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Induction of transcription factors, miRNAs and cytokines involved in T lymphocyte differentiation in BCG-vaccinated subjects

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ABSTRACT

The BCG vaccine induces a Th1 phenotype, which is essential for protection against *Mycobacterium tuberculosis*. However, the effects of BCG vaccination over time on the T helper subpopulation and the microRNAs involved in adulthood have not been studied. In the present study, we explored the involvement of microRNAs, transcription factors and multifunctional cytokines in BCG vaccination by examining their levels both before and after vaccination of healthy adults. Peripheral blood mononuclear cells were obtained at 0, 2 and 6 months after vaccination. Cells were cultured in the presence or absence of ESAT-6 and CFP-10 or *M. tuberculosis* filtrate. The expression levels of miRNAs and transcription factors were evaluated using qRT-PCR. Cytokine production in supernatants and serum samples was evaluated using ELISA. Multifunctional CD4+ T cells were analyzed using multiparametric flow cytometry. We observed a decrease in the expression levels of *T-BET*, *GATA3* and *FOXP3* at 2 months and miR-146a, miR-326 and miR-155 at 6 months after receiving the vaccine. In the supernatant, the production of IL-17 was increased after 6 months, with both stimuli. In contrast, IL-10, TNF-α and IFN-γ increased at 2 months. In the serum, high levels of IL-10 were found after 2 months compared to time 0 and 6 months. The production of multifunctional cells that expressed the cytokine profiles CD4+TNF-α+IFN-γ-IL-10-, CD4+TNF-α+IL-1IFN-γ-, CD4+IL-10+IFN-γ-TNF-α- and CD4+IL-17+IFN-γ- predominantly increased after 2 months with and without the stimulus. Correlation analysis revealed a negative association between *FOXP3* and miR-155 ($r = -0.5120$, $p = 0.0176$) and between IL-17 and miR-326 ($r = -0.5832$, $p = 0.0364$). This study is the first to demonstrate roles for microRNAs, transcription factors and cytokines in the T helper differentiation lineage and to describe the possible mechanism by which their expression is modulated by the presence of the BCG vaccine in adulthood. In conclusion, our results suggest that the BCG vaccine induces a modulation in transcription factors and miRNAs with high production of multifunctional cells CD4+TNF-α+IL-10+IFN-γ-.

1. Introduction

Mycobaterium tuberculosis, the agent that causes tuberculosis, it is estimated that has infected one-third of the world's population. The only vaccine available contains live attenuated *M. bovis bacilli Calmette-Guerin* (BCG), which is given to newborns in endemic areas, such as Africa, Asia and México (Pereira et al., 2007). Vaccination with BCG confers protection in children against the disseminated forms of tuberculosis infection (Hanekom, 2005); however, it only confers partial protection against the most common form of tuberculosis, pulmonary tuberculosis, with a protection range between 0 and 80%. This variation is the result of various factors, mainly including differences in the strains used in the BCG vaccine, the geographical region, and genetic factors in the mycobacteria (Luca and Mihaescu, 2013).

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The immune system plays an important role in protective immunity against *M. tuberculosis*. CD4+ and CD8+ T cells dominate this process by restricting the growth of the bacilli (Dheda et al., 2010). CD4+ T cells differentiate into several types of effector cells, such as Th1, Th2, Th17 and regulatory T cells (Tregs). Th1 cells mainly produce IFN-γ, which controls the intracellular infection caused by *M. tuberculosis*, whereas Th2 cells produce IL-4 and IL-5, which mediate humoral immunity (Gopal et al., 2012). The role of Th17 cells is controversial: in mice, these cells are involved in the early control of the infection in the absence of IFN-γ (Wozniak et al., 2010), whereas in patients who are actively infected, the Th17 response is suppressed (Chen et al., 2010). Tregs have the ability to control tissue damage while dampening the adequate control of mycobacterial infection (Marin et al., 2010). Multifunctional cells, which are CD4+ T cells that simultaneously secrete IFN-γ, TNF-α and IL-2, are associated with the control of chronic bacterial infection. Several studies have shown that their presence might allow latent and active tuberculosis to be discriminated (Caccamo et al., 2010; Yamashita et al., 2013) or might be useful to monitor the efficacy of a treatment (Qiu et al., 2012). However, their role in BCG vaccination remains unknown.

MicroRNAs belong to the most abundant class of small RNAs in animals. They are non-coding RNAs that play key roles in regulating gene expression (Lu and Liston, 2009). They are short, single-stranded RNA molecules that are approximately 21–23 nucleotides in length, and are partially complementary to one or more mRNAs. Their main function is to downregulate gene expression by inhibiting translation or by targeting mRNA for degradation or deadenylation (Felekkis et al., 2010). It has been shown that microRNAs can regulate the transcription factors that promote the differentiation of the Th phenotypes. For example, miR-29 targets T-bet (Th1) (Steiner et al., 2011), and miR-326 targets Ets-1 (Th17) (Du et al., 2009). Other microRNAs that target Th cell types have also been identified (Kroesen et al., 2014).

Vaccination with BCG induces a Th1 phenotype in children by increasing the production and accumulation of IFN-γ over time (Djuardi et al., 2010). However, this phenotype is dependent on when the vaccine is administered. For example, delaying the vaccination by 6 weeks decreases the proportion of CD4+IFN-γ+cells (Lutwama et al., 2014), and delaying vaccination for 4 months reduces the number of cells with the Th1/Th17 phenotype (Burl et al., 2010). MicroRNAs have been used as biomarkers, and alterations in their levels have been found to depend on the disease state. Several microRNAs have been identified in serum that can discriminate between active and latent tuberculosis (Wang et al., 2011) and BCG-vaccinated subjects (Zhang et al., 2014). However, the effects of BCG vaccination over time on the Th subpopulation and the microRNAs involved in adulthood have not been studied. Therefore, the aim of this study was to evaluate the levels of microRNAs, transcription factors and multifunctional cells that are involved in BCG vaccination, both before and after its administration to healthy adults.

2. Material and methods

2.1. Subjects and BCG vaccination

A total of 25 healthy subjects were recruited from the School of Medicine of Autonomous University of San Luis Potosí within their second year of study. The subjects included 13 males and 12 females aged 18–23 years. Vaccination with BCG was performed at the Hospital Central "Ignacio Morones Prieto" by qualified personnel. All subjects were found to be purified protein derivative-(PPD)-negative before they received the vaccine. The BCG vaccine contains the attenuated live Bacillus Calmette-Guerin strain Tokio (Laboratorios Imperiales, Juitepec, Morelos, México). A volume of 0.1 mL was administered by intradermal injection. The bioethical committee of the Hospital Central "Dr. Ignacio Morones Prieto" approved this study (Project No. 43-12), and written informed consent was obtained from all of the participants.

2.2. TST and QFT

Tuberculin skin tests (TSTs) were used to demonstrate the level of host reactivity to *M. tuberculosis* antigens. The subjects were intradermally challenged with 5 UI of PPD. An absence of induration in the skin was associated with the lack of a delayed-type hypersensitivity reaction. Additionally, reactivity to other specific *M. tuberculosis* antigens that are not contained in BCG or most non-tuberculous mycobacteria [primarily early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10)] was assessed by analyzing the production of IFN-γ using a QuantiFERON®-TB test (QFT) (Cellestis Limited, Carnegie, Victoria, Australia). This assay was performed using aliquots of heparinized whole blood cells that were incubated with the test antigens for 16–24 h at 37 °C with 5% CO_2. Tests for phytohemagglutinin were included as a positive assay in controls and as a negative control in saline solutions. After incubation, the concentration of IFN-γ in the separated plasma samples was determined using an enzyme-linked immunosorbent assay (ELISA).

2.3. Peripheral blood mononuclear cell (PBMC) isolation and cell culture

Samples of peripheral blood and serum were obtained via venous puncture from healthy subjects at time 0 (before receiving the vaccine) and 2 and 6 months after administration of the vaccine. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St. Louis, MO, USA). Then, 1.5×10^6 cells were cultivated in RPMI 1640 complete culture medium (GIBCO BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (GIBCO), 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma). Then, ESAT-6 (10 μg/mL) and CFP-10 (10 μg/mL) or *Mycobacterium tuberculosis* filtrate (25 μg/mL) from the H37Rv strain (donated by Dr. Rivas-Santiago, Medical Research Unit-Zacatecas-IMSS, México) were added to the cultures and the cells were incubated for 48 h at 37 °C in 5% CO_2. Both ESAT-6 and CFP-10 are proteins that elicit a strong T-cell response in subjects with tuberculosis, whereas the *M. tuberculosis* filtrate contains a mixture of several proteins that are involved in the protective immune response. The cells were then centrifuged to obtain a cell pellet, which was used for total RNA extraction. The supernatant was used for cytokine measurements, which were performed using ELISA.

2.4. Intracellular cytokine staining and flow cytometry

The percentage of cells expressing the intracellular cytokines of interest was evaluated as follows. First, anti-CD3 (10 μg/mL) and anti-CD28 (10 μg/mL) antibodies were adhered to the plate for 1 h at 37 °C in 5% CO_2. Then, 1.5×10^6 cells were cultivated in RPMI 1640 complete culture medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma) with ESAT-6 (10 μg/mL) and CFP-10 (10 μg/mL) or *Mycobacterium tuberculosis* filtrate (25 μg/mL) from the H37Rv strain for 48 h at 37 °C in 5% CO_2. Cells that were not stimulated were used as the negative control. Brefeldin A (10 μg/mL) (Sigma) was added 4 h before the end of incubation. Then, 3×10^5 cells were stained using anti-CD4-PerCP antibodies (eBioscience, San Diego, CA) for 20 min at 4 °C in the dark. Subsequently, to intracellularly stain the cells, they were fixed with 4% paraformaldehyde, per-

meabilized with 0.1% saponin solution and then incubated with anti-IFN-γ-FITC, anti-TNF-α-APC and anti-IL-10-PE or anti-IFN-γ-FITC, anti-TNF-α-APC and anti-IL-17-PE (eBioscience) antibodies for 30 min at 4 °C in the dark. The cells were then fixed with 1% paraformaldehyde and were analyzed in a FACS Canto II (Becton Dickinson, San Jose, CA, USA) using FACS Diva software.

2.5. Total RNA and microRNA extraction and quantitative real-time reverse transcription

Extraction of RNA and microRNAs was performed using TRIzol reagent(Invitrogen). The concentration and integrity of the RNA were determined using a spectrophotometer (Synergy HT, BioTek). A total of 10 ng of RNA was used to produce cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) or a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A total of 100 ng of cDNA was used to perform real-time PCR using TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assay primers for human miR-146a, miR-155, miR-326 and miR-29a. Probes with the following Applied Biosystems assay numbers were used: *GATA3*, Hs00231122_m1; *RORC*, Hs01076122_m1; *TBX21*, Hs00203436_m1; and *FOXP3*, Hs01085834_m1. All reactions were analyzed using a CFX 96 Real-Time PCR machine (BioRad, Hercules, CA, USA), and microRNA levels were normalized to snU6, which was used as an endogenous control, whereas for transcription factor mRNA levels, 18S RNA was used as the endogenous control. The cycle threshold (Ct) values corresponding to the PCR cycle number at which fluorescence emission reached a threshold above baseline emission were used to determine the basal expression levels of microRNAs and transcription factors. Relative microRNA expression levels and transcription factors were calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001)

2.6. Enzyme-Linked InmunoSorbent Assay (ELISA)

Serum from whole blood and supernatants from cells cultured in the presence or absence of antigens or the filtrates of *M. tuberculosis* after 48 h to measure IL-4, IL-10, IL-17, TNF-α and IFN-γ levels using ELISA kits (Biolegend) according to the manufacturer's instructions. The data were analyzed using a Synergy HT reader (BioTek).

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.01 (GraphPad Software, Inc.). The data are expressed as boxes with the median for cytokines and whiskers showing the minimum and maximum values to describe the data for multifunctional cells. ANOVA was used to identify differences between the groups and Pearsonís correlation analysis was used to identify correlations between sets of data. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Subject characteristics

Twenty five healthy subjects (18–23 years old) who met the inclusion criteria were recruited. All of the subjects tested negative for TST and QFT before they were vaccinated with BCG. Two months after vaccination, the QFT and TST assays were repeated, and results were obtained after 48 and 72 h (Table 1). At that time, all of the subjects presented TST responses with up to >5 mm induration. According to Mexican guidelines (NOM-006-SSA2-2013), induration above 10 mm indicates a reaction to TST; in this case, it indicated a reaction to BCG vaccination. At 6 months after vaccination, the TST and QFT assays were again performed, and all of the subjects were negative for TST (<5 mm induration) and QFT (Table 1).

Table 1
TST and QFIN results of BCG vaccinated subjects.

TST (mm of induration)	2 months (No. students)		6 months (No. students)	
	48 h	72 h	48 h	72 h
0	0	0	0	0
0–5	0	0	23	23
5–10	3	5	0	0
10–15	9	9	0	0
15–20	12	10	0	0
Total	24	24	23	23
QFIN				
Positive	0		0	
Negative	24		23	

TST is consider reactive above 10 mm of induration.

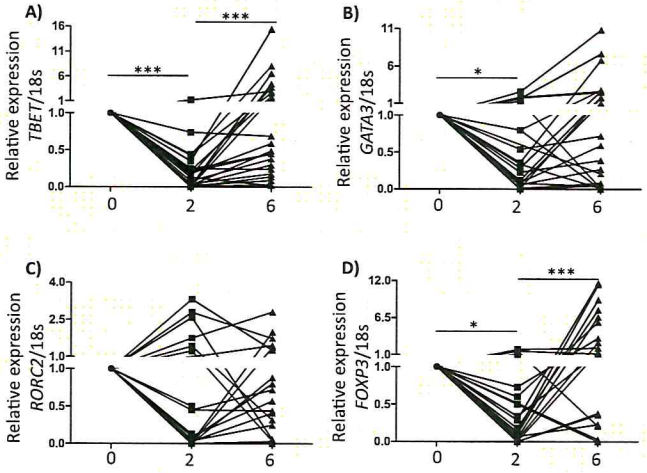

Fig. 1. Relative expression levels of transcription factors in BCG-vaccinated subjects. The levels of the transcription factors *TBET* (A), *GATA* (B), *RORC2* (C) and *FOXP3* (D) were evaluated in peripheral blood mononuclear cells obtained from subjects before receiving the vaccine at 0 (●) months, and at 2 (■) and 6 (▲) months after receiving the vaccine using real-time PCR. Expression levels were normalized to those of 18 s RNA. *$p < 0.05$, ***$p < 0.001$.

3.2. Transcription factors and microRNA expression

First, we determined the relative expression levels of transcription factors known to be involved in the differentiation of the Th lineage in non-stimulated cells obtained from the study subjects. As shown in Fig. 1, PCR analysis revealed a decrease in *TBET, GATA3 and FOXP3* expression at 2 months after vaccination compared to levels before vaccination and a further increase at 6 months compared to levels at 2 months. However, this increase at 6 months was significant only for *FOXP3* and *TBET* and no difference was detected in the expression of *RORC2*.

Next we explored if the miRNAs involved in the differentiation of Th lineages are related to these changes in transcription factors. On this way, the expression of microRNAs was assessed in non-stimulated cells from the studied subjects (Fig. 2). In contrast with transcription factors, we observed two different responses in the expression of all evaluated miRNAs (miR-29a, miR-146a, miR-326, and miR-155) at 2 months after vaccination when compared to existing levels before vaccination, one group of subjects showed lower levels of miRNAs. Besides we observed an increase in these microRNAs, with high levels in some subjects in a second group. Nevertheless, the high variability between subjects did not

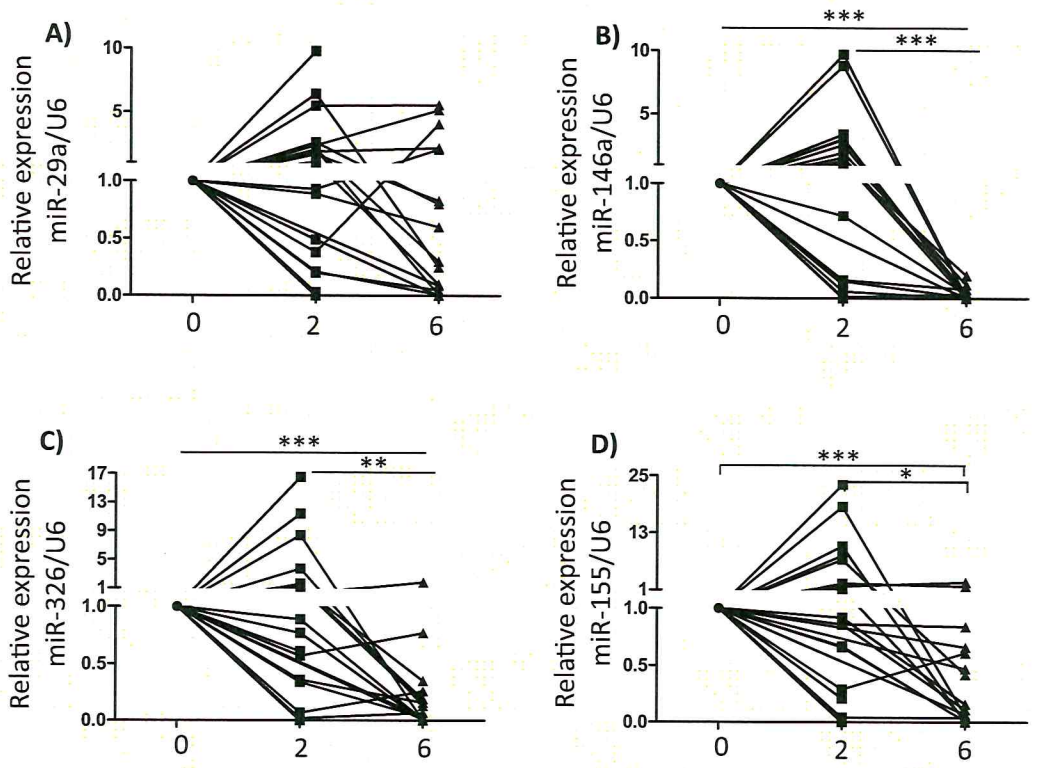

Fig. 2. Relative expression levels of microRNAs in BCG-vaccinated subjects. The levels of the microRNAs miR-29a (A), miR-146a (B), miR-326 (C) and miR-155 (D) were evaluated in peripheral blood mononuclear cells PBMC obtained from subjects before receiving the vaccine at 0 (●) months, and at 2 (■) and 6 (▲) months after receiving the vaccine using real-time PCR. Expression levels were normalized to U6 RNA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

allow observing significant differences. At 6 months the expression of miRNAs showed a very similar pattern with a statistically decrease of miR-146a, miR-326, and miR-155 when compared at 0 and 2 months. No significant changes were detected in the miR-29a expression levels; however the data follow the same pattern as the other evaluated miRNAs. After stimulating the cells with antigens and *Mycobacterium tuberculosis* filtrates, we also observed no differences in the studied transcription factors or the microRNAs (Supplemental Fig. S1).

3.3. Cytokines in serum and culture supernatants

We evaluated cytokine production in serum and culture supernatants of PBMC in the presence or absence of stimulation with ESAT-6 and CFP-10 or *M. tuberculosis* at the sampled times. Significantly higher levels of IL-17 were observed following stimulation with *M. tuberculosis* filtrate and with the antigens (ESAT-6 and CFP-10) at 6 months than at 2 months after vaccination (Fig. 3A), but these increases were not observed when levels were evaluated in serum (Fig. 3B), which could be indicating a specific response against *M. tuberculosis* antigens. In contrast, we observed higher levels of IL-10 at 2 months than at 0 months following stimulation with the filtrate, and we observed a subsequent decrease following stimulation with both the antigens and the filtrate at 6 months (Fig. 3C) compared to at 2 months. In the serum, we detected again high levels of IL-10 at 2 months compared to the level before vaccination, but not at 6 months (Fig. 3D).

In relation to IFN-γ, we observed significantly increased levels of this cytokine at 2 months after stimulation with the antigens compared to the level at time 0 (Fig. 3E). Similarly, this cytokine showed a tendency to increase in the serum of the BCG-vaccinated subjects at 2 months after vaccination. However, the levels of IFN-γ significantly decrease at 6 months (Fig. 3F). The IL-4, a Th2 cytokine, showed no change in either the culture supernatant or the serum (Fig. 3G–H).

Finally, we analyzed the production of TNF-α and observed the same tendency followed by IL-10 and IFN-γ, increased levels of this cytokine at 2 months after stimulation with the antigens and the filtrate and a subsequent decrease in cells exposed to only the filtrate (Fig. 3I). However, no differences were observed in the serum TNF-α concentrations at the studied time points (Fig. 3J).

3.4. Multifunctional cells

To further assess the role of the Th population in BCG-vaccinated subjects, we evaluated the percentage of CD4-positive cells that produced one or more cytokines in the presence or absence of the antigens ESAT-6 and CFP-10 and the *M. tuberculosis* filtrate (Fig. 4). Fig. 4A is a representative dot plot that shows the CD4+ cells (Fig. 4A) and the populations of IFN-γ negative-cells that were analyzed (Fig. 4B), the percentage of CD4+ cells that were also IL-17+ (Fig. 4C) or were double-positive for TNF-α+ and IL-10+ (Fig. 4D). In addition, Fig. 4E shows a comparative dot plot of the percentage of cells that were positive for CD4+/IL-10+/TNF-α+ and negative for IFN-γ in the presence of the stimulus at the evaluated times and we observed an increase in the percentage of these triple-positive cells at 2 and 6 months, particularly in the presence of ESAT-6 and CFP-10.

We analyzed the cells that produced only one cytokine, and no significant differences were detected (data not shown). Then, we analyzed the percentage of cells that were positive for two and three cytokines but were negative for two and one cytokine. For example, for CD4+TNF-α+IFN-γ-IL-10- (Fig. 4F), an increase in the percentage of positive cells was found only without the stimulus at time 2 and 6 compared to at time 0. We detected an increase in triple-positive cells (CD4+TNF-α+IL-10+IFN-γ-) at time 2 and

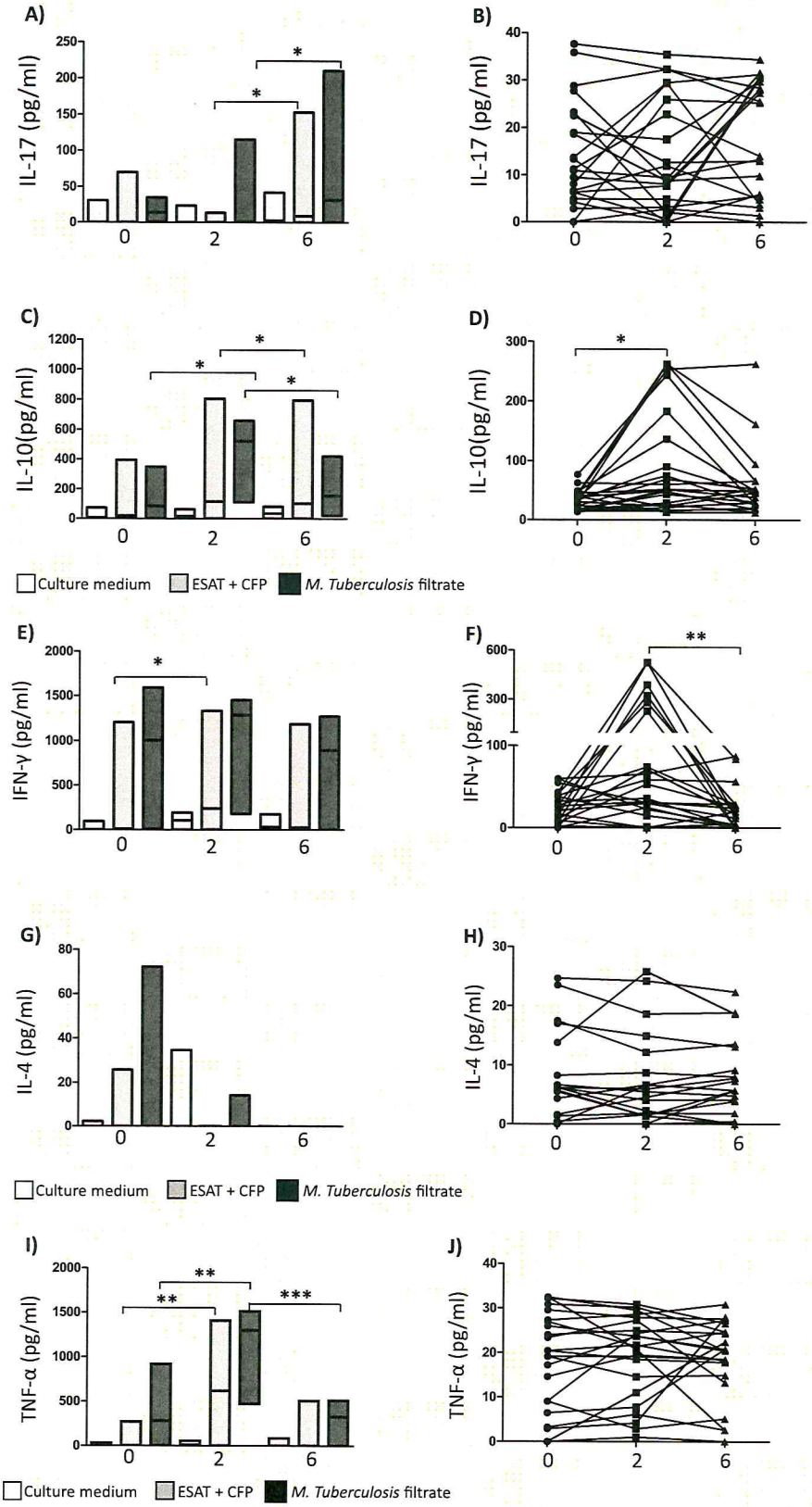

Fig. 3. Cytokine levels in peripheral blood mononuclear cell (PBMC) culture supernatants and serum samples obtained from BCG-vaccinated subjects. The PBMC were incubated in the presence or absence of ESAT-6 (10 μg/mL) and CFP-10 (10 μg/mL) or *Mycobacterium tuberculosis* filtrate (25 μg/mL) from the H37Rv strain at 0 months (before the vaccine), or 2 months and 6 months after receiving the vaccine. Cytokine synthesis was evaluated using ELISA. The supernatant levels were determined for IL-17 (A), IL-10 (C), IFN-γ (E), IL-4 (G) and TNF-α (I), as shown. White bars represent levels in the culture medium; gray bars indicate the presence of antigens and dark gray bars indicate the presence of *M. tuberculosis* filtrate. Serum levels were determined for IL-17 (B), IL-10 (D), IFN-γ (F), IL-4 (H) and TNF-α (J) and are shown for times 0 (●), 2 (■), and 6 (▲). Horizontal lines correspond to the arithmetic median. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

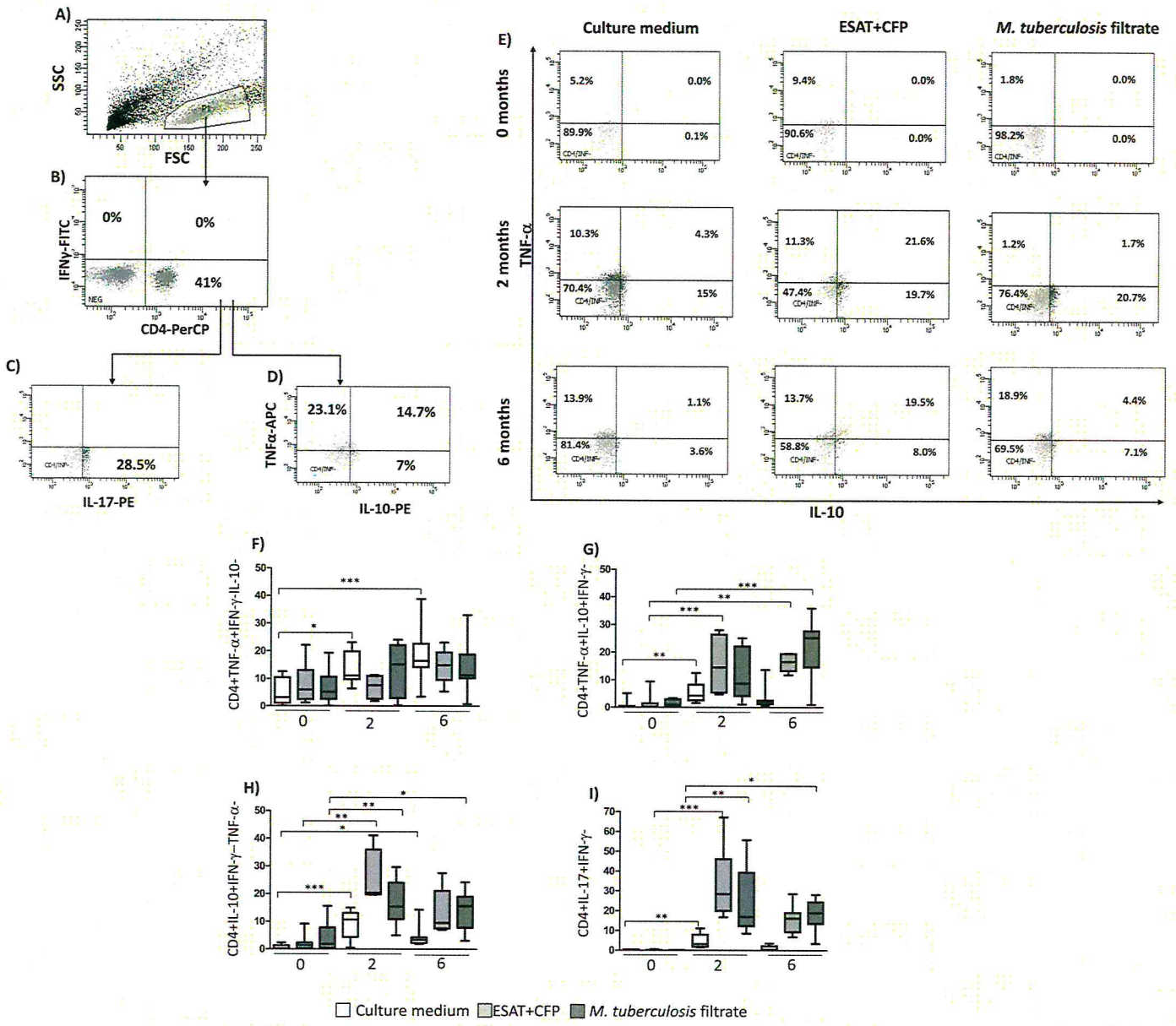

Fig. 4. Cytokine profiles of CD4T cells in BCG-vaccinated subjects. Peripheral blood mononuclear cells (PBMC) from the BCG-vaccinated subjects were obtained at times 0, 2 and 6 months and stimulated with or without ESAT-6 and CFP-10 or *Mycobacterium tuberculosis* filtrate. The cells were then analyzed using flow cytometry for the intracellular expression of IFN-γ, TNF-α, IL-10 and IL-17. Cells were obtained from a representative BCG-vaccinated subject at 6 months after vaccination. The cut-off level was determined using unstimulated T cells obtained from PBMC that were incubated with medium alone. Representative dot plots show the gating procedure used to analyze cytokine production in CD4+ T cells (A-D). A Representative dot plot of CD4+IL-10+TNF-α+IFN-γ- cells in a BCG-vaccinated subject over time and with the different stimuli used showing the different percentages of each population (4E). The percentage of CD4+TNF-α+IFN-γ-IL-10- (F), CD4+TNF-α+IL-10+IFN-γ- (G), CD4+IL-10+IFN-γ-TNF-α- (H) and CD4+IL-17+IFN-γ- (I). White bars represent the culture medium, light gray bars show the antigens and dark gray bars, the filtrate. The minimum and maximum values are shown as whiskers. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

6 compared to at time 0 in the absence of stimuli and with both antigens and filtrate (Fig. 4G). In contrast, an increase at 2 months compared to at 0 time was observed with CD4+IL-10+IFN-γ-TNF-α- (Fig. 4H) and CD4+IL-17+IFN-γ- (Fig. 4I), but these levels of positive cells tended to diminished after 6 months.

3.5. Association between transcription factors, microRNAs and cytokines

To identify associations between transcription factors, microRNAs and cytokine production, we performed a correlation analysis (Table 2). We found a significant negative correlation between the expression of *FOXP3* and miR-155 when cells were stimulated with the antigens at 2 months after vaccination ($r = -0.5120$, $p = 0.0176$) and between the production of IL-17 and the expression of miR-326 when cells were stimulated with the *M. tuberculosis* filtrate ($r = -0.5832$, $p = 0.0364$) prior to vaccination.

Table 2
Correlations between microRNAs, transcription factors and cytokines.

Variables	r value	p value
FOXP3 vs miR-155 EC time 2	−0.5120	0.0176*
IL-17 vs miR-326 M. tb time 0	−0.5832	0.0364*
GATA3 vs miR-146a NS time 0	0.3556	0.1613
TBET vs miR-29 NS time 2	0.2613	0.2950
IL-10 vs *FOXP3* EC time 2	0.3307	0.1233

EC: ESAT-6+CFP-10, M.tb: *Mycobacterium tuberculosis* filtrate, NS: non-stimulated.

4. Discussion

The BCG vaccine has been shown to be effective in children. However, few studies have examined its efficacy in later life. This study included medical students who had negative TST results and who had not been in contact with hospital patients. As expected, the participants showed positive TST results at 2 months after receiving the vaccine, whereas no positive results were recorded 6 months after vaccination. The QFT results remained negative for the duration of the study. These results are similar to previously published data in which medical students of a similar age showed positive TST results and were QFT-negative, 5 months after receiving the vaccine, which differed from results at the beginning of the study (Johnson et al., 1999).

The BCG vaccine induces predominantly Th1-like immunity and it has been shown that these type of cells confer resistance against mycobacterial infections, especially in the presence of IFN-γ, which controls infection (Hoft et al., 1998). In this study, we analyzed the relative expression levels of the master regulators of the Th lineage that are known to be involved in the immune response, both before and after BCG vaccination. We found that the vaccine induced a modulated response by upregulating the expression of *TBET* in some subjects (8/21) and *FOXP3* (11/21) at 6 months after vaccination. However, these data are not in agreement with changes in the expression of the evaluated miRNAs. Further studies exploring other microRNAs, such as miR-21, that could modify this polarization process would be interesting to evaluate. In mice immunized with BCG, a higher percentage of Tregs (CD4+CD25+FOXP3+) cells in the lymph nodes have been observed 3 weeks after immunization. However, reducing this subset of cells did not contribute to a higher protection in this mouse model (Jaron et al., 2008). These results are similar to those here in which low levels of FOXP3 were observed at 2 months, but high levels at 6 months, suggesting that the vaccine might induce an elevated Treg response that might contribute to the efficacy of the BCG vaccination and to a long-term protection.

MicroRNAs have been reported to be regulators of Th linage differentiation (Baumjohann and Ansel, 2013). Both miR-29a and miR-29b inhibit Th1 differentiation through the transcription factors Eomes and T-bet (Steiner et al., 2011), miR-326 increases Th17 differentiation by targeting Ets-1 (Du et al., 2009) and miR-155 regulates the homeostasis of Tregs by directly targeting SOCS1, which allows a higher FOXP3 expression (Yao et al., 2012). Here, the expression levels of these microRNAs (miR-146a, miR-326 and miR-155) are affected by the presence of the BCG vaccine. To our knowledge, this is the first report of this phenomenon in adult humans. Previous studies have reported a high induction of microRNAs for example Wang et al. found that miR-155 targeted SHIP1 to enhance the mycobacterial effect in murine macrophage-like cells that were infected with BCG (Wang et al., 2014) including miR-146a, which regulates inflammation in macrophages through the NF-kB pathway (Liu et al., 2014). In contrast, Zhang et al. reported that the expression of several serum miRNAs decreased in subjects vaccinated with BCG when compared to healthy subjects (Zhang et al., 2014). Our results indicate that the levels of the studied microRNAs were downregulated due to the BCG vaccination in adults. Further studies are required to evaluate other transcription factors related to Th polarization, to provide more information and to understand the mechanism by which the BCG vaccine functions.

Th1 cytokines, such as IFN-γ, play important roles in the protection against mycobacteria and facilitate the activation of the macrophage, the synthesis of NO and the killing of the bacteria. The Th2 cytokine, IL-4, regulates inflammation, IL-17 recruits neutrophils and also activates macrophages (Zygmunt and Veldhoen, 2011) and IL-10, a Treg cytokine, limits pathogen clearance (Redford et al., 2011). It has been reported that following BCG vaccination, the production of IFN-γ first increases and then decreases over time in a mouse model (Singhal et al., 2011). In addition, in BCG-vaccinated mice that were deficient in IL-12, an increased production of IL-17 and TNF-α were found to be correlated with the protection of the lungs (Freches et al., 2011). In infants who received the vaccine, higher levels of pro-inflammatory cytokines (e.g., IFN-γ and TNF-α), IL-17 (Th17), IL-4 (Th2) and IL-10 (Treg) have been observed (Lalor et al., 2010). These findings are mostly consistent with our results, with the exception of IL-4, which we did not detect in our population, and we observed no change in the level of serum TNF-α. With the challenge of the antigens and the filtrate, we demonstrated that the cells from subjects with BCG could synthesize relevant cytokines to participate in controlling the bacteria. The high levels of IL-10 and IFN-γ in the serum might indicate a state of protection induced by the vaccine after 2 months that might act in collaboration with transcription factors and miRNAs.

Multifunctional CD4 T cells can produce multiple cytokines that correspond to different Th subsets (Caccamo et al., 2010) and these have been associated with protection against tuberculosis. For the first time, we have described the profiles of multifunctional cells in a human BCG-vaccinated population; all other recent studies have focused on tuberculosis patients or infected mice. In our population, we observed an increase in the production of CD4+TNF-α+IL-10+IFN-γ-, CD4+IL-10+IFN-γ-TNF-α- and CD4+IL-17+IFN-γ-predominantly after 2 months with ESAT-6+CFP-10. These results in a BCG-vaccinated population show that in the presence of wall antigens of *M. tuberculosis*, a higher percentage of CD4+IFN-γ+IL-2+TNF-α+ cells are observed than in the absence of the stimuli (Adekambi et al., 2012). In a study of BCG-vaccinated mice, a higher percentage of CD4+IL-17+ cells in the lung are produced after 120 days of receiving the vaccine, but CD4+TNF-α+ cells remain unchanged over time (Cruz et al., 2015), similar to the results here. Unlike other studies, we observed a high percentage of multifunctional cells, which might have been the result of various factors, such as differences in the culture time to activate cells or the use of PBMC instead of whole blood (Qiu et al., 2012). Hence, the induction of multifunctional cells such as CD4+TNF-α+IL-10+IFN-γ- might indicate the protection state provided by the vaccine.

A critical player in both innate and adaptive immune responses is miR-155, which can modulate Treg differentiation by targeting *SOCS1* to increase the expression of *FOXP3* in Tregs (Yao et al., 2012). In addition, miR-155 is a direct target of FOXP3 (Zheng et al., 2007), and in our BCG-vaccinated subjects, we observed a negative correlation between miR-155 and *FOXP3* at 2 months after vaccination in peptide-stimulated cells. Thus, the vaccine inhibited differentiation towards the Tregs phenotype in these culture conditions. Another relevant miRNA is miR-326, which is associated with Th17 cells (Du et al., 2009) in patients with multiple sclerosis. The expression of miR-326 is increased and is positively correlated with high levels of *Il17a* expression. In experimental models of autoimmune encephalomyelitis, the overexpression of miR-326 induces high levels of IL-17, and in this study, we found that the higher expression of miR-326 was negatively correlated with IL-17 production in cells stimulated with the filtrate before the administration of the vaccine. Therefore, the filtrate induced the expression of miR-326, but not to a marked degree of differentiation, since the production of IL-17 was reduced. Our results suggest that the BCG vaccine induces modulated response by downregulating miR-155, which targets Treg differentiation with high levels of IL-10 and IFN-γ and by the high production of the multifunctional cytokines CD4+TNF-α+IL-10+IFN-γ-. In conclusion, this study demonstrates for the first time, the roles of microRNAs, transcription factors and cytokines involved in the differentiation of the Th linage and the possible mechanisms by which their expression is modulated in the presence of the BCG vaccine.

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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.molimm.2016.07.006.

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