Expression of CD27 and CD28 on γδ T cells from the peripheral blood of patients with allergic rhinitis

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Abstract. The costimulatory receptors CD27 and CD28 have pivotal and non-redundant roles in the activation and differentiation of γδ T-cells. However, the roles of CD27 and CD28 on γδ T-cells in allergic rhinitis (AR) have remained elusive. The aim of the present study was to investigate the expression of CD27 and CD28 on γδ T cells in patients with AR. Peripheral blood mononuclear cells from 14 patients with AR and 12 healthy subjects were isolated and analyzed by flow cytometry to determine the percentage of γδ T cells and regulatory T cells (Tregs), and the expression of IFN-γ, IL-17A, CD27 and CD28 on γδ T cells. The correlations between the expression of CD27 and CD28, and the percentages of IFN-$γ^+$ and IL-17A^+ γδ T-cell subsets and Tregs in AR were analyzed. It was observed that the percentages of γδ T cells, and the IL-17A^+, CD27-CD28+ and CD27-CD28- γδ T-cell subsets were significantly increased, while the percentages of Tregs and IFN-$γ^+$ and CD27+CD28+ γδ T-cell subsets were significantly decreased in AR. Of note, the percentage of CD27+CD28+ γδ T-cell subsets was positively correlated with that of the IFN-$γ^+$ γδ T-cell subset and the percentage of the CD27-CD28+ γδ T-cell subset was positively correlated with that of the IL-17A^+ γδ T-cell subset. Furthermore, the percentages of γδ T cells and the CD27-CD28+ γδ T-cell subset were both negatively correlated with that of Tregs. Therefore, the results of the present study indicate that CD27 and CD28 may be the key signals for activation of different γδ T-cell subsets and may contribute to the immune regulatory function of γδ T cells in the peripheral blood of patients with AR.

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Abbreviations: AR, allergic rhinitis; HC, healthy control; PBMCs, peripheral blood mononuclear cells; TCR, T-cell receptor; Th, T helper; TNFR, tumor necrosis factor receptor; Treg, T regulatory cell

Key words: allergic rhinitis, γδ T cells, CD27, CD28

Introduction

Allergic rhinitis (AR) is an inflammatory reaction mainly mediated by immunoglobulin (Ig)E after inhaled allergens enter the body through the nasal mucosa and it is the manifestation of a systemic allergic disease in the upper respiratory tract (1). The pathogenesis of allergic inflammation of the nasal mucosa is the result of the interaction among environmental pathogenic factors, genetic susceptibility and the local and systemic immune defense system, in which the imbalance of immune response is a critical factor (2). However, the mechanism driving the immunological imbalance in AR remains elusive.

The γδ T cells, which are predominantly located in the mucosa, have an important role in mucosal immune processes and are considered to act as the connection between innate and acquired immunity (3). γδ T cells are characterized by the expression of the T-cell receptor (TCR) γδ and may be further divided into different functional subsets, exerting their regulatory effects on immune balance by secreting IFN-γ or IL-17A (4,5), as well as by affecting the differentiation and function of other types of immune cell, such as regulatory T cells (Tregs) (6,7). It was previously demonstrated that different subsets of γδ T cells predominantly expressed in the mucosa and epithelium of the respiratory tract may have a pro- or anti-inflammatory role under different conditions, and they are crucial for regulating the occurrence and persistence of allergic inflammatory conditions, including AR (8-10). However, the molecular factors that steer γδ T cells to differentiate into different functional subsets in AR remain elusive.

CD27 and CD28 are two major costimulatory molecules with independent and non-redundant roles in the activation, proliferation and survival of γδ T cells (11,12). Studies in mice have demonstrated that CD27+ γδ T cells produce IFN-γ, whereas CD27- γδ T cells produce IL-17 (13), while the population of IFN-$γ^+$ and IL-17+ γδ T cells failed to expand during infection in CD28-deficient mice (14,15), indicating the essential roles of CD27 and CD28 co-stimulatory signals in the activation of specific functional subsets of γδ T cells. However, the roles of CD27 and CD28 on γδ T cells have not

been extensively investigated in humans, and have not yet been reported in AR. The aim of the present study was to explore the expression of CD27 and CD28 on γδ T cells in patients with AR.

Materials and methods

Subjects. The present study was approved by the Ethics Committee of the Shiyan People's Hospital of Baoan District in Shenzhen City (Shenzhen, China). Written informed consent was provided by all subjects or their legal guardians prior to participation. A total of 14 volunteers with AR were enrolled at the Department of Otolaryngology, Shiyan People's Hospital of Baoan District (Shenzhen, China) between December 2018 and October 2019. The diagnosis of AR was made based on the Allergic Rhinitis and Its Impact on Asthma guidelines (2). To evaluate the atopic status, the concentrations of serum IgE specific to local common inhalant allergens were detected using the automatic system of immunofluorescence quantitative analysis (UniCAP 100E; Pharmacia Biotech). A concentration >0.7 IU/ml was considered as the positive diagnostic criterion for AR. In addition, 12 healthy control (HC) volunteers were recruited, each with no clinical symptoms of any allergic disease and negative serum IgE. Subjects who received oral glucocorticoids, antihistamines or immunotherapy within 1 month were excluded. The characteristics of AR and HC subjects are listed in Table I. Peripheral venous blood samples anticoagulated with heparin were collected from each subject.

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using the Ficoll-Hypaque gradient centrifugation method. First, the cells were suspended in PBS with 0.5% bovine serum albumin (BSA; MP Biomedicals, LLC) to prevent unspecific binding. Prior to fluorescence antibody staining, cells were first incubated with Fixable Viability Dye eFluor 780 (1 µg/ml; cat. no. 65-0865-14) for 20 min at room temperature to confirm their viability. For cell surface staining, cells were incubated with APC-conjugated CD3 (clone: UCHT1) monoclonal antibody (2.5 µg/ml; cat. no. 17-0038-41), FITC-conjugated TCR γ/δ (clone: 5A6.E91) monoclonal antibody (10 µg/ml; cat. no. TCR2061), PE-Cyanine5-conjugated CD27 (clone: O323) monoclonal antibody (2.5 µg/ml; cat. no. 15-0279-42) and PE-conjugated CD28 (clone: CD28.2) monoclonal antibody (2.5 µg/ml; cat. no. 12-0289-42) (all from eBioscience) for 30 min at 4˚C. For transcription factor staining, cells were first incubated with PE-Cyanine5-conjugated CD3 (clone: UCHT1) monoclonal antibody (0.6 µg/ml; cat. no. 15-0038-42) for 30 min at 4˚C, then fixed and permeabilized with the Forkhead box p3 (Foxp3)/Transcription Factor Staining Buffer Set following the manufacturer's protocol at room temperature, and incubated with APC-conjugated Foxp3 (clone: PCH101) monoclonal antibody (5 µg/ml; cat. no. 17-4776-42) for 60 min at room temperature (all from eBioscience). For intracellular cytokine staining, cells were first cultured in RPMI-1640 medium supplemented with heat-inactivated fetal bovine serum (10%) and penicillin/streptomycin (1%) (all from Gibco; Thermo Fisher Scientific, Inc.) for 5 h at 37˚C in humidified air containing 5% CO_2, in the presence of Cell Stimulation Cocktail (plus protein transport inhibitors;

eBioscience) consisting of phorbol 12-myristate 13-acetate (81 nM), ionomycin (1.34 µM), brefeldin A (10.6 µM) and monensin (2 µM). After 5 h, cells were collected and incubated with PE-Cyanine5-conjugated CD3 (clone: UCHT1) monoclonal antibody (0.6 µg/ml; cat. no. 15-0038-42) for 30 min at 4˚C. The cells were then fixed and permeabilized with the Intracellular Fixation and Permeabilization Buffer Set following the manufacturer's protocol at room temperature and incubated with PE-conjugated IFN-γ (clone: XMG1.2) monoclonal antibody (2.5 µg/ml; cat. no. 12-7311-82) and APC-conjugated IL-17A (clone: eBio64DEC17) monoclonal antibody (0.6 µg/ml; cat. no. 17-7179-42) for 30 min at room temperature (all from eBioscience). The same concentrations of species- and subtype-matched antibodies were used as negative controls: APC-conjugated Mouse IgG1 kappa Isotype Control (2.5 or 0.6 µg/ml; cat. no. 17-4714-82), FITC-conjugated Mouse IgG1 Isotype Control (10 µg/ml; cat. no. MA1-10413), PE-Cyanine 5-conjugated Mouse IgG1 kappa Isotype Control (2.5 or 0.6 µg/ml; cat. no. 15-4714-81), PE-conjugated Mouse IgG1 kappa Isotype Control (2.5 µg/ml; cat. no. 12-4714-82), APC-conjugated Rat IgG12a kappa Isotype Control (5 µg/ml; cat. no. 17-4321-81) and PE-conjugated Rat IgG1 kappa Isotype Control (2.5 µg/ml; cat. no. 12-4301-82) (all from eBioscience). Flow cytometry was performed on a Gallios flow cytometer (Beckman Coulter, Inc.), $5x10^4$ events were acquired in each panel and data were analyzed using Kaluza Analysis software (version 2.1; Kaluza Software).

Statistical analysis. GraphPad Prism 6.0 statistical software (GraphPad Software, Inc.) was used for statistical analysis. Data were tested for normality using the Shapiro-Wilk test and are presented as the mean with standard deviation. Comparisons of the percentages of TCR γ/δ+ CD3+ cells, Foxp3+ CD3+ cells, IFN-γ+ CD3+ cells, IL-17A+ CD3+ cells, IFN-γ+ γδ T cells, and IL-17A+ γδ T cells, and the expression pattern of CD27 and CD28 on γδ T cells between groups were performed using Student's t-test. Correlations were analyzed using Pearson's correlation analysis. P<0.05 was considered to indicate statistically significant differences.

Results

γδ T cells are increased and Tregs are decreased in AR. The percentages of γδ T cells and Tregs in PBMCs of HC subjects and patients with AR were first detected. As expected, compared with HC subjects, the percentage of γδ T cells was significantly increased, while the percentage of Tregs was significantly decreased in patients with AR (Fig. 1), suggesting that γδ T cells may have an important role in the pathogenesis of AR, whereas the ability of Tregs to regulate immune homeostasis may be suppressed.

IFN-γ+ γδ T-cell subsets are decreased and IL-17A+ γδ T-cell subsets are increased in AR. To further explore the role of γδ T cells in AR, the percentages of IFN-γ+ and IL-17A+ γδ T cells in PBMCs of HC and AR subjects were tested. Compared with that in HC subjects, the percentage of IFN-γ+ T cells was significantly decreased, while the percentage of IL-17A+ T cells was significantly increased in patients with AR (Fig. 2A-C). Unexpectedly, the expression of TCR γδ

Table I. Clinical characteristics of HC and AR subjects.

Item	HC subjects (n=12)	AR subjects (n=14)	P-value
Age (years)	21 (9-39)	16 (8-41)	0.381
Sex (male/female)	6/6	8/6	N/A
HDM-specific IgE (IU/ml)	0.21 (0.17)	24.44 (26.96)	<0.001

Values are expressed as n, the median (range) or the mean ± standard deviation. HC, healthy controls; AR, allergic rhinitis; HDM, house dust mite; N/A, not applicable.
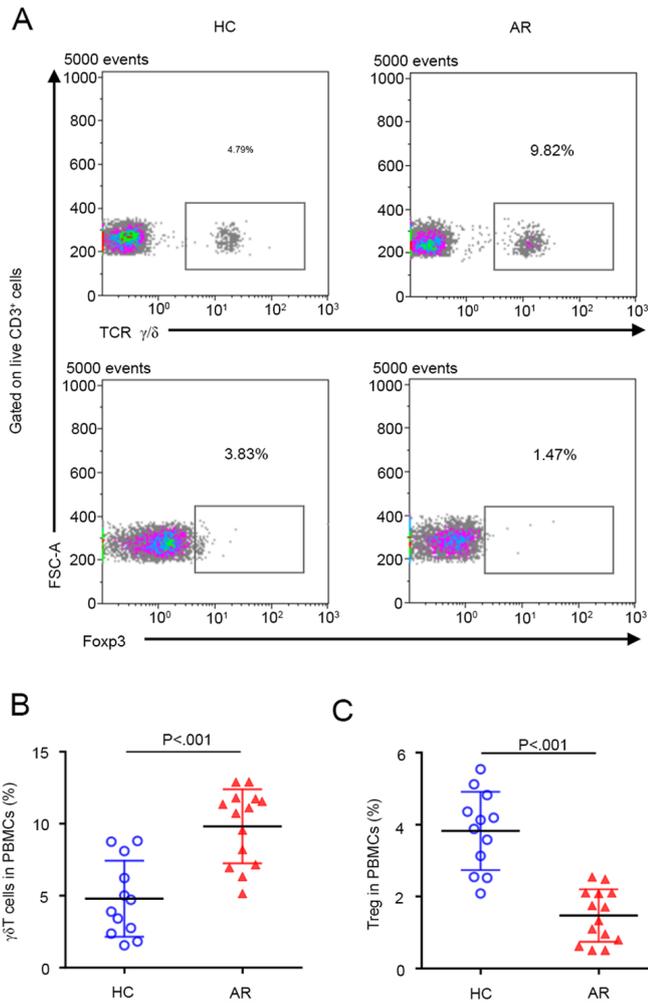

Figure 1. Increased γδ T cells and decreased Tregs in AR. (A) Representative flow cytometry plots of the percentage of T-cell receptor γ/δ+ and Foxp3+ cells gated from live CD3+ T cells in PBMCs from HC (n=12) and AR (n=14) subjects. (B,C) Quantification of the percentage of γδ T cells and Tregs in PBMCs. Values are expressed as the mean ± standard deviation. AR, allergic rhinitis; HC, healthy controls; Fox, forkhead box; PBMCs, peripheral blood mononuclear cells; Treg, regulatory T cell.

was increased on both IFN-γ+ and IL-17A+ T cells in AR (Fig. 2A, D and E). Approximately half of the IFN-γ+ T cells were TCR γδ-positive, suggesting that γδ T cells may be the major source of IFN-γ in AR. Furthermore, the expression of TCR γδ on IL-17A+ T cells in AR subjects was more than 2-fold higher compared with that in HC subjects, suggesting a non-negligible role of γδ T cells in promoting inflammation in

AR by significantly increasing the secretion of IL-17A. More importantly, the percentage of IFN-γ+ γδ T cells was significantly decreased and the percentage of IL-17A+ γδ T cells was increased in AR (Fig. 2F-H), further supporting that the IFN-γ+ and IL-17A+ γδ T-cell subsets have important immune regulatory roles in AR.

Expression pattern of CD27 and CD28 on γδ T cells in AR. The expression pattern of CD27 and CD28 on γδ T cells was CD27+CD28+>CD27-CD28->CD27+CD28->CD27-CD28+ in PBMCs from HC subjects. By contrast, this pattern was CD27-CD28->CD27+CD28+>CD27+CD28->CD27-CD28+ in AR subjects (Fig. 3A). Overall, compared with HC subjects, the percentage of CD27+ γδ T cells was significantly decreased, while the percentage of CD28+ γδ T cells was significantly increased in patients with AR (Fig. 3A-C). Further analysis demonstrated that the percentage of CD27+CD28+ γδ T-cell subsets was significantly decreased in AR (Fig. 3D). The percentage of CD27+CD28- γδ T-cell subsets in patients with AR was similar to that in HC subjects (Fig. 3E). The percentages of CD27-CD28+ and CD27-CD28- γδ T-cell subsets were both significantly increased in AR (Fig. 3F and G).

Correlation of CD27 and CD28 with γδ T-cell subsets in AR. Next, the correlations between CD27 and CD28 expression on γδ T cells and IFN-γ+ or IL-17A+ γδ T-cell subsets from patients with AR were analyzed. The results demonstrated that the percentage of the CD27+CD28+ γδ T-cell subset was positively correlated with that of the IFN-γ+ γδ T-cell subset (Fig. 4A) and the percentage of the CD27-CD28+ γδ T-cell subset was positively correlated with that of the IL-17A+ γδ T-cell subset (Fig. 4B). There was no correlation between the respective other combinations, for instance, between the CD27+CD28+ γδ T-cell subset with the IL-17A+ γδ T-cell subset and the CD27-CD28+ γδ T-cell subset with the IFN-γ+ γδ T-cell subset (data not shown). These results suggested that CD28 is necessary for γδ T cells to produce cytokines, with the CD27+ subset producing IFN-γ and the CD27- subset producing IL-17A.

Correlation of γδ T-cell subsets with Tregs in AR. Finally, the correlation between γδ T-cell subsets and Tregs among the PBMCs of patients with AR was analyzed. The percentage of γδ T cells was negatively correlated with that of Tregs (Fig. 4C). In addition, the percentage of the CD27-CD28+ γδ T-cell subset was negatively correlated with that of Tregs (Fig. 4D), suggesting that the CD27-CD28+ γδ T-cell subset
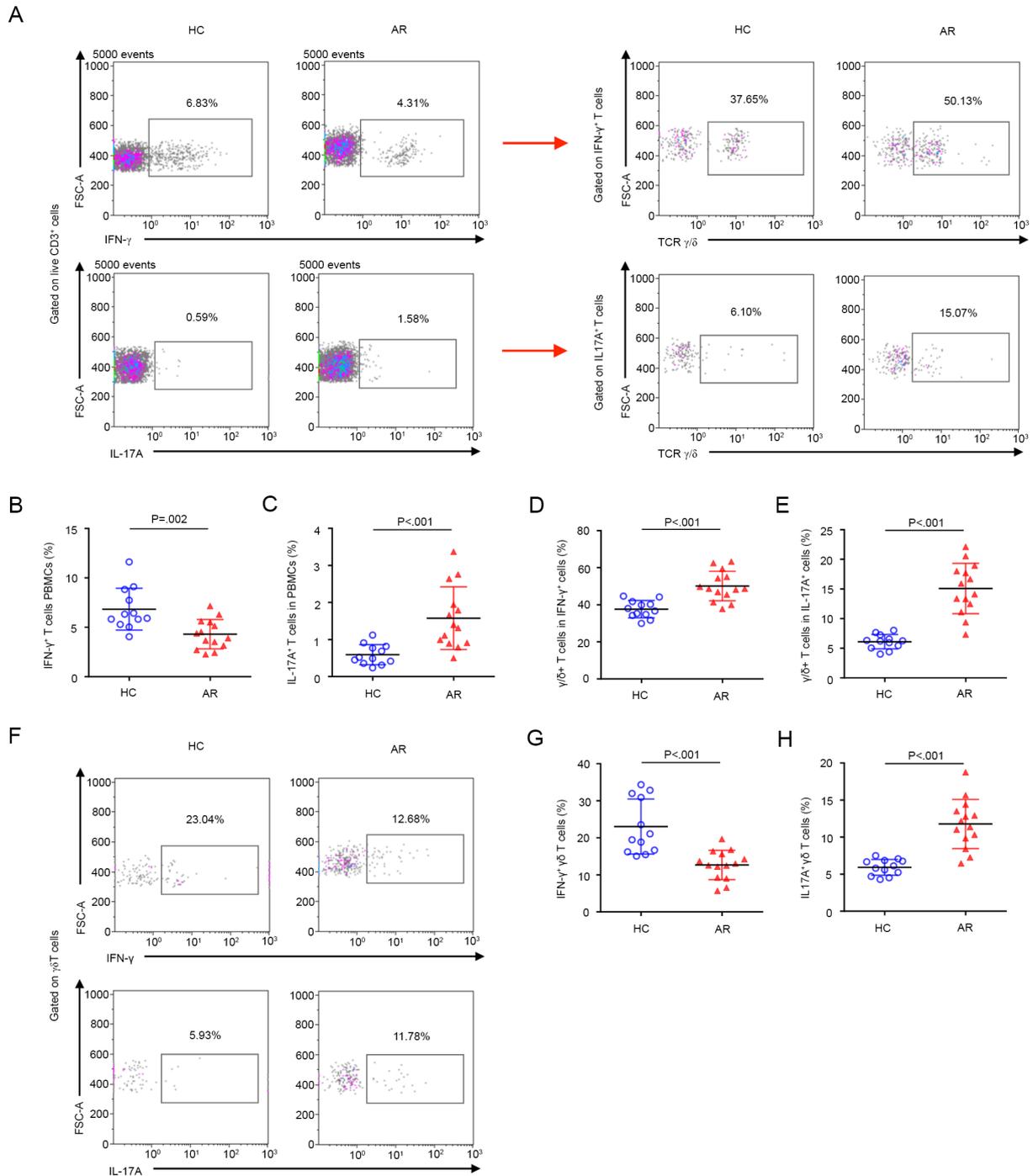
Figure 2. Decreased IFN-γ⁺ and increased IL-17A⁺ γδ T-cell subsets in AR. (A) Representative flow cytometry plots of the percentage of IFN-γ⁺ and IL-17A⁺ cells gated on live CD3⁺ T cells and the percentage of TCR γ/δ⁺ cells gated from IFN-γ⁺ and IL-17A⁺ T cells in PBMCs from HC (n=12) and AR (n=14) subjects. (B-E) Quantification of the percentage of (B) IFN-γ⁺ and (C) IL-17A⁺ T cells in PBMCs and the percentage of (D) γδ⁺ T cells in IFN-γ⁺ and (E) IL-17A⁺ T cells. (F) Representative flow cytometry plots of IFN-γ⁺ and IL-17A⁺ cells gated from γδ T cells in PBMCs from HC (n=12) and AR (n=14) subjects. (G and H) Quantification of the percentage of (G) IFN-γ⁺ and (H) IL-17A⁺ γδ T cells in PBMCs. Values are expressed as the mean ± standard deviation. AR, allergic rhinitis; HC, healthy controls; PBMCs, peripheral blood mononuclear cells.

may be involved with the downregulation of the number and function of Tregs in AR.

Discussion

The results of the present study demonstrated that the elevated γδ T cells in the peripheral blood of patients with AR had an increased percentage of the CD27⁻CD28⁺ γδ T-cell subset, which was positively correlated with the percentage of IL-17A⁺ γδ T cells and negatively correlated with Tregs. Furthermore, the percentage of the CD27⁺CD28⁺ γδ T-cell subset was significantly decreased and positively correlated with the percentage of IFN-γ⁺ γδ T cells. Given that the IFN-γ⁺ and IL-17A⁺ γδ T-cell subsets have been indicated to exert anti- or pro-inflammatory effects on autoimmune diseases (16), the present results provide evidence supporting a potential
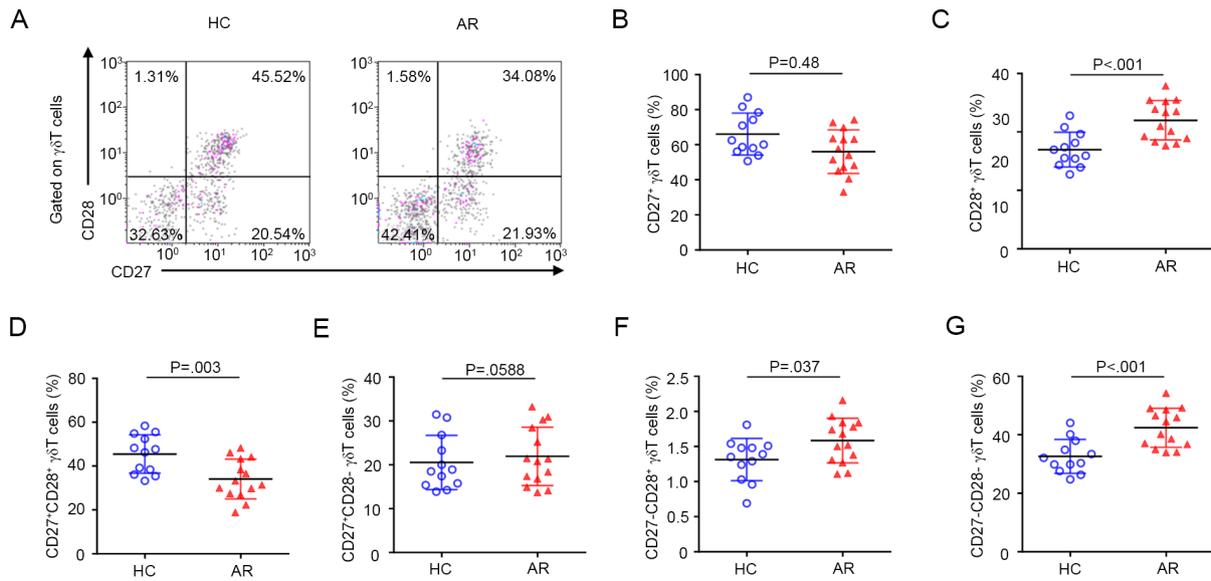
Figure 3. Expression pattern of CD27 and CD28 on γδ T cells in AR. (A) Representative flow cytometry plots of the expression of CD27 and CD28 on γδ T cells among PBMCs from HC (n=12) and AR (n=14) subjects. (B-G) Quantification of the percentage of (B) CD27+, (C) CD28+, (D) CD27+CD28+, (E) CD27+CD28-, (F) CD27-CD28+ and (G) CD27-CD28- γδ+ T cells in PBMCs. Values are expressed as the mean ± standard deviation. AR, allergic rhinitis; HC, healthy controls; PBMCs, peripheral blood mononuclear cells.
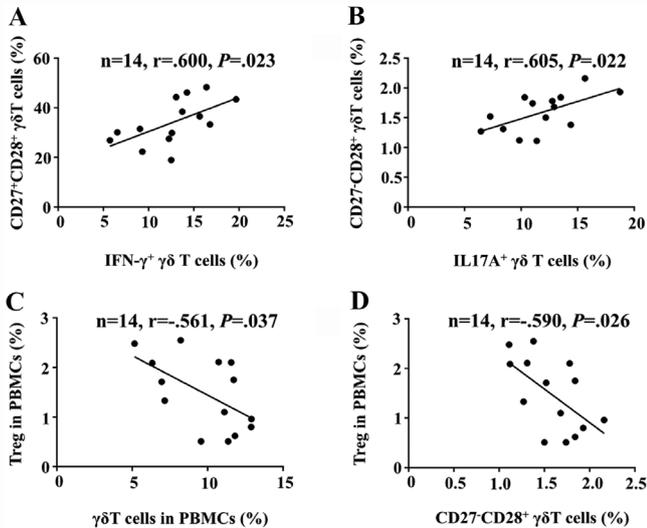

Figure 4. Correlation analysis of CD27 and CD28 γδ T-cell subsets and Tregs in allergic rhinitis. (A) Correlation of the proportion of the CD27+CD28+ γδ T-cell subset with that of the IFN-γ+ γδ T-cell subset. (B) Correlation of the proportion of the CD27-CD28+ γδ T-cell subset with that of the IL-17A+ γδ T-cell subset. (C) Correlation of the proportion of γδ T cells with that of Tregs. (D) Correlation of the proportion of the CD27-CD28+ γδ T-cell subset with that of Tregs. PBMCs, peripheral blood mononuclear cells; Treg, regulatory T cell.

important role for CD27 and CD28 in the immune regulatory function of γδ T cells in AR.

The major mechanism underlying AR has been indicated to be the dysfunction of the mucosal barrier, producing an overactive, unbalanced immune response against certain substances that are originally 'harmless' *in vitro*, such as allergens (1). Studies have proven that γδ T cells predominantly occur in the epithelium and mucosa and have an indispensable role in

orchestrating the immune response of allergic diseases (17-20). In the present study, a significant elevation in the percentage of γδ T cells in PBMCs from patients with AR was observed when compared with the control subjects, which is largely consistent with previous studies (21,22), confirming the important role of γδ T cells in the pathogenesis of AR.

Previous studies have indicated that AR is characterized by a T helper type (Th) 2-skewed inflammation, with decreased levels of the Th1-associated cytokine IFN-γ and increased levels of the Th17-associated cytokine IL-17A (23,24). Consistently, the data of the present study also demonstrated a significant decrease in the percentage of IFN-γ+ and an increase in the percentage of IL-17A+ T cells in PBMCs of patients with AR compared with control subjects. The average percentage of IFN-γ+ T cells in HC subjects was 6.83%, which was slightly lower than the range reported by other studies using similar experimental conditions (25,26). This may be due to individual differences between the donors and future experiments with larger sample sizes may eliminate this difference. γδ T cells are a major source of IFN-γ and IL-17A in several autoimmune responses and lower airway allergic inflammations (16,27); this may explain why the percentage of TCR γδ+ cells was significantly increased in the IFN-γ- and IL-17A-producing T cells in patients with AR in the present study. Of note, the percentage of the IFN-γ+ γδ T-cell subset was significantly decreased and that of the IL-17A+ γδ T-cell subset was significantly increased in AR, further supporting that the IL-17A+ γδ T-cell subset may have an important pro-inflammatory role in AR.

CD27, a member of the TNF receptor (TNFR) superfamily, and CD28, a member of the Ig superfamily, are typical T-cell costimulatory receptors that have specific roles in γδ T cells. They display different modes of intracellular signaling: CD27 requires the adaptor protein TNFR-associated factor, whereas CD28 associates directly with protein kinases to

link to downstream signaling mediators (6). Studies in mice have demonstrated that CD27 and its ligand, CD70, are absolutely required for the survival, development, differentiation and function of γδ T cells (28,29). Another study in humans suggested that the majority of circulating Vγ9Vδ2$^+$ T cells express CD27, which acts as a coreceptor that promotes, in conjunction with TCR-mediated signals, the production of IFN-γ (30). In αβ T cells, the interaction of CD28 and its ligands, B7-1 (CD80) and B7-2 (CD86), provide a 'second signal' that is crucial for T-cell activation, proliferation and survival (31,32). Although the requirement of CD28/B7 signaling for the activation of γδ T cells remains controversial (29), certain studies have revealed that CD28 is expressed on γδ T cells in the lymph nodes and has the ability to promote the activation, proliferation and survival of γδ T cells, mainly via IL-2 secretion (12,15). Data from a study on CD28-deficient mice revealed that the IFN-γ$^+$ and IL-17$^+$ γδ T-cell populations failed to expand during infection (15), suggesting that CD28 is also required for the expansion of γδ T cells. In the present study, the decreased proportion of CD27$^+$CD28$^+$ and increased proportion of CD27$^-$CD28$^+$ and CD27$^-$CD28$^-$ γδ T cells in the PBMCs of patients with AR, as well as the positive correlations of the CD27$^+$CD28$^+$ γδ T-cell subset with the IFN-γ$^+$ γδ T-cell subset, and of the CD27$^-$CD28$^+$ γδ T-cell subset with the IL-17A$^+$ γδ T-cell subset, suggest an aberrant activation and proliferation pattern of γδ T cells in AR and a different cytokine profile compared with that of healthy subjects.

Since first identified in 1995, the immunomodulatory effect of CD4$^+$CD25$^+$ Tregs on the maintenance of immune homeostasis has been proven (33). It has been demonstrated that Tregs may inhibit excessive type I hypersensitivity by producing IL-10 and TGF-β, thereby having a pivotal role in the immune tolerance of allergens (34). It has been reported that the number and function of Tregs are significantly decreased in the peripheral blood during allergic airway inflammation (35). Furthermore, γδ T cells and Tregs may exert a synergistic regulatory effect in the pathogenesis of AR (22,36). Since the specific transcription factor Foxp3 has a key role in the development and functional maintenance of Tregs (37), and is stably and constitutively expressed at a high level in CD4$^+$CD25$^+$ regulatory T cells, Foxp3$^+$CD3$^+$ cells in PBMCs were identified as Tregs in numerous studies (38,39). Therefore, in the present study, only Foxp3 expression on CD3$^+$ T cells was analyzed. It was observed that the percentage of Tregs was significantly decreased in patients with AR. More importantly, negative correlations were obtained between the percentage of Tregs with that of the CD27$^-$CD28$^+$ γδ T-cell subset. These results suggest that the CD27$^-$CD28$^+$ γδ T-cell subset may have a role in the decrease of the number and function of Tregs in AR. However, as the percentage of the CD27$^-$CD28$^+$ γδ T-cell subset was low, the function of CD27$^-$CD28$^+$ γδ T cells must still be proven by further experimental studies.

In conclusion, the increased numbers of circulating γδ T cells were characterized by an increased percentage of the CD27$^-$CD28$^+$ γδ T-cell subset, which was indicated to be positively correlated with the percentage of IL-17A$^+$ γδ T cells and negatively correlated with Tregs in patients with AR. Furthermore, the decreased CD27$^+$CD28$^+$ γδ T-cell subset was positively correlated with the percentage of IFN-γ$^+$ γδ

T cells. These data indicate the potentially important roles of CD27 and CD28 in the immune regulatory role of γδ T cells in AR. Given the limitations of the correlation analysis, it is necessary to fully verify the specific functions of CD27 and CD28 on γδ T cells in AR via further experiments, such as cell experiments.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QW contributed to the conceptualization and design of the study and drafted the manuscript. QS and QC performed the flow cytometry assays and helped draft the manuscript. HL and DL helped to analyze data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Shiyan People's Hospital of Baoan District in Shenzhen City (Shenzhen, China). Written informed consent was provided by all subjects or their legal guardians prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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