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Rv3615c-Specific CD4+ T Cells Express IL-21 To Help B Cells Produce Immunoglobulins in Tuberculosis Pleurisy

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Abstract

Rv3615c is a new vaccine candidate in fighting against *Mycobacterium tuberculosis* (*M.tb*) in active Tuberculosis (TB). However, the humoral immune response mediated by Rv3615c remains unclear. In the present study, we found that Rv3615c-specific $CD4^+$ T cells produced IL-21, IFN-γ, TNF-α and IL-17A. IL-21-producing $CD4^+$ T cells were predominantly $CD69^+$ T cells, and displayed effector memory phenotype. The majority of Rv3615c-specific IL-21-expresing $CD4^+$ T cells co-expressed IFN-γ and $IL\text{-}21^+IFN\text{-}\gamma^+CD4^+$ T cells exhibited obviously poly functionality, and correlation of IL-21 expression with Th1 but not Th17. Moreover, Rv3615c-specific $CD4^+$ T cells expressed high levels of Tfh markers CD40L, CXCR5 and BCL-6. The frequency of Bcl-6-expression was higher in IL-21-expressing but not in non-IL-21-expressing $CD4^+$ T cells. Purified $CD4^+$ T cells produced IL-21 after Rv3615c stimulation, which expressed high levels of CD40L. On the other hand, B cells from PFMCs expressed high levels of CD40 and IL-21R. Importantly, Rv3615c-specific $CD4^+$ T cells provided help to B cells for the production of IgG, IgM and IgA. Taken together, our data demonstrate that Rv3615c-specific $CD4^+$ T cells from a local site of *M.tb* infection produced IL-21 and expressed CD40L, help B cells to secrete immunoglobulins, and may participate in local immune responses against *M.tb* infection.

Keywords: B cells; Humoral immunity; IL-21; Immunoglobulins; *M.tb*; Rv3615c

Introduction

Mycobacterium tuberculosis (*M.tb*), the causative agent of human Tuberculosis (TB) is one of the most widely spread human pathogens and is responsible for 9 million newly reported TB cases every year worldwide [1]. Although the currently available *M. bovis* Bacillus Calmette-Guerin (BCG) vaccine, based on an attenuated *M. bovis* strain, has nearly been used for a century in many countries, it does not protect adult pulmonary tuberculosis and provides insufficient protection against disease [2]. At present, many promising TB vaccine candidates and/or vaccine strategies have been and are currently being developed but efficacy in animals and humans remain to be proven.

M.tb specific antigen, Rv3615c, encoded outside RD1, which is the ESAT-6-like protein that originally identified as an Esx-1 substrate protein C, is actively expressed and is accessible to antigen-processing pathways during intracellular infection *in vivo* [3]. What's more, Rv3615c, was identified to be as immune dominant as the well-known ESAT-6 and CFP-10, and has brought promising expectations to more sensitive T-cell based diagnosis and vaccine development, suggesting it might be a target of cellular immunity in TB [4]. Thus, Rv3615c, significant as a previously unrecognized *M.tb* antigen where high diagnostic specific is conferred by RD1-dependent secretion, being as immune dominant and as highly specific for *M.tb* infection as ESAT-6 and CFP-10, is indicating potential for immune diagnosis as well as vaccine

development [5-7]. We therefore hypothesized that Rv3615c may be recognized by *M.tb*-specific T cells from patients infected with *M.tb* and potentiating the anti-TB vaccine candidate.

$CD4^+$ T cells and Th1 cytokines, such as IFN-γ and TNF-α, are important in the cell-mediated responses to *M.tb* infection [6,8]. IFN-γ is an important cytokine for macrophage activation and contributes to the major effector response to *M.tb*. TNF-α is also a key cytokine in host immunity to intracellular bacteria, most notably *M.tb* [9]. Clearly, various $CD4^+$ T cell effector subsets exist, ranging from early activated cells making only IL-2, to cells making IFN-γ, to multifunctional cells expressing IL-2, IFN-γ and TNF-α, and the presence of these multifunctional cells is associated with protection [10]. Other subsets of functional T cells, such as those producing IL-17A and IL-21, and these cells have been seen in the mouse model and in humans exposed to tuberculosis [11,12]. Whether this response is equally important in humans is as yet unknown. However, $CD4^+$ antigen-specific IL-17- and IL-21- producing cells can clearly be detected in humans exposed to *M.tb*, although only the majority of IL-21 is detected in the lung [13]. IL-21 is produced by activated NKT and multiple $CD4^+$ T cell subsets and differentiated T helper cell subsets polarized towards Th17 and follicular helper (Tfh) phenotypes [14-16]. IL-21 plays important protection roles in the regulation of hematopoiesis and both innate and adaptive immune responses. IL-21 co-stimulates the proliferation and differentiation of $CD4^+$ T cells, and enhances the proliferation and cytotoxicity mediated by NK cells and $CD8^+$ T cells. In addition, IL-21 co-stimulates B cell proliferation and differentiation into plasma cells producing immunoglobulins. The immunological components and their interactions are necessary to prevent or control *M. tuberculosis* infection in humans [17-19].

In this study, we demonstrate for the first time that antigen-specific $CD4^+$ T cells isolated from Pleural Fluid Mononuclear Cells (PFMCs) from TB patients produce IL-21 following stimulation with Rv3615c and that IL-21 is able to induce the production of immunoglobulins by B cells, which might influence the local immune response to *M.tb* in TB patients.

Materials and Methods

Study Participants

Eight patients with tuberculous pleurisy (3 females and 5 males, 23-71 years old) were recruited from the Chest Hospital of Guangzhou, China (Table 1). The diagnosis of pleural effusion from TB etiology was based on the following criteria: (i) *M. tuberculosis* on a pleural fluid smear (by Ziehl-Neelsen method); (ii) pleural fluid or pleural biopsy specimens growing *M. tuberculosis* on Lowenstein-Jensen medium; (iii) histological evidence of caseating granuloma on biopsy specimens of pleural tissue with positive staining for *M. tuberculosis*. Patients with HIV, HBV, HCV or a history of autoimmune diseases were excluded from the study. Informed written consent was obtained from all patients. The study protocol was approved by the Ethics Committee of the Zhongshan School of Medicine, Sun Yat-sen University (Guangzhou, China) and the Chest Hospital of Guangzhou (Guangzhou, China).

Demographic and clinical characteristic of patients with TB	
	Patients with TB
Total (n)	8
Age (year) (mean ± SD)	31.33 ± 12.52
Sex (male) (n%)	5M （62.5%）
Pulmonary TB	8
New pulmonary TB	8
Sputum smear/ culture positive	7
Tuberculosis pleurisy	8
Tuberculosis pleural effusion	8
Antituberculosis therapy	Less than one week
TB=Tuberculosis; TB=Tuberculosis Pleurisy; M=Male; ND=Not Done.	

Preparation of Pfmcs and Pbmcs

Pleural Fluid (PF) from TBP patients were centrifuged at 2000 rpm for 10 min, and collected the cell pellets. Cell pellets from PF and peripheral blood were suspended and isolated by Ficoll-Hypaque (Tianjin HaoYang Biological Manufacture, Tianjin, China) density gradient centrifugation at 2000 rpm for 20 min. Pleural Fluid Mononuclear Cells (PFMCs) and Peripheral Blood Mononuclear Cells (PBMCs) were collected and washed twice by using Hank's balanced salt solution. Then re-suspended the cells at a final concentration of 2×10^6 cells/ml in complete RPMI 1640 medium (Life Technologies, Grand Island, USA) supplemented with 10% heated-inactivation fatal calf serum (Sijiqing, Hangzhou, China), 100 μg/ml streptomycin, 100 μ/ml penicillin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol (Life Technologies, Grand Island, USA).

Isolation of T Cells, B Cells and Monocytes

$CD4^+$ T cells, B cells ($CD19^+$) and monocytes ($CD14^+$) were positively purified from freshly isolated PFMCs by using of anti-CD4 microbeads, anti-CD19 microbeads and anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according to the manufacturer's protocol. Briefly, PFMCs were washed twice in Magnetic Activated Cell Sorter (MACS) buffer Phosphate-Buffered Saline (PBS) supplemented with 2 mM EDTA and 0.5% Bovine Serum Albumin (BSA), and were re-suspended in the buffer, then mixed well with anti-CD14 microbeads, anti-CD4 microbeads and anti-CD19 microbeads,

and incubated on ice for 15 min. The cells were washed and magnetically separated on a MACS magnet fitted with a MACS LS column. The purity of $CD4^+$ T cells, B cells and monocytes were 96.0%, 98.7% and 96.4%, respectively, as assessed by flow cytometry.

Peptide, Reagents and Mabs

Total Rv3615c protein (Rv3615c$_{1-103}$: MTENLTVQPERLGVLASHHDNAAVDAS SGVEAAAGLGESVAITHGPYCSQFNDTLNVYLTAHNALGSSLHTAGVDLAKSLRIAAKIYSEADEAWRKAIDGLFT.) were all synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The purity of the peptides was high than 95%, as assayed by HPLC, and their compositions were verified by mass spectrometry. Lyophilized peptides were dissolved in 10% dimethyl sulfoxide to obtain a concentration of 10mg/ml. CD3-PE-CF594, CD4-APC-Cy7, IFN-γ-PE-Cy7, TNF-α-PE, IL-17A-FITC, CD45RO-AF700, CD62L-PE, CD27-APC, CCR7-PE-Cy7, CXCR5-AF488, CD40L-PE, IL-21-AF647, IL-21R-PE and purified anti-CD28 (clone CD28.2) mAbs (BD Biosciences, San Jose, USA).

Flow Cytometry

The cells were washed twice with PBS buffer containing 0.1% BSA and 0.05% sodium azide. For surface staining, cells were incubated with the respective mAbs at 4°C in the dark for 30 min. Cells were washed twice and fixed in 0.5% paraformaldehyde before acquisition. For the detection of intracellular cytokines, cells were incubated with Rv3615c plus anti- CD28 mAb for 6-8 hrs in the presence of brefeldin A (10 μg/mL; Sigma-Aldrich, St Louis, MO). After stimulation, cells were washed twice with PBS and fixed in 4% paraformaldehyde, followed by permeabilization, and stained for the intracellular cytokines and molecules in PBS buffer containing 0.1% saponin. Lymphocytes were gated on forward- and side-scatter profiles. Flow cytometry was performed using a BD FACS Calibur (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (TreeStar, San Carlos, CA).

ELISA for Cytokines

Purified $CD4^+$ T cells with or without purified monocytes were re-suspended in complete RPMI 1640 medium and cultured in the presence or absence of Rv3615c plus anti-CD28 mAbs for 72 hrs. The supernatants were harvested and assayed for the production of IFN-γ, TNF-α, IL-17A and IL-21 by Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's protocol (BD Pharmingen, San Diego, CA). Purified $CD4^+$ T cells with or without purified B cells were re-suspended in complete RPMI 1640 medium and cultured in the presence or absence of Rv3615c or sCD40L, IL-21, and neutralizing antibody anti-CD40, anti-IL-21R for 10 days. The supernatants were harvested and assayed for the production of IgG, IgM and IgA by ELISA according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Purified $CD4^+$ T cells with or without purified monocytes were re-suspended in complete RPMI 1640 medium and cultured in the presence Rv3615c (5 μg/ml) plus anti-CD28 (1 μg/ml) for 72h. The supernatants were harvested and assayed for the production of IFN-γ, IL-21 and IL-17A by ELISA according to the manufacturer's instructions (BD Biosciences). Purified B cells with or without purified $CD4^+$ T cells were re-suspended in complete RPMI 1640 medium and cultured in the presence or absence of Rv3615c (5 μg/ml) for 10 days. The supernatants were harvested and assayed for the production of IgG, IgM and IgA by ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

Statistical Analysis

All statistical tests were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA). Significant differences between data sets were performed with either the unpaired Student's t test when comparing two groups, one-way ANOVA for more than two groups or two-way ANOVA for two variables (GraphPad Software Inc, San Diego, CA, USA). Data are represented as mean ± SD. $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; and $P>0.05$, not significant, as stated in Figure legends.

Results

Rv3615c-specific peptide induces IL-21 production by $CD4^+$ T cells from PFMCs

Interleukin-21 (IL-21) is a cytokine that has broad effects on both innate and adaptive immune responses. To determine whether the MTB-specific antigen Rv3615c could induce IL-21 production by $CD4^+$ T cells from PFMCs, we collected the PFMCs and PBMCs from TB patients (n=8), and cultured them in the presence of medium alone, or Rv3615c peptide. Following stimulation with Rv3615c, PFMCs and PBMCs could produce IL-21, IFN-γ, TNF-α and IL-17A, and the levels of cytokines in PFMCs higher than those in PBMCs (Figure 1A). Rv3615c-specific Th1, Th17 and Tfh cells in PFMCs were much higher than those in PBMCs. The same results were detected in ELISPOT assay for IL-21 production (Figure 1B, C). To further identify which subsets of cells from PFMCs and PBMCs could produce IL-21, freshly isolated PFMCs and PBMCs were stimulated in vitro with Rv3615c peptide, and conducted by fluorescence-activated cell sorting (FACS). The results showed that, without any stimulation, $CD4^+$ and $CD8^+$ T cells from PFMCs and PBMCs did not express IL-21. Following stimulation with Rv3615c peptide, a very low frequency of IL-21 expression by PBMCs, but high levels of IL-21 expression by PFMCs. $CD4^+$ T cells, but not $CD8^+$ T cells from PFMCs and PBMCs expressed IL-21 (Figure 1D). The mean frequency of $CD4^+IL\text{-}21^+$ T cells in PFMCs and PBMC was 1.8% (ranging from 0.1% to 3.0%) and 0.4% (ranging from 0.0% to 1.0%), respectively (Figure 1E). Furthermore, the frequency of IL-21-expressing $CD4^+$ T cells

in PFMCs was significantly higher than in PBMCs (P<0.001). These results indicated that Rv3615c peptide induced significantly higher percentages of IL-21-expressing CD4+ T cells, but not CD8+ T cells in PFMCs.

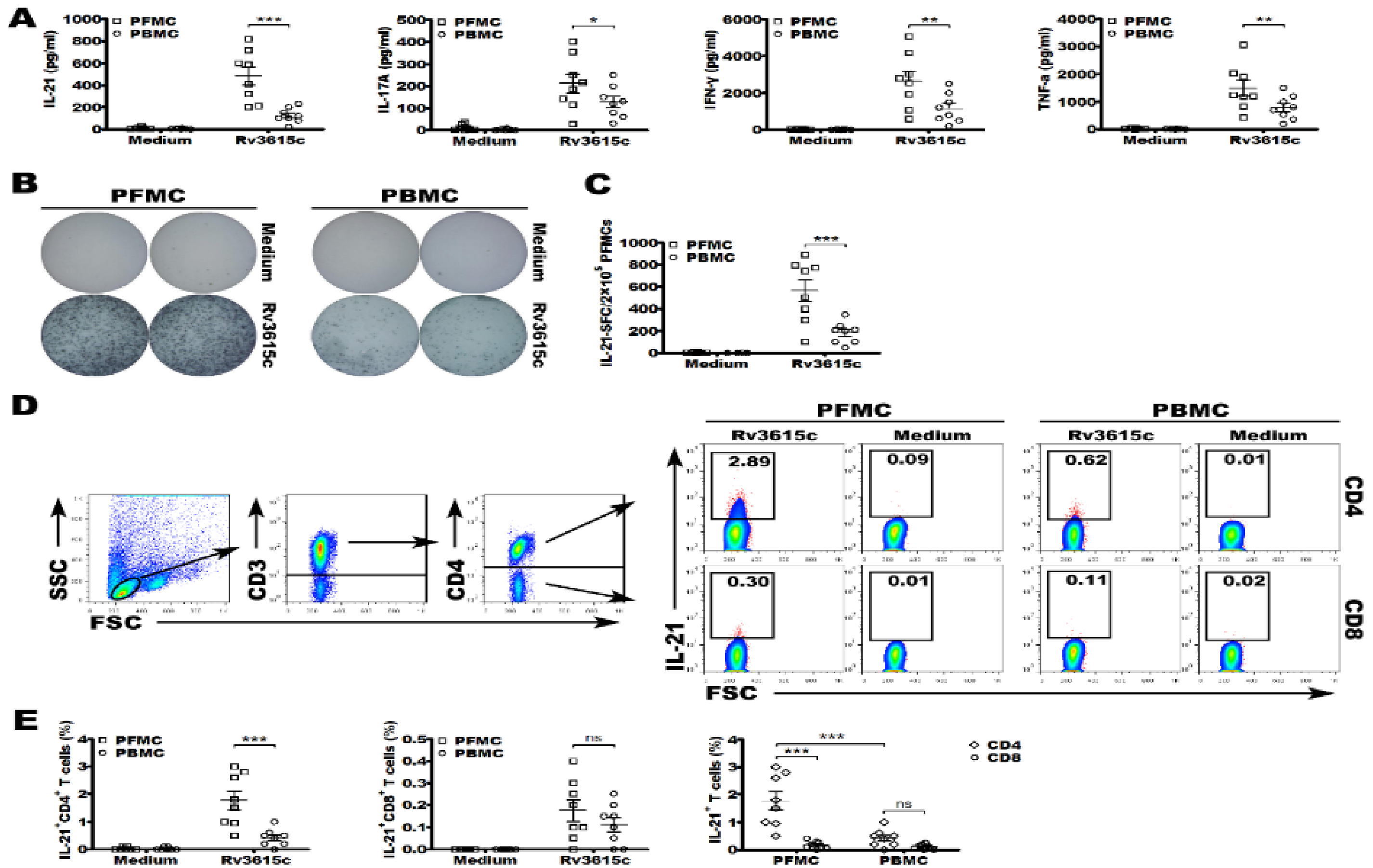

Figure 1: Rv3615c-specific peptide induces IL-21 production by CD4+ T cells from PFMCs. **(A):** PFMC from patients with TBP (n = 8) and PBMC from HD (Healthy donors) (n = 8) were cultured with or without Rv3615c stimulation in the presence of anti-CD28 for 72 hrs. IL-21, IL-17A, IFN-γ and TNF-α in culture supernatants were quantitatively assessed by ELISA and expressed as mean with individual data point, and were compared with Student's t-test. Each dot represented one patient or donor. **(B-C):** PFMC from patients with TBP (n = 8) and PBMC from healthy donors (n = 8) were cultured with or without Rv3615c stimulation in the presence of anti-CD28 for 24 hrs. The frequency of IFN-γ-producing cells were assessed by ELISPOT. The left panel showed representative counting of Spot-Forming Cells (SFP) and the right panel showed frequency of IFN-γ-producing cells as mean with individual data point, and were compared with Student's t-test. Each dot represented individual patient or donor. **(D-E):** PFMCs from patients with TBP (n = 8) were cultured with or without Rv3615c stimulation in the presence of anti-CD28 for 12 hrs. Cells were harvested and stained with fluorochrome-conjugated monoclonal antibodies, and studied for lineage differentiation and intracellular cytokine expression with flow cytometry. Each dot represented one patient. Data were expressed as mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

The Rv3615c-specific IL-21-expressing CD4+ T cells were tissue resident memory T cells in pleural fluid

To demonstrate phenotypic characteristics of IL-21-expressing CD4+ T cells, freshly isolated PFMCs were stimulated with Rv3615c peptides. Following stimulation with Rv3615c peptide, high levels of CD69 on CD4+ T cells at day1 and high levels of CD25 on CD4+ T cells at day3 (Figure 2A). At day 1 (early phase), the expression of earliest activation marker CD69+ in CD4 cells was high, there was a slight decline of the expression of earliest activation marker CD69, and CD69 continued to be highly expressed through 72h after peptide stimulation (Figure 2A, B), suggesting there were a vast number of effector cells formation in the early stage. At the same time, at day 3 (late phase), there is a significant increase in the expression of later activation marker CD25+ in CD4 cells (Figure 2A, B), suggesting that there was a large proportion of resting memory T cells in the local lesions. The previous explanation of the expression of CD69 was that they were in an activated

state, perhaps as a result of retained antigen; however, we now know that CD69 expression is a generic characteristic of resting tissue resident memory (TRM) T cells. The IL-21$^+$CD4$^+$ T cells were predominately on CD69$^+$ and CD25$^+$ T cells. The IL-21$^+$CD4$^+$ T cells expressed significantly higher levels of CD69 and CD25 compared with counterparts IL-21$^-$CD4$^+$ T cells (Figure 2C). Collectively, this suggested that Rv3615c-specific IL-21$^+$CD4$^+$ T cells were predominately produced by tissue resident memory T cells in pleural fluid.

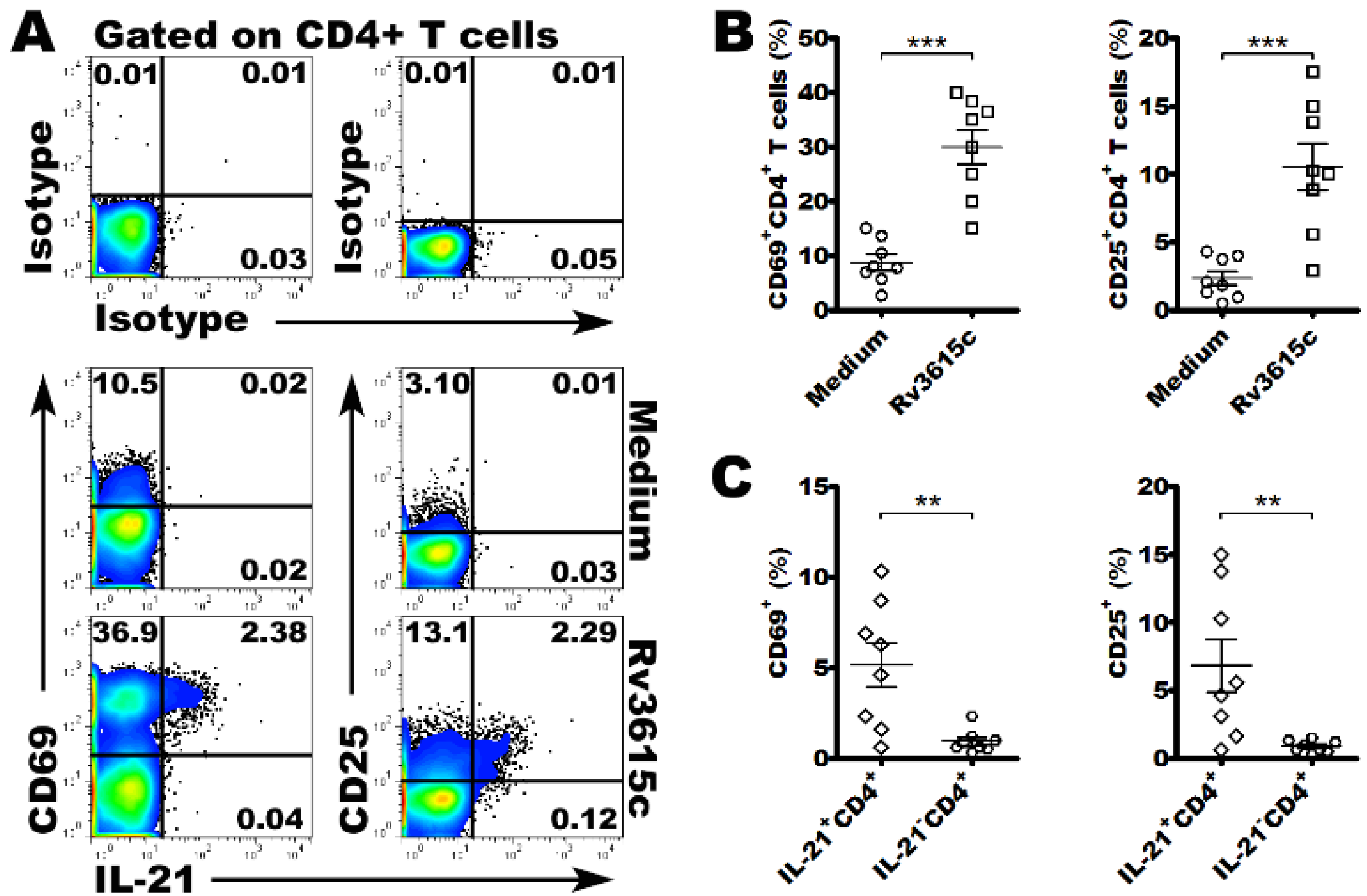

Figure 2: The Rv3615c-specific IL-21-expressing CD4$^+$ T cells were tissue resident memory T cells in pleural fluid**.** PFMCs from Patients with TBP (n = 8) were cultured with or without Rv3615c stimulation in the presence of anti-CD28 for 1 day and 3 days. Cells were harvested and stained with fluorochrome-conjugated monoclonal antibodies, identified by lineage differentiation, and studied for CD69 and CD25 expressions with flow cytometry. **(A):** Representative histograms showed the CD69 and CD25 expressions in CD4$^+$ T cells. **(B):** Frequency of CD69 and CD25 expressions in CD4$^+$ T cells after Rv3615c stimulation. **(C):** Frequency CD69 and CD25 expressions in IL-21$^+$CD4$^+$ and IL-21$^-$CD4$^+$T cells. Data were expressed as mean ± SD, and compared with Student's t-test (n = 6). **P<0.01; ***P<0.001.

The Rv3615c-specific IL-21-expressing CD4$^+$ T cells were effector or effector memory T cells

To examine the phenotype of Rv3615c-specific IL-21-expressing CD4$^+$ T cells, IL-21-expressing CD4$^+$ T cells were gated for the analysis of the expression of CD45RO, CD62L, CD27 and CCR7. The results showed that the IL-21$^+$CD4$^+$ T cells were found to be predominantly CD45RO$^+$, thereby exhibiting a memory cell phenotype. In contrast, the IL-21$^-$CD4$^+$ T cells consisted of a significantly low percentage of CD45RO$^+$ cells. We also found that IL-21$^+$CD4$^+$ T cells expressed significantly lower levels of CD62L, CD27 and CCR7 than IL-21$^-$CD4$^+$ T cells (Figure 3A). Furthermore, IL-21$^+$CD4$^+$ T cells showed higher levels of CD45RO$^+$CD62L$^-$, CD45RO$^+$CD27$^-$, CD45RO$^+$CCR7$^-$ than IL-21$^-$CD4$^+$ T cells (Figure 3B, C), suggesting that Rv3615c-specific IL-21$^+$CD4$^+$ T cells were effector or effector memory T cells.

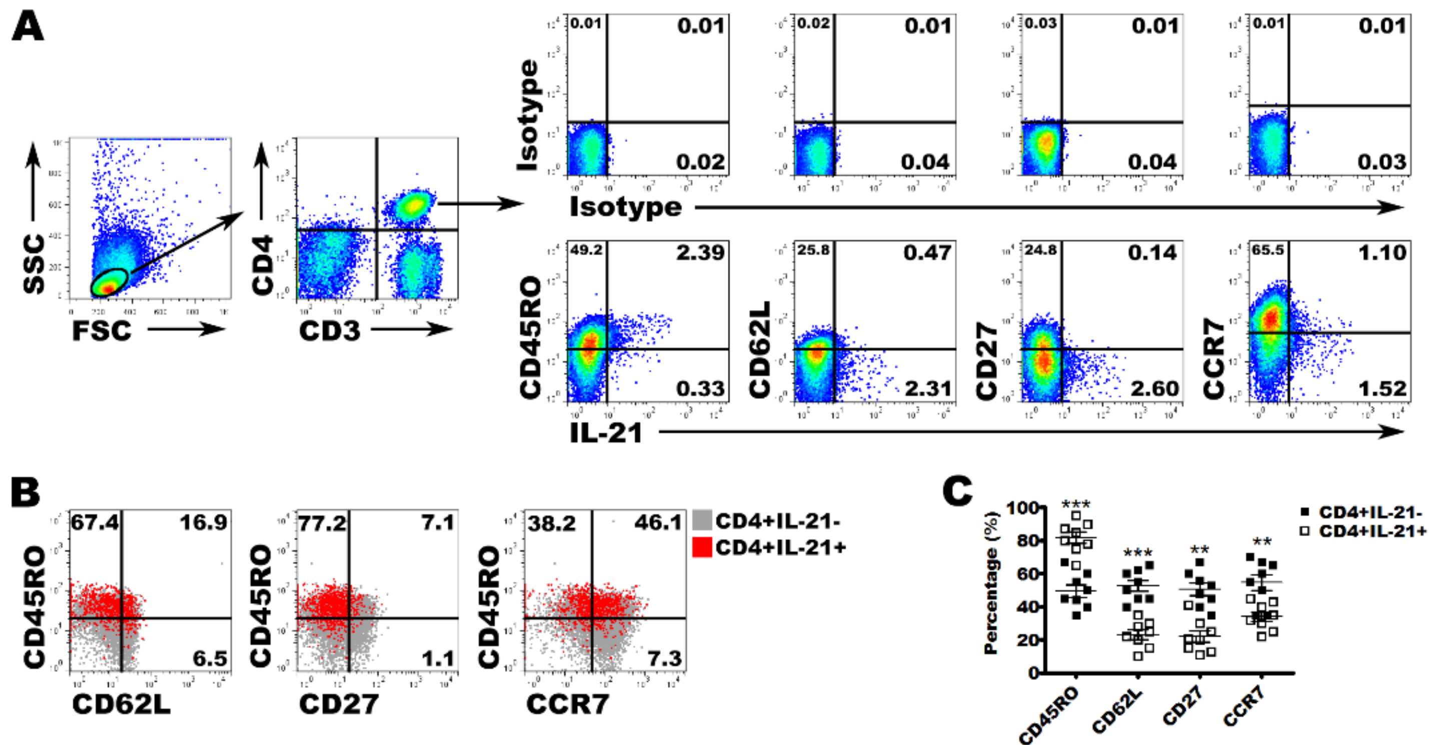

Figure 3: The Rv3615c-specific IL-21-expressing CD4⁺ T cells were effector or effector memory T cells. PFMCs from patients with TBP (n = 8) were cultured with or without Rv3615c in the presence of anti-CD28 for 12 hrs. Cells were harvested and stained with fluorochrome-conjugated monoclonal antibodies extracellularly and intracellularly. The CD4⁺ T cells responding to Rv3615c were identified by lineage differentiation and intracellular cytokine expression, and studied for differentiation with flow cytometry. **(A):** Representative dot plots showed the gating strategy and expression of differentiation markers in CD4⁺ T cells with or without cytokine production. **(B):** Representative dot plots showed the expression of differentiation marker on Rv3615c-expanded CD4⁺ T cells. **(C):** Frequency of differentiation marker-expressing cells and their combinations in cytokine-producing CD4⁺ T cells. Data were expressed as mean ± SD, and compared with Student's t-test (n = 5). *P<0.05; **P<0.01; ***P<0.001.

The subsets of IL-21-expressing CD4⁺ T cells is different from Th1 and Th17 subpopulations

To determine whether Rv3615c-specific IL-21-expressing CD4⁺ T cells are related to Th1 and Th17 cell populations, freshly isolated PFMCs were stimulated in vitro with Rv3615c peptide, and the expression of cytokines was analyzed by flow cytometry. Following stimulation with Rv3615c peptide, CD4⁺ T cells from PFMCs could co-express IL-21 with IFN-γ, TNF-α and IL-17A (Figure 4A). The statistical results showed that after stimulation with Rv3615c peptide, about 2.5% of CD4⁺ T cells expressed IL-21, 5.7% of CD4⁺ T cells expressed IFN-γ, 3.9% of CD4⁺ T cells expressed TNF-α and 1.1% of CD4⁺ T cells expressed IL-17A (Figure 4B). In addition, the representative data demonstrated that Rv3615c induced co-expression of IL-21 with IFN-γ, TNF-α and IL-17A by CD4⁺ T cells. In the correlation between IL-21 and IFN-γ, TNF-α or IL-17A expression, cytokine-expressing cells could be clearly divided into three subsets. As shown in the pie charts that IFN-γ or TNF-α single-positive cells had the largest proportion of cytokine-secreting cells, followed by IL-21⁺IFN-γ⁺/IL-21⁺ TNF-α⁺ cells and IL-21 single-positive cells. However, a fraction of IL-21-expressing cells did not or scarcely stimultaneously expresses IL-17A (Figure 4C). To further evaluate the poly functionality of IL-21⁺IFN-γ⁺ cells, CD4⁺ T cells were divided into four subsets according to the production of IL-21 and IFN-γ. The results indicated that IL-21⁺IFN-γ⁺ T cells co-expressed high levels of TNF-α, but rarely IL-17A compared to IL-21⁺IFN-γ⁻ T cells, suggesting the obviously poly functionality of these cells (Figure 4D, E). Taken together, these results indicate that a subset of IL-21-expressing CD4⁺ T cells from PFMCs co-expressed Th1 cytokines.

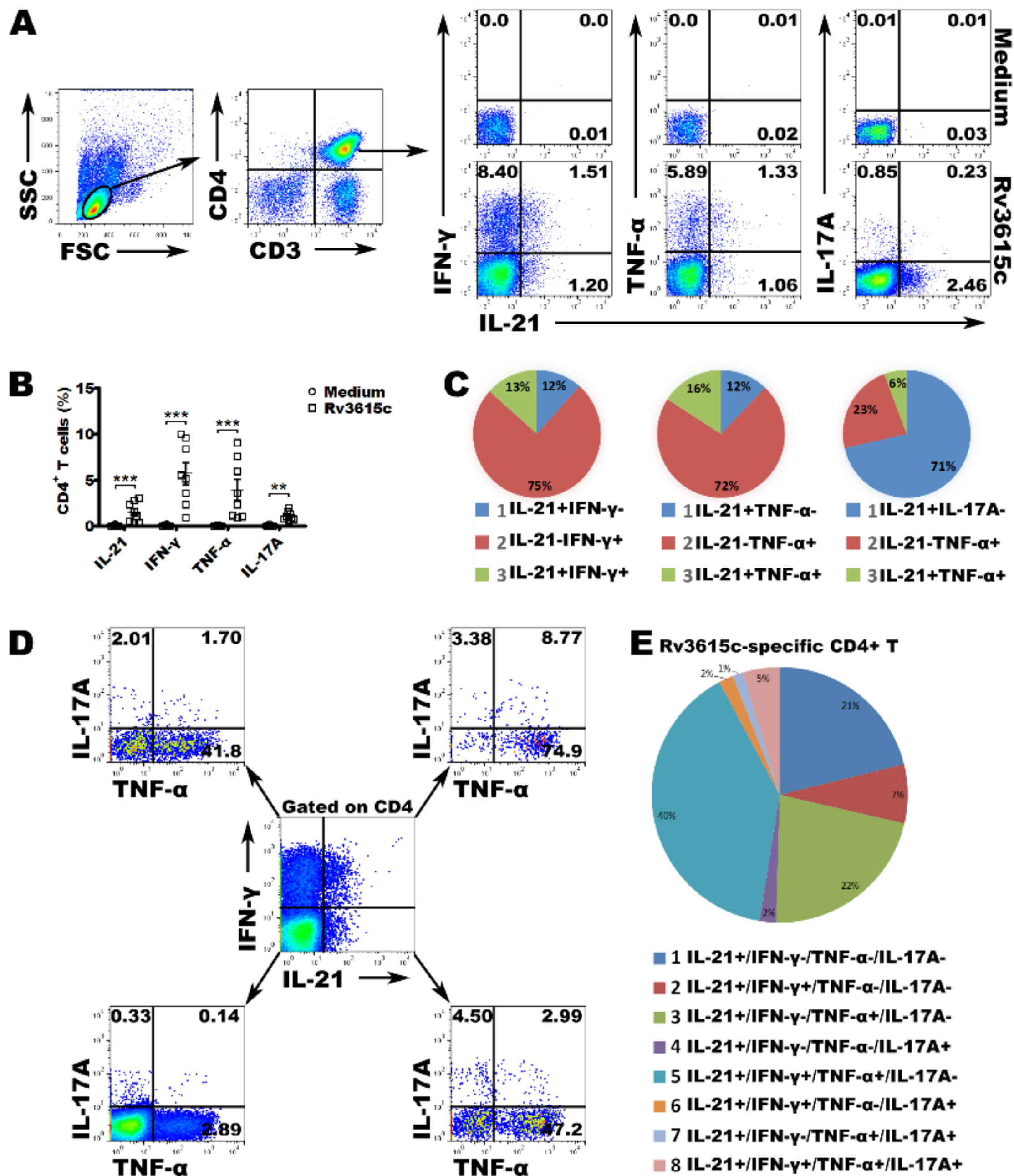

Figure 4: The subsets of IL-21-expressing CD4+ T cells is different from Th1 and Th17 subpopulations. **(A):** PFMCs (n= 8) were stimulated with Rv3615c peptide. The expression of cytokines was determined by FACS. Expression of IL-21 and the IFN-γ, TNF-α and IL-17A was assessed by FACS. Numbers in quadrants indicate percentages of cells in each population. **(B):** Summary data of the frequency of IL-21, IFN-γ, TNF-α and IL-17A expression by CD4+ T cells after Rv3615c stimulation. **(C):** Data are quantified and presented in a pie chart; each slice of the pie represents the fraction of the mean value of a given quadrant. Independent experiments were repeated at least eight times. **(D):** PFMCs (n= 8) were stimulated with Rv3615c peptide. CD4+ T cells were gated in lymphocytes from PFMCs. The expression of IL-21and IFN-γ was detected by FACS. The expression of IL-17A and TNF-α within each cell subset was analyzed. **(E):** Corresponding statistical results are shown in pie charts. Data were expressed as mean ± SD, and compared with Student's t-test (n = 5). **P<0.01; ***P<0.001.

The Rv3615c-specific IL-21-expressing CD4+ T cells from PFMCs have the characteristics of "Tfh like" cells

We next examined whether Rv3615c-specific IL-21-expressing CD4+ T cells from PFMCs have the characteristics of "Tfh like" cells, the expression of Tfh cell-specific markers on IL-21+CD4+ T cells was analyzed. Freshly isolated PFMCs were stimulated with or without Rv3615c peptide. The results indicated that without any stimulation, CD4+ T cells from PFMCs could express neither IL-21 nor CD40L. However, after stimulation, Rv3615c could induce high levels of IL-21, CD40L, CXCR5 and PD-1, and almost all of IL-21-producing CD4+ T cells expressed CD40L, and CXCR5 (Figure 5A, B). What's more, the IL-21+CD4+ T cells co-expressed higher levels of CD40L, CXCR5 and PD-1 compared to counterparts (Figure 5C). Because the contribution of transcription factor of cytokines expression, we examined the expression of Bcl-6 (for IL-21) in CD4+ T cells of PFMCs. The results showed that nearly 64.3% of CD4+ T cells expressing IL-21 also expressed Bcl-6, which was significantly greater than the frequency of Bcl-6-expressing cells among non-IL-21-expressing CD4+ T cells (22.7%) (Figure 5C). However, IL-21+CD4+ T cells expressed significantly higher amount of CD40L, CXCR5, and Bcl-6 compared to IL-21-CD4+ T cells.

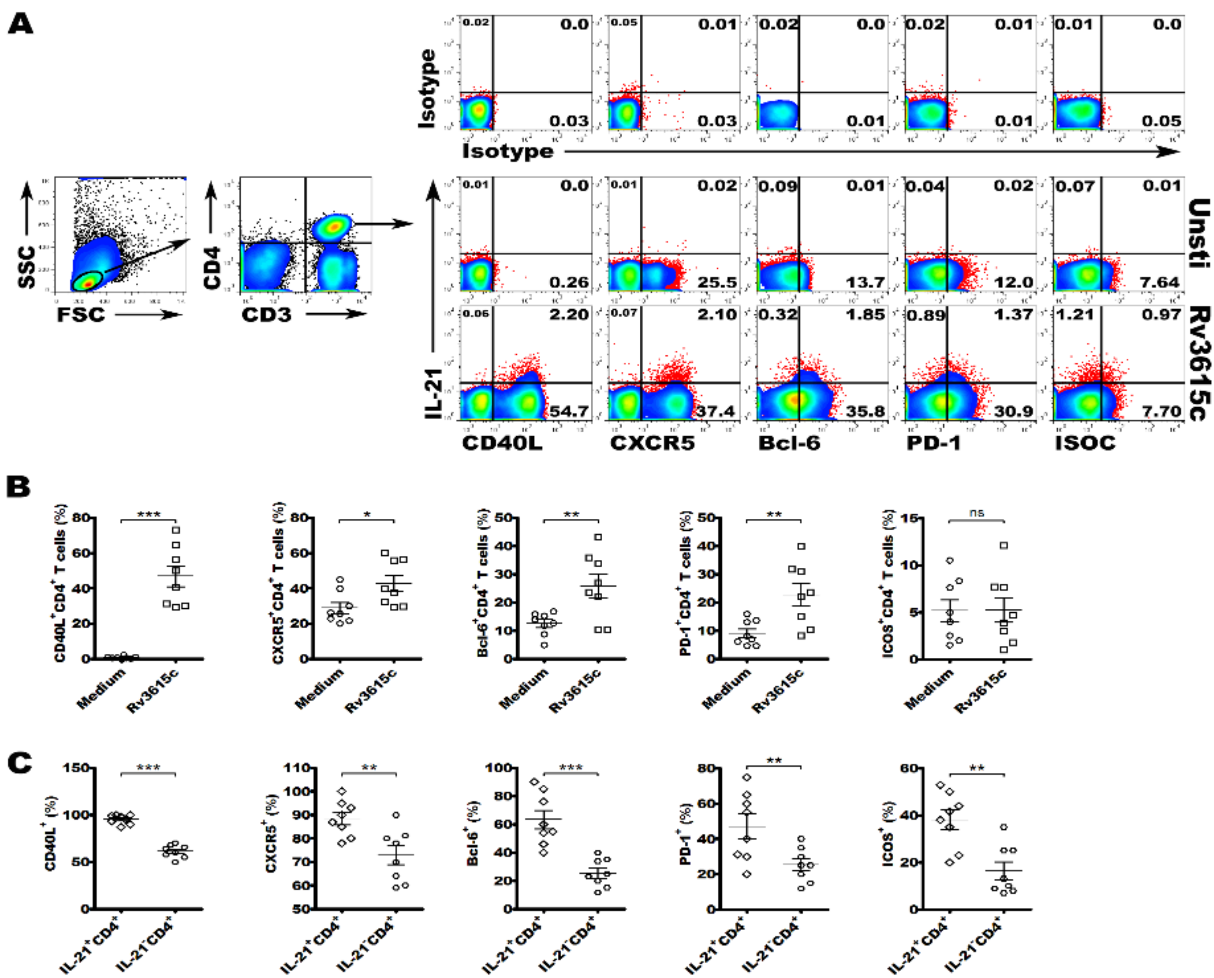

Figure 5: The Rv3615c-specific IL-21-expressing CD4+ T cells from PFMCs have the characteristics of "Tfh like" cells **(A):** PFMCs (n= 8) were stimulated with Rv3615c peptide. IL-21 and surface markers (CD40L, CXCR5, Bcl-6, PD-1 and ISOC) expressed by CD4+ T cells were evaluated by FACS. **(B):** Frequency of CD40L, CXCR5, Bcl-6, PD-1 and ISOC expressions in CD4+ T cells after Rv3615c stimulation. **(C):** Frequency CD40L, CXCR5, Bcl-6, PD-1 and ISOC expressions in IL-21+CD4+ and IL-21-CD4+T cells. Data were expressed as mean ± SD, and compared with Student's t-test (n = 8). **$P<0.01$; ***$P<0.001$.

The Rv3615c-specific CD4$^+$ T cells produce IL-21 to help B cells for the production of immunoglobulins

To explore the humoral immunity in local infection lesion elicited by the Rv3615c peptide, we detected the production of immunoglobulins by B cells stimulated by Rv3615c with the help of CD4$^+$ T cells. First, we detected the expression of CD40 and IL-21R on CD19$^+$ B cells from freshly isolated PFMCs. The results indicated that significantly high levels of CD40 and IL-21R expression on B cells (Figure 6A, B). To further define the functions of CD4$^+$ T cells and B cells with the Rv3615c stimulation, we isolated CD4$^+$ T cells, B cells (CD19$^+$) and monocytes (CD14$^+$) from PFMCs by microbeads, respectively (Figure 6C). Purified CD4$^+$ T cells co-cultured with monocytes, and can produce IL-21, IFN-γ and IL-17A following the stimulation with Rv3615c (Figure 6D). Furthermore, we cultured purified B cells with or without purified CD4$^+$ T cells following the stimulation with Rv3615c. It showed that purified B cells could have secreted large amounts of IgG, IgM and IgA when co-cultured with purified CD4$^+$ T cells following the stimulation with Rv3615c (Figure 6E). To test the promotive effect of recombinant IL-21 on the production of immunoglobulins by B cells, we cultured purified B cells with purified CD4$^+$ T cells following the stimulation with Rv3615c, and added simultaneously IL-21 alone and/or IL-21R in vitro. After stimulation for 10 days, we found that recombinant IL-21 could promote the production of IgG, IgM and IgA in cultured purified B cells, and this promoting effects were largely blocked by neutralizing antibody (anti-IL-21R) (Figure 6F). These in vitro findings confirmed that IL-21 was capable of promoting B cell maturation and immunoglobulins production in local infection lesion. Based on these results, we speculate that at the local site of *M.tb* infection, Rv3615c-specific CD4$^+$ T cells secreted IL-21, and help B cells secret immunoglobulins in order to protect against *M.tb* infection.

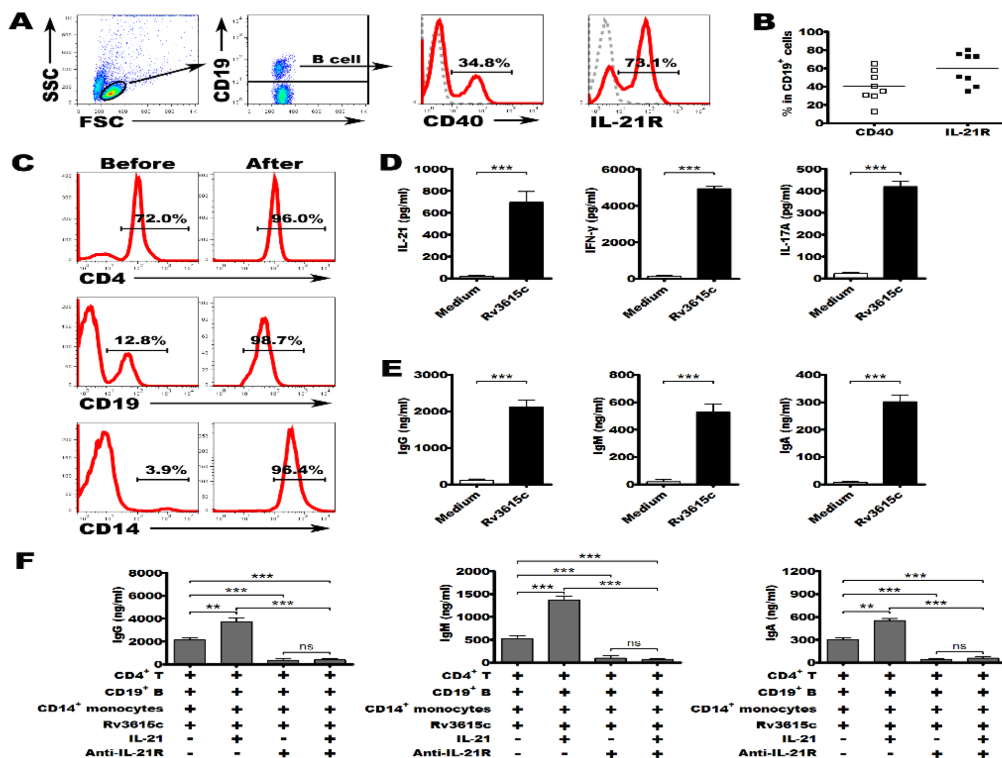

Figure 6: The Rv3615c-specific CD4$^+$ T cells produce IL-21 to help B cells for the production of immunoglobulins. **(A):** Representative FACS data and **(B):** Statistic results showed the expression of CD40 and IL-21R on CD19$^+$ B cells in PFMCs (n= 8). **(C):** Representative FACS data showed that the purity of CD4$^+$, CD19$^+$ and CD14$^+$ cells isolated from fresh PFMCs (n= 4). **(D):** Isolated CD4$^+$ T cells were cultured with or without monocytes and stimulated by Rv3615c for 3 days, statistic results showed for the production of IFN-γ, and IL-21 by isolated CD4$^+$ T cells. **(E):** Isolated CD19$^+$ cells were cultured with or without CD4$^+$ T cells and stimulated by Rv3615c for 10 days, statistic results showed for the production of IgG, IgM and IgA by isolated CD19$^+$ cells. **(F):** Isolated CD19$^+$ cells were cultured with or without CD4$^+$ T cells and stimulated by Rv3615c, plus soluble CD40L and/or recombination IL-21, or blocking antibody anti-CD40 and/or anti-IL-21R for 10 days, statistic results showed for the production of IgG, IgM and IgA by isolated CD19$^+$ cells. Representative data of three independent experiments were shown. Data were shown as mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

Discussion

Animal and human studies of intracellular pathogens have extensively evaluated poly functional $CD4^+$ T cells producing multiple pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-2) as a possible correlate of protection from infection and disease [20,21]. In the past few years, we published our efforts in determining the phenotype, function, and regulation of *M.tb*-specific $CD4^+$ T cells [22-24]. Our findings here were consistent with our earlier studies that the phenotypic and functional characteristics of IL-21-expressing $CD8^+$ T cells in human nasal polyps [25], and the first time that antigen-specific human NKT cells from tuberculosis patients produce IL-21 to help B cells for the production of immunoglobulins [14]. However, at present, little data exists characterizing the production and function of IL-21-producing $CD4^+$ T cells during tuberculosis pleurisy.

Currently, we evaluated whether or not novel TB vaccine candidate Rv3615c, which is a highly immune dominant RD1 (Region of Difference 1)-dependent secreted antigen specific for *M.tb* infection, induced poly functional $IL\text{-}21^+CD4^+$ T cells in PFMCs and if these T cell responses correlate with vaccine-mediated immunoglobulin production. The further studies showed that novel TB vaccine candidate Rv3615c peptide induced significantly higher levels of IL-21 expression on $CD4^+$ T cells than on $CD8^+$ T cells in PFMCs. In concordance with others previous work, our study demonstrated that the Tfh cell-type cytokine IL-21 was predominantly expressed by $CD4^+$ T cells in PFMCs at the local infection sites. These discrepancies of cell sources of IL-21 production might be due to differences in species, stimuli, or stimulation conditions. Next, we examined the correlation of IL-21-producing $CD4^+$ T cells with other cell types, and found that the subset of Rv3615c-specific IL-21-expressing $CD4^+$ T cells was clearly different from the *M.tb*-specific Th1, Th2, and Th17 subpopulations. A fraction of IL-21-producing $CD4^+$ T cells simultaneously produced Th1 cytokines, however, not Th2 or Th17 cytokines. Therefore, $IL\text{-}21^+IFN\text{-}\gamma^+CD4^+$ T cells exhibited obviously polyfunctionality than IL-21 single-expressing $CD4^+$ T cells.

It is well known that IL-21 is a marker for Tfh cells [26]. However, IL-21-producing cells cannot be classified as Tfh cells because other cell types can express substantial amounts of IL-21 [27,28]. In addition to the expression of IL-21, some important markers, such as ICOS, CXCR5, PD-1, as well as Bcl-6, are also critical in the identification of Tfh cells [29]. The effects of IL-21 include promotion of antibody production, NK cell differentiation, proliferation and differentiation of cells of monocyte and granulocyte lineages, costimulation of T cells and augmentation of antitumor activity of $CD8^+$ T cells [17]. IL-21, produced by Tfh cells, plays a critical role in B-cell proliferation, class switching and Ig production. It has been speculated that IL-21, through its varies positive and negative regulatory effects on cell-mediated and humoral immunity, may play a role in infection diseases [30].

Accumulating evidence suggest that B cells also play a role in the orchestration of an immune response against *M.tb* by interacting with various immune cells. B cells and antibodies have a variety of potential protective roles at each stage of *M.tb* infection and postulate that such roles should be considered in the development strategies for TB vaccines and other immune-based interventions [31,32]. We found that IL-21R is preferentially expressed by B cells. Binding of its ligand, IL-21, in these cells results in the activation of the Jak/Stat signal transduction pathway [33]. The effects IL-21 ligand binding has pleiotropic actions such as augmenting the proliferation of T cells, driving of B cells into memory cells, terminally differentiating plasma cells and augmenting the activity of natural killer cells. Our study showed that IL-21 upregulated the secretion of Ig by activated PFMCs. Therefore, we speculated that $CD4^+IL\text{-}21^+$ cells shared functional properties with Tfh cells from secondary lymphoid organs. In concordance with Tfh cells, the cells required activation to provide help to B cells and induce their differentiation into Ig-producing cells.

In summary, we found that Rv3615c induced production of IL-21 in addition to IFN-γ, TNF-α, IL-2 and IL-17 by $CD4^+$ T cells in pleural fluids from TB patients. IL-21-expressing $CD4^+$ T cells expressed phenotypes of effector memory cells and co-expressed CXCR5 and CD40L, and $CXCR5^+$ T helper cells mediate protective immunity against tuberculosis. CD40L is expressed on a variety of cell types including activated $CD4^+$ T cells and some $CD8^+$ T cells, NK cells. CD40L is also known as CD154; it serves as a ligand for CD40 that is expressed on B cells, macrophages, and dendritic cells. The expression of CD40L by activated T-helper cells costimulates B-cell activation and proliferation through binding to CD40 expressed on B cells. In response to T-dependent antigens, the CD40L and CD40 interaction is required for B-lymphocyte differentiation, including immunoglobulin production and isotype switching and memory B cell generation, suggesting that engaging the CD40-CD40L pathway augments T-helper cell responses and improves control of *M.tb* infection [34]. IL-21 is mainly produced by activated $CD4^+$ T cells including T Follicular Helper (Tfh) cells, and has a broad range of immune regulatory functions on B cells, $CD8^+$ T cells, NK cells and dendritic cells. Furthermore, IL-21 produced by $CD4^+$ T cells promoted B cells to secrete IgG, IgM and IgA. Our data deepen information on local immune responses against *M.tb* infection and provide a further rationale for the evaluation of $CD4^+$ T cells in host defense against tuberculosis. All of the above characteristics are distinguishing features of Tfh cells. However, in contrast to Tfh cells, these cells did not localize within B-cell follicles, so it is still challenging to address whether *M.tb*-specific IL-21-expressing cells are circulating Tfh cells or "Tfh-like" cells. Nevertheless, evaluation of *M.tb*-specific IL-21 -expressing $CD4^+$ T cells could help us understand cellular and humoral immune responses to *M.tb*.

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