

Original Article

Aberrant expression of miR-16, B12 and CD272 in peripheral blood mononuclear cells from patients with active tuberculosis

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Abstract: Tuberculosis (TB) immunity is affected by complex immune regulation processes, which involve various immune cells, immune molecules, and cytokines. Here, we evaluated the expression of B12, CD272 and miR-16 in peripheral blood mononuclear cells (PBMC) of patients with active pulmonary tuberculosis. The results showed that monocytes expressing CD272 or B12 were down-regulated in patients with tuberculosis. The expression of B12 and CD272 in T cells and monocytes is related to tuberculosis. In TB patients, the up-regulation of miR-16 was negatively correlated with B12 mRNA expression, miR-16 was mainly expressed in CD14⁺ monocytes, and CD272 mRNA was mainly expressed in CD19⁺ B cells. It is worth noting that the overexpression of miR-16 inhibits the expression of CD272 and B12 in monocytes of TB patients. After BCG stimulation, miR-16 expression of CD14⁺ monocytes was up-regulated and B12 mRNA and CD272 mRNA expressions were down-regulated in TB patients. Finally, we found that miR-16 may participate in the TB immunization process through targeted regulation of B12 expression. These studies indicate that the expression of B12, CD272 and miR-16 in PBMC may be related to tuberculosis.

Keywords: Tuberculosis, miR-16, peripheral blood mononuclear cells, CD272, B12

Introduction

According to data from the World Health Organization (WHO, 2017), there were 10,400,000 new cases of tuberculosis (TB) in 2016, of which approximately 1,670,000 died [1]. In China, the annual number of new cases of TB is about 895,000, while India and Pakistan have 284,000 and 51,000 respectively [2, 3]. Therefore, TB is still a major threat to public health, and its prevention and control are still urgent around the world.

At present, it is not clear the detailed mechanism of the occurrence, outcome and prognosis of TB. T and B lymphocytes are the main cells responsible for cellular and humoral immunity to *Mycobacterium tuberculosis* (MTB), respectively. After MTB infection, B cells recognize MTB through various surface molecules,

such as B cell antigen receptor (BCR), Ig α and CD40 [4-6]. In this process, B cells not only activate their own signal channels to secrete antibodies that target MTB, but also transmit antigen information to T cells to activate MTB-specific cellular immunity. Meanwhile, MTB inhibits the apoptosis of CD4⁺ and CD8⁺ lymphocytes and antigen-presenting cells [7]. In addition, MTB-specific Treg cells, signal channels mediated by IFN- γ and signal attenuation channels mediated by MTB secreted antigens (such as ESAT-6) also participate in the immune escape process of MTB [8]. Therefore, TB immunity is affected by complex immune regulation processes, which involve various immune cells, immune molecules and cytokines.

In recent years, scholars have proved the important role of miRNA in the immunity against TB [9-13]. Human Has-miR-16 is a member of the

miR-15 family located on chromosome 13q14. It participates in the body's immune response by regulating the expression of multiple genes. miR-16 is highly expressed in immunocytes, such as macrophages (M Φ), neutrophils, and lymphocytes [14-16]. The miR-16 overexpressed in M2 polarized M Φ promotes the transformation of M Φ to the M1 state, which is similar to the activation process of M0 polarized M Φ [15]. miR-16 targets the 3'-UTR region of the PD-L1 gene, down-regulates the expression of PD-L1, on the one hand, regulates the differentiation of M Φ , and on the other hand, weakens the antigen presentation signal, thereby affecting the function of CD4⁺ T cells [17]. In addition, miR-16 targets the 3'-UTR region of PDCD4 and affects the function of M Φ by down-regulating PDCD4 expression. In M Φ , overexpressed miR-16 can activate MAPK and NF- κ B signaling pathways, thereby down-regulating the levels of IL-6 and TNF- α and up-regulating the level of IL-10, thereby contributing to the immune response of M Φ [18].

CD272, also known as B and T lymphocyte attenuator (BTLA), acts like a co-suppressor molecule like other members of the CD28 family (PD-1, CTLA-4, CD160, and Tim-3). CD272 is expressed in T lymphocytes, B lymphocytes, M Φ , DC cells, and NK cells [19, 20]. B12, also known as TNFAIP1 and hBACURD2, is the first identified TNF- α -inducible protein and belongs to the hBACURD and PDIP1 family. B12 expression is related to the development of T lymphocyte subsets [21]. Under stimulation of the core hepatitis B virus (HBV) antigen, B12 expression in peripheral blood mononuclear cells (PBMCs) is downregulated in immunological subjects but slightly upregulated in HBV carriers, which suggests that B12 plays a vital role in HBV infection [22]. However, to our knowledge, the interaction of miR-16 with CD272 and B12 in tuberculosis has not been reported.

In a previous study, our team found that CD272 expression in the monocytes of patients with pulmonary TB (PTB) was significantly lower than that in those of healthy volunteers and that CD272 mediated the development, exhaustion, and functional weakening of MTB antigen memory T cells [19]. In that study, T cells highly expressing CD272 (CD272^{hi}) and lowly expressing CD272 (CD272^{lo}) were separated using flow cytometry. After total RNA extraction, 6 rela-

tively conservative miRNAs that possibly targeted human CD272 (miR-15a, miR-15b, miR-195, miR-424, miR-497, and miR-16) were predicted with TargetScan Release 6.2 software. The verification outcomes revealed that only miR-16 showed a significant difference in expression between CD272^{hi} and CD272^{lo} T cells, which suggested that miR-16 downregulates the functions of CD272 in T cells. Based on the TargetScan (www.targetscan.org/), miRDB (www.mirdb.org/) and miRanda (www.microrna.org/) databanks as well as on bioinformatics analysis, CD272 and B12 might be a target gene of miR-16. Based on the above information, this study aims to explore the role of miR-16 and its possible targets CD272 and B12 in TB immunity. The results of this study may help deepen our understanding of the molecular mechanism of miR-16-mediated TB immune response and provide a new perspective for TB biomarkers and therapeutic targets.

Materials and methods

Subjects

A total of 47 TB patients who received treatment at Dongguan Sixth People's Hospital (a TB designated hospital of Dongguan) between November 2015 and October 2016 were enrolled in this study. The diagnosis was in accordance with the WS 288-2008 TB diagnostic criteria. The exclusion criteria included the following: 1) patients < 16 years or > 65 years; 2) females in the gestation or lactation period; 3) patients who were alcoholic or smoked seriously; 4) patients with severe kidney diseases, heart diseases, or other diseases in a serious condition; 5) patients administered sedatives or antipsychotics; 6) patients complicated by AIDS infection, other infectious diseases, tumors, or other chronic diseases, such as diabetes mellitus; 7) patients taking immune modulators or recently receiving hormonal therapy; and 8) patients refusing to sign the informed consent form. In addition, 52 healthy volunteers that underwent a health examination at the same hospital during the same period constituted the HV group (normal control). Basic information on the participants is summarized in **Table 1**. This study was approved by the ethics committees of the Dongguan Sixth People's Hospital (Approval no. GDLY20150312) and the Guangdong Provincial Key Laboratory of

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Table 1. Basic clinical data of the TB and HV groups

	TB (n=47)	HV (n=52)	t/p
Age	41.06±1.983	36.42±1.382	1.950/0.0541
Sex (male/female)	34/13	41/11	-
Tubercle bacillus (+/-)	15/32	-	-
WBC (10 ⁹ /L)	6.975±0.3423	6.194±0.2384	1.838/0.0696
MCV (fL)	85.87±0.9932	88.94±0.9900	2.182/0.0320
MCH (pg)	27.85±0.4496	28.63±0.4037	1.289/0.2009
MCHC (g/L)	323.5±2.185	321.7±1.870	0.6174/0.5387
PLT (10 ⁹ /L)	268.7±11.81	225.5±8.229	2.949/0.0042
LYMPH (%)	25.73±1.280	31.79±1.108	3.549/0.0006
MONO (%)	8.464±0.4658	6.813±0.3070	2.899/0.0048
NEUT (%)	62.48±1.324	58.70±1.134	2.146/0.0348
EO (%)	2.423±0.2760	1.985±0.2182	1.228/0.2230
LYMPH (10 ⁹ /L)	1.730±0.09116	1.942±0.07772	1.750/0.0838
MONO (10 ⁹ /L)	0.5761±0.03315	0.4185±0.02036	3.958/0.0002
NEUT (10 ⁹ /L)	4.477±0.2913	3.706±0.1884	2.176/0.0324
EO (10 ⁹ /L)	0.1705±0.02096	0.1336±0.01672	1.352/0.1803
BASO (10 ⁹ /L)	0.06159±0.004632	0.04075±0.003912	3.402/0.0010
RDW-CV (%)	12.28±0.2670	11.59±0.1744	2.138/0.0355
PDW (fL)	18.92±0.1708	18.84±0.4137	0.1753/0.8613
MPV (fL)	7.625±0.2299	9.035±0.2573	4.099/< 0.0001
PCT (%)	0.2045±0.008574	0.2034±0.007287	0.09823/0.9220
RBC (10 ¹² /L)	4.553±0.1135	4.986±0.08317	3.025/0.0033
HGB (g/L)	125.9±2.977	142.1±2.304	4.241/< 0.0001
HCT (%)	38.89±0.8624	44.18±0.6414	4.848/< 0.0001
sCD14 (pg/mL)	370.1±16.130	315.1±12.45	2.502/0.0147

Medical Molecular Diagnostics (Approval no. GMMD201500007). Informed consent was obtained from all participants.

Sample collection and cell separation

Plasma and peripheral blood mononuclear cells (PBMCs) were collected from the participants. The T cell subset CD3⁺ (MojoSort™ human CD3 T cell selection kit; B205647; Biolegend, San Diego, CA, USA), B cell subset CD19⁺ (EasySep™ human B cell enrichment kit; 16C70018; STEMCELL Technologies Inc., Vancouver, Canada), and monocyte CD14⁺ (MojoSort™ human CD14 selection kit; B208411; Biolegend, USA) were separated using immunomagnetic beads. The procedures were conducted in accordance with the manufacturer's instructions.

Real-time PCR

The MiR-XTM miRNA First-Strand Synthesis kit, SYBR® qRT-PCR user manual, Prime Script TM

reagent kit, and SYBR Premix EX Taq™ kit (Tli RNaseH Plus) were utilized to detect the mRNA expression of miR-16, CD272, and B12 mRNA in PBMCs, T cells, B cells, and monocytes, and the primers are listed in [Table S1](#). All kits were purchased from Takara (New York, NY, USA), and the procedures were performed according to the manufacturer's instructions.

Routine blood examination

Routine blood examination was performed to determine the mean corpuscular volume (MCV), lymphocyte percentage (LYMPH %), mean platelet volume (MPV), red blood cell count (RBC), hematocrit level (HCT), hemoglobin concentration (HGB), blood platelet count (PLT), monocyte percentage (MONO %) and count (MONO), neutrophil percentage (NEUT %) and count (NEUT), basophil count (BASO), and coefficient of variation of the red cell distribution width (RDW-CV) by Department of Laboratory Medicine (Dongguan Sixth People's Hospital).

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Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) were used to detect sCD14 expression in plasma, and the procedures were performed according to the manufacturer's instructions (HUMAN sCD14 ELISA kit; Hycult Biotech, Uden, Netherlands).

Flow cytometry

Flow cytometry was performed using our previously established methods to determine the expression of CD272 and B12 in PMBC T cells, B cells and monocytes [19, 23, 24].

miR-16 lentiviral transfection

miR-16 lentivirus was established, and the transfection conditions were optimized according to the literature [25]. Flow cytometry was performed to detect the influence of miR-16 lentiviral transfection on the expression of B12 and CD272 in PTB patients. Real-time PCR was performed to detect the influence of bacille Calmette-Guérin (BCG) lysates on the expression of miR-16, B12 mRNA, and CD272 mRNA in PBMCs.

Dual-luciferase reporter plasmid construction and miR-16 target verification

The mature sequences of miR-16 were obtained from the online miRNAbase database (<http://www.mirbase.org/>). The target genes of miR-16 were analyzed and predicted based on the online miRDB (<http://mirdb.org/>), miRanda (<http://www.microrna.org/>), and TargetScan (<http://www.targetscan.org/>) databases. The mRNA sequences of the target genes and the 3'-UTR contained in the genes were obtained from GenBank. The recombinant plasmids B12-3'-UTR, B12-mutant-3'-UTR, CD272-3'-UTR, and CD272-mutant-3'-UTR were constructed [26]. miR-16 targeting of the B12 and CD272 3'-UTR regions was verified using dual-luciferase reporter assays [26, 27].

Statistical analysis

Data were processed with SPSS 18.0 and GraphPad Prism 5 software. Experiments were repeated at least three times. Measurement data are presented as Mean \pm SEM, and a two-sided test was used for results analysis. A *t* or chi square test was used for group data. Correlation analysis was performed using Pear-

son's method. $P < 0.05$ was considered statistically significant.

Results

Clinical data

The TB group (n=47) consisted of 34 males and 13 females, with a mean age of 41.06 ± 1.983 years. A positive sputum examination outcome was found in 15 patients, while 32 patients had a negative outcome. The HV group (n=52) comprised 41 males and 11 females, with a mean age of 36.42 ± 1.382 years. The TB and HV groups did not show significant differences in gender or age. The basic clinical information of the two groups is summarized in **Table 1**. Compared with the HV group, the TB group showed significantly lower MCV, LYMPH %, MPV, RBC, HCT, and HGB values but higher PLT, MON %, MONO, NEUT %, NEUT, BASO, and RDW-CV values ($P < 0.05$). ELISA method found that the content of sCD14 in the TB group was significantly higher than that in the HV group ($P < 0.05$).

Monocytes expressed CD272 or B12 were down-regulated in TB patients

To study the role of CD272 and B12 in anti-tuberculosis immunity, we used flow cytometry to detect CD272 and B12 expression in T cells, monocytes and B cells in PBMCs. The results showed that the percentage of T cells and B12⁺ T cells in tuberculosis patients increased significantly, while there was no significant difference in the percentages of CD272⁺ T cells and CD272⁺B12⁺ T cells between tuberculosis patients and healthy volunteers (**Figure 1A-D**). It is worth noting that for the first time, we found that CD272⁺ Monocytes, B12⁺ Monocytes and CD272⁺B12⁺ Monocytes were all reduced in TB patients, even though total Monocytes were elevated (**Figure 1B, 1E, 1F**). However, in B cells, CD272⁺ B cells, B12⁺ B cells and CD272⁺B12⁺ B cells were no significantly different between TB patients and healthy volunteers, even though total B cells were elevated (**Figure 1B, 1G, 1H**). These studies indicate that the expression of CD272 and B12 in T cells and monocytes may be related to TB.

B12 and CD272 expression in T cells and monocytes is related to TB

Based on the clinical data (sputum examination outcomes, gender and age) of the TB patients,

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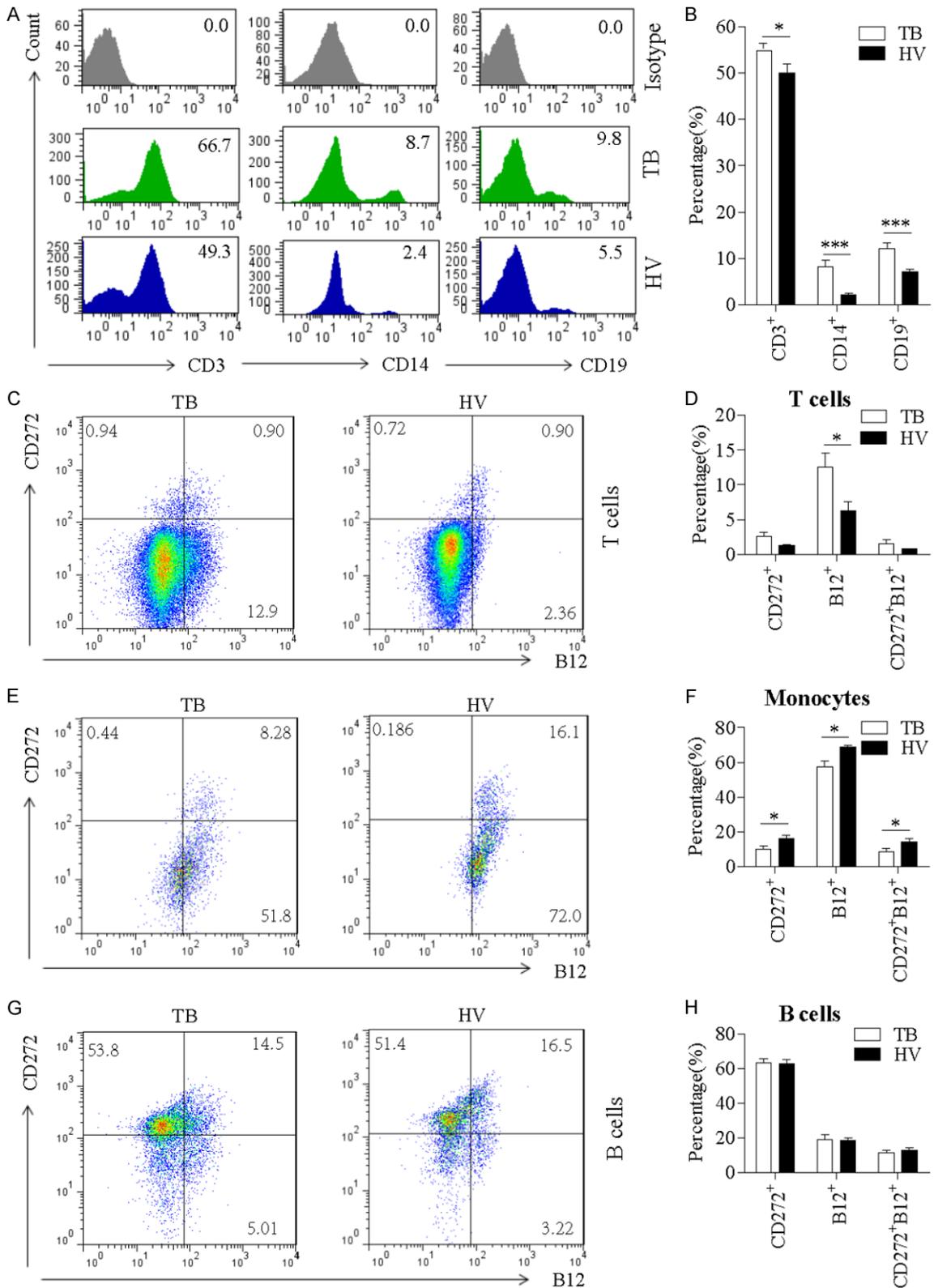


Figure 1. The expression of B12 and CD272 in CD3⁺, CD14⁺, and CD19⁺ cells. A. Representative flow cytometry plots of CD3⁺, CD14⁺, and CD19⁺ cells in PBMCs. B. The percentages of CD3⁺, CD14⁺, and CD19⁺ cells between TB and HV groups. C-H. The expression of B12 and CD272 in T cells, CD14⁺ monocytes and B cells. *P < 0.05, **P < 0.01, and ***P < 0.0001.

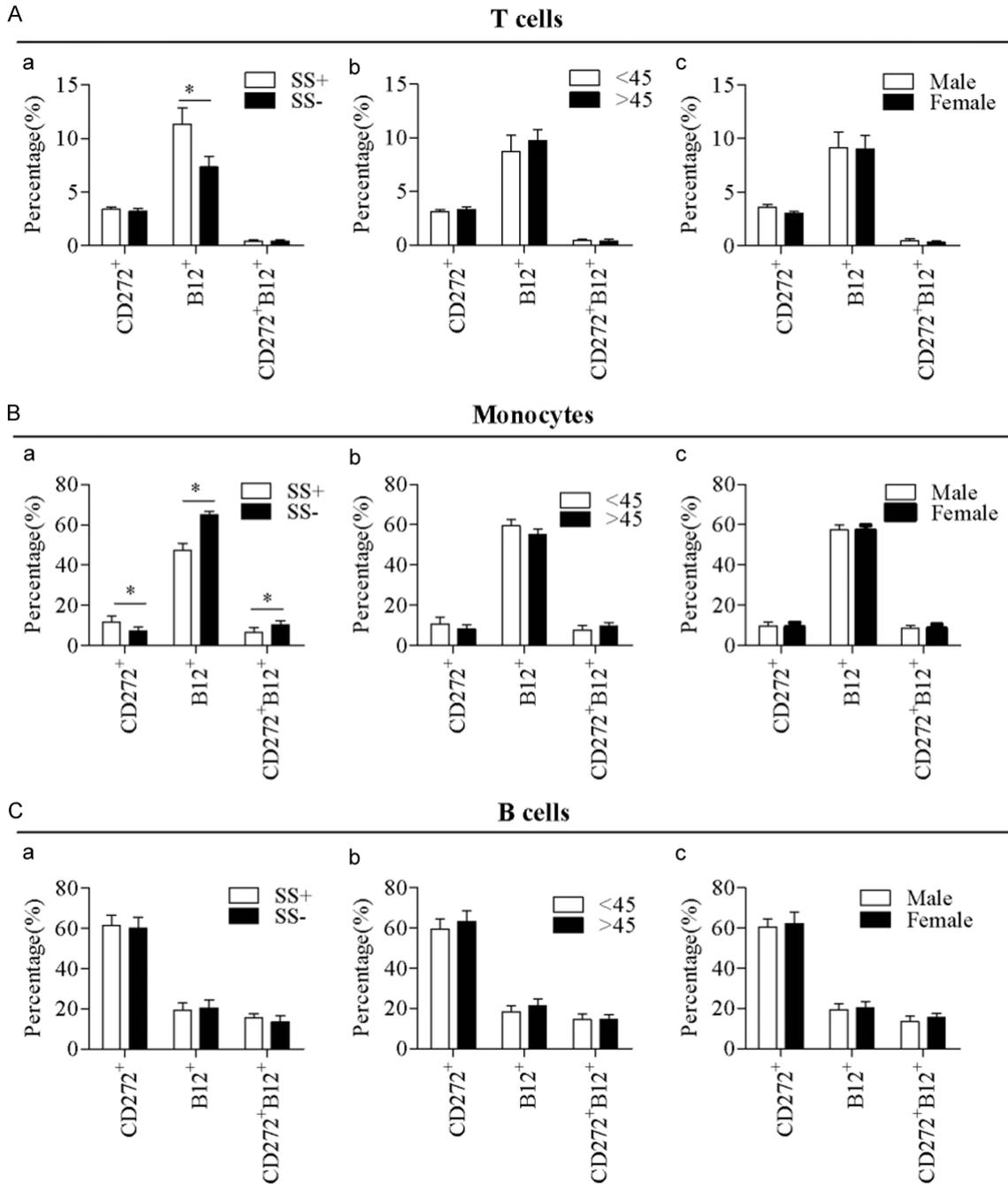


Figure 2. Correlation analysis of B12 and CD272 expression with the clinicopathological characteristics of the TB patients. A-C. Correlation of B12 and CD272 expression with the clinicopathological characteristics in T cells, monocytes and B cells. *P < 0.05, **P < 0.01, and ***P < 0.0001.

the associations of B12⁺CD3⁺ T cells, CD272⁺CD3⁺ T cells, CD272⁺B12⁺CD3⁺ T cells, B12⁺CD14⁺ monocytes, CD272⁺CD14⁺ monocytes, CD272⁺B12⁺CD14⁺ monocytes, B12⁺CD19⁺ B cells, CD272⁺CD19⁺ B cells, and CD272⁺B12⁺CD19⁺ B cells with the clinicopathological characteristics were further analyzed, and the

results are shown in **Figure 2**. The percentage of B12⁺CD3⁺ T cells and CD272⁺CD14⁺ monocytes in TB patients with a positive sputum examination outcome (SS+) was significantly higher than that in patients with a negative outcome (SS-) (**Figure 2Aa, 2Ba**), whereas the percentages of B12⁺CD14⁺ and CD272⁺B12⁺CD14⁺

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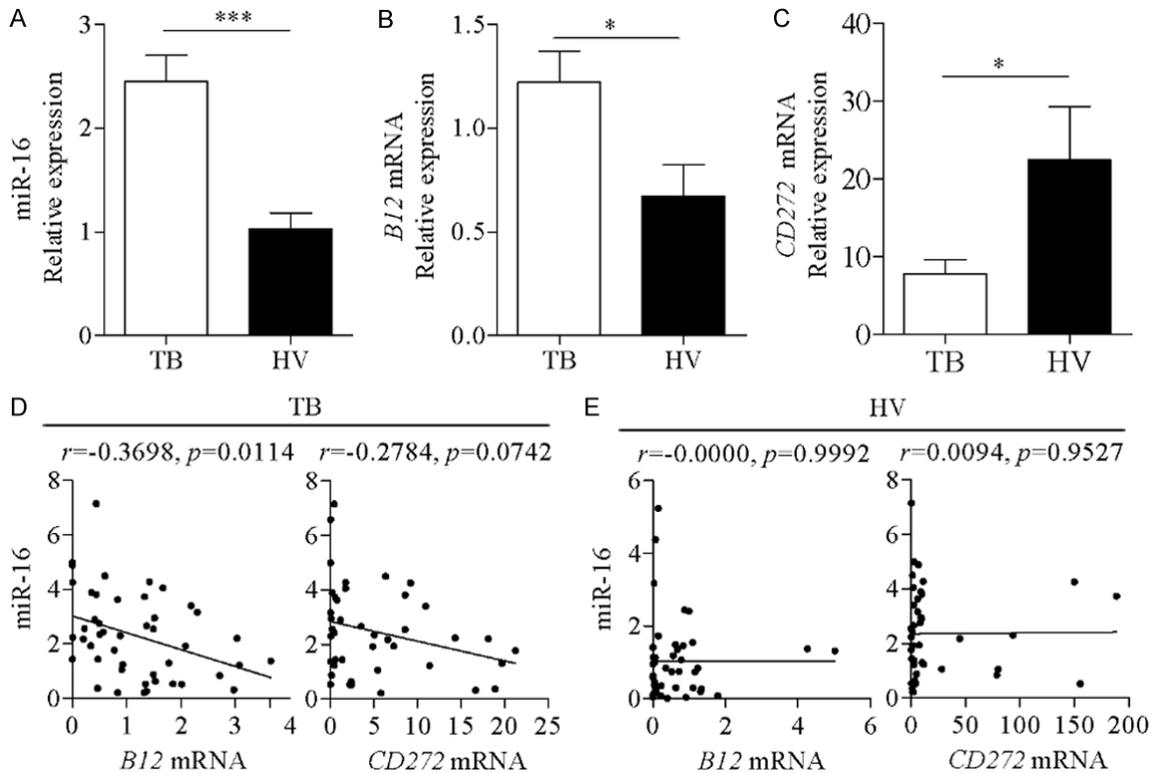


Figure 3. The relative expression of miR-16, *B12* mRNA, and *CD272* mRNA in PBMCs of the TB and HV groups. A-C. The expression of miR-16, *B12* mRNA, and *CD272* mRNA between TB and HV groups. D, E. Analysis of the correlation of miR-16 expression with that of *B12* mRNA and *CD272* mRNA in the TB and HV group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

monocytes in the SS+ patients were significantly lower than those in the SS- patients (Figure 2Ba). The percentages of $CD272^+CD3^+$ T cells, $CD272^+B12^+CD3^+$ T cells, $B12^+CD19^+$ B cells, $CD272^+CD19^+$ B cells, and $CD272^+B12^+CD19^+$ B cells had no correlation with sputum examination outcomes (Figure 2Aa, 2Ca). The percentages of $B12^+CD3^+$ T cells, $CD272^+CD3^+$ T cells, $CD272^+B12^+CD3^+$ T cells, $B12^+CD14^+$ monocytes, $CD272^+CD14^+$ monocytes, $CD272^+B12^+CD14^+$ monocytes, $B12^+CD19^+$ B cells, $CD272^+CD19^+$ B cells, and $CD272^+B12^+CD19^+$ B cells had no correlation with the gender or age of the TB patients (Figure 2Ab and 2Ac, 2Bb and 2Bc, 2Cb and 2Cc). These studies suggest that the expression of *B12* and *CD272* expression in T cells and monocytes is indeed related to TB, especially active TB.

Upregulation of miR-16 was negatively correlated with B12 mRNA expression in TB patients

Based on the TargetScan (www.targetscan.org/), miRDB (www.mirdb.org/) and miRanda

(www.microrna.org/) databanks, we predicted that miR-16 directly recognizes the 3'-UTR of the *CD272* and *B12* transcript. Therefore, the relative expression of miR-16, *B12* mRNA, and *CD272* mRNA in PBMCs of the TB and HV groups were detected using real-time PCR, and the results are shown in Figure 3. The relative expression levels of miR-16 and *B12* mRNA in the TB group were significantly higher than those in the HV group ($P < 0.05$) (Figure 3A, 3B), whereas the relative expression of *CD272* mRNA in the TB group was significantly lower than that in the HV group ($P < 0.05$) (Figure 3C). Correlation analysis showed that the relative expression of miR-16 was negatively correlated with that of *B12* mRNA but not with that of *CD272* mRNA in the TB group (Figure 3D). By contrast, no correlation between the relative expression of miR-16 and of *B12* mRNA or of *CD272* mRNA was found in the HV group (Figure 3E). These studies indicate that the expression of miR-16, *B12* mRNA and *CD272* mRNA in peripheral blood is related to TB, especially the expression of *B12* mRNA may be related to the expression of miR-16.

miR-16 mediates TB immunity via B12 and CD272

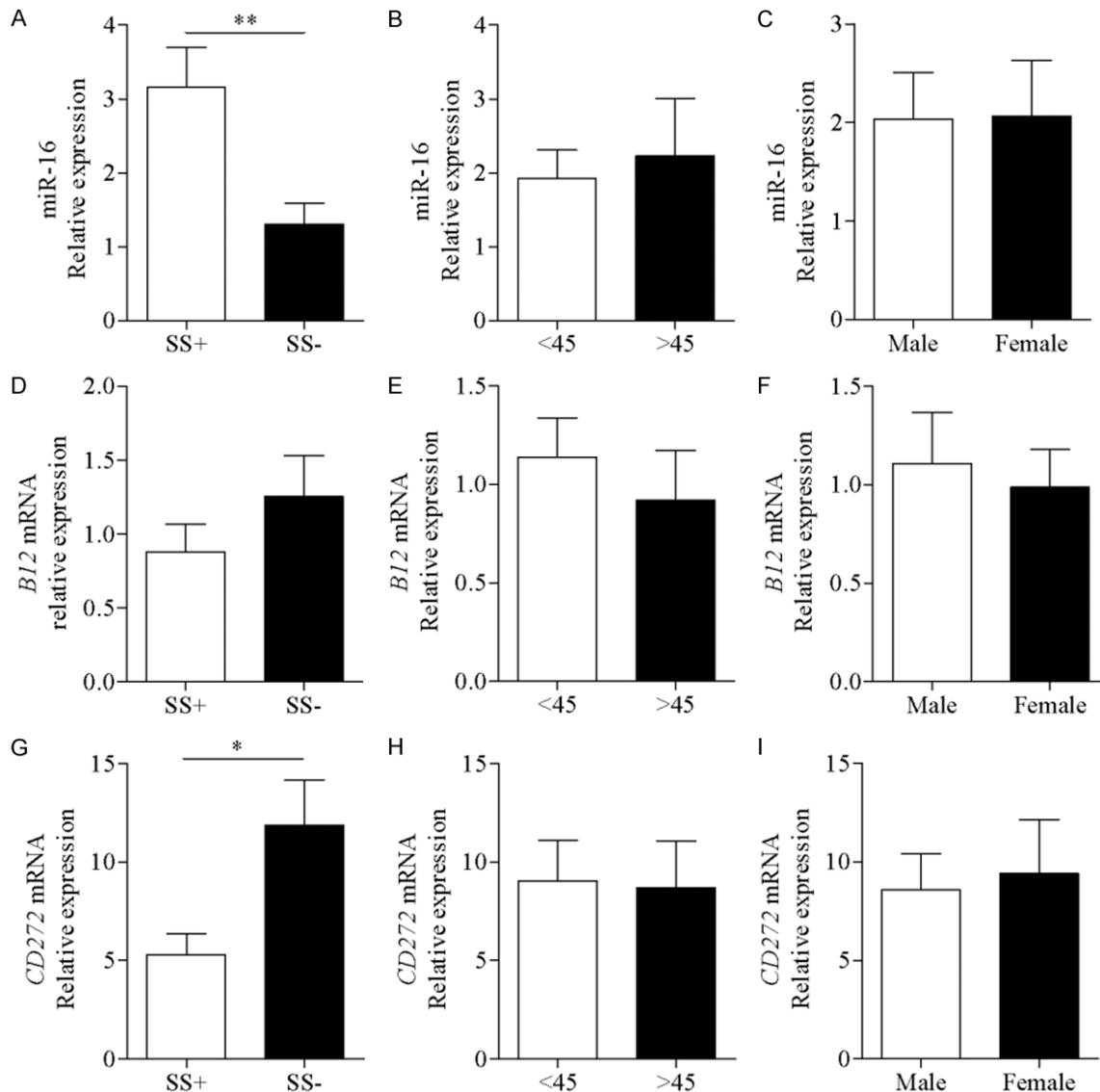


Figure 4. Correlation analysis of the relative expression of miR-16, *B12* mRNA, and *CD272* mRNA and clinicopathological characteristics of the TB patients. A-C. Comparison of miR-16 expression in TB patients with clinicopathological characteristics (SS+, age and genders). D-F. Comparison of *B12* mRNA expression in TB patients with clinicopathological characteristics. G-I. Comparison of *CD272* mRNA expression in TB patients with clinicopathological characteristics. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

miR-16 and *CD272* mRNA are related to *MTB* infection

As shown in **Figure 4**, the relative expression of miR-16 in the SS+ TB group was significantly higher than that in the SS- TB group ($P < 0.05$) (**Figure 4A**), and no correlation between the relative expression of miR-16 and patients' gender or age was found (**Figure 4B, 4C**). Although the relative expression of *B12* mRNA in the SS+ TB group was lower than that in the SS- TB group, no significant difference was

observed (**Figure 4D**). No correlation between the relative expression of *B12* mRNA and patients' gender or age was found (**Figure 4E, 4F**). The relative expression of *CD272* mRNA in the SS+ TB group was significantly lower than that in the SS- TB group ($P < 0.05$) (**Figure 4G**), and no correlation between the relative expression of *CD272* mRNA and patients' gender or age was found (**Figure 4H, 4I**). These studies suggest that miR-16 and *CD272* mRNA are related to *MTB* infection, and *MTB* may induce miR-16 expression.

miR-16 mediates TB immunity via B12 and CD272

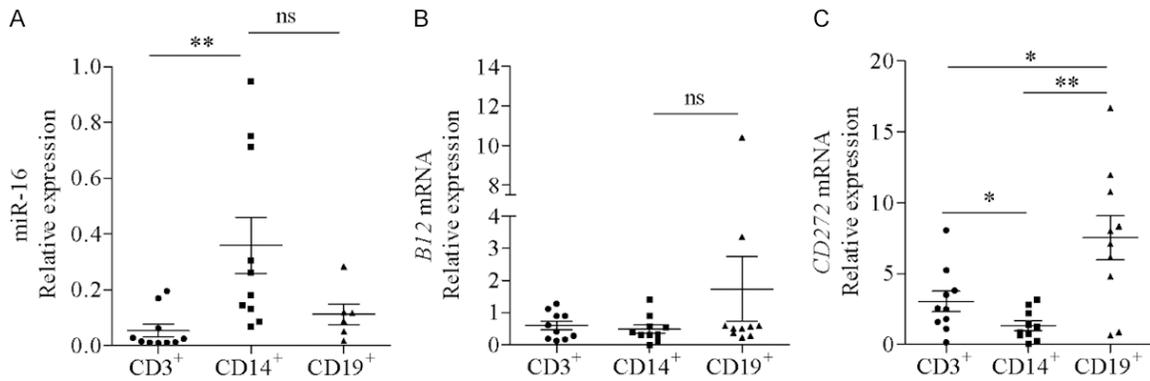


Figure 5. The expression of miR-16, *B12* mRNA, and *CD272* mRNA in $CD3^+$, $CD14^+$, and $CD19^+$ cells of TB patients. A-C. Comparison of miR-16, *B12* mRNA and *CD272* mRNA expression among $CD3^+$, $CD14^+$, and $CD19^+$ cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

miR-16 is mainly expressed in $CD14^+$ monocytes, and *CD272* mRNA is mainly expressed in $CD19^+$ B cells

T cells, monocytes, and B cells were obtained from 10 TB patients with $CD3$, $CD14$, and $CD19$ immunomagnetic beads, respectively. Total RNA was extracted using the TRIzol method, and the expression of miR-16, *B12* mRNA, and *CD272* mRNA in $CD3^+$, $CD14^+$, and $CD19^+$ cells was detected using real-time PCR (Figure 5). The relative expression of miR-16 in $CD14^+$ monocytes was significantly higher than that in $CD3^+$ T cells ($P < 0.005$) (Figure 5A), whereas no significant difference was found between $CD3^+$ T cells and $CD19^+$ B cells or between $CD14^+$ monocytes and $CD19^+$ B cells (both $P > 0.05$) (Figure 5A). The relative expression of *B12* mRNA was not significantly different among $CD3^+$ T cells, $CD14^+$ monocytes, and $CD19^+$ B cells (Figure 5B). The relative expression of *CD272* mRNA in $CD19^+$ B cells was significantly higher than that in $CD3^+$ T cells and $CD14^+$ monocytes, and $CD3^+$ T cells showed significantly higher *CD272* mRNA expression than $CD14^+$ monocytes ($P < 0.05$) (Figure 5C). These studies suggest that miR-16 is mainly expressed in $CD14^+$ monocytes, and *CD272* mRNA is mainly expressed in $CD19^+$ B cells.

Overexpression of miR-16 inhibits *CD272* and *B12* expression in monocytes of TB patients

To explore the influence of miR-16 transfection on *B12* and *CD272* expression in TB patients, the following groups were designed: BCG lysates combined with the miR-16 transfection group (BCG+LV-miR-16), BCG lysates combined

with the normal control lentivirus transfection group (BCG+LV-NC), the BCG lysates group (BCG), the miR-16 transfection group (LV-miR-16), the normal control lentivirus transfection group (LV-NC), and the PBS control group (PBS). At 72 h after PBMC treatment, the expression of *B12* and *CD272* in $CD3^+$ T cells, $CD14^+$ monocytes, and $CD19^+$ B cells was detected using flow cytometry. As shown in Figure 6, both the stimulation of BCG lysates and miR-16 transfection significantly decreased the percentages of $CD272^+CD3^+$ and $B12^+CD272^+CD3^+$ T cells (Figure 6A). BCG lysates significantly increased the percentage of $B12^+CD3^+$ T cells, whereas miR-16 decreased this percentage. These results suggested that BCG lysate stimulation might inhibit the expression of *CD272* in $CD3^+$ T cells or the survival of $CD272^+CD3^+$ T cells. In addition, BCG stimulation might promote the expression of *B12* in $CD3^+$ T cells or the survival of $B12^+CD3^+$ T cells. However, these processes could be reversed by miR-16 overexpression. BCG lysates significantly decreased the percentages of $CD272^+CD14^+$ and $B12^+CD272^+CD14^+$ monocytes, whereas miR-16 transfection did not significantly influence their percentages (Figure 6B). After stimulation with BCG lysate, the percentage of $B12^+CD14^+$ monocytes increased significantly, but decreased after miR-16 transfection. These results suggested that BCG lysate stimulation might inhibit the expression of *CD272* in $CD14^+$ monocytes or the survival of $CD272^+CD14^+$ monocytes. In addition, BCG stimulation might promote the expression of *B12* in $CD14^+$ monocytes or the survival of $B12^+CD14^+$ monocytes. Similarly, miR-16 overexpression might reverse these

miR-16 mediates TB immunity via B12 and CD272

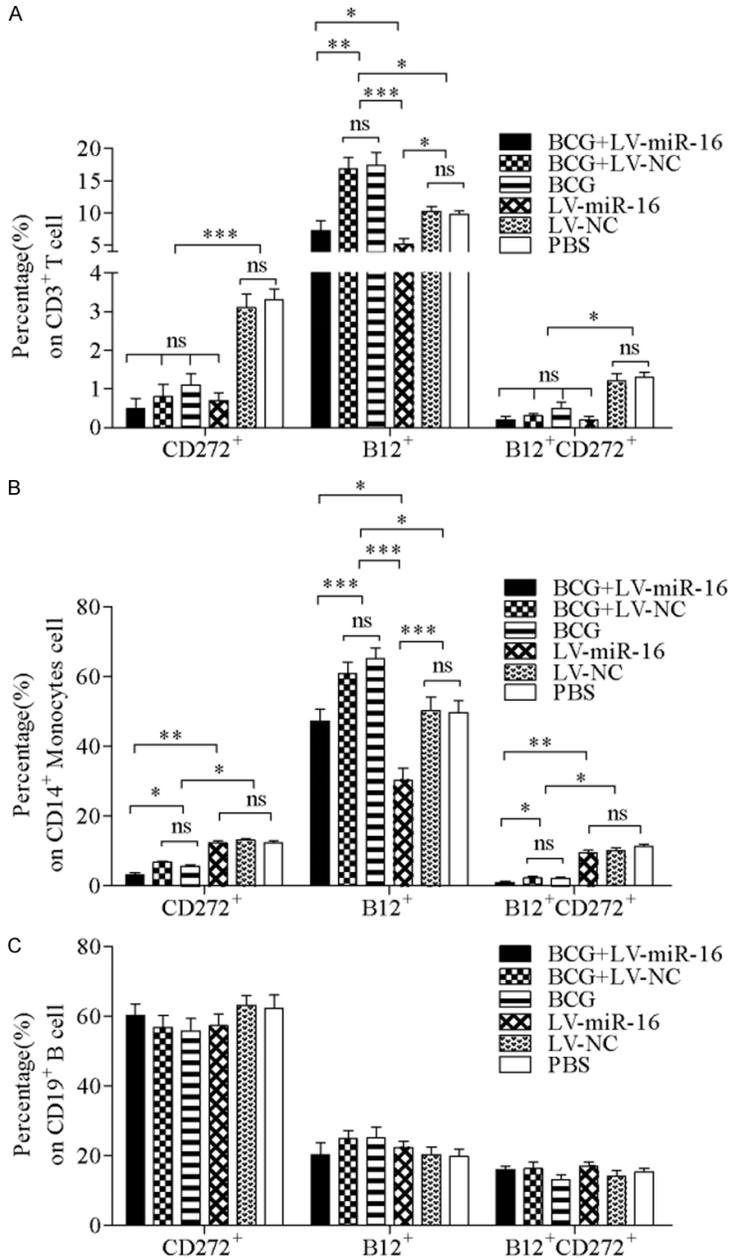


Figure 6. Effects of miR-16 transfection on B12 and CD272 expression in PBMC of TB patients. A-C. Expression of B12 and CD272 in T cells, monocytes and B cells after BCG infection and miR-16 transfection. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

processes. At 72 h after BCG stimulation or miR-16 transfection, no abnormal changes in the expression of B12 and CD272 in CD19⁺ B cells were observed (Figure 6C). These studies indicate that the overexpression of miR-16 mainly inhibits the expression of CD272 and B12 in monocytes of TB patients.

miR-16 expression was up-regulated while B12 mRNA and CD272 mRNA expression was down regulated in CD14⁺ monocytes of TB patients after BCG stimulation

CD3⁺ T cells and CD14⁺ monocytes were obtained from 10 TB patients at 27 h after BCG lysate stimulation using the immunomagnetic bead method. The expression of miR-16, B12 mRNA, and CD272 mRNA in PBMCs, CD3⁺ T cells, and CD14⁺ monocytes was detected using real-time PCR (Figure 7). Compared with those in the PBS group, the relative expression of miR-16 in PBMCs, CD3⁺ T cells, and CD14⁺ monocytes increased (Figure 7A-C), which suggested that BCG lysates can induce the expression of miR-16 in the PBMCs of TB patients. BCG lysates also increased the relative expression of B12 mRNA in PBMCs, CD3⁺ T cells, and CD14⁺ monocytes but downregulated that of CD272 mRNA in these cells compared with the levels in the PBS group (Figure 7D-I).

miR-16 targeted the 3'-UTR regions of B12 mRNA but not CD272 mRNA

Dual-luciferase reporter assays was used to verify whether miR-16 targeted the 3'-UTR region of B12 and CD272. The results are shown in Figure 8. The relative luciferase activity in the B12-3'-UTR+miR-16 group was only 66% of that in the B12-3'-UTR-NC+miR-16 group ($t=29.44$, $P < 0.0001$). No significant differences were observed among the B12-3'-UTR-Mut+miR-16, TRAF6-3'-UTR+miR-146b-NC (positive control), and B12-3'-UTR-NC+miR-16 groups ($P > 0.05$). The relative luciferase activity in the TRAF6-3'-UTR+miR-146b group was merely 56.33% of that in the B12-3'-UTR-NC+miR-16 group

miR-16 mediates TB immunity via B12 and CD272

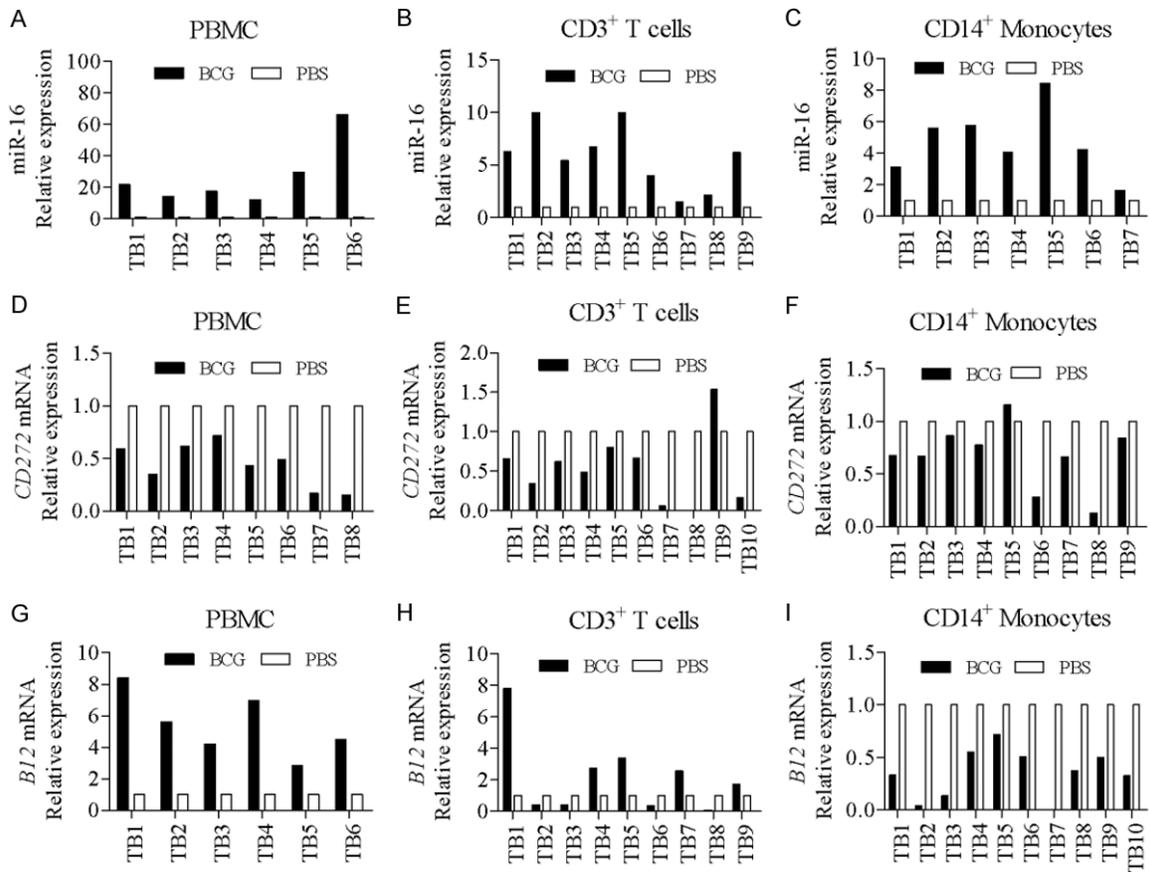


Figure 7. Effects of BCG lysate stimulation on the expression of miR-16, *B12* mRNA, and *CD272* mRNA in the PBMCs of TB patients. A-C. miR-16 expression in PBMCs, T cells and monocytes after BCG stimulation. D-F. *B12* mRNA expression in PBMCs, T cells and monocytes after BCG stimulation. G-I. *CD272* mRNA expression in PBMCs, T cells and monocytes after BCG stimulation.

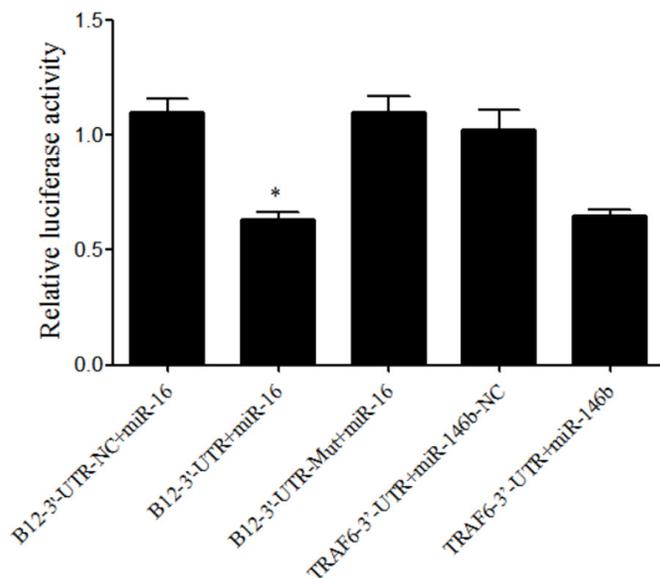


Figure 8. miR-16 targeted the 3'-UTR regions of *B12* mRNA. Relative luciferase activities in the *B12*-3'-UTR+miR-16 and *B12*-Mut-3'-UTR+miR-16 groups. * $P < 0.05$, *B12*-3'-UTR+miR-16 vs. *B12*-3'-UTR-NC+miR-16.

($t=10.56$, $P=0.0005$). No significant difference was observed between the *B12*-3'-UTR+miR-16 group (0.66) and the *TRAF6*-3'-UTR+miR-146b group (0.56) ($t=2.293$, $P > 0.05$). There was no significant difference in the relative luciferase activities of the *CD272*-3'-UTR+miR-16 group and the *CD272*-3'-UTR-Mut+miR-16 group ($t=2.00$, $P > 0.05$).

Discussion

CD272 belongs to a family of immunosuppressive molecules and plays an important role in suppressing/down-regulating T cell activation and cytokine production. *CD272* is involved in the occurrence and development of various infectious diseases [28]. A previous study conducted by our current team

found that the low expression of CD272 in peripheral blood T cells of tuberculosis patients is closely related to the clinicopathological characteristics of tuberculosis [19]. In this study, flow cytometry showed that the percentage of CD272⁺CD14⁺ cells in the TB group was significantly lower than that in the HV group. Real-time PCR showed that the relative expression of CD272 mRNA in the peripheral blood of the TB group was noticeably lower than that of the HV group. Furthermore, the relative expression of CD272 mRNA in SS+ TB patients was significantly lower than that in SS- TB patients. These results indicate that CD272 mediates the process of anti-TB immunity, which is consistent with our previous reports [19].

TNF- α is a proinflammatory cytokine produced by macrophages and monocytes [29]. Numerous studies have shown that TNF- α expression is increased in tumors [30], cardiovascular diseases [31], and infectious diseases [32]. B12 is also known as a TNF- α -induced protein 1 (TNFAIP1) and as a hBACURD2 member. B12 is involved in the pathophysiological processes of various diseases. B12 mediates tumorigenesis through the Rho/KCTD10 pathway, chronic HBV infection through the ZAP-70 pathway, cerebral cortical development through the Rnd2 pathway, and insulin resistance through the INSR pathway [33]. In this study, real-time PCR showed that the relative expression of *B12* mRNA in TB patients' PBMCs was significantly higher than that in the HV group. Flow cytometry analysis showed that the percentage of B12⁺CD3⁺ T cells in the TB group was significantly higher than that in the HV group ($P < 0.05$), whereas the percentage of B12⁺CD14⁺ monocytes was significantly lower than that in the latter group ($P < 0.05$). Furthermore, the percentage of B12⁺CD3⁺ T cells in the SS+ TB group was significantly higher than that in the SS- TB group, while the percentage of B12⁺CD14⁺ monocytes was significantly lower than that in the latter group ($P < 0.05$). Together, these results suggest that B12 may mediate the anti-TB immune response process and is closely related to the development of tuberculosis.

miRNAs are noncoding micromolecular RNAs ranging in length from 19-24 nt, and they play a regulatory role in gene expression after transcription in eukaryotes. miRNAs have recently become a hotspot in biological research, and

many studies have demonstrated their key regulatory role in the time series of organisms and disease development [34-37]. Recent studies have proven that miRNAs play a major role in the host-pathogen host-immunological network [15, 17, 18]. In addition, miRNA-mediated anti-TB immune process has also attracted the attention of scholars [9, 24]. miRNAs play an important control and management role in the dormant infection and active infection of TB. In addition, in patients with MTB infection, miR-155, miR-29a, miR-361-5p, miR-889, miR-125b, miR-576-3p, miR-147, miR-30c, miR-146a and miR-93 is abnormally expressed and is closely related to the development of tuberculosis [38-44]. These miRNAs may become potential biomarkers for the diagnosis of tuberculosis. In this study, the relative expression of miR-16 in PBMCs was detected using real-time PCR. We found that the relative expression of miR-16 in the TB group was significantly higher than that in the HV group, and the SS+ TB patients had significantly higher miR-16 expression levels than the SS- TB patients. Furthermore, T cells, monocytes, and B cells were separated from 10 TB patients with CD3, CD14, and CD19 immunomagnetic beads. Real-time PCR showed that the relative expression of miR-16 in CD14⁺ monocytes was significantly higher than that in CD3⁺ T cells ($P < 0.05$). However, no significant difference was observed between CD3⁺ T cells and CD19⁺ B cells or between CD14⁺ monocytes and CD19⁺ B cells. These results suggested that miR-16 is associated with the development of TB. To further investigate TB immunological mechanisms mediated by miR-16, mature miR-16 sequences were searched online using miRNAbase (<http://www.mirbase.org/>), and miR-16-regulated target genes were analyzed and predicted via miRDB (<http://mirdb.org/>), iRanda (<http://www.microrna.org/>), and TargetScan (<http://www.targetscan.org/>). miR-16 might target the 3'-UTR region of *CD272* and *B12* mRNA. Correlation analysis revealed that the relative expression of miR-16 was negatively correlated with that of *B12* mRNA in TB patients' PBMCs ($r = -0.3698$, $P = 0.0114$), whereas no such correlation was found in the HV group ($r = -0.0000$, $P = 0.9992$). Furthermore, no noticeable correlation was found between the relative expression of miR-16 and that of *CD272* mRNA in either group. Together, these results suggested that miR-16 mediates the development of TB by

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regulating the expression of B12 or CD272 in PBMCs.

To further explore the miR-16-mediated anti-TB immune mechanism and the association of miR-16 with CD272 and B12, the transfection efficiency of miR-16-1 and miR-16-2 in Jurkat cells and PBMC was compared. Based on the results, miR-16-2 was used as the vector to overexpress miR-16 in subsequent experiments. After lentiviral transfection, the percentages of CD272⁺CD3⁺ T cells and B12⁺CD272⁺CD3⁺ T cells significantly decreased, which was also proven by the BCG lysate stimulation experiment. miR-16-2 transfection downregulated the expression of B12 in CD3⁺ T cells, but BCG lysate stimulation upregulated its expression, which suggested that overexpressing miR-16 might reverse the upregulatory effect of BCG lysates on B12 expression in CD3⁺ T cells. Presumably, both BCG lysate stimulation and miR-16 lentiviral transfection affect the expression of CD272 and B12 in CD3⁺ T cells. However, the underlying mechanisms need to be explored in the future. In addition, the effect of miR-16 lentiviral transfection on CD272 and B12 expression in CD14⁺ monocytes and CD19⁺ B cells was also explored. miR-16 lentiviral transfection decreased the expression of B12 in CD14⁺ monocytes but did not affect the expression of CD272 in CD14⁺ monocytes. miR-16 lentivirus transfection did not affect the expression of B12 and CD272 in CD19⁺ B cells. Together, these results suggest that miR-16 mediates TB immunity by regulating CD272 and B12 expression in CD3⁺ T cells and B12 expression in CD14⁺ monocytes.

At 24 h after BCG lysate stimulation in PBMCs isolated from 10 TB patients, CD3⁺ T cells and CD14⁺ monocytes were separated using the immunomagnetic bead method. Real-time PCR showed that the expression of miR-16 in PBMCs, CD3⁺ T cells, and CD14⁺ monocytes increased, which suggested that BCG lysate stimulation could induce the expression of miR-16 in PBMCs from TB patients' peripheral blood. Similarly, the relative expression of *B12* mRNA in PBMCs, CD3⁺ T cells, and CD14⁺ monocytes also increased after BCG stimulation. However, the stimulation downregulated the expression of *CD272* mRNA in PBMCs, CD3⁺ T cells, and CD14⁺ monocytes. Based on these results, it is reasonable to conclude that

MTB infection may upregulate miR-16 expression in the PBMCs of TB patients, which further affects CD272 and B12 expression in CD3⁺ T cells and B12 expression in CD14⁺ monocytes, thereby mediating the occurrence and development of TB.

Based on online database analysis (miRDB, iRanda, and TargetScan), the mRNA 3'-UTR regions of *CD272* and *B12* might be miR-16 targets. To assess this prediction, recombinant B12-3'-UTR, B12-mutant-3'-UTR, CD272-3'-UTR, and CD272-mutant-3'-UTR plasmids were constructed. Dual-luciferase reporter assays showed that miR-16 targeted the mRNA 3'-UTR region of *B12* rather than that of *CD272*. These results suggest that miR-16 targets the 3'-UTR region of *B12* to affect B12 expression in CD3⁺ T cells and CD14⁺ monocytes, thereby mediating the development of TB. However, whether miR-16 affects CD272 expression in CD3⁺ T cells by regulating B12 expression or other signaling pathways remains to be explored.

This study conclusion the following for the first time: Abnormal miR-16, B12, and CD272 expression is closely associated with the pathogenesis of TB, and miR-16 may participate in the TB immunity process by regulating B12 expression. The results of this study may further enhance our understanding of the molecular mechanism of miR-16-mediated immune response to TB and provide a new perspective for seeking biomarkers and treatment targets for TB in the future.

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Disclosure of conflict of interest

None.

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Table S1. A list of primers used in the reactions for real-time RT-PCR

Genes	primers
miR-16	5'-CTAGCAGCACGTAATATTGG-3'
B12	F: 5'-TTACCTCCGAGATGACACCATCAC-3' R: 5'-TCCTCATCTTCACTGGGGGAA-3'
CD272	F: 5'-CAGTCTAGAGCCACCATGAAGACAGTGCCTGCCATGC-3' R: 5'-GTCAAGCTTTCAGCCTGGCCTCTCTCCATGGTG-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' R: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'