

Increased $V\delta 1\gamma\delta T$ cells Predominantly Contributed to IL-17 Production in the Development of Adult Human Post-infectious Irritable Bowel Syndrome

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Abstract

Background: $\gamma\delta$ T cells play an important role in the mucosa inflammation and immunity-associated disorders. Our previous study reported that $\gamma\delta$ T cells producing IL-17 were involved in the pathogenesis of post-infectious irritable bowel syndrome (PI-IBS). However, their subset characteristic profile in this kind of disease remains unclear. Thus the current study's aim is to investigate the functionally predominant subset and its role in PI-IBS.

Methods: The total T cells were collected from the peripheral blood of patients with PI-IBS. The peripheral proportion of V δ 1 and V δ 2 subset was detected by FACS after stained with anti δ 1-PE and anti δ 2-APC. The local colonic proportion of these two subsets were measured under laser confocal fluorescence microscope. V δ 1 $\gamma\delta$ T cells were enriched from the total peripheral T cells by microantibody-immunomicrobeads (MACS) method, and cultured, functionally evaluated by CCK-8 assay (proliferation), CD69/CD62L molecules expression assay (activation) and ELISA (IL-17 production) respectively.

Results: 1. V δ 1 $\gamma\delta$ T cells significantly increased while V δ 2 $\gamma\delta$ T cells remained unchanged in both the peripheral blood and local colonic tissue from PI-IBS patients ($P < 0.05$). 2. When cultured in vitro, the V δ 1 $\gamma\delta$ T cells remarkably proliferated, activated and produced IL-17 ($P < 0.05$).

Conclusions: Our results suggest that V δ 1 $\gamma\delta$ T cells was the predominant $\gamma\delta$ T cells subset in both peripheral and intestinal tissue, and was the major IL-17 producing $\gamma\delta$ T cells in PI-IBS.

1. Background

Post-infectious irritable bowel syndrome (PI-IBS) is a kind of functional gastrointestinal disorders, with clinical feature of abdominal pain or discomfort accompanied with abnormal defecate habit and /or stool character. The incidence of this disease ranges from 10% to 60%, predominantly in the developed western industrialized countries. During the last three decades, it is reported to occur in the developing countries [1]. Irritable bowel syndrome (IBS) which occurred after an initial episode of acute gastrointestinal infection was defined as postinfectious irritable bowel syndrome (PI-IBS) [2]. Suffering from the refractory symptoms, the patients are living with low life quality and have to be charged with expensive medical bills. Without obvious morphological changes and biochemistry abnormality, this kind of disease is short of specific and effective therapy [3]. Basically, it is regarded as a post-inflammatory immune disturbance [4].

Regulation of inflammatory response might contribute to alleviate symptom of PI-IBS. Research reported that EphA2, a member of Eph receptor family, could regulate inflammation and oxidative stress via Nrf2 and NF- κ B signaling pathway. Upregulating of EphA2 and activation of NF- κ B signaling pathway exerts beneficial effect in PI-IBS [5]. Moreover, non-coding RNAs also showed the pivotal role in the regulation of inflammatory response in PI-IBS. Zhang et al found that miRNA-510, a widely investigated miRNA in cancer progression, downregulated in intestinal tissue might contribute to inflammatory injury and PI-IBS via targeting PRDX1 [6]. Accounting for only 0.5% [5] of the whole T cells, $\gamma\delta$ T cells are located in

peripheral and local mucosa-associated lymphoid tissue(MALT), such as derma, respiratory tract, reproductive tract and digestive tract, recognizing the pathogens directly or presenting antigen to effect cells like B cells , thus triggering the specific immune response[7-10]. In some disorders, gdT cells are proved to be the major resource of IL-17 [11-13]. Additionally, IL-17 is also involved in the development of PI-IBS. For instance, Rifaximin could alleviated visceral hypersensitivity, recovered intestinal barrier function, and inhibited low-grade inflammation in colon and ileum of PI-IBS mouse model via suppressing the expression of IL-17 and promoting the expression of the major tight junction protein occluding [14]. It has been proved that gdT cells are involved in the pathogenesis of experimental autoimmune encephalomyelitis, rheumatoid arthritis, Non-obese diabetes[15-17]. On the other hand, their protective role was observed in rats with colitis and sepsis, and mouse with experimental autoimmune uveitis[18][19].

Recently, the role of the gdT cells' subset in the diseases is becoming a research hotspot. The gdTCR is constituted by g and d variable regions. The Vg and Vd genetic locus possess recombinant multiformity, which include g1- g9 and d1- d3 variable regions. According to the various expression of V gene segments, gd T cells can be divided into various subsets, the heterogeneity of which determinates their functional diversity. Vd1 gd T cells is mostly located in the thymus and mucosa epithelium, while a small amount exist in the peripheral blood. Vd1 gd T cells recognize the stress molecule by its TCR and activate as a producer of some pro-inflammatory cytokines to triger the fast immune response. For example, Vd1 gd T cells produce IL-17 in pneumococcus infection and protect mice from Listeria infection[20,21]. Vd2 gd T cells are the major gd T cells in the peripheral circulation of healthy human, accounting for 50%-90% of the total gdT cells, presenting the antigen to the B and NK lymphocytes and producing IFN- γ [22,23]. Vd3 gd T cells are mainly enriched in the liver, accounting for the minimum of the total gd T cells (0.2%) [24]. We previously reported that the intestinal gd T cells could exert an important role in a PI-IBS mouse model[25]. However, the precise mechanism of gdT cells subset in this kind of disease remain unclear, thus the current study aims to investigate the changes of the gdT cells subsets and its functional meaning in PI-IBS.

2. Methods

2.1. PI-IBS ptients

From January 2018 to December 2019, thirty-five IBS patients including inpatients and outpatients were recruited for the study at Hainan General Hospital. The inpatients met the criteria that having abdominal pain, diarrhea, or abdominal pain accompanied by changes in stool characteristics. No obvious biochemical or pathological abnormalities were observed after multiple examinations, including colonoscopy. All hospitalized patients excluded other organic diseases and were clearly diagnosed with irritable bowel syndrome. All 35 patients met the Rome III dignosis criteria for IBS and were positively confirmed onset after an episode of acute gastroenteritis with diarrhea and/or vomiting in addition to a series of examinations such as microbiology test, hence, meeting the definition of PI-IBS [26]. Moreover, microscopic colitis and other inflammation GI diseases such as celiac disease were excluded by

histological screening of mucosal biopsies throughout the entire colon, obtained by colonoscopy prior to inclusion according to established diagnostic criteria. The exclusive criteria for patients were as follows: 1) experienced major abdominal surgery. 2) evidence of metabolic, gastrointestinal, cardiovascular, psychological or malignant disease. 3) gastrointestinal organic disease including peptic ulcer, Crohn's disease, ulcerative colitis and pancreatitis. 4) pregnancy or lactating. 5) patients who are taking nonsteroidal anti-inflammatory drugs, steroids, or antibiotics. The patients' age ranged from 17 to 53 years old, with the mean age of 33.9 years, including 19 female and 16 male. In this study, 33 voluntary healthy controls were recruited by advertisement and had regular examination including colonoscopy to exclude any inflammatory diseases. None of them had GI complaints, chronic pain conditions, infectious or inflammatory disorders such as rheumatoid arthritis, psychiatric illnesses, or were taking pharmaceutical agents. The age of health controls ranged from 19 to 55 years old, with the mean age of 32.9 years, including 15 female and 18 male. The peripheral blood and colonic tissue were collected through colon biopsy and preserved in -80°C freezer for further examination. In addition, some tissue was processed into frozen sections. Some tissue was smashed into powder within ultrasonic disintegrator and the supernatant was preserved under -80°C freezer for further examination.

2.2. Morphological analysis

The colonic tissue was collected by colonoscopy biopsy and processed into ultra-thin frozen sections by liquid nitrogen quick-frozen slicing. The sections were treated by immunofluorescence histochemical staining. The primary antibody was fluorescence-conjugated rabbit anti-mouse anti- $\delta 1$ TCR or $\delta 2$ TCR monoclonal antibodies. The stained tissue sections were scanned under Laser scanning confocal microscope (Olympus FV10i Olympus Tokyo, Japan). The intensity of fluorescence was calculated automatically using the image analysis software.

2.3. Enrichment of V $\delta 1$ $\gamma\delta$ T cells

The enrichment of T cells were conducted as previously described by Cheng et, al [10]. Briefly, 5ml peripheral blood of PI-IBS patients was collected and the total T cells were isolated by centrifuging with lymphocyte separation medium Ficoll (Sigma-Aldrich, ST. Louis, MO, USA). The total T cells were stained with anti human V $\delta 1$ -PE and anti human V $\delta 2$ -APC (BD Biosciences, Franklin Lakes, NJ, USA) and the proportion of V $\delta 1$ and V $\delta 2$ $\gamma\delta$ T cells was measured by FACS. In conclusion, the proportion of V $\delta 1$ T cells were all increased in 35 PI-IBS patients compared with control group.

The total T cells were stained with FITC conjugated anti- $\delta 1$ TCR mAb (100ul antibodies per 1×10^8 cells, incubated at 4°C for 30 min), followed by stained with microbeads conjugated anti-FITC mAb (100ul antibodies per 1×10^8 cells, incubated at 4°C for 30 min). The V $\delta 1$ $\gamma\delta$ T cells were positively selected by MACS Miltenyi Biotec GmbH, Cologne, NRW, Germany). For further purification of V $\delta 1$ $\gamma\delta$ T cells, the residual $\alpha\beta^+$ T cells were depleted using PE-conjugated anti- $\alpha\beta$ TCR antibody with anti-PE microbeads.

2.4. Functional Evaluation of V $\delta 1$ $\gamma\delta$ T cells

The enriched V δ 1 γ δ T cells were incubated in the presence of IL-23 (10 ng/ml) under 37°C, 5%CO₂ for 48 hours followed by functional evaluation including proliferation, activation and cytokine producing capability.

Proliferation

Cell Counting Kit -8 (CCK-8) Assay was used to evaluate the enriched V δ 1

γ δ T cells' proliferation. Briefly, the cells were seeded at 4×10^3 cells/well in 96-well plates, then incubated at 37 °C for 24 h in a total volume of 100 μ l medium. The cells were added with 10 μ l CCK8/well and cultured at 37°C 5% for 8 hours. The absorbance value at 450nm was measured on a Thermomax Microplate Reader (Menlo Park, CA, U.S.A). The proliferation response was expressed as the OD value of mean \pm standard deviation (SD) of triplicate determinations.

Activation

V δ 1 γ δ T cells were stained with PE conjugated anti-CD69 mAb or anti-CD62L mAb (100ul antibodies per 1×10^8 cells, incubated with IL-23 and TLR4 at 4°C for 30 min) . The expression of CD69 and CD62L on V δ 1 γ δ T cells was determined by FACS as described previously [10, 13].

Production of proinflammatory cytokines

The concentration of IFN γ and IL-17 in the supernatants from the cultured V δ 1 γ δ T cells and the colonic tissue were measured by ELISA in accordance with the manufacture's instruction. Briefly, the supernatant from cultured cells and tissues were removed and replaced with fresh media (RPMI-1640) and were then returned to standard culture conditions for 24hours. Subsequently, the IL-17 protein of cell supernatant was analyzed by IL-17 high-sensitivity (0.25–16pg/mL sensitivity range) ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol.

2.5. Statistics analysis

Experimental data were analyzed with Kolmogorov-Smirnov test by SPSS software to explore whether data complied with a normal distribution. The result of K-S analysis indicated a normality distribution of all data. The unpaired Student's t-test was used to compare differences between two groups (SPSS 19.0 software). Data were expressed as the mean \pm standard error of the mean. Values in the same row with different superscripts are significant ($P < 0.05$), while values with same superscripts are not significant ($P > 0.05$).

3. Results

3.1 Proportion of γ δ T cells subset

3.1.1 Proportion of γ δ T cells subset in peripheral blood from PI-IBS patients

Proportion of $\gamma\delta$ T cells subset in peripheral blood from PI-IBS patients was detected by FACS (PE-d1TCR/APC- d2TCR). Usually, V δ 1 $\gamma\delta$ T cells dominate in local mucosa tissue and V δ 2 $\gamma\delta$ T cells were the major peripheral $\gamma\delta$ T cells. However, in PI-IBS patients' peripheral blood, V δ 1 $\gamma\delta$ T cells significantly increased and became the predominant subset, while V δ 2 $\gamma\delta$ T cells decreased and became the minor subset (Figure1, Table 1). The results suggest that the $\gamma\delta$ T cells' characteristic subset profile shifting from V δ 2 to V δ 1 could have potential functional significance.

3.1.2. Enrichment and identifying of V δ 1 $\gamma\delta$ T cells

The V δ 1 $\gamma\delta$ T cells were isolated by positively selection protocol (MACS) from the total T cells from the peripheral blood of the PI-IBS patients. After the contaminant $\alpha\beta$ T cells were depleted, the proportion of the purified V δ 1 $\gamma\delta$ T cells was up to 99.7%. And the cell viability remained up to more than 90%. Thus the high degree of purity of the enriched V δ 1 $\gamma\delta$ T cells guaranteed their functional analysis in vitro.

3.2 Functional analysis of V δ 1 $\gamma\delta$ T cells

The function of the enriched V δ 1 $\gamma\delta$ T cells was evaluated by proliferation assay, activation assay and pro-inflammatory cytokines production assay. Firstly, CCK-8 assay show that V δ 1 $\gamma\delta$ T cells from PI-IBS patients significantly proliferated, with the absorbance OD value almost two times more than that from the control group (Figure. 4). Secondly, compared with the healthy human, the expression of CD62L molecule remarkably decreased while that of CD69 molecule increased (Figure.5). The changes of the expression of these two molecules indicated that the cells' activation occurred. Furthermore, stimulated with IL-23, the enriched V δ 1 $\gamma\delta$ T cells from PI-IBS patients produced much more IL-17 but not IFN- γ , suggesting that the capability of producing pro-inflammatory cytokines, especially IL-17 of this subset boosted(Figure.6). These results revealed the functional profile of V δ 1 $\gamma\delta$ T cells in line with their quantitative changes in PI-IBS.

3.3 Pro-inflammatory cytokines level in PI-IBS patients

The peripheral blood serum was collected and the colon tissue from PI-IBS was smashed by ultrasonic disintegrator. The IL-17 and IFN- γ concentration in the serum and tissue supernatants was measured by ELISA. As shown in Figure 7, compared with the control group, the peripheral IL-17 level in PI-IBS patients significantly increased ($P < 0.05$) while IFN- γ remain unchanged ($P > 0.05$), suggesting that peripheral IL-17 could be involved in PI-IBS. As shown in Figure 8, compared with the control group, the intestinal IL-17 level in PI-IBS patients significantly increased ($P < 0.05$), while the IFN- γ level remained unchanged ($P > 0.05$), suggesting that the local IL-17 could participate in the intestinal pathological disorder during PI-IBS. Furthermore, it was intriguing that the increase amplitude of IL-17 in colon tissue was far more than that in peripheral blood, implicating that the triggering and activating event did occur in the local intestine. All these results proved that during PI-IBS, IL-17 but not IFN- γ is the major proinflammatory cytokine.

4. Discussion

Infection and inflammation are considered involved in the pathogenesis of IBS, whereas antibiotic therapy failed to induce expected improvement in all IBS patients, especially in those patients with refractory and prolonged symptoms [27]. This condition suggests that the immunity disturbance after infection could exert more crucial role in this disorder. gdT cells participate in keeping the immune balance in the intestinal mucosa and associated with some digestive diseases [28-30]. Recently it is proved that gd T cells present different function due to their various subsets' distribution and function. For example, Costa et al reported that Murine IL-17 +Vg4 T lymphocytes accumulate in the lungs and play a protective role during severe sepsis. It is reported that murine IL-17 +V g4 T lymphocytes accumulate in the lungs and play a protective role during severe sepsis., and that a novel proinflammatory human skin-homing Vg9Vd2 T cell subset was identified with a potential role in psoriasis. The microbiota were associated with the development of gdT cells subset. [31-33]. Thus it is important to investigate the precise role of the subset of gd T cells in PI-IBS.

We found that the Vd1 gdT cells was the predominant subset both in the peripheral blood and colon tissue. Usually in peripheral blood, Vd2 gdT cells dominated but in PI-IBS condition, the major subset changed from Vd2 to Vd1. So where did the Vd1 gdT cells come from? Firstly, the possible origin was intestine. In PI-IBS, the intestinal activated and proliferated Vd1 gdT cells traveled to the peripheral blood. Secondly, was other peripheral organ like lympho-node, spleen. Thirdly, they came directly from the the peripheral Vd2 gdT cells.

Did the subset polarity drifting have some functional meaning? We isolated and cultured Vd1gdT cells subset, investigated their immune function in vitro. It is surprising that these Vd1gdT cells subset from PI-IBS patients remarkably proliferated, activated and produced abundant IL-17. As for the pro-inflammatory cytokines, we found that the IL-17 level increased in the peripheral blood but IFN- γ remained unchanged, we also observed the similar phenomena as in the local intestine. These results suggested that the IL-17 both in peripheral and local intestine could come from Vd1gdT cells. Interestingly, the increasing degree of IL-17 in peripheral blood was less than that in the local intestine. Because we cannot enrich Vd1gdT cells from the patients'intestine, we speculate that the local tissue microenvironment could promote the resident Vd1gdT cells and contribute to the difference.

Sometimes the cells' behavior in vivo could be different from that in vitro. Thus we explored the morphological alteration of the gdT cells subset in the local colon from the PI-IBS patients. We found that Vd1 gdT cells significantly increased and Vd2 gdT cells decreased relatively. Simultaneously the IL-17 level in the colon tissue expanded. We previously reported that gdT cells' Th17 response participated in the development of PI-IBS [25]. Thus our results suggested that Vd1 gdT cells could functionally be involved in Th17 response during PI-IBS.

An interesting problem is that whether the gdT cells subset in peripheral blood share the same biological behavior with their counterpart in local colon tissue. Perhaps T lymphocyte homing assay could help to solve this puzzle problem. On the other hand, the gdT cells subset could regulate each other via some unknown pathway. Probably Vd2 gdT cells participate in the pathological event with their own manner,

not just as a bystander. Thus the precise role of the gdT cell subset in the pathogenesis of PI-IBS needs an in-depth study.

There are also some shortcomings and limitations in our study. First of all, the number of samples used in this study is relative small and needs to be increased in further study. Although the low number of functional experiments is one limitation of our study, the results were very consistent between peripheral blood and local environment. Moreover, the deep molecular mechanisms of the regulation of gdT cells on IL-17 need to be well investigated in future.

5. Conclusions

In conclusion, the current study results show the pivotal role of Vd1 gd T cells and its stimulation product IL-17 in the development of PI-IBS. Various variable regions determine different immune response by gd T cells subset in diverse pathological conditions. The Vδ1 γδ T cells are mainly presented in local mucosa tissue and Vδ2 γδ T cells were the majority in peripheral γδ T cells. In patients with post-infectious irritable bowel Syndrome (PI-IBS), we found that Vd1 gd T cells, instead of Vd2 gd T, dominated in both the peripheral blood and colonic tissue. Moreover, we also observed the increased ability of proliferation, activation and IL-17 production of Vd1 gd T cells in PI-IBS patients after IL-23 stimulation. Taken together, these results suggest that Vδ1 gd T cells was the predominant gd T cells in both peripheral and intestinal tissue and was the major IL-17 producing gd T cells in PI-IBS, which would be a novel therapeutic target for PI-IBS.

Abbreviations

PI-IBS Post-infectious irritable bowel syndrome; IL-17: interleukin 17; IFN-γ interferon gamma; mAb: monoclonal antibody; FACS Fluorescence Activating Cell Sorter; TCR: T cell receptor; ELISA enzyme linked immunosorbent assay

Declarations

1. Ethics approval and consent to participate

The research protocol in the current manuscript was permitted by Ethnic Committee of Hainan General Hospital and obtained the written informed consent of the patients and volunteers.

2. Consent for publication

All of the individual persons participating in the current study approved that their data in the current manuscript 'Increased Vδ1 gdT cells Predominantly Contributed to IL-17 Production in the Development of Adult Human Post-infectious Irritable Bowel Syndrome' will be published.

3. Competing interests

We declare no financial competing interests conflict.

4. Funding

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5. Authors' contributions

Corresponding author: CL : the conception and design of the work, guarantor of integrity of entire study

LD: clinical and experimental studies, the acquisition and statistical analysis of data, manuscript preparation

XS: design of the work

ZM: the acquisition of data

JF: the acquisition of data

FL: the acquisition of data

BH: the acquisition of data

DL: the analysis and interpretation of data

DS: interpretation of data and substantively revised the manuscript,

***all authors have read and approved the manuscript 'Increased V δ 1gdT cells Predominantly Contributed to IL-17 Production in the Development of Adult Human Post-infectious Irritable Bowel Syndrome'**

6. Acknowledgements

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7. Availability of data and materials

The regarding supporting data and materials in our manuscript is available from the corresponding author.

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Table

Table 1. Proportion of $\gamma\delta$ T cells subset in peripheral blood from PHBS patients

	number	V δ 1 [%]	V δ 2 [%]
Control	n=33	12.46 \pm 2.10	76.87 \pm 7.93
PHBS	n=35	80.18 \pm 6.24 ^a	17.48 \pm 3.18 ^b

a, compared with control group, t=47.86, P=0.000;

b, compared with control group, t=35.73, P=0.000

Figures

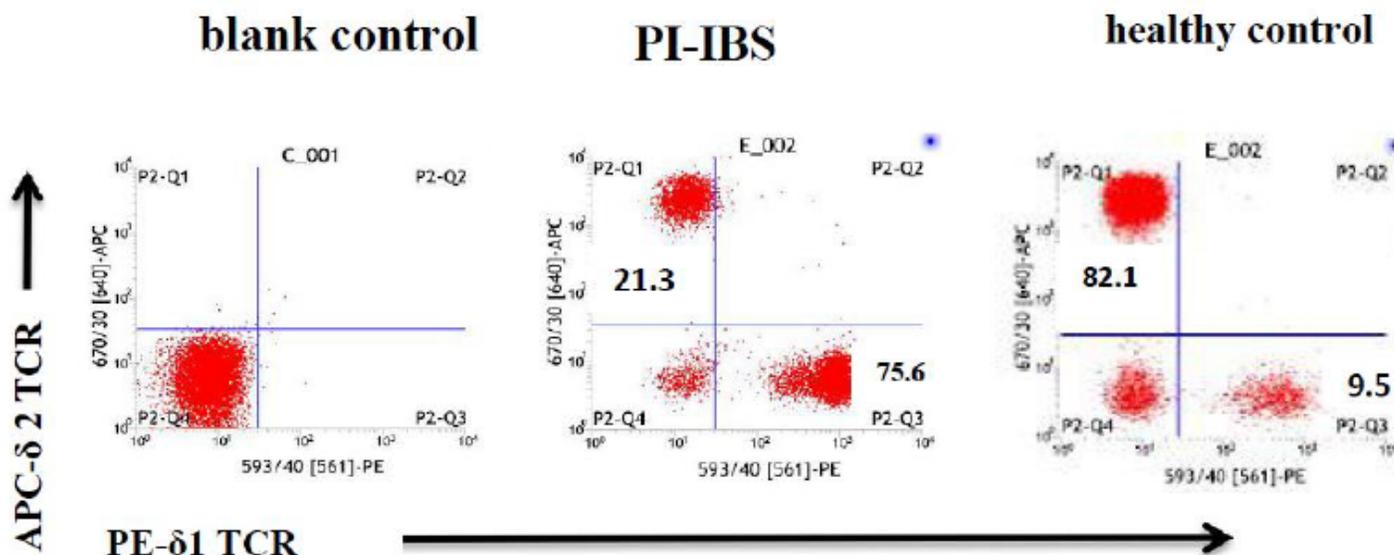


Figure 1

Proportion of $\gamma\delta$ T cells subset in peripheral blood from PI-IBS patients was detected by FACS (PE- $\delta 1$ TCR/APC- $\delta 2$ TCR). The results indicated that V $\delta 1$ $\gamma\delta$ T cells significantly increased and became the predominant subset compared with healthy controls, while V $\delta 2$ $\gamma\delta$ T cells decreased and became the minor subset in PI-IBS patients, $P < 0.01$. Representative graphs were shown in the current figure.

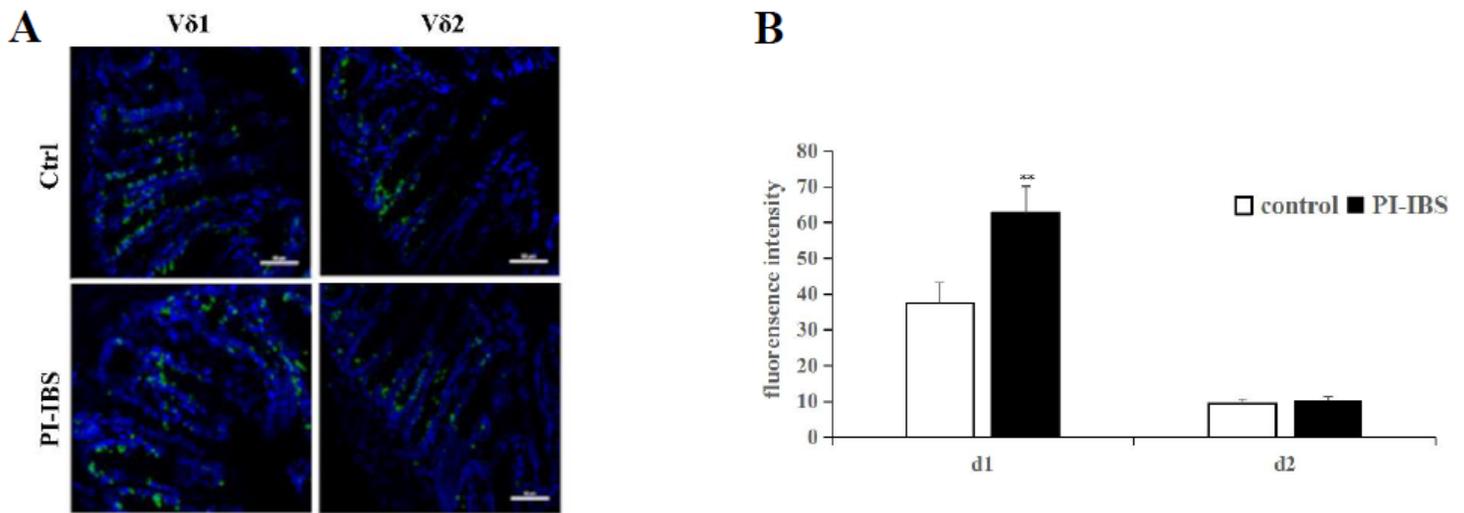


Figure 2

The colonic tissue was collected from PI-IBS patient by colonic endoscopic biopsy and processed into frozen sections followed by immunofluorescent staining with anti- $\delta 1/\delta 2$ monoclonal antibody. Under laser scanning confocal microscope (200x), the intestinal V $\delta 1$ $\gamma\delta$ T cells from PI-IBS patients significantly increased while V $\delta 2$ $\gamma\delta$ T cells decreased compared with that from control group, $p < 0.01$. Representative graphs were shown in the current figure.

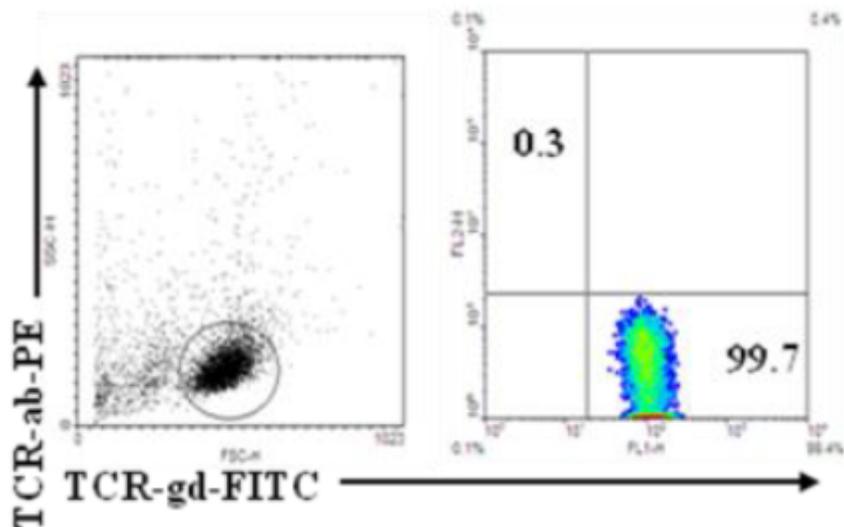


Figure 3

The V $\delta 1$ $\gamma\delta$ T cells were isolated by positively selection protocol (MACS) from the total T cells from the peripheral blood of the PI-IBS patients, the purity degree up to 99.7%.

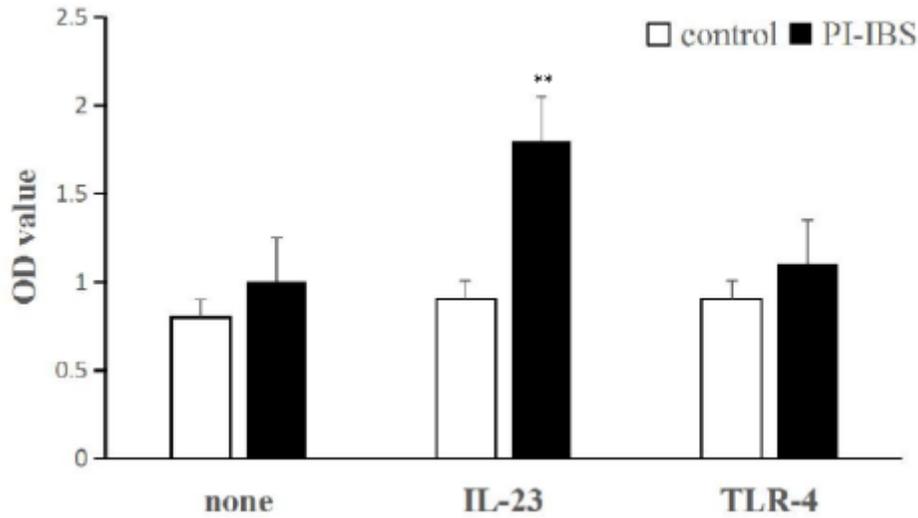


Figure 4

CCK-8 Assay was used to evaluate the enriched Vδ1 γδ T cells' proliferation stimulated with IL-23 or TLR-4. As shown in figure4, compared with the control group, in the presence of IL-23, the Vδ1 γδ T cells from PI-IBS patients significantly proliferated. All experiments were repeated three times. $p < 0.05^*$, $p < 0.01^{**}$.

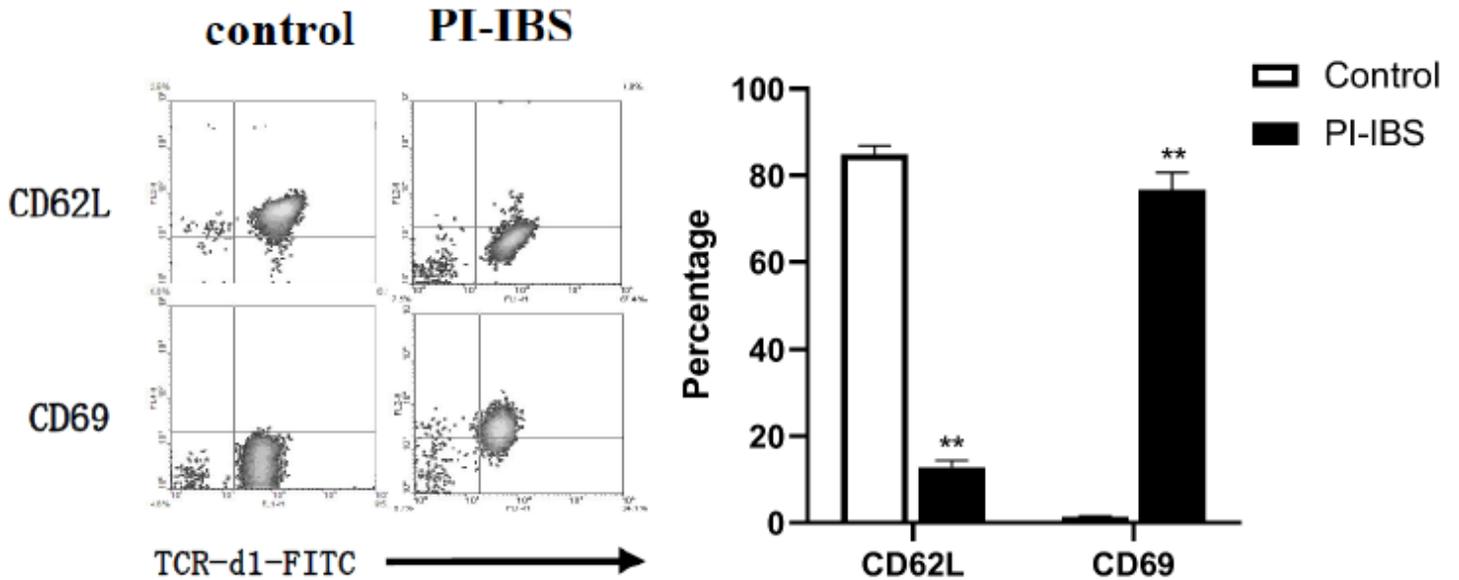


Figure 5

The expression of CD62L and CD69 molecules on the enriched Vδ1 γδ T cells were detected to evaluate the activation status of the subset. Expression level of CD62L remarkably decreased while that of CD69 increased on the surface of Vδ1 γδ T cells from PI-IBS patients compared with healthy controls. All experiments were repeated three times. $p < 0.05^*$, $p < 0.01^{**}$.

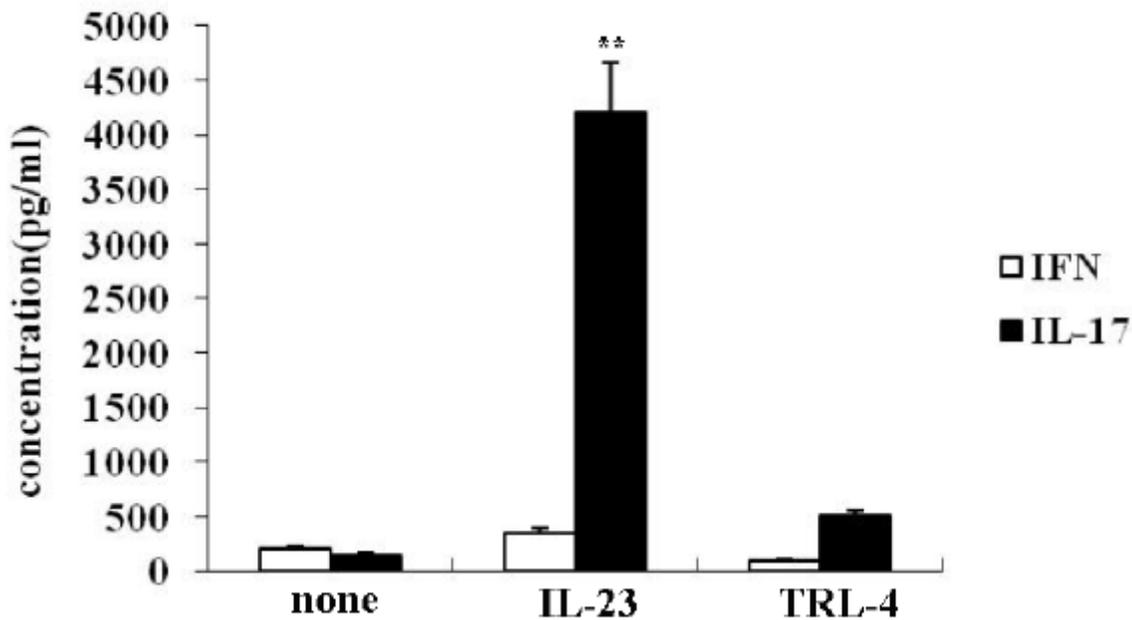


Figure 6

The capability of producing inflammatory cytokines by the V δ 1 $\gamma\delta$ T cells was evaluated by detecting the concentration of IL-17 and IFN- γ in the supernatants of the cultured subset. The V δ 1 $\gamma\delta$ T cells of PI-BS patients significantly produced more IL-17 than control groups after stimulated by IL-23. All experiments were repeated three times. $p < 0.05^*$, $p < 0.01^{**}$.

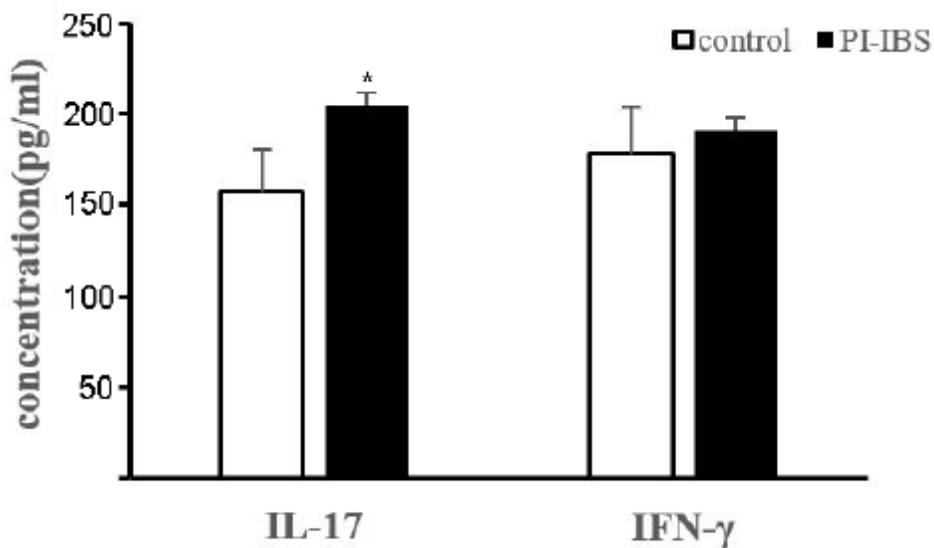


Figure 7

The concentration of IL-17 and IFN- γ in the peripheral blood was measured by ELISA. Compared with the control group, the peripheral IL-17 level significantly increased, suggesting the V δ 1 $\gamma\delta$ T cells' potential role. All experiments were repeated three times. $p < 0.05^*$, $p < 0.01^{**}$.

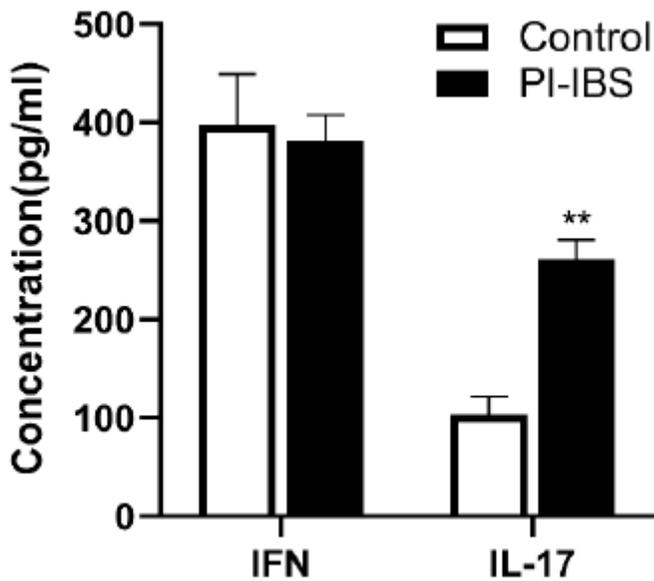


Figure 8

The concentration of IL-17 and IFN- γ in the colon tissue was measured by ELISA. Compared with the control group, the colonic IL-17 but not IFN- γ level significantly increased in PI-IBS. The change of pro-inflammatory cytokines in the patients accompanied with the increased and activated V δ 1 $\gamma\delta$ T cells suggested that this kind of subset could participated in PI-IBS through producing IL-17. All experiments were repeated three times. $p < 0.05^*$, $p < 0.01^{**}$.