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Original Article

ILC2s induce Treg but not Th2-type immunity through IL-33/ST2 pathway in pulmonary tuberculosis

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Abstract

Introduction: We investigated the function of type 2 innate lymphoid cells (ILC2s) and IL-33 in pulmonary tuberculosis (PTB).

Methodology: Peripheral blood samples were collected from PTB patients and healthy controls. The cytometric bead array was used to detect plasma IL-33, TGF-β, IL-4, IL-5, IL-6, IL-10, IL-13, and soluble ST2 (sST2). ILC2s, Th2, and Treg cells were detected with flow cytometry. Quantitative real-time PCR was used to measure mRNA levels. ILC2s were co-cultured with peripheral blood mononuclear cells and then intervened with IL-33 or anti-ST2 antibody + IL-33 in vitro. IL-4, IL-6, IL-5, IL-10, IL-13, and TGF-β levels were measured by enzyme-linked immunosorbent assay.

Results: Compared with healthy controls, the levels of IL-33, sST2, TGF-β, IL-10, and IL-6 in the plasma of PTB patients were significantly higher. No significant difference was found in the plasma IL-4, IL-5, and IL-13 levels. Patients with PTB had significantly increased ILC2s proportion and mRNA levels of RAR-related orphan receptor α and GATA binding protein 3. After 48 h of IL-33 stimulation in vitro, Treg cell proportion significantly increased and the IL-10 level was significantly elevated. Treatment with anti-ST2 abolished these effects. No significant difference was found in cytokines of IL-4, IL-6, IL-5, IL-13, and TGF-β, or Th2 cells before and after IL-33 treatment. ILC2s proportion in peripheral blood was increased and plasma IL-33 was upregulated in PTB patients.

Conclusions: IL-33 may promote the growth of ILC2s and the production of Treg-related cell cytokines, but not Th2-related cell cytokines, to participate in immune response to PTB.

Key words: tuberculosis; ILC2s; IL-33/ST2; Treg; Th2.

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Introduction

Tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis* [1]. Cellular immunity, especially T cell immunity, plays an important role in anti-tuberculosis immunity. Under the combined stimulation of *Mycobacterium tuberculosis* antigens and costimulatory molecules, naive CD4+ T lymphocytes can differentiate into T helper cells (including Th1, Th2, and Th17) and regulatory T (Treg) cells. Due to the functional antagonism between Treg cells and Th17 cells, they can induce immune tolerance and result in uncontrolled *Mycobacterium tuberculosis* infection [2]. Additionally, *Mycobacterium tuberculosis* can induce hyperactivation of Treg cells and the production of anti-inflammatory mediators, which have immunosuppressive functions and can promote the growth and survival of *Mycobacterium tuberculosis* [3,4].

Interleukin-33 (IL-33) is a member of the IL-1 family [5] and an endogenous cytokine produced by damaged or necrotic barrier cells (endothelial and epithelial cells). IL-33 signal transduction depends on recognition by the specific receptor of growth stimulation expressed gene 2 (ST2), which is mainly expressed in immune cells. IL-33/ST2 signaling affects peripheral homeostasis, phenotypic diversity, and function of Treg cells, which are necessary for the establishment and maintenance of immune self-tolerance [6,7].

IL-33/ST2 signaling can promote type 2 effector responses, and ST2 can regulate Treg cells in the type 2 immune response of allergic pulmonary inflammation in a mouse model [8]. IL-33/ST2 signaling can increase Treg cells and decrease IL-17 and IFN-γ production [9,10]. In a mouse model with graft-versus-host disease (GVHD), treatment with IL-33 enhanced proportions of ST2+ Treg cells and ameliorated GVHD [11]. After

skin grafting, IL-33-treated mouse grafts are reported to have increased Treg cell numbers, decreased IFN-γ and IL-17 production, and increased IL-10 production, thus promoting skin graft survival [12].

IL-33 can regulate type 2 immune responses by targeting Th2 cells, type 2 innate lymphoid cells (ILC2s), mast cells, and eosinophils. ILC2s can regulate metabolic homeostasis and resist helminth infection [13]. In humans, ILC2s are found in the lung and gut [14]. Enhanced IL-33/ST2 signaling can expand ILC2s in vivo [15,16]. During lung inflammation after *Nippostrongylus brasiliensis* infection, IL-33 can promote the production of cytokines by ILC2s [17]. Blocking IL-33/ST2 signaling in influenza-infected mice could reduce ILC2s in the lung and result in reduced lung function and loss of airway epithelial integrity, indicating that IL-33/ST2 signaling in ILC2s is also critical for resisting lung infection [18]. The above findings show that whether the effects of ST2/IL-33 stimulation in ILC2s are beneficial or harmful depends on the specific disease type. However, the regulation of ILC2s on immune cell differentiation through the IL-33/ST2 pathway in tuberculosis has not been reported.

Herein, we investigated the regulatory effect of ILC2s on Treg cells in patients with pulmonary tuberculosis (PTB) through the IL-33/ST2 pathway. Both in vivo and in vitro experiments were performed.

Methodology

Participants

A total of 26 patients with PTB who were hospitalized in the Eighth Affiliated Hospital of Xinjiang Medical University in Urumqi, China from November 2021 to March 2022 were enrolled. PTB diagnosis was made following the diagnostic criteria of the National Health and Family Planning Commission of China (WS288-2017) [19]. The control group consisted of 22 healthy individuals who received physical examinations and were without active clinical signs. This study was approved by the Ethics Committee of the People's Hospital of Xinjiang Uygur Autonomous Region (No.2019030622). Each participant provided informed consent.

Samples

A 10 mL sample of peripheral blood was collected before antibiotic or hormone therapy. The Ficoll density gradient method was used to isolate peripheral blood mononuclear cells (PBMCs). ILC2s were isolated from the peripheral blood samples of 26 patients with PTB using the EasySep™ Human ILC2 Enrichment kit (cat. no. 17972, STEMCELL Technologies, Vancouver, Canada). The plasma was collected after centrifugation and stored at -80 °C until use.

Quantitative real-time polymerase chain reaction (PCR)

The total RNA of PBMCs was isolated using the RNeasy Mini Kit (74104, Qiagen, Hilden, German). The chemoattractant receptor homologous molecule of Th2 cells (CRTH2), GATA binding protein 3 (GATA3), and retinoic acid receptor-related orphan receptor α (RORα) mRNA levels were measured on Applied Biosystems QuantStudion 6 Flex (Life Technologies, Carlsbad, California, USA), with β-actin as internal control. The primers [20] are listed in Table 1. The PCR conditions were: 95 °C for 2 min; followed by 40 cycles of 95°C for 5s and 60°C for 10s. The 2-ΔΔCt method was used for calculating the relative mRNA levels [21].

Cell co-culture, grouping, and stimulation

The cells were divided into the following groups according to treatments: PBMCs control group, PBMCs + ILC2s group, PBMCs + ILC2s + IL-33 group, and PBMCs + ILC2s + IL-33 + anti-ST2 group. In the control group, PBMCs from the healthy individuals were treated with 1640 medium. In the PBMCs + ILC2s group, the ILC2s from patients with PTB were seeded at 500 cells/well in 96-well plates [20] and co-cultured with PBMCs from healthy individuals for 48 h. In the PBMCs + ILC2s + IL-33 group, the co-cultured PBMCs and ILC2s were stimulated with human rIL-33 (30 ng/mL) by the concentration gradient experimentally (200-33-10UG, PeproTech, Rocky Hill, New Jersey, USA) for 48 h. In the PBMCs + ILC2s + IL-33 + anti-ST2 group, the co-cultured PBMCs and ILC2s were treated with human ST2/IL-33R

Table 1. Primer sequences used in quantitative real-time PCR.

Gene	GenBank accession	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)	Tm (°C)
RORα	NM_002943.3	CTGGTGTGCATAGCGGAGGTTG	CCTGCGGACTGGCAATAATCGG	101	60
GATA3	NM_001002295.1	GTGCATGACTCACTGGAGGACTTC	CATGTGGCTGGAGTGGCTGAAG	114	60
CRTH2	NM_004778.2	CGCCACACTGAAGCCACTCTG	GCGTGGTCGATGTAGCGGATG	90	60
β-Actin		CATGTACGTTGCTATCCAGC	CATGTACGTTGCTATCCAGC	138	60

CRTH2: chemoattractant receptor homologous molecule of Th2 cells; GATA3: GATA binding protein 3; RORα: retinoic acid receptor-related orphan receptor α.

neutralizing antibody (1 μg/mL) [22] (AF523, R&D Systems, Minneapolis, MN, USA) and human rIL-33 (30 ng/mL) for 48 h. The proportion of Th2 and Treg cells was detected by flow cytometry. The culture supernatants were collected for cytokine analysis by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry

In order to detect the proportion of ILC2s in peripheral blood, as well as the proportion of Treg and Th2 cells in the co-culture system, samples were incubated at room temperature with Lin-(CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD203c, FcεRIα)-FITC, CD127-PE, CD45-APC, CRTH2-PE-CF594, CD117-PE-Cy™5, CD25-BB515, CD4-PE-Cy™7, CD3-APC, CD183-PE-Cy™5, CD196-PE, and, CCR4-PE-Cy™7, in the dark for 30 min. The following antibodies were from BD (Franklin Lake, New Jersey, USA): CD3-FITC (cat. no. 561807), CD4-FITC (cat. no. 555346), CD8-FITC (cat. no. 555366), CD14-FITC (cat. no. 555397), CD15-FITC (cat. no. 555401), CD16-FITC (cat. no. 555406), CD19-FITC (cat. no. 555412), CD20-FITC (cat. no. 555622), CD33-FITC (cat. no. 555626), CD127-PE (cat. no. 557938), CRTH2-PE-CF594 (cat. no. 563501), CD117-PE-Cy™5 (cat. no. 559879), CD45-APC (cat. no. 555485), CD25-BB515 (cat. no. 564467), CD4-PE-Cy™7 (cat. no. 557852), CD3-APC (cat. no. 561810), CD183-PE-Cy™5 (cat. no. 551128), CD196-PE (cat. no. 559562), and, CCR4-PE-Cy™7 (cat. no. 557864). FITC-labeled antibodies against CD34 (cat. no. 343504) and CD203c (cat. no. 324614), and FcεRIα (cat. no. 334608) were from Biolegend (San Diego, California, USA). In the case of the peripheral blood sample, red blood cell lysis was performed for 15-30 min by using the red blood cell lysate (cat. no. 555899; R&D Systems, Minneapolis, Minnesota, USA) for 15-30 min. Finally, flow cytometry was conducted on the DXflex flow cytometer (Beckman, Brea, California, USA). Data was analyzed with the Kaluza software (Beckman, Brea, California, USA). The total ILC2s were Lin−CD45+CD127+CRTH2+CD117- [22]. Treg was defined as CD4+CD25+CD127low. Th2 was defined as CD4+CCR4+CD196-CD183- [23].

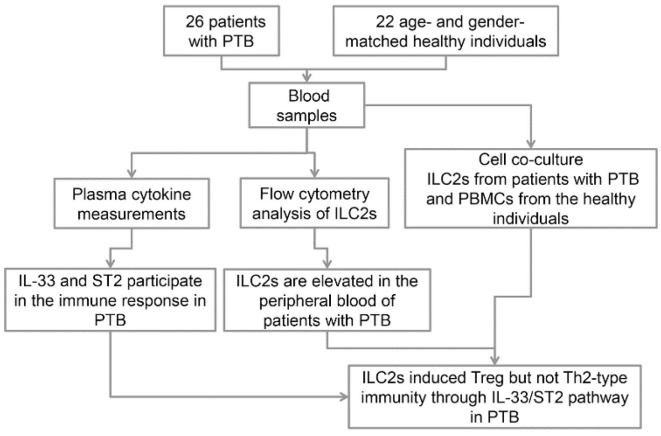
Measurement of cytokines

The concentration of IL-4, IL-5, IL-6, IL-10, IL-13, IL-33, TGF-β, and soluble(s) ST2 in plasma and cell culture supernatant were determined by the cytometric bead array (CBA) method. The Human Custom 32-plex kit (T1C329432) and TGF-beta 1-plex (B111206) from Beijing QuantoBio Biotechnology Co., Ltd (Beijing, China) were used. The analysis was performed on a flow cytometer (Beamcyte-1026, Changzhou Bidake Biology Science and Technology Co., Ltd, Changzhou, China) following the manufacturer's instructions.

Statistical analysis

Data analysis was performed using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Data were described by mean ± standard deviation (SD), median (interquartile range), or percentages, as appropriate. The *t*-test, one-way ANOVA, Mann-Whitney U test, and χ^2 test were used for data comparison. Pearson correlation analysis was used to evaluate the correlation. $p < 0.05$ indicated a significant difference.

Figure 1. Study flowchart illustrating the study procedures.



PTB: pulmonary tuberculosis; ILC2s: type 2 innate lymphoid cells; PBMCs: peripheral blood mononuclear cells; ST2: growth stimulation expressed gene 2.

Results

Basic clinical information

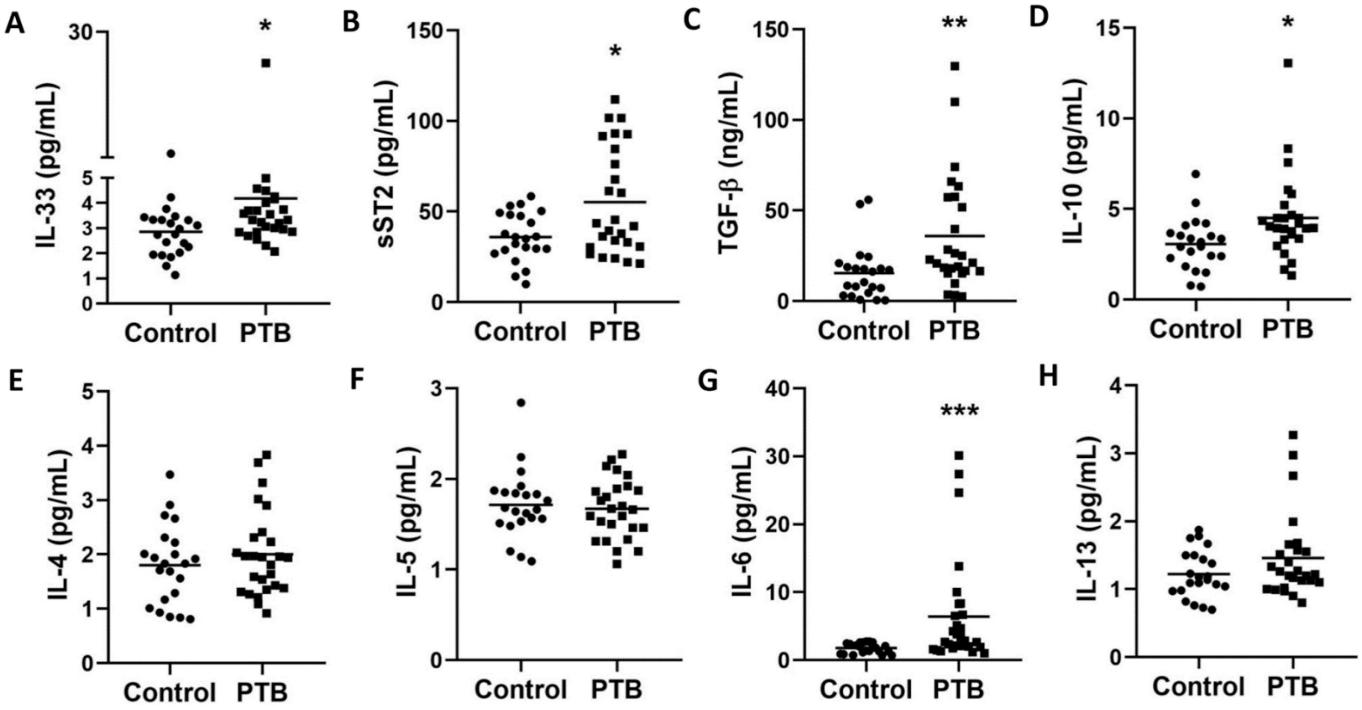
The study flowchart is presented in Figure 1. Table 2 summarizes the basic clinical information of all participants. A total of 26 PTB patients (15 males and 11 females) were included, with an average age of 45 ± 3.7 years.

Table 2. Clinical data of the study cohort.

Groups	Healthy individuals (n = 22)	Patients with pulmonary tuberculosis (n = 26)
Age (years)	41 ± 3.5	45 ± 3.7
Male/female	13/9	15/11
BCG inoculation	22	26
TST+ or T-SPOT.TB+	0	16
Sputum smear +	0	13
Culture positive	0	17

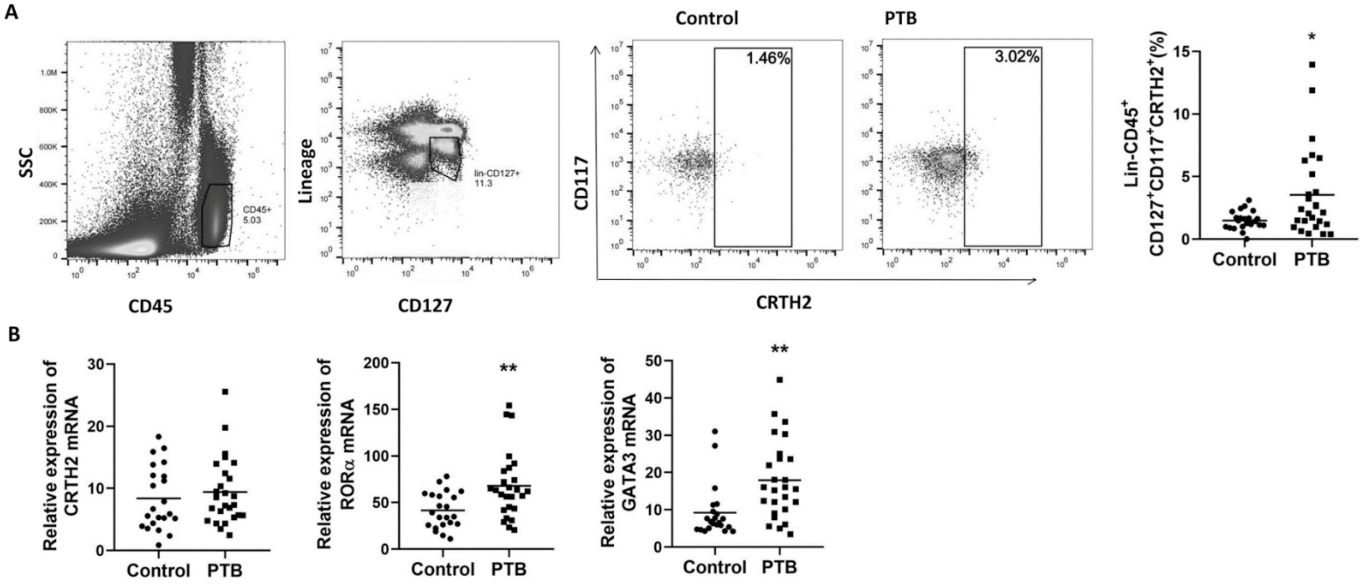
BCG: Bacillus Calmette-Guérin; T-SPOT/TB: T cell spot test for tuberculosis infection; TST: tuberculin skin test.1

Figure 2. Plasma levels of IL-33, sST2, Treg-related cytokines, and Th2-related cytokines. Plasma levels of (A) IL-33, (B) sST2, (C) TGF-β, (D) IL-10, (E) IL-4, (F) IL-5, (G) IL-6 and (H) IL-13 in control and patients with PTB.



Values are expressed as the mean ± standard deviation. *p < 0.05, **p < 0.01, *** p < 0.001 vs. control group. IL: interleukin; PTB: pulmonary tuberculosis.

Figure 3. Analysis of proportion of ILC2s and transcription factor mRNA levels. (A) The proportion of ILC2s in the peripheral blood of control subjects and patients with PTB was analyzed with flow cytometry. Representative flow cytometry results with a gating strategy and quantitative flow cytometry results were shown. (B) Relative mRNA expressions of CRTH2, RORα, and GATA3 in PBMCs of control subjects and patients with PTB were shown. Data were normalized to the control group.



Values are expressed as the mean ± standard deviation. *p < 0.05, **p < 0.01 vs. control group. CRTH2: chemoattractant receptor homologous molecule of Th2 cells; ILC2s: type 2 innate lymphoid cells; GATA3: GATA binding protein 3; Lin: lineage; PBMCs: peripheral blood mononuclear cells; PTB: pulmonary tuberculosis; RORα: RAR-related orphan receptor α.

The control set included 22 healthy individuals (13 males and 9 females), with an average age of 41 ± 3.5 years. All participants had received Bacillus Calmette-Guérin (BCG) inoculation. However, positive tuberculin skin test or T cell spot test for tuberculosis infection, or sputum smears and cultures were only found in some PTB patients (Table 2).

IL-33 and ST2 participate in the immune response in PTB

The plasma levels of IL-33 (Figure 2A), sST2 (Figure 2B), TGF-β (Figure 2C), IL-10 (Figure 2D), and IL-6 (Figure 2G) in patients with PTB were significantly higher compared to the control. No significant difference was identified in the IL-4 (Figure 2E), IL-5 (Figure 2F), and IL-13 (Figure 2H) levels between the two groups. This result indicates that IL-33 and ST2 may participate in the immune response of PTB.

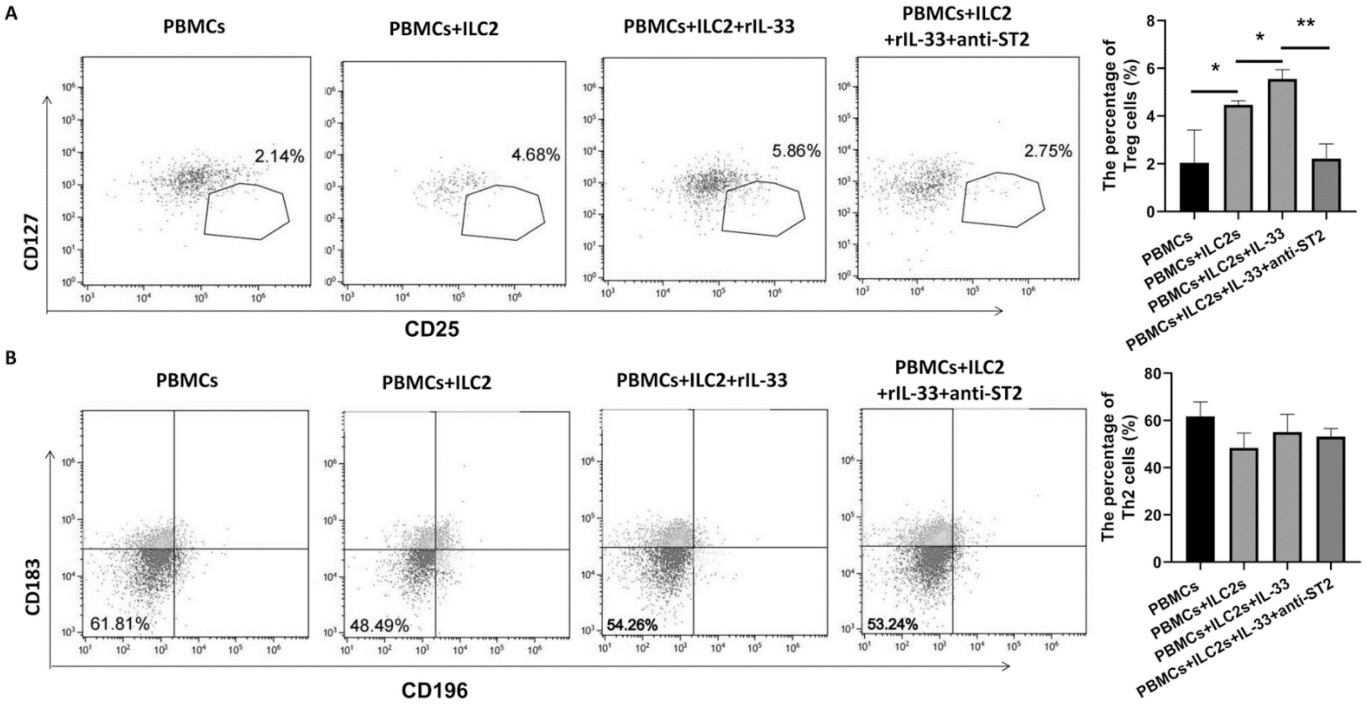
ILC2s are elevated in the peripheral blood of patients with PTB

To study whether ILC2s are involved in the pathogenesis of PTB, the proportion of ILC2s was analyzed with flow cytometry. The results showed that patients with PTB had significantly higher ILC2s proportion than the control group (Figure 3A). Furthermore, quantitative real-time PCR revealed that compared to control, there were significantly higher GATA3 and RORα mRNA in PBMCs of patients with PTB (Figure 3B). No significant difference was identified in CRTH2 mRNA levels between the two groups (Figure 2B). These results suggest that ILC2s may have a role in PTB pathogenesis.

ILC2s can induce Treg while not Th2-type immunity through IL-33/ST2 in patients with PTB in vitro

To assess the involvement of the IL-33/ST2 pathway in the role of ILC2s in regulating Treg or Th2-type immune response, we first co-cultured ILC2s and PBMCs in the presence of IL-33 or anti-ST2 in vitro. Then, we detected the proportion of Treg and Th2 cells with flow cytometry. As shown in Figure 4A, the proportion of Treg cells was significantly increased in PBMCs + ILC2s group than in only PBMCs (control) group, suggesting that ILC2s from patients with PTB may stimulate the differentiation of PBMCs from healthy controls into Treg cells. Moreover, the proportion of Treg cells in the PBMCs + ILC2s + IL-33 group was further increased after stimulation by rIL-33.

Figure 4. Analysis of the proportion of Treg and Th2 cells. PBMCs from healthy individuals were co-cultured with ILC2s from patients with PTB in the presence of rIL-13 or anti-ST2 in vitro. The proportion of Treg and Th2 cells was detected with flow cytometry (n = 6). Representative and quantitative flow cytometry results were shown. (A) Treg was defined as CD4+CD25+CD127low. (B) Th2 was defined as CD4+CCR4+CD196-CD183-.



*p < 0.05, **p < 0.01. PBMCs, peripheral blood mononuclear cells; PTB: pulmonary tuberculosis; rIL: recombinant interleukin; sST2: soluble ST2.

However, simultaneous anti-ST2 treatment reversed this increase. Interestingly, the proportion of Th2 was not significantly changed in the different groups (Figure 4B).

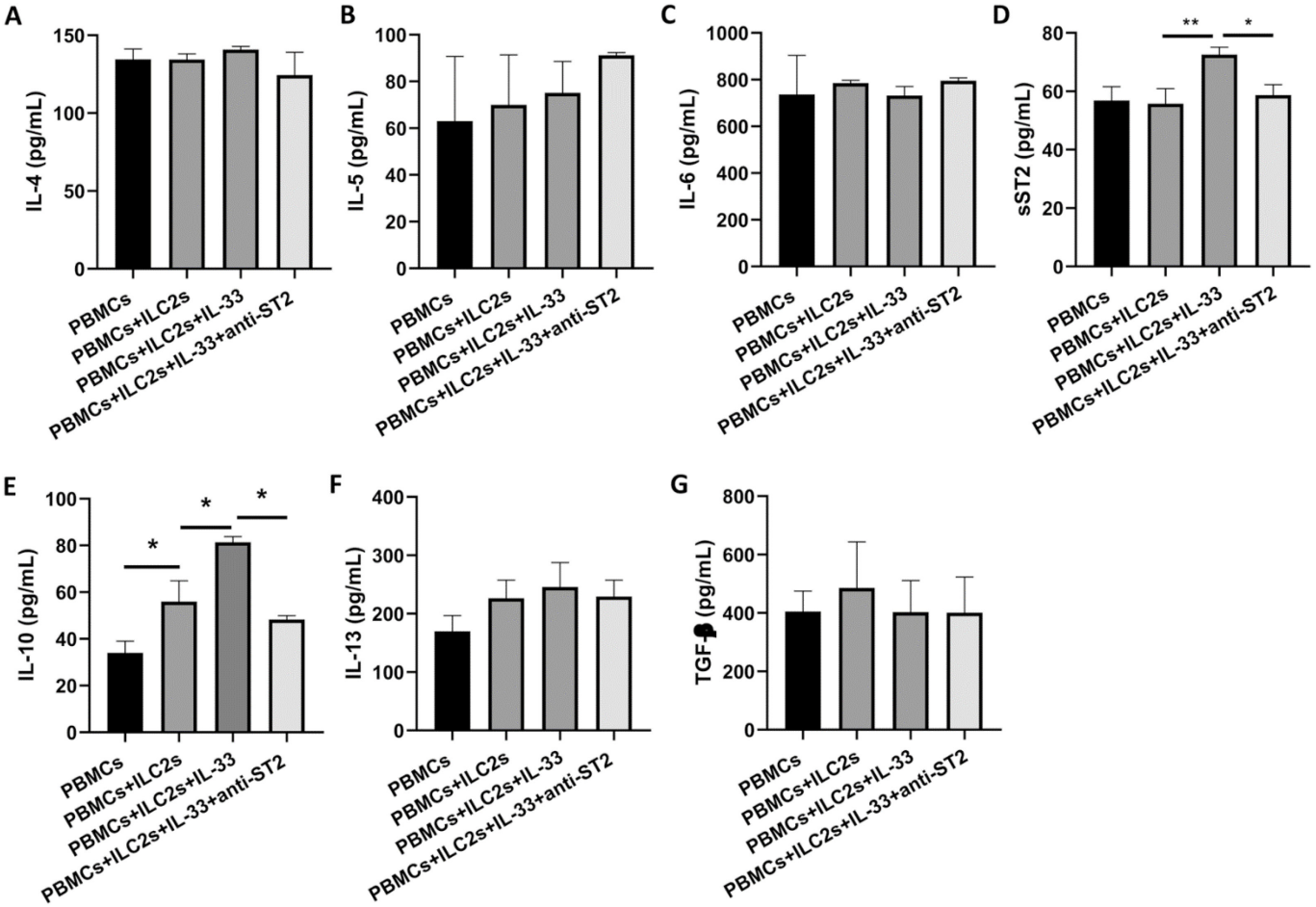
Next, we measured the concentration of Treg-related cytokines (TGF-β and IL-10) and Th2-related cytokines (IL-4, IL-13, IL-6, and IL-5) in the culture supernatant. The level of sST2 was increased after IL-33 stimulation and was decreased after simultaneous anti-ST2 treatment (Figure 5D). The level of IL-10 (Figure 5E) in the PBMCs + ILC2s group and PBMCs + ILC2s + IL-33 group was significantly higher than that in the control group and PBMCs + ILC2s group, respectively. After the addition of ST2 neutralizing antibody, IL-10 level decreased significantly in the PBMCs + ILC2s + IL-33 + anti-ST2 group than in the PBMCs + ILC2s + IL-33 group. However, the levels of other cytokines (Figure 5A-5C and Figure 5F-5G) were not significantly changed among groups.

Thus, in patients with PTB, IL-33 could stimulate the differentiation of PBMCs into ILC2s and further induce ILC2s to secrete the Treg-related cytokines, but not Th2-related cytokines. These effects may partly act through the IL-33/ST2 signaling pathway.

Discussion

IL-33 is a nuclear factor that plays an important role in allergic reactions, parasitic infections, chronic inflammation, etc. It can activate various immune cells (such as ILC2s, Treg cells, Th2 cells, etc.), and have pleiotropic effects in different immune microenvironments. For example, IL-33 promotes the activation of Treg cells and the repair of barrier epithelium [24]. The IL-33/ST2 signaling has been shown to adversely affect autoimmune disease [25,26]. The IL-33/ST2 signaling also plays an important protective role in colitis by regulating the number of ILC2 and Treg [24].

Figure 5. Analysis of cytokine levels in the culture supernatant. PBMCs from healthy individuals were co-cultured ILC2s from patients with PTB in the presence of rIL-13 or anti-ST2 in vitro. The concentration of (A) IL-4, (B) IL-5, (C) IL-6, (D) sST2, (E) IL-10, (F) IL-13, and (G) TGF-β and in the culture supernatant was detected with ELISA (n = 6).



*p < 0.05. ILC2s: type 2 innate lymphoid cells; PBMCs: peripheral blood mononuclear cells; ST2: growth stimulation expressed gene 2; sST2: soluble ST2.

Currently, there is no report on how IL-33/ST2 regulates the role of ILC2s in tuberculosis. Here, we found significantly higher plasma levels of IL-33, sST2, TGF-β, IL-6, and IL-10 in patients with PTB, demonstrating that IL-33 and ST2 may be involved in the pathogenesis of PTB. Researchers first proposed in an experimental colitis model that ST2/IL-33 signaling in Treg cells could enhance their protective ability, and IL-33 treatment could improve colon tissue damage and colitis symptoms [27]. It has been shown that IL-33 systemic treatment could effectively reduce *Mycobacterium tuberculosis* infection [28]. ILC2s, a newly identified innate immune cell type, are mainly located on lung mucosa, skin, gut, and adipose tissue [29,30], and are importantly involved in IL-33-related allergic inflammatory diseases. ILC2s accumulate at sites of airway inflammation in chronic rhinosinusitis with nasal polyps and interact with Treg cells to counteract Treg-mediated inhibitory functions in allergic inflammation [31]. Our previous study found that the ILC2 proportion was elevated in the peripheral blood of PTB [22].

The CRTH2 expression on ILC2s can promote IL-13 production following IL-33 stimulation [14]. We showed that compared with healthy controls, ILC2s in the peripheral blood of patients with PTB were significantly increased, and GATA3 and RORα mRNA were significantly higher in PBMCs of patients with PTB. These findings indicate that ILC2s may play a role in the development of PTB.

In this study, ILC2s induced the number of Treg cells and the concentration of IL-10 increased in vitro. This effect was more obvious after IL-33 stimulation and was abolished by anti-ST2 treatment. Similarly, the level of sST2 was increased after IL-33 stimulation but decreased after simultaneous anti-ST2 treatment. These results indicate that ILC2s may induce Treg but not Th2-type immunity through the IL-33/ST2 pathway in PTB.

Conclusions

In summary, IL-33 can promote IL-10 and TGF-β production, and ILC2 differentiation in patients with PTB. IL-33 and ILC2s may participate in PTB development. This study provides a theoretical basis for immunotherapy of PTB. However, the mechanisms underlying the roles of IL-33 and ILC2s need to be further studied.

Acknowledgements

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Authors' contributions

QL: study design, data collection, data interpretation, literature search, funds collection, manuscript preparation; QW: data collection; ZX: data collection.

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