIV antibody test and for study participation. The informed consent form was approved by Universidad del Cauca Ethics Committee for Scientific Research.

2.2. Tuberculin skin test

The TST was carried out by a trained nurse, who administered two units of PPD (Tuberculin RT23, Statens Serum Institute Copenhagen, Denmark) intradermal on left forearm and measured induration between 48 and 72 h after injection. A reaction was considered positive with an induration diameter $\geq$ 10 mm [21,22].

2.3. Anti-HIV antibody test

Anti-HIV antibodies were determined in serum using commercial kit Determine HIV-1/2® (Inverness Medical Innovations, Bedford, MK, UK), according to manufacturer's instructions.

2.4. Isolation of PBMCs and cell culture

PBMCs from venous blood sample were separated on a Ficoll-Hypaque density gradient (Cambrex, Charles City, IA, US). Two million PBMCs were stained with 5(6)-Carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes/invitrogen Carlsbad, CA, USA) at final concentration of 1 μM as described by Lyons [23]. PBMCs, stained or not with CFSE, were cultured 1.5×10^5 cells/well in 96-well-plates (Fisher Scientific) and stimulated or not with PPD (10 μg/ml) (Statens Serum Institute; Copenhagen, Denmark), CFP-10 (5 μg/ml) (kindly donated by John Belisle and Patrick J. Brennan, NIH Contract HHSN266200400091c/ADB Contract NO1-AI-40091, "TB Vaccine Testing and Research Materials" Colorado University) or Phytohemagglutinin (5 μg/ml) (PHA, Sigma Immunochemicals, St. Louis, MO, USA) as positive control. The cultures were incubated at 37 °C, 5% CO_2.

2.5. Quantification of the percentage of $CD4^+$, $CD8^+$, $CD56^+CD3^+$ and $CD56^+CD3^-$ expressing CD69, producing IFN-γ and proliferating fraction cells by flow cytometry

The percentage of cells expressing CD69 was determined in 48 h cultures, since in preliminary kinetics experiment this was the time with highest CD69 expression (data not shown). Therefore, 5×10^5 PBMCs, stimulated or not with PPD, CFP-10 or PHA were collected at 48 h and stained with anti-CD4-FITC and anti-CD8-PECy5.5 plus anti-CD69-PE or with anti-CD3-FITC and anti-CD56-PECy5.5 plus anti-CD69-PE, or their respective isotype controls (eBioscience, San Diego, CA, USA). Ten thousand events were acquired in a FACSCalibur flow cytometer (Becton Dickinson Franklin Lakes, NJ, USA). The software FCS express (De Novo Software, Thornhill, Canada) was used to perform analysis.

For quantification of the percentage of IFN-γ producing cells, PBMCs were stimulated under same conditions described above and treated with 5 μg/ml of Brefeldin A (Sigma) for 4 h before being harvested for intracellular staining. The optimal time for measuring IFN-γ was determined in kinetics experiments for each cell type, with 96 h being the best for $CD56^+CD3^-$ and $CD56^+CD3^+$ cells and 120 h for $CD4^+$ and $CD8^+$ cells (data not shown). Therefore, after treatment with Brefeldin A, at these time periods, cells were harvested and stained with either anti-CD4-FITC, anti-CD8-PECy5.5 or anti-CD3-FITC plus anti-CD56-PECy5.5 with their respective isotype controls (eBioscience). Cells were treated with a permeabilizing solution 2 (Becton Dickinson, Franklin Lakes, NJ, USA) and then anti-IFN-γ-PE and Mouse IgG1*k*-PE (eBioscience) isotype control were added. One hundred thousand events were acquired in the flow cytometer and the percentage of IFN-γ^+ cells were quantified.

For quantification of the percentage of proliferating cells was performed regardless of CD69 expression and the percentage of IFN-γ producing cells. The cells previously stained with CFSE were stimulated as described above. The stimulation time was 96 h for $CD56^+CD3^-$ and $CD56^+CD3^+$ cells and 144 h for $CD4^+$ or $CD8^+$ cells according to kinetics experiment previously performed (data not shown). Thereafter, cells were collected and stained with either anti-CD3-PE plus anti-CD56-PECy5.5 or anti-CD4-PE plus anti-CD8-