Cellular Immunology 289 (2014) 167–173

Contents lists available at ScienceDirect

Cellular Immunology

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

Analysis of Th1, Th17 and regulatory T cells in tuberculosis case contacts

R.E. García Jacobo [a], C.J. Serrano [a], J.A. Enciso Moreno [a], O. Gaspar Ramírez [c], J.L. Trujillo Ochoa [a], E.E. Uresti Rivera [b], D.P. Portales Pérez [b], R. González-Amaro [d], M.H. García Hernández [a,*]

[a] Unidad de Investigación Médica-Zacatecas, Instituto Mexicano del Seguro Social, IMSS, Mexico
[b] Departamento de Inmunología y Biología Celular y Molecular, Facultad de Ciencias Químicas, UASLP, San Luis Potosí, SLP, Mexico
[c] Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. Unidad Noreste., Monterrey, Nuevo León, Mexico
[d] Departamento de Inmunología, Facultad de Medicina, UASLP, San Luis Potosí, SLP, Mexico

ARTICLE INFO

Article history:
Received 5 November 2013
Accepted 24 March 2014
Available online 1 April 2014

Keywords:
Tuberculosis case contacts
Immune response
T lymphocytes
Mycobacteria
TB spectrum

ABSTRACT

We have hypothesized that individuals infected with *Mycobacterium tuberculosis* that exhibit different patterns of immune reactivity in serial interferon (IFN)-γ release assays (IGRA's) correspond to different status within the immune spectrum of latent tuberculosis (TB). Accordingly, we analyzed the possible association between the consistent results (negative or positive) in an IGRA test and relevant immune parameters, mainly the levels of Th1 and Th17 lymphocytes and T regulatory (Treg) cells in the peripheral blood of TB case contacts. We found that individuals with a persistently positive IGRA showed increased levels of Th1 and Th17 lymphocytes upon *in vitro* stimulation with MTB antigens. In addition, a significant increase in the proportion of CD4+CTLA-4+ and CD4+Foxp3+ cells was detected in assays with blood samples from these individuals. Our data support that different immune phenotypes can be identified into the spectrum of latent TB, by combining different parameters of immune reactivity against MTB.

1. Introduction

Tuberculosis (TB) remains as one of the leading causes of mortality worldwide, with approximately 1.5 million deaths and 9.2 million new cases per year [1]. In addition, it has been estimated that up to one-third of the human population is latently infected with *Mycobacterium tuberculosis* (MTB), and that a significant fraction of them will develop the disease [2,3]. It has been suggested that individuals with latent TB, similar to active TB, exhibit a wide spectrum of immune resistance, from individuals who have completely cleared the infection to those with an active replication of mycobacteria [4,5]. Although the current immune tests employed to detect MTB latent infection are unable to identify individuals with low and high resistance to the mycobacteria [6–9], it is possible that persistent and no persistent positive results of assays of immune reactivity against MTB may correspond to different degrees of immunity [4,10]. In this regard, it has been proposed that since an IGRA positive test indicates the presence of viable mycobacterias in the host [11], then, the most resistant individuals are those showing a persistent IGRA negative test despite repeated exposure to MTB [12]. In this regard, it has been proposed that a positive result in an IGRA test require the sustained exposure to antigens from MTB, to maintain of the presence of IFN-γ producing cells [13].

Two types of tests are currently available to detect latent TB, the tuberculin skin test (TST) and the interferon-γ release assays (IGRAs). IGRAs are based on the *in vitro* release of IFN-γ by T lymphocytes stimulated with antigens from MTB [14–16]. It has been recently proposed that four different groups of individuals can be identified on the basis of serial determinations of an IGRA test: (1) Persistently positive, (2) Persistently negative, (3) Stable conversion, and (4) Unstable conversion [10,17,18]. Although it has been suggested that these four groups correspond to different immune phenotypes of latent TB, this possibility remains as an interesting point to be elucidated.

The immune response elicited by MTB infection is mainly mediated by effector CD4+ T cells. In this regard, it has been stated that Th1 cells play an important role in granuloma formation and clearance of MTB infection [19,20], and that deficiencies in the

Abbreviations: TB, tuberculosis; MTB, *Mycobacterium tuberculosis*; TST, tuberculin skin test; IGRAs, interferon-γ release assays; PPD, purified protein derivative; IFN-γ, interferon-γ; Treg, T regulatory; Foxp3, forkhead box P3; CTLA-4, cytotoxic T-lymphocyte antigen 4; Th1, T helper 1; QFT, Quantiferon-TB-Gold test; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline.

* Corresponding author. Unidad de Investigación Médica Zacatecas, Instituto Mexicano del Seguro Social, IMSS, Interior Alameda No. 45, 98000 Zacatecas, Zac, Mexico.
E-mail address: mariana.haydee.gh@gmail.com (M.H. García Hernández).

http://dx.doi.org/10.1016/j.cellimm.2014.03.010
0008-8749/

IL-12-IFN-γ-STAT1 signaling pathway lead to the dissemination of mycobacterial infections [21]. In contrast, the role of Th17 cells in MTB infection remains to be elucidated. However, it has been reported that the immune response against MTB includes the production of IL-17, which contributes to the formation of granuloma and the control of bacterial growth [22]. Moreover, it is very likely that T regulatory (Treg) cells play an important role in the pathogenesis of MTB infection. In this regard, it is feasible that these lymphocytes may exert a beneficial role in some patients, preventing tissue damage during TB infection. However, it is also possible that the expansion of Treg cells may delay the onset of adaptive immunity against MTB [23].

In this work, we evaluated the possible association between the blood levels of different T helper cell subsets or Treg lymphocytes and a persistently positive or negative result in an IGRA test in TB case contacts.

2. Materials and methods

2.1. Individuals and IGRA tests

Nineteen TB case contacts detected by the Health Department of Zacatecas, Zac., México, from 2008 to 2010 were included in the study. Fourteen were females and five were males, with a mean of age 41.0 years. A TB case contact was defined as a household contact with the TB patient by at least three times per week, 6 h each time. The time of exposition with the TB case was 10.16 ± 17.06 months. All case contacts were apparently healthy, with no clinical manifestations of TB. In order to determinate the absence of type 2 diabetes mellitus, the criteria for the diagnosis of this condition of the American Diabetes Association were employed, including the presence of polyuria, polydipsia or polyphagia, and random plasma glucose levels ($\geqslant$11.1 mmol/l) [24]. Additional demographic and clinical data of patients included in the study are shown in Table 1.

Quantiferon-TB-Gold I-Tube test (QFT) (Cellestis Limited, Chadstone, Vic., Australia) was carried out in all cases, and at three different times (0, 12, and 18 months). A value >0.35 IU/mL was considered as positive. According to the results of the three IGRA tests performed, case contacts were classified into two groups: persistently positive or persistently negative. An additional blood sample was obtained at month 18 from all these case contacts to perform the cell cultures and flow cytometry analyzes described below. Furthermore, in all cases a candidin skin test was performed at month 18, and a value $\geqslant$5 mm of induration was considered positive. This test was used to assess the immunological cellular competence of case contacts, and those individuals with a negative result were excluded of this study.

Table 1
TB case contacts demographics and clinical characteristics.

Group positive	Persistently negative	Persistently positive
N	10	9
Age (years)	46.30 ± 19.22	40.67 ± 15.78
Gender (female/male)	(7/3)	(7/2)
Random glucose (mg/dL)	102.80 ± 62.30	76.27 ± 25.62
WBC (K/μL)	7.48 ± 1.81	7.07 ± 1.48
Body mass index (Kg/m^2)	27.95 ± 3.531	26.58 ± 2.371
Life condition (urban/rural)	(10/0)	(9/0)
BCG vaccination (scar present/absent)	(9/1)	(8/1)
Ethnicity	Mexican mestizo (10/10)	Mexican mestizo (9/9)

Data correspond to the arithmetic mean ± SD, urban: >2500 residents, rural: <2500 residents.

2.2. Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St. Louis, MO, USA). Cells were washed with phosphate buffer saline (PBS), analyzed for viability (always >95%), and resuspended at a density of 1×10^6 cells/mL in complete RPMI 1640 culture medium (Hyclone, Laboratories Inc., Logan, UT, USA), supplemented with 10% fetal bovine serum (GIBCO BRL, Rockville, MD, USA), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (Sigma). PBMCs (1×10^6) were placed in 48-well plates and incubated with mouse anti-human CD3 and CD28 (10 μg/mL) (eBioscience, San Diego, CA, USA) plus a rabbit anti-mouse IgG (4 μg/mL) (AbDseroTec, Killington, UK) as a cross-linker. Moreover, 1 ml of heparinized whole blood was cultured for 24 h at 37 °C, 5% CO_2 and 100% humidity in the presence of a peptide cocktail containing the ESAT-6, CFP-10 and TB7.7 (p4) antigens from *M. tuberculosis* (Quantiferon-TB-Gold IT kit). Brefeldin (1 μg/mL, Sigma) was added 3 h before the end of the incubation period. Finally, PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and analyzed by flow cytometry [25,26].

2.3. Flow cytometry analysis

Stimulated PBMCs were incubated with an anti-human CD4-FITC mAb, followed by fixation and permeabilization (4% p-formaldehyde and 0.01% saponin) and addition of an anti-human-CTLA-4-PE, anti-human Foxp3-PE (Becton-Dickinson, San Jose, CA, USA), anti-human IL-17-PE, or anti-human IFN-γ-PE (eBioscience) for 20 min at 4 °C. Then cells were washed and fixed with 1% PFA, and analyzed in a FACSCanto II flow cytometer (Becton-Dickinson). Results were expressed as the percentage of positive cells.

2.4. Statistical analysis

Statistical analysis was performed by using the InStat Graph Pad software (InStat Graph Pad Inc., v. 3.0. San Diego, CA, USA). Differences between groups were determined via parametric and non-parametric analysis using the un-paired *T* test and Mann Whitney *U* test. The association between cell numbers and IFN-γ release levels was determined by using the Spearman's correlation analysis.

3. Results

3.1. QFT reactivity and Th1 lymphocyte response

According to the three serial results of QFT (Fig. 1a), case contacts were classified as persistently positive (three positive QFT, $n = 9$) and persistently negative (three negative QFT, $n = 10$). In all these case contacts we did not observe conversions (a QFT+ test in a contact with a previous QFT-negative result) or reversions (a QFT-negative test in a contact with a previous QFT+ result) regarding QFT reactivity. No significant differences were observed between the two groups of contacts when age, weight, gender ratio, plasma glucose levels or white cell count were analyzed (Table 1). Interestingly, when the levels of IFN-γ released in the QFT were analyzed, we detected that persistently positive case contacts showed a significant increase in the release of this cytokine through the study ($p < 0.05$, 1st vs 3rd determination, Fig. 1a). In contrast, cells from QFT persistently negative case contacts tended to diminish the release of IFN-γ through the study (Fig. 1a); however, no significant differences were detected in this group.