Increased Vδ1 γδT cells Predominantly Contributed to IL-17 Production in the Development of Adult Human Post-infectious Irritable Bowel Syndrome

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Research article

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Abstract

Background: gdT cells play an important role in the mucosa inflammation and immunity-associated disorders. Our previous study reported that gd 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 Vδ1 and Vδ2 subset was detected by FACS after stained with anti δ1-PE and anti δ2-APC. The local colonic proportion of this two subsets were measured under laser confocal fluorescence microscope. Vδ1 gd T cells were enriched from the total peripheral T cells by minoantibody-immuno-microbeads (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. Vδ1 gd T cells significantly increased while Vδ2 gd 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 Vδ1 gd T cells remarkably proliferated, activated and produced IL-17 ($P<0.05$).

Conclusions: Our results suggest that Vδ1 gd T cells was the predominant gd T cells subset in both peripheral and intestinal tissue, and was the major IL-17 producing gd T cells in PI-IBS.

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 morbidity 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]. 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 abnormity, this kind of disease is short of specific and effective therapy[2]. Basically, it is regarded as a post-inflammatory immune disturbance [3]. Accounting for only 0.5%[5]of the whole T cells. gdT cells are located in peripheral and local mucosal lymphoma tissue, 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[4-7]. In some disorders, gdT cells are proved to be the major resource of IL-17 [8-10]. It has been proved that gdT cells are involved in the pathogenesis of experimental autoimmune encephalomyelitis, rheumatoid arthritis, Non-obese diabetes [11-13]. On the other hand, their protective role was observed in rats with colitis and sepsis, and mouse with experimental autoimmune uveitis[14][15].

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[16,17]. 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[18,19]. Vd3 gd T cells are mainly enriched in the liver, accounting for the minimum of the total gd T cells (0.2%) [20]. We previously reported that the intestinal gd T cells could exert an important role in a PI-IBS mouse model[21]. 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.

Methods

2.1. PI-IBS ptients

35 patients (inpatients and outpatients) were enrolled, diagnosed in accordance with the diagnostic criteria of IBS (Rome III[2006) [22] from January 2018 to December 2019 at Hainan General Hospital . The patients' age ranged from 18 to 50 years old, with the mean age of 34.5 years, including 18 female and 17 male. The patients had at least once intestinal infection history but did not receive standard and enough antibiotics treatment. All the patients had no any organic disorders and reported abdominal discomfort-associated abnormal defecation when enrolled. 33 healthy volunteers were assigned into the control group. The research protocol in the current study was permitted by Ethnic Committee of Hainan General Hospital. The written informed consent was obtained from all patients and volunteers.

The patients were checked by colonoscopy to exclude obvious organic diseases. 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 ultrosonic 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 florescence- conjugated rabbit anti-mouse anti-δ1 TCR or δ2 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 Vδ1 γδ T cells

The enrichment of T cells were conducted as previously described by Cheng et, al [7]. 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 humanVδ1-PE and anti humanVδ2-APC (BD Biosciences, Franklin Lakes, NJ, USA) and the proportion of Vδ1 and Vδ2 γδ T cells was measured by FACS.

The total T cells were stained with FITC conjugated anti-δ1 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 Vδ1 γδT cells were positively selected by MACS[Miltenyi Biotec GmbH, Cologne, NRW, Germany). For further purification of Vδ1 γδ T cells, the residual αβ+ T cells were depleted using PE-conjugated anti-αβTCR antibody with anti-PE microbeads.

2.4. Functional Evaluation of Vδ1 γδ T cells

The enriched Vδ1 γδ T cells were incubated in the presence of IL-23 (10 ng/ml) under 37°C, 5%CO_2 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 Vδ1 γδ 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 μl medium. The cells were added with 10μl 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±standard deviation (SD) of triplicate determinations.

Activation

Vδ1γδT 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 Vδ1γδT cells was determined by FACSas described previously[7, 9].

Production of proinflammatory cytokines

The concentration of IFNγ and IL-17 in the supernatants from the cultured Vd1 γδ T cells and the colonic tissue were measured by ELISA in accordance with the manufacture's instruction.

2.5. Statistics analysis

Data were analyzed using Student's t-test (SPSS 19.0 software). Data were expressed as the mean ± 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 ($P > 0.05$).

Results

3.1 Proportion of gd T cells subset

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

Proportion of gd T cells subset in peripheral blood from PI-IBS patients was detected by FACS (PE-d1TCR/APC- d2TCR). Usually, Vδ1 γδ T cells dominate in local mucosa tissue and Vδ2 γδ T cells were the major peripheral γδ T cells. However, in PI-IBS patients' peripheral blood, Vδ1 γδ T cells significantly increased and became the predominant subset, while Vδ1 γδ T cells decreased and became the minor subset (Figure1, Table 1). The results suggest that the γδ T cells' characteristic subset profile shifting from Vδ2 to Vδ1 could have potential functional significance.

3.1.2. Enrichment and identifying of Vd1 gd T cells

The Vd1 gd 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 αβ T cells were depleted, the proportion of the purified Vd1 gd 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 Vd1 gd T cells guaranteed their functional analysis in vitro.

3.2 Functional analysis of Vd1 gd T cells

The function of the enriched Vd1 gd T cells was evaluated by proliferation assay, activation assay and pro-inflammatory cytokines production assay. Firstly, CCK-8 assay show that Vd1 gd 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. The changes of the expression of these two molecules indicated that the cells' activation occurred. Furthermore, stimulated with IL-23, the enriched Vd1 gd 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. These results revealed the functional profile of Vd1 gd 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 ultrosonic 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.

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[23]. 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[24-26]. 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 servere sepsis. It is reported that murine IL-17 +V g4 T lymphocytes accumulate in the lungs and play a protective role during servere 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. [27-29].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. Theses 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 [21]. Thus our results suggested that Vd1 gdT cells could functionally is 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 helps 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.

Conclusions

Various variable regions determine different immune response by gd T cells subset in diverse pathological conditions. In patients with post-infectious irritable bowel Syndrome (PI-IBS), we found that Vd1 gd T cells dominated in the peripheral blood and colonic tissue, proliferating, activating and producing IL-17. The 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.

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 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**gd**T 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: LAN Cheng : the conception and design of the work, guarantor of integrity of entire study

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

XN Sun: design of the work

ZC Ma: the acquisition of data

J Fu: the acquisition of data

FJ Liu: the acquisition of data

BL Huang: the acquisition of data

DC Liang: the analysis and interpretation of data

DM Sun: interpretation of data and substantively revised the manuscript,

***all authors have read and approved the manuscript**

6. **Acknowledgements**

We express our gratitude to Dr. Willi Born for the supplication of anti-δTCR antibodies (GL3 clone).

7. **Availability of data and materials**

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

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

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Table

Table 1. Proportion of gd T cells subset in peripheral blood from PI-IBS patients

	number	Vδ1 (%)	Vδ2 (%)
Control	n=33	12.46±2.10	76.87±7.93
PI-IBS	n=35	80.18±6.24[a]	17.48±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

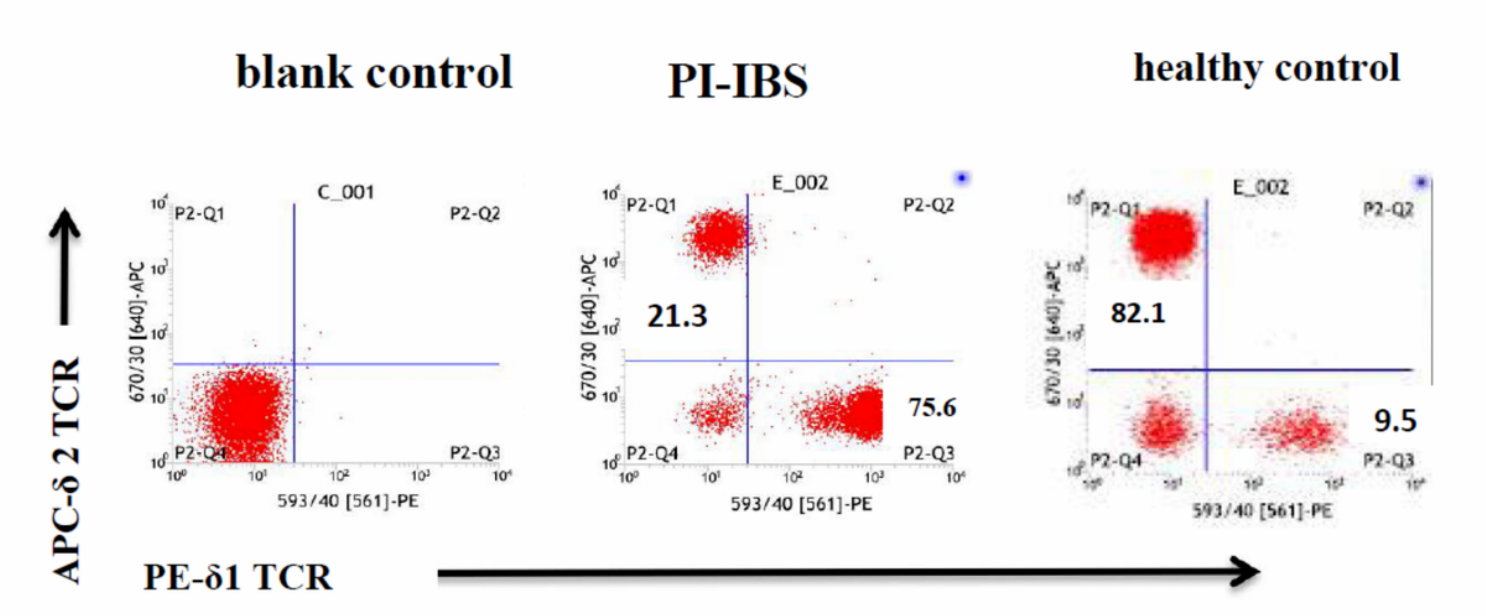

Figure 1

Proportion of ▯▯ T cells subset in peripheral blood from PI-IBS patients

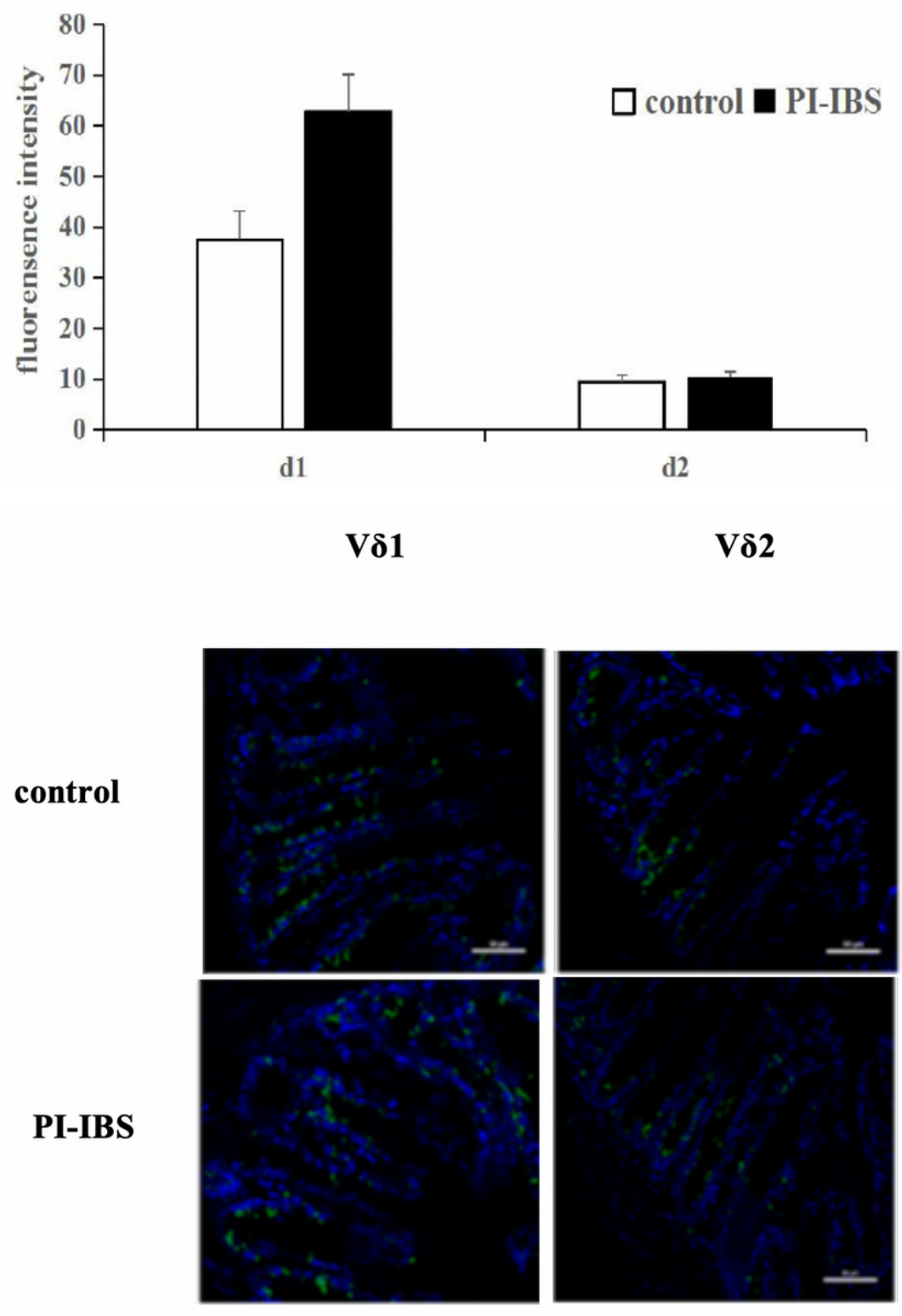

Figure 2

Proportion of ▯▯ T cells subset in colonic tissue from PI-IBS patients Figure2a. the subset phenotype of the intestinal ▯▯ T cells (under confocal laser scanning microscopy, oringinal magnification ×200). Figure 2b the quantitative analysis of the fluorescence intensity

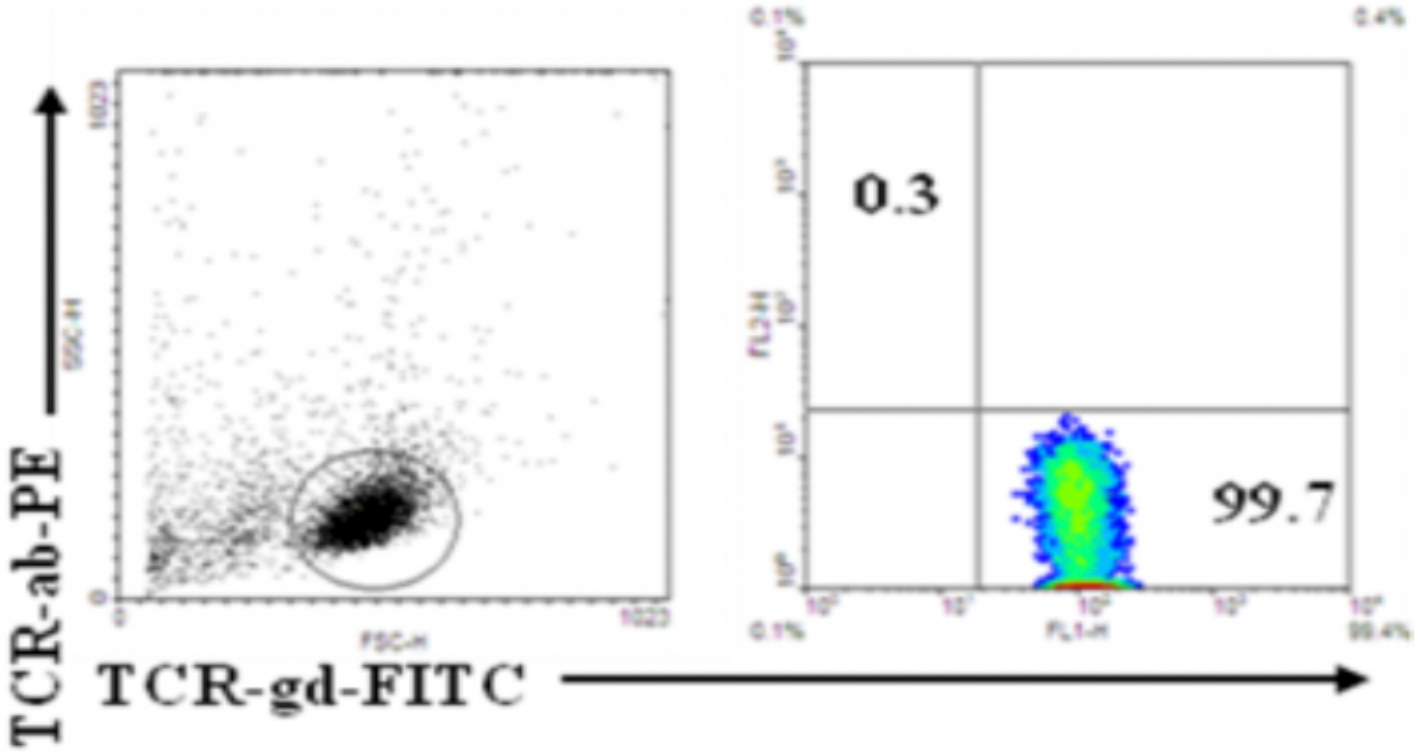

Figure 3

Enrichment of V□1 □□ T cells

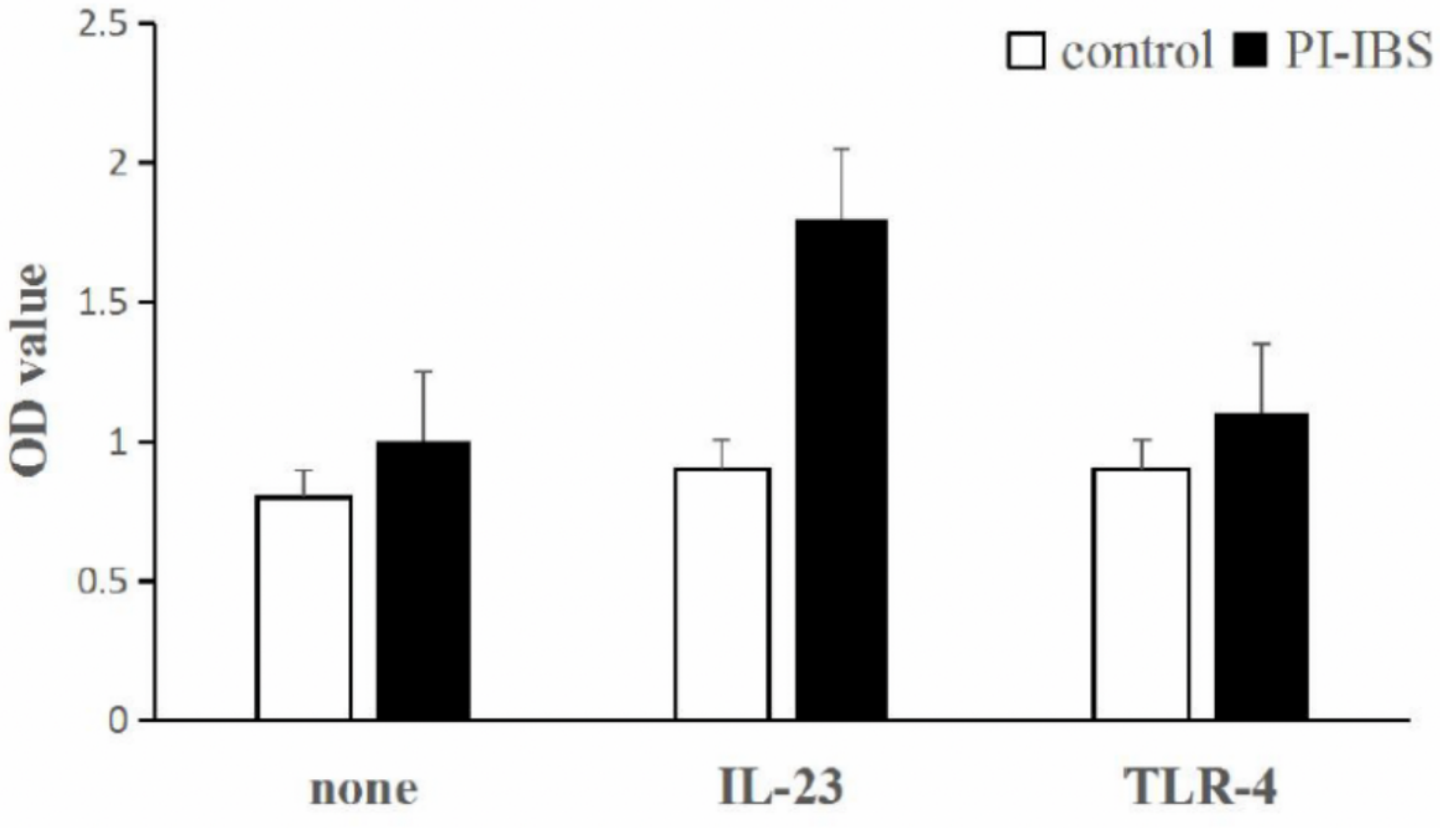

Figure 4

CCK-8 Assay was used to evaluate the enriched Vδ1 γδ T cells' proliferation stimulated with IL-23 orTLR-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.

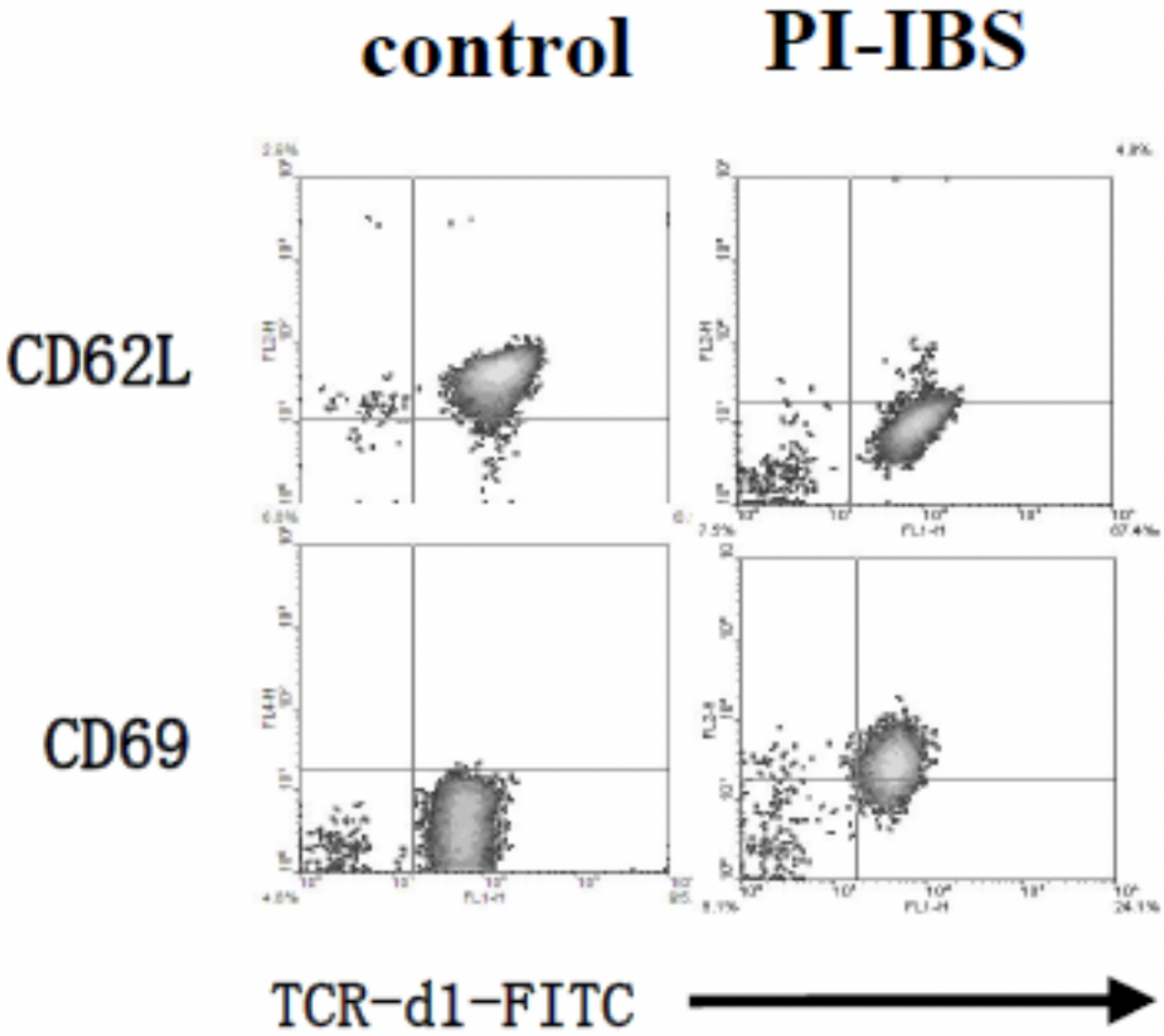

Figure 5

The expression of CD62L and CD69 molecules on the enriched Vδ1 γδ T cells were detected to evaluate the activation status of the subset. As shown in figure5, compared with the healthy human, CD62L remarkably decreased while that of CD69 molecule increased on the Vδ1 γδ T cells from PI-IBS patients, indicating that the subset cells' activation occurred.

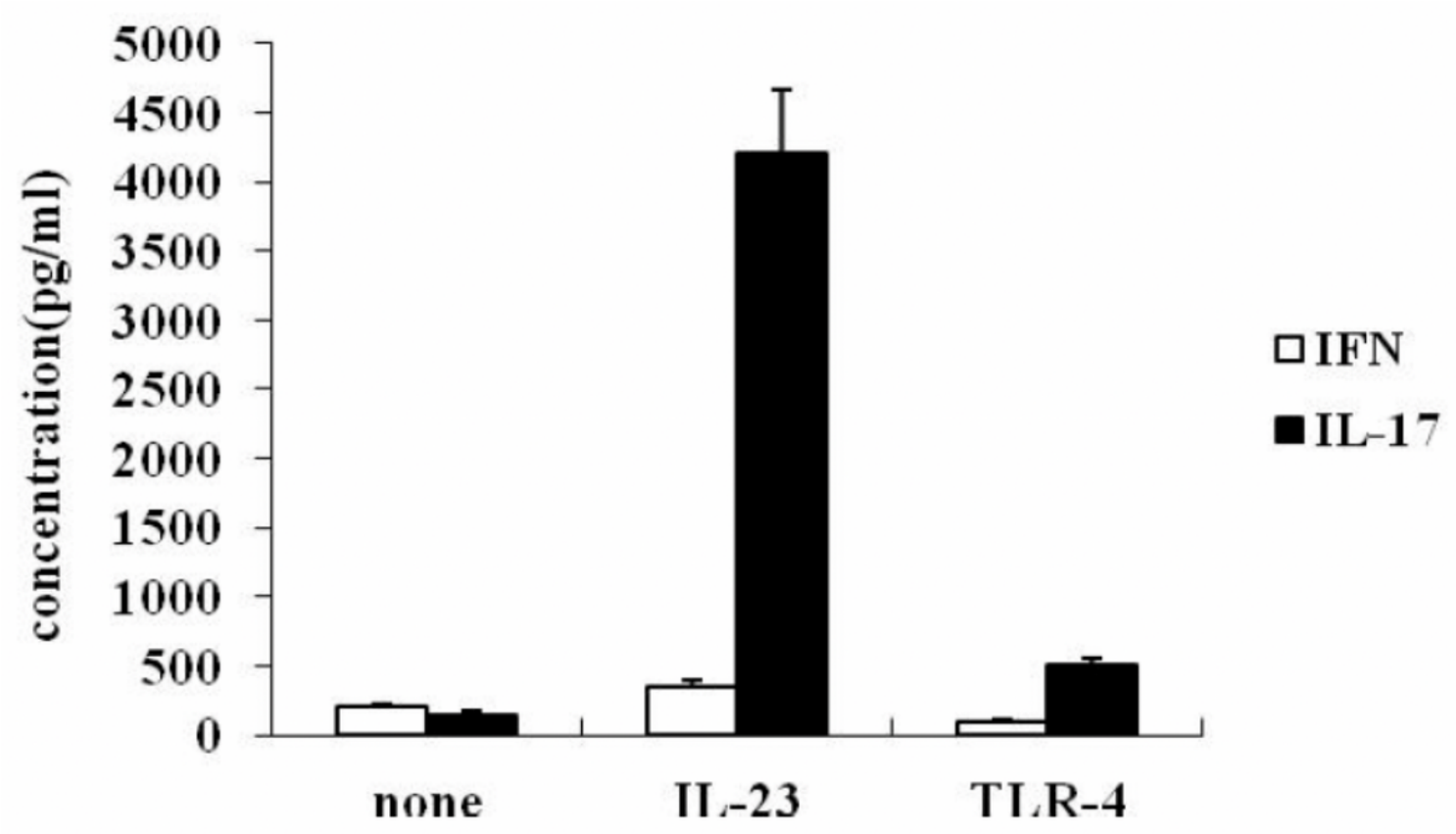

Figure 6

The capability of producing inflammatory cytokines by the Vδ1 γδ T cells was evaluated by detecting the concentration of IL-17 and IFN-γ in the supernatants of the cultured subset. As shown in figure6, compared with the control group, the Vδ1 γδ T cells from the PI-IBS patients produced abundant IL-17 but not IFN-γ after stimulated by IL-23.

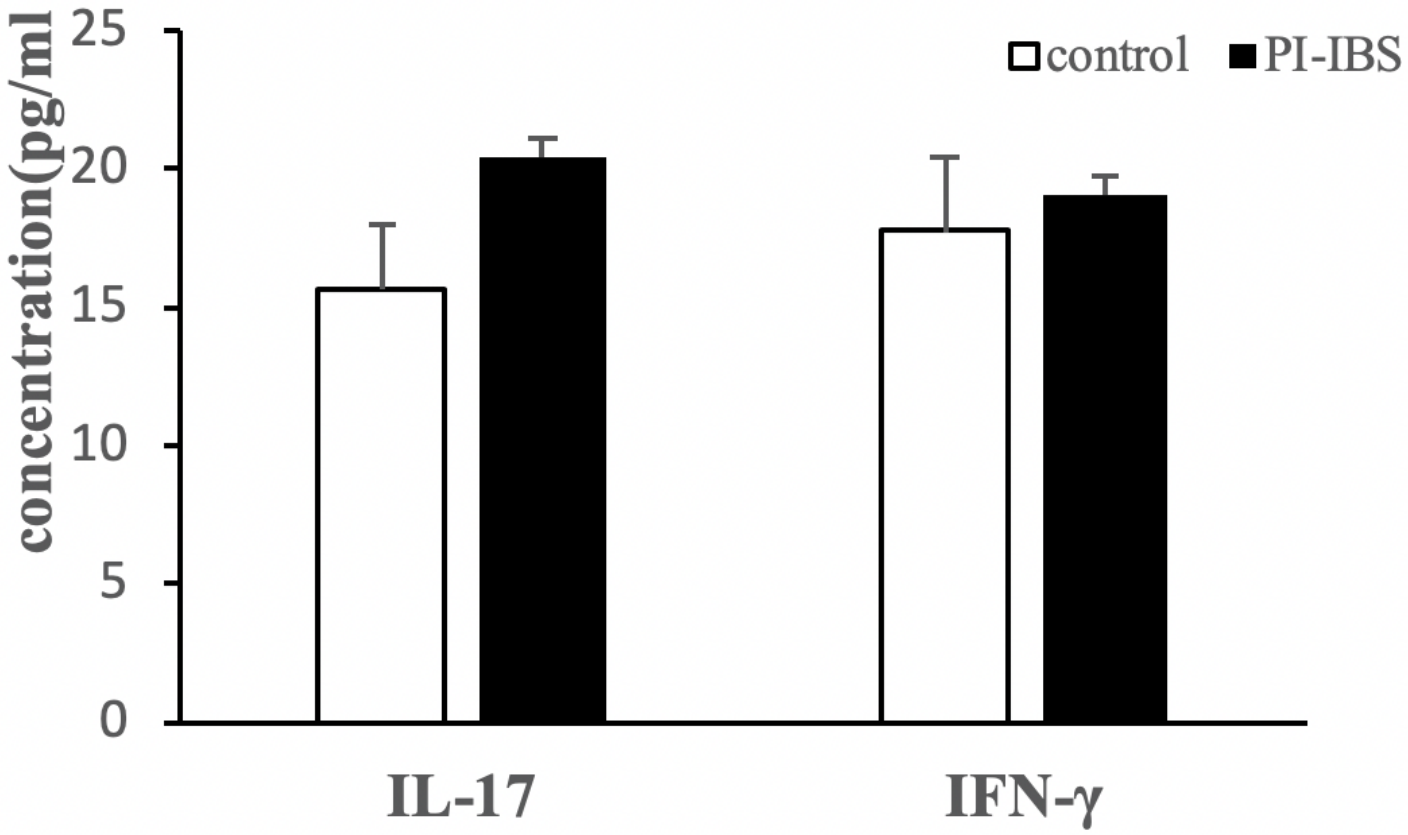

Figure 7

The concentration of IL-17 and IFN-γ in the peripheral blood was measured by ELISA. As shown in figure7, compared with the control group, the peripheral IL-17 level significantly increased, suggesting the Vδ1 γδ T cells' potential role.

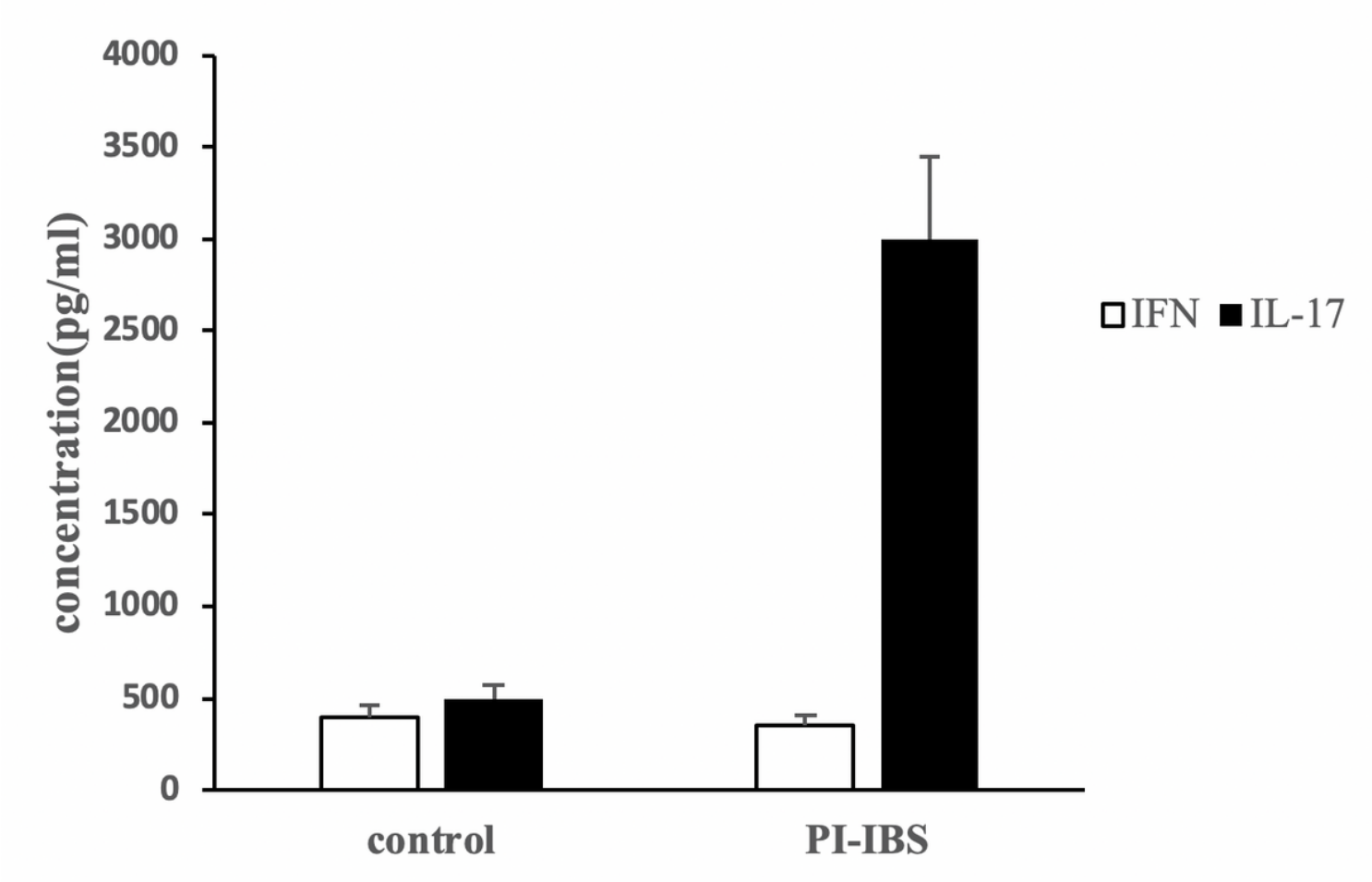

Figure 8

The concentration of IL-17 and IFN-γ in the colon tissue was measured by ELISA. As shown in figure7, 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 Vδ1 γδ T cells suggested that this kind of subset could participated in PI-IBS through producing IL-17.