ORIGINAL ARTICLE

Poly-functional T helper cells in human tonsillar mononuclear cells

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ABSTRACT. Tonsils are important lymphoid organs in which B cells and T cells complete their maturation and identify cells that are infected by pathogens. However, the functions of T cells in human tonsils remain unclear, especially the characteristics of polyfunctional $CD4^+$ T helper cells. In this study, we used multi-color flow cytometry to analyze the expression or co-expression of effector cytokines in $CD4^+$ T cells from tonsillar tissues. We have demonstrated that tonsillar $CD4^+$ T cell can express various Th effector cytokines after short-term polyclonal stimulation, and that cytokine-producing $CD4^+$ T cells were $CD45RO^+$ T cells. In addition, we analyzed the co-expression of two or more kinds of cytokines at the level of a single cell. The results showed that tonsillar $CD4^+$ T cells exhibited polyfunctionality by co-expressing two to five kinds of cytokines in the same time. These data furnished a basic theory for further understanding the differentiation of polyfunctional Th cells in human tonsils and their functions in resisting invasive microorganisms.

Key words: poly-functional Th cells, cytokines, tonsils

Effector $CD4^+$ T cells play critical roles in mediating adaptive immunity to a variety of pathogens via the secretion of cytokines, helping B cells to make antibodies, enhancing and maintaining $CD8^+$ T cell responses, regulating macrophage function, orchestrating immune responses both to control autoimmunity and to adjust the magnitude and persistence of responses [1]. During a particular cytokine-polarizing condition, naive $CD4^+$ T cells may differentiate into one of several lineages of T helper cells under the regulation of special transcription factors, including classical Th1 and Th2 cells, the more recently defined Th9, Th17, follicular helper (Tfh) and Th22 cells [1, 2]. Recently, the polyfunctionality of the $CD4^+$ T cell lineage has been demonstrated in numerous studies. It has been reported that IL-12, acting *via* the transcription factor STAT-4, induced both *Il-21* and *Ifng* genes, generating cells with features of both Tfh and Th1 cells [3]. After stimulation with *Mycobacterium tuberculosis* (MTB)-specific antigens, $CD4^+CD69^+$ T cells in pleural fluid cells (PFCs) from patients with tuberculous pleurisy co-expressed significantly higher levels of IFN-γ, IL-2 and TNF-α than $CD4^+CD69^-$ T cells, demonstrating that $CD4^+CD69^+$ T cells are MTB-specific Th1 cells [4]. It was also demonstrated that the percentage of cells able to produce IL-17 or IFN-γ was significantly higher within $CD4^+IL\text{-}21^+$ T cells than in their $CD4^+IL\text{-}21^-$ T cell counterpart in SIV-infected or uninfected rhesus macaques (RMs) and sooty mangabeys (SMs) [5]. Most articles report the possible function of triple cytokine-producing $CD4^+$ T cells in many infectious diseases, and differentiation has been proved in *in vitro* investigations: however, the characterization of polyfunctional $CD4^+$ T cells, which can produce two or more cytokines at the same time, remains unclear.

Palatine tonsils, located on the inside of the throat, form part of the first major barrier protecting the digestive and respiratory tracts from potentially invading microorganisms. Tonsils, as important second lymphoid organs, are also the major tissue location of effector T cells, where T helper cells complete their differentiation and maturation, and execute their functions of responding to antigens [6]. Histologically, the tonsils have a surface of stratified squamous epithelium and follicular germinal centers with a mantle zone and an interfollicular area. Organized in the sub-epithelial space are B cell-rich lymphoid follicles: T cells with a very high $CD4^+$:$CD8^+$ T cell ratio are mostly located in the extra-follicular spaces [7, 8]. For many years, tonsillectomy has been used routinely in children to treat chronic or recurrent acute tonsillitis, and they have also been used as sources of lymphoid tissues [9, 10]. Previous studies on tonsils mostly focus on the formation of germinal centers (GCs) where high-affinity B cells are selected and differentiate into long-lived memory B cells and plasma cells. However, the phenotypical property of

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cytokine production by CD4+ T cells in tonsils remains to be established, especially the polyfunctionality of tonsillar CD4+ T cells.

In our studies, we analyzed the characteristics of multiple cytokine-producing CD4+ T cells from tonsillar tissues by multi-color flow cytometry. Our data demonstrated that a single activated tonsillar CD4+ T cell could express one to five kinds of cytokines at the same time, which isuggests that tonsillar CD4+ T cells exhibit polyfunctionality.

MATERIALS AND METHODS

Subjects

The current investigation was approved by the Medical School Review Board at the Zhongshan School of Medicine, Sun Yat-sen University, China. Appropriate written informed consent was obtained from all individuals involved in this study. A group of 10 patients with chronic tonsillitis, consisting of four men and six women aged from 15 to 41 years were recruited from The First Affiliated Hospital, Sun Yat-sen University. Individuals that had been diagnosed with autoimmune diseases, tumor or any other serious diseases were excluded from the study.

Antibodies and reagents

The following monoclonal antibodies were used for phenotypic, intracellular cytokine staining: phycoerythrin (PE)-labeled anti-CD45RO, fluorescein isothiocyanate (FITC)-labeled anti-IL-17A, Alexa Fluor 647-labeled anti-IL-21, PE-Cy7-labeled anti-IFN-γ, PE-Cy7-labeled anti-TNF-α, PE-CF594-labelled anti-CD3, APC-Cy7-labeled anti-CD4, peridin-chlorophyll protein-Cy5.5 (PerCP-Cy5.5)-labeled anti-IL-2 and (PerCP-Cy5.5)-labeled anti-IFN-γ were purchased from BD Biosciences (San Jose, CA, USA); allophycocyanin (APC)-labeled anti-IL-22, PE-labeled anti-IL-21 and PE-labeled anti-IL-22 were purchased from eBioscience (San Diego, CA, USA). PMA, ionomycin and Brefeldin A (BFA) were purchased from Sigma-Aldrich (St. Louis, USA).

Preparation of tonsillar mononuclear cells

Tonsils obtained from tonsillectomies were washed twice with sterile PBS, cut manually into small pieces and incubated for 1 h at 37°C with incomplete RPMI1640 medium including collagenase type I and DNase. Digested tissues were filtered through a 2 μm filter and washed twice with Hank's balanced salt solution. Mononuclear cells were isolated by Ficoll–Hypaque (Hao Yang Biological Manufacture, Tianjin, China) gradient centrifugation, washed twice with Hank's and suspended at a final concentration of 2×10^6/mL in complete RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal calf serum (Sijiqing, Hangzhou, China), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol (all from Gibco).

Cells culture and intracellular staining analysis

Cells were suspended in complete RPMI-1640 medium (2×10^6/mL) and stimulated for six h with PMA (20 ng/mL) plus ionomycin (1 μg/mL) in the presence of BFA (10 μg/mL) at 37°C with 5%CO_2. The cells were washed twice with PBS buffer containing 0.1% BSA and 0.05% sodium azide, and incubated with monoclonal antibodies for CD3, CD4 with or without CD45RO at 4°C in the dark for 30 min, washed twice and fixed in 4% paraformaldehyde at room temperature in the dark for 8 min, followed by permeabilization, and stained with monoclonal antibodies against the intracellular cytokines in PBS buffer containing 0.1% saponin at 4°C in the dark for 30 min, washed twice and fixed in 0.5% paraformaldehyde before acquisition. These cells were collected by BD FACS Calibur or Aria II (Becton Dickinson, San Jose, CA, USA) and analyzed using FlowJo software (Treestar, San Carlos, CA, USA).

RESULTS

Cytokine production of tonsillar mononuclear cells

Tonsillar mononuclear cells were stimulated for six h with or without PMA plus ionomycin in the presence of the secretion inhibitor BFA. Cytokine expression was analyzed by intracellular staining (*figure 1*). We gated on lymphocytes and then gated on $CD3^+CD4^+$ population cells. Cytokine expression on $CD3^+CD4^+$ population cells was analyzed. We found that CD4+ T cells from tonsils expressed effector cytokines as peripheral blood mononuclear cells (PBMCs), such as Th1 effector cytokines IFN-γ, IL-2 and TNF-α; Th17 effector cytokine IL-17; Tfh effector cytokine IL-21 and Th22 effector cytokine IL-22. Without any stimulation, tonsillar CD4+ T cells spontaneously generated low levels of pro-inflammatory cytokines, including IFN-γ, IL-17 and IL-22, which might be closely associated with the progression of chronic tonsillar inflammation. However, the frequencies of $IFN\text{-}\gamma^+CD4^+$, $IL\text{-}17^+CD4^+$ and $IL\text{-}22^+CD4^+$ T cells were extremely up-regulated under the polyclonal stimulation with PMA plus ionomycin. Statistical data showed that 8.530 ± 1.111% of CD4+ T cells expressed IFN-γ, 19.490 ± 4.022% of CD4+ T cells expressed TNF-α, 16.130 ± 1.929% of CD4+ T cells expressed IL-2, 3.100 ± 0.640% of CD4+ T cells expressed IL-17, 9.119 ± 2.339% of CD4+ T cells expressed IL-21 and 0.711 ± 0.134% of CD4+ T cells expressed IL-22.

Phenotype of cytokine-producing CD4+ T cells in tonsillar tissue

Previous studies have found that naive and memory CD4+ T cells can recirculate though T and B zones of tonsillar tissues where they can encounter antigens [11]. Up-regulation of surface marker CD45RO and down-regulation of CD45RA are observed during the development of naive T cells into memory T cells. Memory T cells are always $CD45RO^{high}CD45RA^-$ while naive T cells are $CD45RO^-CD45RA^{high}$. To assess further the possible phenotype of the cytokine-producing cells, we analyzed the correlation between CD45RO and cytokines. The results of flow cytometry showed that most of CD4+ T cells were $CD45RO^+$ T cells that are memory CD4+ T cells (*figure 2*). The main immunological functions of tonsils are to recognize and eliminate pathogens, we found that cytokine-producing CD4+ T cells from tonsils co-expressed memory phenotype CD45RO, suggesting that

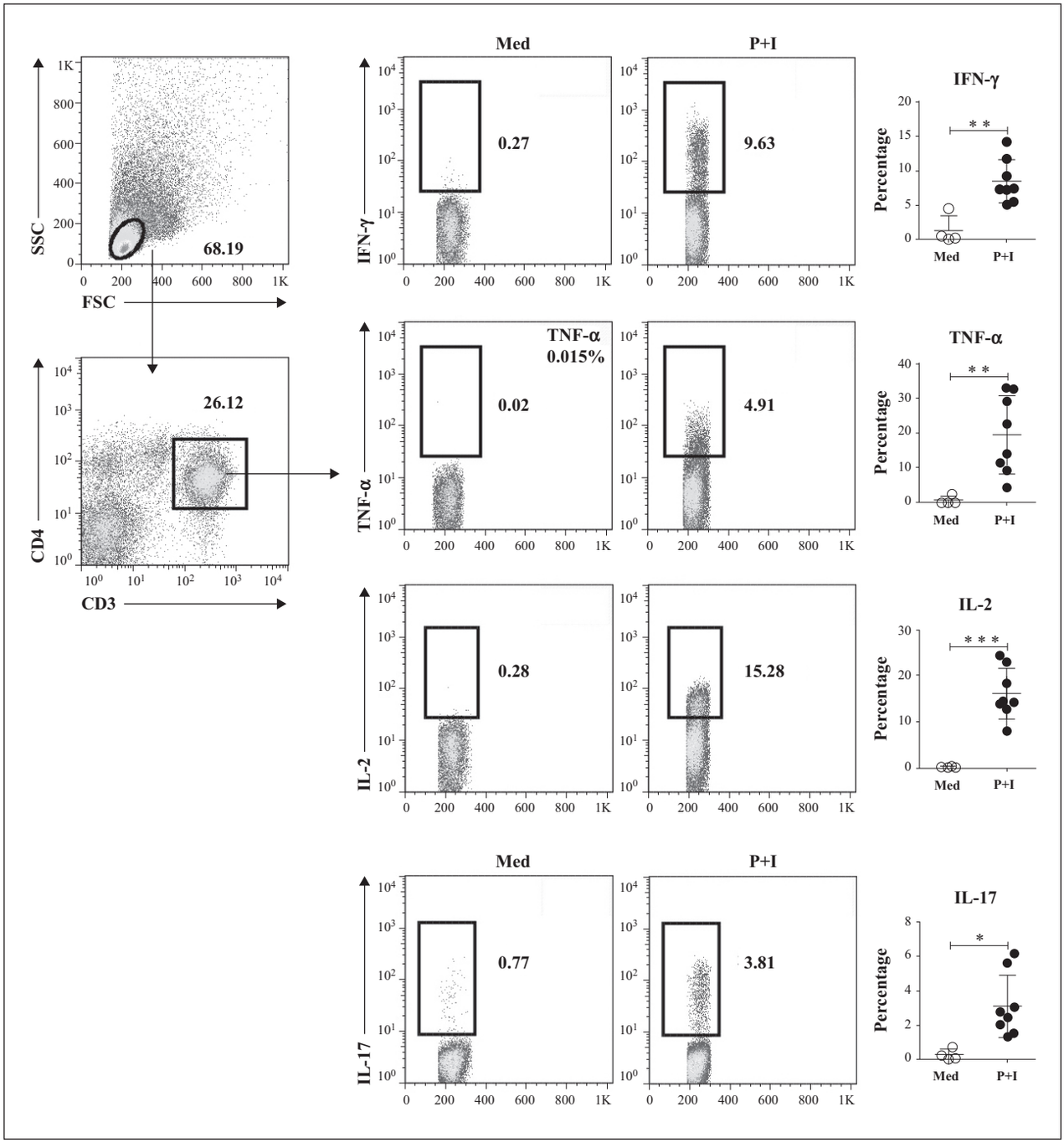

Figure 1

Cytokine expression by tonsillar CD4$^+$ T cells. Mononuclear cells were separated from chronic inflammatory tonsillar tissues and stimulated for 6 hours with PMA plus ionomycin (P+I) or without PMA plus ionomycin (Med) in the presence of BFA. The expression of cytokines by CD4$^+$ T cells was analyzed by intracellular staining. The representative dot graphs showed the frequencies of IFN-γ$^+$, IL-2$^+$, TNF-α$^+$, IL-17$^+$, IL-21$^+$ and IL-22$^+$CD4$^+$ T cells and statistical data were mean ± SD from eight independent experiments.

tonsillar tissues as key secondary lymphoid organs might be an important location of memory T cells. The other features of cytokine-expressing CD45RO$^+$CD4$^+$ T cells need further study.

Single tonsillar CD4$^+$ T cell co-expressed two kinds of cytokines

In many infectious and autoimmune diseases, previous studies have demonstrated that Ag-specific or non-specific CD4$^+$ T cells could produce and express two effector cytokines of different Th subsets [12, 13]. We analyzed the co-expression of two cytokines by a single CD4$^+$ T cell from tonsillar mononuclear cells. After stimulation for six h with PMA plus ionomycin, a definite proportion of CD4$^+$ T cells exhibited the capacity to express two kinds of cytokines (*figure 3A*), IFN-γ$^+$TNF-α$^+$, IFN-γ$^+$IL-2$^+$, IFN-γ$^+$IL-17$^+$, IFN-γ$^+$IL-21$^+$, IFN-γ$^+$IL-22$^+$, TNF-α$^+$IL-2$^+$, TNF-α$^+$IL-17$^+$, TNF-α$^+$IL-21$^+$, TNF-α$^+$IL-22$^+$, IL-2$^+$IL-17$^+$, IL-2$^+$IL-21$^+$, IL-2$^+$IL-22$^+$, IL-17$^+$IL-21$^+$, IL-17$^+$IL-22$^+$ and IL-21$^+$IL-22$^+$CD4$^+$ T cells were detected in tonsillar cells. The statistical results of six separate experiments are showed in *table 1* and *figure 3B*. Furthermore, we calculated that 84.596% IFN-γ$^+$CD4$^+$ T

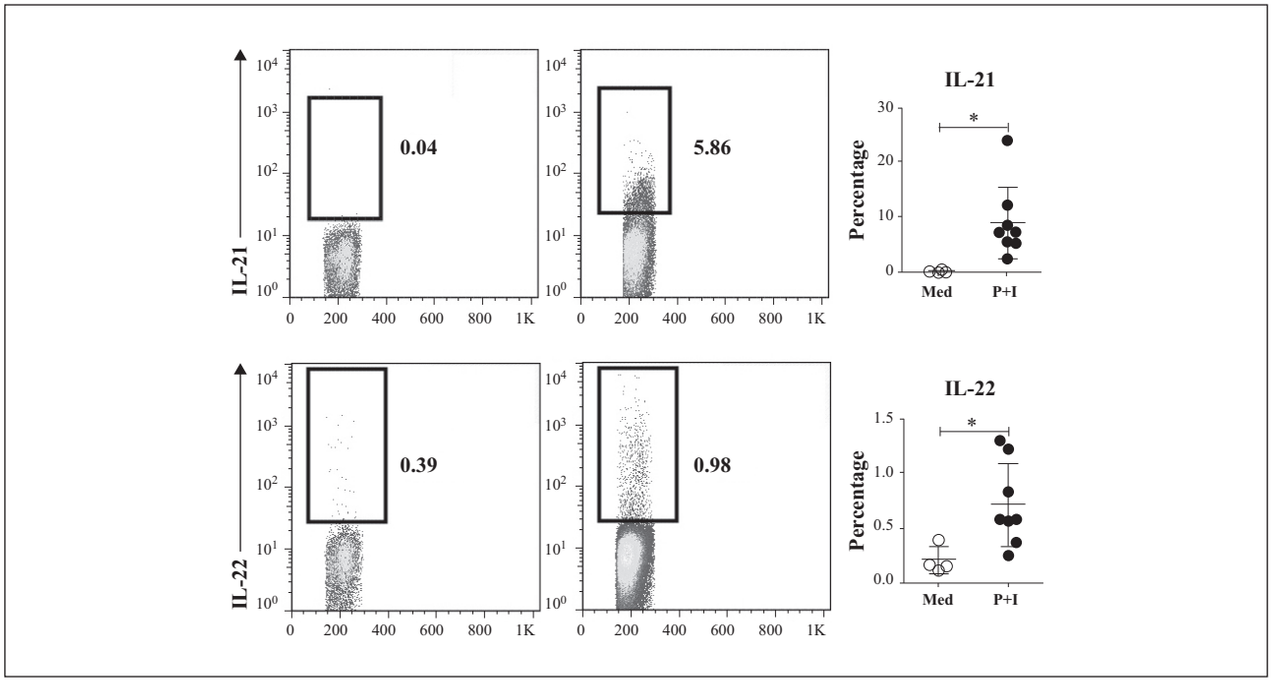

Figure 1 (Continued)

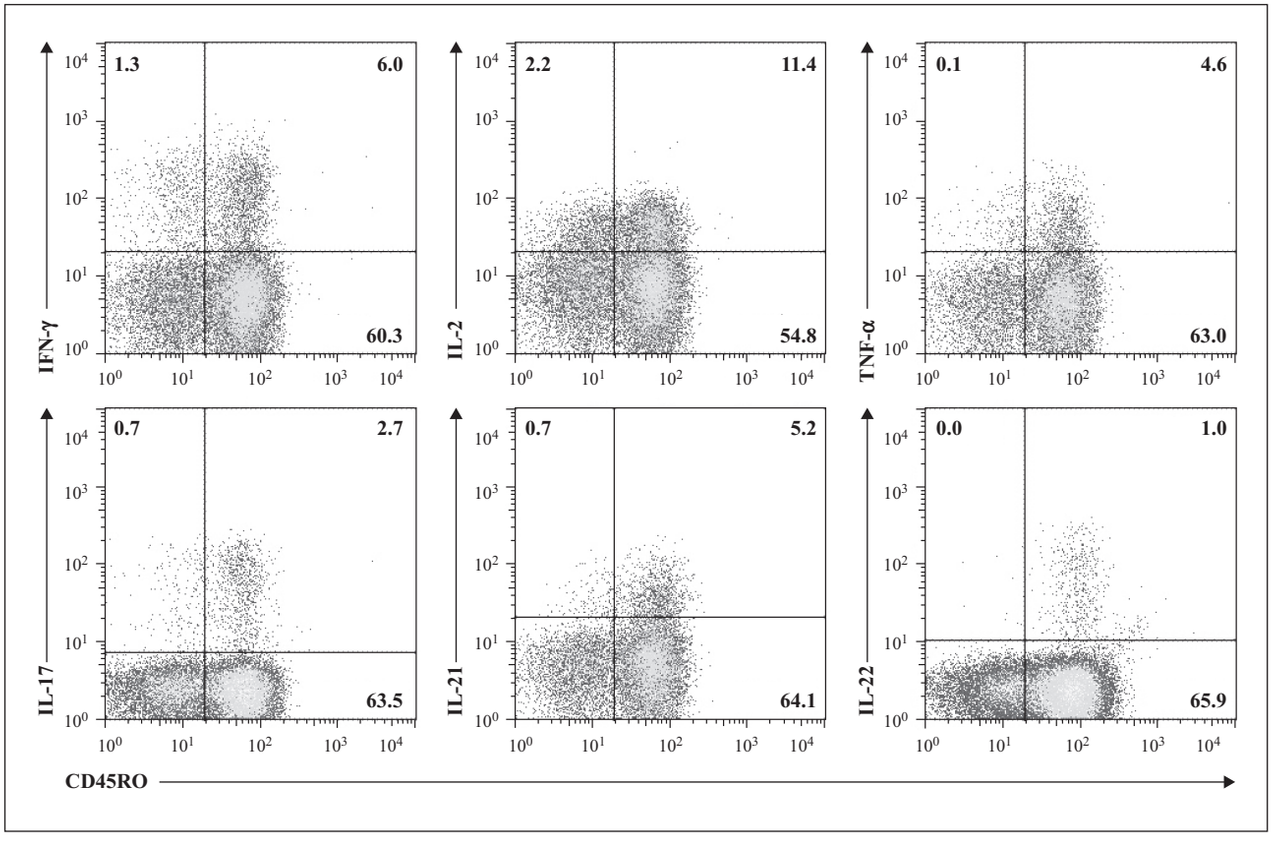

Figure 2

Phenotype of cytokine-producing CD4+ T cells in tonsillar tissues. Tonsillar mononuclear cells were stimulated for 6 hours with PMA plus ionomycin in the presence of BFA, the relationship between the expression of cytokines by CD4+ T cells and the expression of surface marker CD45RO was analyzed by FACS. The representative dot graphs showed the co-expression of IFN-γ and CD45RO, IL-2 and CD45RO, TNF-α and CD45RO, IL-17 and CD45RO, IL-21 and CD45RO and IL-22 and CD45RO.

cells co-expressed TNF-α, 16.706% co-expressed IL-2, 3.470% co-expressed IL-17, 14.138% co-expressed IL-21, and 1.829% co-expressed IL-22. 25.736% TNF-α$^+$CD4$^+$ T cells co-expressed IL-2, 6.424% co-expressed IL-17, 21.016% co-expressed IL-21, and 1.473% co-expressed IL-22. 3.466% IL-2$^+$CD4$^+$ T cells co-expressed IL-17, 22.331% co-expressed IL-21, and 1.166% co-expressed IL-22. 39.228% IL-17$^+$CD4$^+$ T cells co-expressed IL-21, and 10.096% co-expressed IL-22. 3.081% IL-21$^+$CD4$^+$ T cells co-expressed IL-22.

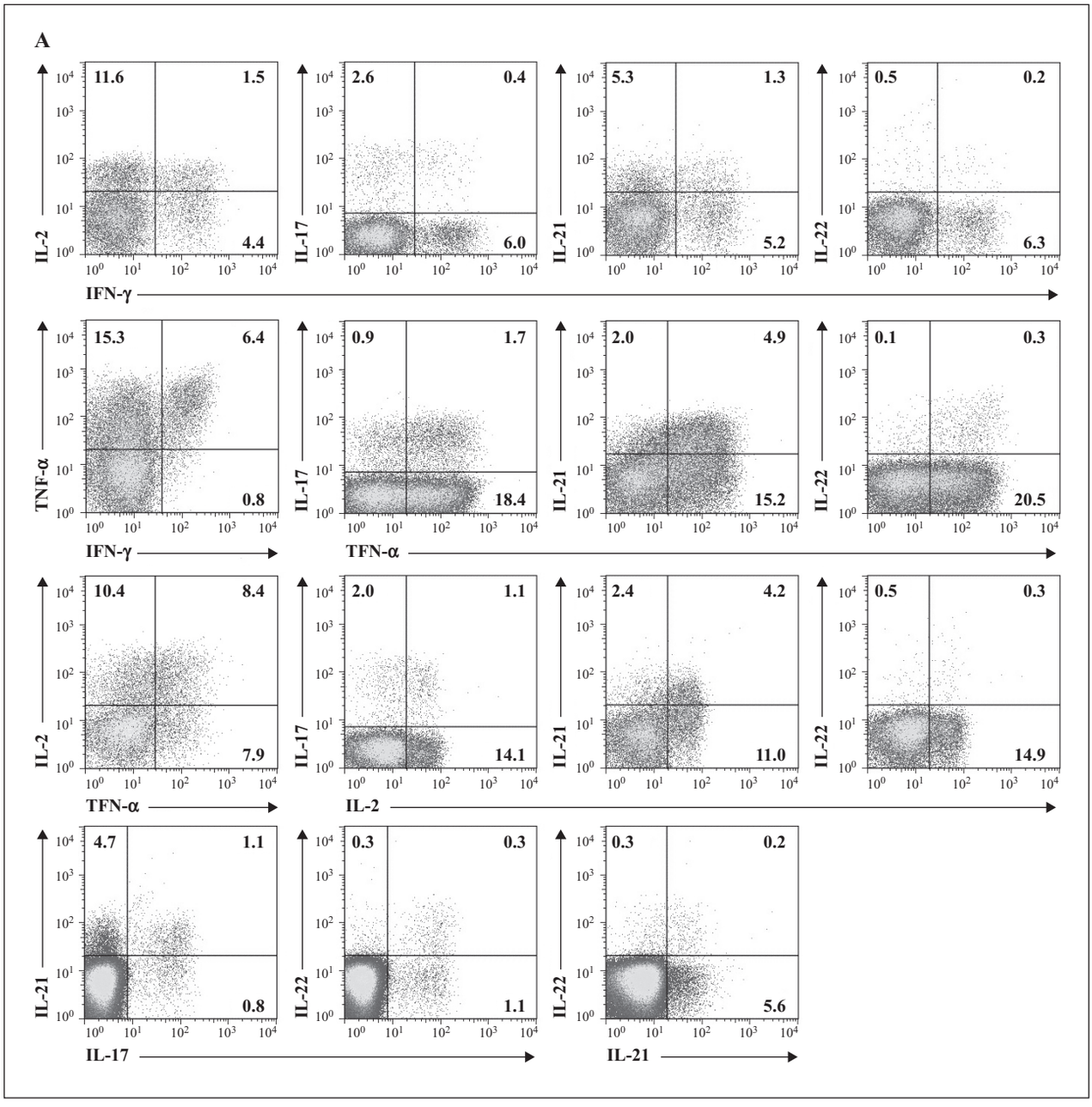

Figure 3

Single tonsillar CD4$^+$ T cell co-expressed two cytokines. Tonsillar mononuclear cells were stimulated for 6 hours with PMA plus ionomycin in the presence of BFA. The expression of cytokines was detected by FACS and co-expression of two different Th subset-related effector cytokines was analyzed by software FlowJo. The representative dot graphs showed the frequencies of IFN-γ^+TNF-α^+, IFN-γ^+IL-2$^+$, IFN-γ^+IL-17$^+$, IFN-γ^+IL-21$^+$, IFN-γ^+IL-22$^+$, TNF-α^+IL-2$^+$, TNF-α^+IL-17$^+$, TNF-α^+IL-21$^+$, TNF-α^+IL-22$^+$, IL-2$^+$IL-17$^+$, IL-2$^+$IL-21$^+$, IL-2$^+$IL-22$^+$, IL-17$^+$IL-21$^+$, IL-17$^+$IL-22$^+$ and IL-21$^+$IL-22$^+$CD4$^+$ T cells **(A)**. Statistical data were mean ± SD from eight independent experiments **(B)**.

Single tonsillar CD4$^+$ T cell co-expressed three or more kinds of cytokines

A recent and widely accepted concept suggests that T helper cells are polyfunctional, which means that CD4$^+$ T cells produced two or more different cytokines. Recent findings have demonstrated that the differentiation of CD4$^+$ T cells involves the characteristic of plasticity [14]. Tonsillar cells were stimulated for six h with PMA and ionomycin. The cells were then subjected to a seven-color flow cytometric assay as described above (*figure 4*) and the staining strategy was followed as *table 2*. We first gated on the IFN-γ^+ and IFN-γ^- CD4$^+$ T cells (*figure 4A*) or TNF-α^+ and TNF-α^- CD4$^+$ T cells (*figure 4C*): the expression of IL-2 and IL-21 in the four subsets was analyzed, which identified CD4$^+$ T cells as forming sixteen different subpopulations. The expression of IL-17 and IL-22 were further assayed. The statistical results of five separate experiments are shown in *table 3* and *figure 4*: we found that 0.006 ± 0.004% of tonsillar CD4$^+$ T cells secreted five cytokines IFN-γ, IL-2, IL-21, IL-17 and IL-22 (*figure 4B*), and 0.016 ± 0.011% of tonsillar CD4$^+$ T cells secreted TNF-α, IL-2, IL-21, IL-17 and IL-22 (*figure 4D*). These results showed that CD4$^+$ T cells from tonsils exhibited poly-functionality.

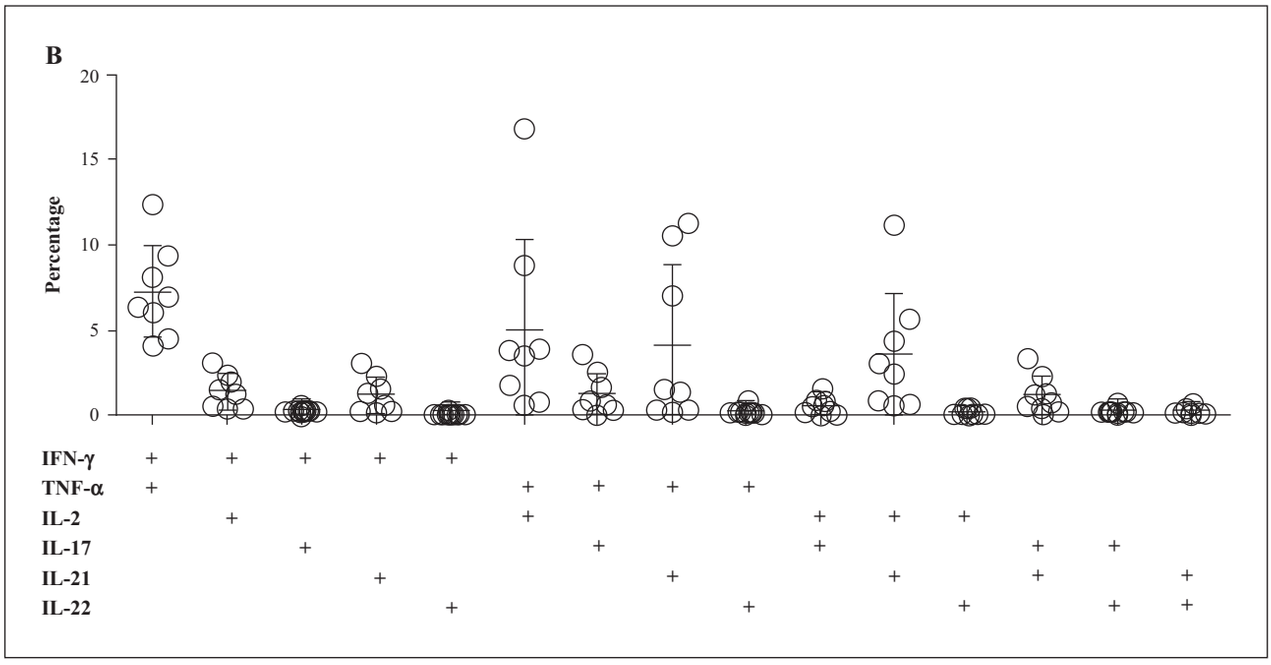

Figure 3 (Continued)

Table 1
Frequency of double cytokine-producing tonsillar CD4+ T cells

Percentages (Mean ± SD)	IFN-γ	TNF-α	IL-2	IL-17	IL-21
TNF-α	7.216 ± 0.950	—	—	—	—
IL-2	1.425 ± 0.360	5.016 ± 1.915	—	—	—
IL-17	0.296 ± 0.060	1.251 ± 0.439	0.559 ± 0.185	—	—
IL-21	1.206 ± 0.375	4.096 ± 1.675	3.602 ± 1.266	1.220 ± 0.391	—
IL-22	0.156 ± 0.026	0.287 ± 0.083	0.188 ± 0.044	0.315 ± 0.070	0.281 ± 0.072

—: the result was reduplicative as before, n=8.

DISCUSSION

In our study, we mainly analyzed the phenotypes of poly-functional T helper cells in tonsils, including Th1, Th17, Tfh and Th22. It is well known that in the presence of IL-12, with or without IFN-γ, naive CD4+ T cells can differentiate into Th1 cells: Th1 cells mostly make IFN-γ, TNF-α and IL-2 that are required for the clearance of intracellular pathogens and bacterial infection [15, 16]. IL-6 and TGF-β are available for Th17 cell differentiation *in vitro*, and these cells are characterized by the production of IL-17 and IL-22 as signature cytokines, which are potent inducers of tissue inflammation and have been associated with the pathogenesis of many autoimmune diseases [17]. The distinguishing features of Tfh cells are the expression of the master regulator transcription factor Bcl-6, cytokine IL-21 and molecule CXCR5, which are the specialized providers of B cell help in GCs [18-20]. Cytokine IL-6 or IL-21 with TCR signaling will induce Tfh cell differentiation, but this mechanism is controversial. CD4+ T cells are important sources of IL-22. IL-22, a member of the IL-10 family of cytokines, plays an important role in host defense, inflammation and tissue repair [21-23]. Otherwise the functions of this cytokine in regulating the development of effector Th cells and as the regulation mechanism of transcription factor have been demonstrated. A reliable means of developing multiple cytokine-producing CD4+ T cells *in vitro* are still being sought.

In this study, we used multi-color flow cytometry to analyze the expression of cytokines in tonsillar CD4+ T cells

Table 2
The staining strategy used for analysis of co-expression of five cytokines

Surface staining		Fluorescent color
Tube 1&2	CD3	PE-CF594
	CD4	APC-Cy7
Intracellular staining		Fluorescent color
Tube 1	IFN-γ	PE-Cy7
	IL-2	PerCP-Cy5.5
	IL-17	FITC
	IL-21	APC
	IL-22	PE
Tube 2	TNF-α	PE-Cy7
	IL-2	PerCP-Cy5.5
	IL-17	FITC
	IL-21	APC
	IL-22	PE

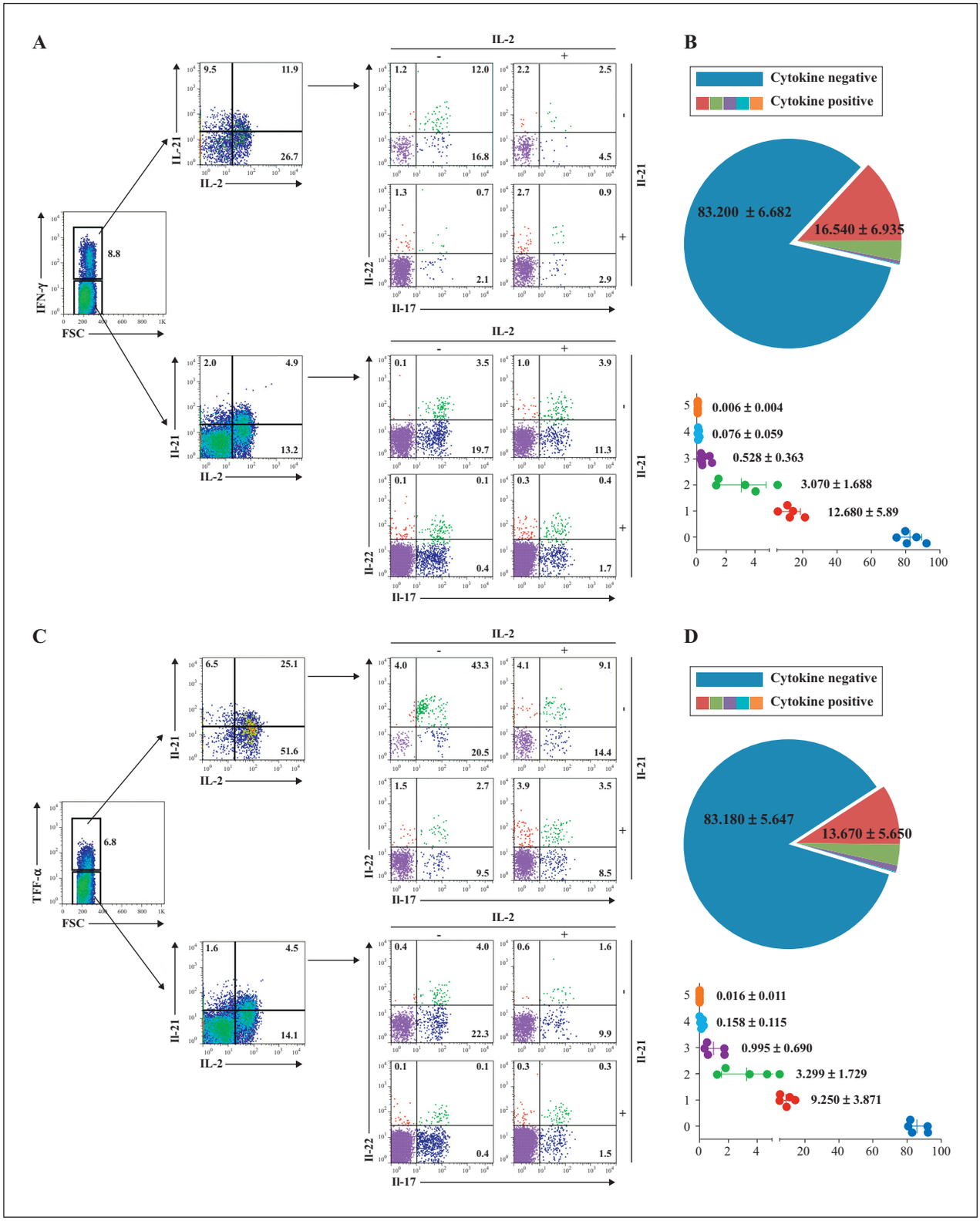

Figure 4

Single tonsillar CD4$^+$ T cell co-expressed multiple cytokines. Tonsillar mononuclear cells were stimulated for 6 hours with PMA plus ionomycin in the presence of BFA. The expression of cytokines was analyzed by FACS and co-expression of 5 cytokines by CD4$^+$ T cells was analyzed by software FlowJo. The representative dot graphs showed the frequencies of IFN-γ$^+$IL-2$^+$IL-17$^+$IL-21$^+$IL-22$^+$CD4$^+$ T cells or TNF-α$^+$IL-2$^+$IL-17$^+$IL-21$^+$IL-22$^+$CD4$^+$ T cells (**A and C**). Statistical data were mean ± SD from five independent experiments (**B and D**).

at the single cell level. We found that tonsillar CD4$^+$ T cells could express cytokines IFN-γ, TNF-α, IL-2, IL-17, IL-21 and IL-22 after short-term stimulation with PMA and ionomycin. Previous results demonstrated that naive and memory T cells could recirculate through T and B cell zone of tonsils where they could encounter antigens, and T_{CM} and T_{EM} were more frequent in the CD4$^+$ T cells compared to CD8$^+$ T cells. Our results also showed that most CD4$^+$ T cells expressed memory surface marker CD45RO, and that the cytokine-producing cells

Table 3
Frequency of multiple cytokine-expressing tonsillar CD4+ T cells

Percentages (Mean ± SD)		IFN-γ+	IFN-γ-	TNF-α+	TNF-α-
IL-21+IL-2-	IL-22+IL-17-	3.372 ± 1.204	0.976 ± 0.345	2.976 ± 1.267	1.288 ± 0.593
	IL-22+IL-17+	4.810 ± 1.683	2.788 ± 1.067	10.33 ± 4.483	3.191 ± 1.30.3
	IL-22-IL-17+	17.940 ± 2.517	18.710 ± 3.226	21.96 ± 1.364	17.030 ± 3.563
	IL-22-IL-17-	73.880 ± 3.731	78.080 ± 3.800	64.720 ± 4.665	78.480 ± 5.167
IL-21+IL-2+	IL-22+IL-17-	2.470 ± 0.495	0.986 ± 0.272	3.030 ± 1.227	0.575 ± 0.119
	IL-22+IL-17+	2.788 ± 0.651	3.482 ± 0.687	3.122 ± 1.025	2.617 ± 1.131
	IL-22-IL-17+	3.818 ± 0.776	8.758 ± 1.581	8.928 ± 1.437	6.540 ± 1.299
	IL-22-IL-17-	90.940 ± 0.765	87.35 ± 1.701	84.940 ± 3.277	90.280 ± 1.319
IL-21-IL-2+	IL-22+IL-17-	1.902 ± 0.427	0.847 ± 0.283	2.691 ± 0.819	0.611 ± 0.145
	IL-22+IL-17+	0.801 ± 0.179	0.795 ± 0.244	1.593 ± 0.508	0.453 ± 0.118
	IL-22-IL-17+	1.838 ± 0.305	2.672 ± 0.529	4.744 ± 1.310	1.940 ± 0.442
	IL-22-IL-17-	95.460 ± 0.837	95.660 ± 0.399	90.960 ± 2.300	97.000 ± 0.396
IL-21-IL-2-	IL-22+IL-17-	0.934 ± 0.249	0.282 ± 0.075	1.506 ± 0.409	0.216 ± 0.057
	IL-22+IL-17+	0.359 ± 0.166	0.064 ± 0.009	1.142 ± 0.410	0.045 ± 0.006
	IL-22-IL-17+	1.534 ± 0.384	1.390 ± 0.648	5.732 ± 1.370	1.074 ± 0.427
	IL-22-IL-17-	97.500 ± 0.497	98.280 ± 0.643	91.620 ± 1.835	98.68 ± 0.410

N = 5

were CD45RO+CD4+ T cells. However, the expression of CCR7 and CD62L, which are used to classify memory T cells as T_{CM} (CCR7+CD62L+) and T_{EM} (CCR7-CD62L-) subpopulations [24, 25], on cytokine-expressing CD4+ T cells requires more study. Interestingly, the results of flow cytometry showed that cytokine-producing CD4+ T cells co-expressed another Th-related effector cytokine, such as cell co-expressed Th1-related cytokine IFN-γ and Th17-related cytokine IL-17, Th1-related cytokine IFN-γ and Tfh-related cytokine IL-21, Th17-related cytokine IL-17 and Tfh-related cytokine IL-21 *et al*. To analyze further the polyfunctional CD4+ T cells in tonsils, we found that tonsillar CD4+ T cells could express five different cytokines, which were the critical effector cytokines of different Th subsets, such as IFN-γ (Th1), IL-2 (Th1), IL-17 (Th17), IL-21 (Tfh) and IL-22 (Th22). Polyfunctional CD4+ T cells were also reported in other inflammatory diseases. CD4+ T cells from nasal polyps co-expressed IL-21, IFN-γ and IL-17 [26]. *Mycobacterium tuberculosis* antigen-specific IFN-γ+IL-2+TNF-α+CD4+ T cells were detected in tuberculous pleural effusion [27]. In the current study, we are the first to report the five cytokine-expressing single CD4+ T cells in tonsillar mononuclear cells. Nevertheless, we still need to understand the possible pathological and physiological functions of multiple cytokine-expressing CD4+ T cells in specific inflammatory diseases, such as chronic tonsillitis.

Although we have demonstrated that cytokine-producing CD4+ T cells are likely to be memory cells, which highly express memory master surface marker CD45RO, most studies support the notion that plasticity is the differentiated state of CD4+ T cells [28]. Polyfunctional CD4+ T cells are thought to exist in the transient state during the plastic differentiation of T helper cells. Moreover, how such CD4+ T cell polyfunctionality is regulated remains poorly understood. Epigenetics is now considered to be one of the key mechanisms that dictate the stability and cellular polyfunctionality of effector T cell subsets [29]. The differentiation of polyfunctional CD4+ T cells remains undefined. Our study provides a novel insight into adaptive immunity in tonsils, and also represents an important basic work for studying the function of tonsils in defending against bacterial infections and other inflammatory diseases.

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