



Changes in peripheral blood inflammatory factors (TNF- α and IL-6) and intestinal flora in AIDS and HIV-positive individuals

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Received Feb. 20, 2019; Revision accepted June 30, 2019; Crosschecked Aug. 8, 2019

Abstract: Objective: In this study, we investigated the changes in peripheral blood inflammatory factors and intestinal flora in acquired immune deficiency syndrome (AIDS) and human immunodeficiency virus (HIV)-positive individuals (AIDS/HIV patients), and explored the relationships among intestinal flora, peripheral blood inflammatory factors, and CD4⁺ T lymphocytes. Methods: Thirty blood and stool samples from an AIDS group and a control group were collected. The levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were determined by enzyme-linked immunosorbent assay (ELISA), and the number of CD4⁺ T lymphocytes by a FACSCount automated instrument. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the messenger RNA (mRNA) levels of *Bifidobacterium*, *Lactobacillus*, *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus faecium*. Correlations among intestinal flora, inflammatory factor levels, and CD4⁺ T lymphocyte values were evaluated using the Spearman correlation coefficient. Results: The levels of TNF- α and IL-6 in the AIDS group were higher than those in the control group, while the number of CD4⁺ T lymphocytes was lower. The amounts of *Bifidobacterium* and *Lactobacillus* in the AIDS group were significantly lower than those in control group, while the amounts of *E. coli*, *E. faecalis*, and *E. faecium* were much higher. The amounts of *Bifidobacterium* and *Lactobacillus* were negatively correlated with the content of TNF- α and IL-6 and the CD4⁺ T lymphocyte count, while those correlations were reversed for *E. coli*, *E. faecalis*, and *E. faecium*. Conclusions: The intestinal microbiota of AIDS/HIV patients were disordered, and there was a correlation between the amount of intestinal flora and the number of CD4⁺ T lymphocytes and the levels of TNF- α and IL-6.

Key words: Acquired immune deficiency syndrome (AIDS); Tumor necrosis factor- α (TNF- α); Interleukin-6 (IL-6); Intestinal flora; CD4⁺ T lymphocytes

<https://doi.org/10.1631/jzus.B1900075>

CLC number: R512.91

1 Introduction

Acquired immune deficiency syndrome (AIDS) is an immune system disease caused by human immunodeficiency virus (HIV) infection and CD4⁺ T cell depletion. AIDS patients have several co-morbidities, including gastrointestinal disease, systemic immune activation, malignancy, liver disease, bone disease, and

neurological complications (Capeau, 2011; Kolgiri and Patil, 2017), which have a clear impact on survival. Previous studies have reported that antiretroviral therapy (ART) successfully controls systemic HIV replication, but immune recovery is variable (Maartens et al., 2014; Monaco et al., 2016). One of the characteristics of AIDS is a rapid depletion of CD4⁺ T cells in the gut-associated lymphoid tissue (Klatt et al., 2008). In addition, AIDS can lead to gastrointestinal disease characterized by increased gastrointestinal inflammation, diarrhea, and malabsorption (Brenchley, 2013).

It has been reported that dysbiosis of the microbiota plays an important role in gut dysfunction and

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gastrointestinal inflammation (Klase et al., 2015). The human intestine is a complex ecosystem inhabited by the microbiome, including bacteria, viruses, archaea, and eukaryotic organisms. Previous studies have suggested that HIV infection may be related to obvious changes in enteric bacterial populations (Dillon et al., 2014; Dinh et al., 2015; Monaco et al., 2016). The bacterial microbiome includes *Bifidobacterium*, *Lactobacillus*, *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus faecium*. *E. faecalis* and *E. faecium* are commonly used in human intestinal colonization (Quiloan et al., 2012). *Lactobacillus* and *Bifidobacterium* can prevent gastrointestinal infections and restore an effective intestinal barrier. They also have a positive effect on the immune function of HIV/AIDS patients (Irvine et al., 2010). *E. coli* is the most common bacterium associated with diarrhea in HIV patients (Shah et al., 2016). Moreover, bacterial microbiome alteration has been reported to be linked to inflammatory bowel disease (IBD) and inflammation in HIV-1 infection (Bäckhed et al., 2012; Lyte, 2013; Márquez et al., 2016). However, a better understanding of the relationships established among inflammatory factors, CD4⁺ T lymphocyte counts, and the intestinal flora compartment in AIDS is urgently needed.

In this study, we investigated the levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), and the amounts of CD4⁺ T lymphocytes, *E. coli*, *Lactobacillus*, *Bifidobacterium*, *E. faecalis*, and *E. faecium*, as well as the correlations among them. Our results revealed that the intestinal microbiota of AIDS/HIV patients were disordered. The results also confirmed that there was a correlation between the amount of intestinal flora and the number of CD4⁺ T lymphocytes and levels of TNF- α and IL-6. The findings of our study may provide a new theoretical foundation for the clinical treatment of AIDS/HIV patients.

2 Materials and methods

2.1 Patients and groups

Thirty AIDS/HIV patients (18 males and 12 females, aged 20–65 years) were included in the AIDS group. They were selected from the outpatient ($n=21$) and inpatient ($n=9$) departments of Qingdao Sixth People's Hospital (Qingdao, China) from April

2016 to April 2018. The statistical characteristics of the patients are shown in Table 1. Subjects who had used antacids within the previous two months were excluded. Patients with an active opportunistic infection or inflammation were also excluded from our cohort. Simultaneously, 30 healthy volunteers (15 males and 15 females, aged 23–61 years), were set as the control group. None of the volunteers had a history of gastrointestinal diseases or hepatobiliary diseases, and none had used antibiotics or microecological preparations within two months before the experiment. This study was approved by the Ethics Committee of Qingdao Sixth People's Hospital and informed consent was signed by the patients and healthy volunteers.

Table 1 Clinical characteristics of AIDS/HIV patients

Characteristics	Patients	
	Number	Percentage (%)
Male	18	60.0
Female	12	40.0
Outpatient	21	70.0
Inpatient	9	30.0
Infection pathway		
Drug abuse	10	33.3
Sexual transmission	16	53.4
Blood transmission	4	13.3
Antiretroviral therapy (ART)		
Yes	10	33.3
No	20	66.7
Antibiotics		
Yes	25	83.3
No	5	16.7
Acute illness		
Yes	3	10.0
No	27	90.0
CD4 ⁺ cell count (cells/ μ L)		
>500	5	16.7
200–500	15	50.0
<200	10	33.3
Median age (year)		39.5
Median CD4 ⁺ cell count (cells/ μ L)		304.5
HIV viral load* (copies/mL plasma)		
ART treatment		20 (0–50)
No treatment		95672 (24455–285548)

*The values for HIV viral load are expressed as median (range)

2.2 Detection of inflammatory factors by ELISA

Fasting venous blood samples (3–5 mL) were collected and stored at 4 °C, centrifuged at 2000 r/min

for 10 min, and the plasma was dispensed into 2-mL Eppendorf (EP) tubes and stored at -80°C . The levels of TNF- α and IL-6 were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp., USA). The absorbance value was measured by an absorbance detector at 450 nm.

2.3 Detection of CD4⁺ T lymphocytes by flow cytometry

The number of CD4⁺ T lymphocytes was assessed using a FACSCalibur type flow cytometer and a tricolor fluorescent antibody labeling kit (BD, USA), as described by Yao et al. (2017) and Zhang SG et al. (2017). According to the reagent specification, each sample was processed in a TruCONT tube dedicated to flow cytometry. A total of 20 μL of monoclonal antibody (mAb) was firstly added to the TruCONT tube, and then 50 μL of whole blood containing ethylenediaminetetraacetic acid (EDTA) was added. After 15 min in the dark, 450 μL of 10% (0.1 g/mL) fluorescence activated cell sorter (FACS) hemolysin was added and the tube was placed in the dark for 15 min. Finally, the number of CD4⁺ T lymphocytes was determined using MultiSET software.

2.4 qRT-PCR

Fresh feces from the two groups were collected and placed on ice. A total of 220 mg of medium stool was weighed, and the fecal genomic DNA was extracted using a bacterial genomic DNA extraction kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). The DNA concentration of each sample was tested and adjusted to 20 $\mu\text{g}/\text{mL}$, and then the amounts of *Bifidobacterium*, *Lactobacillus*, *E. coli*, *E. faecalis*, and *E. faecium* in fecal samples were separately quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Standards were set for simultaneous amplification in each reaction, and the DNA template was replaced with sterile ddH₂O as a negative control. A total of 20 μL of reaction system was used for qRT-PCR, including 10 μL 2 \times SYBR mixture uracil-*N*-glycosylase (UNG; including reactive oxygen species (ROX)), 0.75 μL upstream and downstream primers, 2.0 μL DNA template, and 6.5 μL sterilized ddH₂O. Then, the amplification was carried out according to the following procedures: UNG enzymes acted at 50°C for 5 min, pre-denatured at 95°C for 10 min followed by

35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. The C_T value and the standard curve were calculated as a quantitative result. The target gene sequence was searched from GenBank (<https://www.ncbi.nlm.nih.gov>), and primer design was carried out using Primer Premier 5.0 (Primer, Canada). Five primers were verified online by BLAST software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and synthesized by Shanghai Biotech Engineering Services Co., Ltd., Shanghai, China. The primer sequences are shown in Table 2.

Table 2 Primer sequences

Name	Sequence (5'→3')
<i>Bifidobacterium</i>	F: TCGCCTCCGGGTGAGAGTGG
	R: CGAAGCCATGGTGGGCGGTT
<i>Lactobacillus</i>	F: TGGAAACAGGTGCTAATACCG
	R: GTCCATTGTGGAAGATTCCC
<i>Escherichia coli</i>	F: CTCGCTGGCATTGCGTAC
	R: ATCTTTTGCCGTCGTTTTGG
<i>Enterococcus faecalis</i>	F: TTGGCATTCCACAAGTACCA
	R: AATTGCTCGGGCATCATAAC
<i>Enterococcus faecium</i>	F: TCGGACGACTTTGCTGGCG
	R: TCGCACCGGCTCAATCCGC

F: forward; R: reverse

2.5 Statistical analysis

The experimental measurements are expressed as mean \pm standard deviation (SD), and a sample *t*-test was performed using GraphPad Prism 5.0 software. The correlations between the amount of intestinal flora and the levels of TNF- α and IL-6 and the number of CD4⁺ T lymphocytes were analyzed by Spearman correlation. All experiments were carried out three times. If $P < 0.05$, statistical significance was accepted.

3 Results

3.1 Study population characteristics

The median age of patients in the AIDS group was 39.5 years (range 20–65 years) and 60% were male. Drug abuse (33.3%), sexual transmission (53.4%), and blood transmission (13.3%) were the main routes of infection. Ten subjects (33.3%) in the AIDS group received ART treatment for six months, which consisted of tenofovir (20 mg/kg) and emtricitabine (30 mg/kg). In addition, 25 patients were treated with antibiotics

consisting of vancomycin (15 mg/kg) and enrofloxacin (10 mg/kg) for one month. ART-treated participants had undetectable viral loads (<20 copies/mL plasma) and participants without ART treatment had high viral loads (95 672 copies/mL plasma). Three patients suffering acute illness were clinically diagnosed AIDS patients and the other 27 were HIV-positive patients, including patients not yet under treatment. Among the 30 patients, 10 (33.3%) had a CD4⁺ cell count of <200 cells/ μ L, 15 (50.0%) had a CD4⁺ cell count of 200–500 cells/ μ L, and 5 (16.7%) had a CD4⁺ cell count of >500 cells/ μ L. Three (10.0%) were suffering acute illness (Table 1).

3.2 Higher levels of TNF- α and IL-6 in the peripheral blood of AIDS/HIV patients

The measured levels of inflammatory factors in the peripheral blood of the AIDS group and the control group are shown in Fig. 1. TNF- α and IL-6 levels were markedly higher in subjects in the AIDS group than in the control group ($P<0.05$). The results illustrated that the expression of inflammatory factors in the peripheral blood of AIDS/HIV patients was abnormally high.

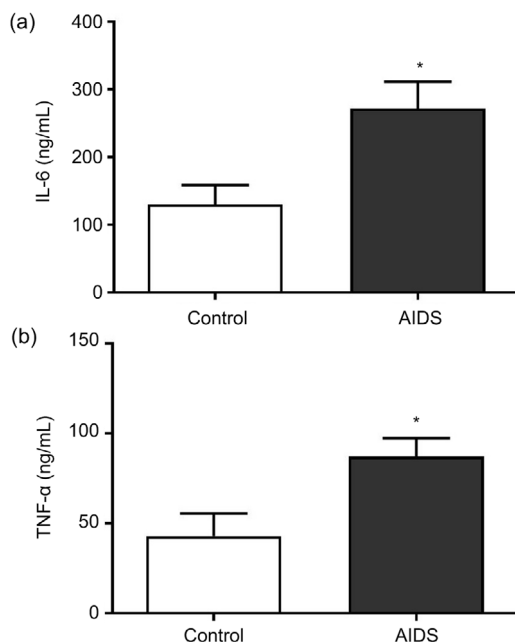


Fig. 1 Expression levels of IL-6 and TNF- α in the AIDS and control groups

(a) The expression level of IL-6; (b) The expression level of TNF- α . Data from experiments carried out three times are presented as mean \pm SD. * $P<0.05$, vs. control

3.3 Lower number of CD4⁺ T lymphocytes in AIDS/HIV patients

The numbers of CD4⁺ T lymphocytes in the AIDS and control groups are shown in Fig. 2. The number of CD4⁺ T lymphocytes in the AIDS group was much lower than that in the control group ($P<0.05$), suggesting that the number of CD4⁺ T lymphocytes was abnormally low in AIDS/HIV patients.

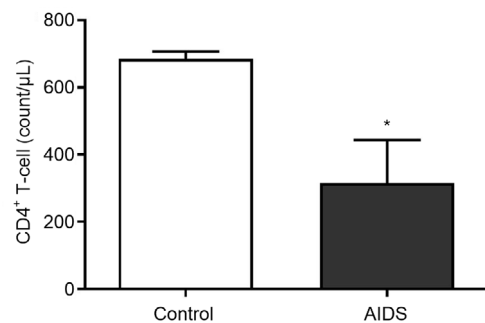


Fig. 2 Numbers of CD4⁺ T lymphocytes in the AIDS and control groups

Data from experiments carried out three times are presented as mean \pm SD. * $P<0.05$, vs. control

3.4 Changes in the amounts of *Bifidobacterium*, *Lactobacillus*, *E. coli*, *E. faecalis*, and *E. faecium*

The relative amounts of *Bifidobacterium* and *Lactobacillus* in the AIDS group were markedly lower than those in the control group ($P<0.05$; Figs. 3a and 3b). However, the amounts of *E. coli*, *E. faecalis*, and *E. faecium* in the AIDS group were significantly higher than those in the control group ($P<0.05$; Figs. 3c–3e). These findings indicated that the amounts of intestinal flora changed abnormally and the intestinal microbiota were destroyed.

3.5 Correlations of the amount of intestinal flora with the levels of TNF- α and IL-6 in peripheral blood

Spearman correlation analysis showed that the amounts of *Bifidobacterium* and *Lactobacillus* in the AIDS group were negatively correlated with the level of TNF- α ($P<0.05$; Figs. 4a and 4b), while the amounts of *E. coli*, *E. faecalis*, and *E. faecium* were positively correlated with the level of TNF- α ($P<0.05$; Figs. 4c–4e). Similarly, the amounts of *Bifidobacterium* and *Lactobacillus* in the AIDS group were negatively

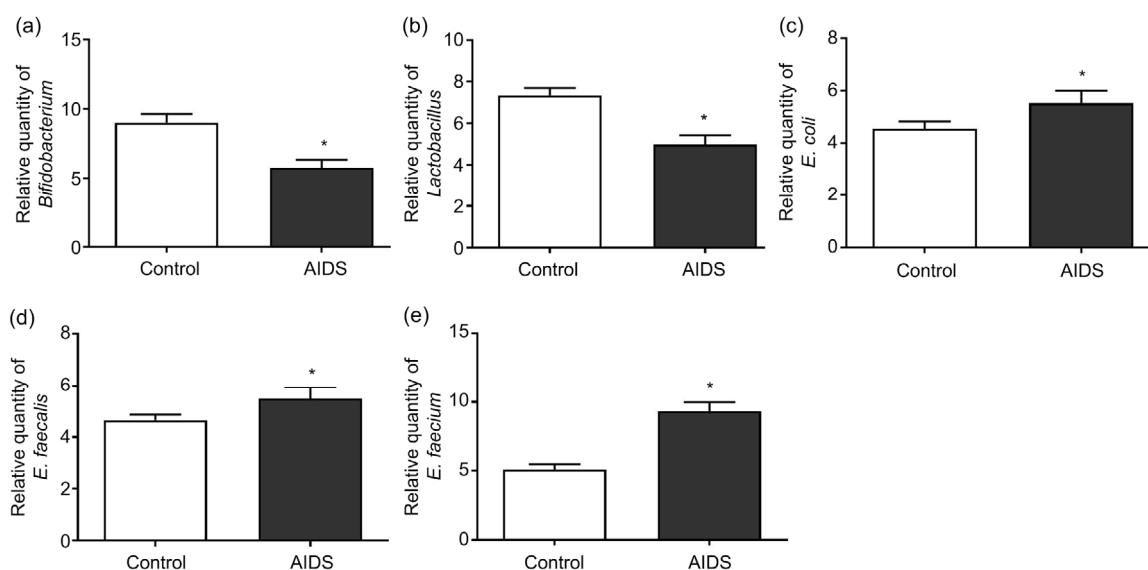


Fig. 3 Amounts of different bacteria in the AIDS and control groups

(a) *Bifidobacterium*; (b) *Lactobacillus*; (c) *Escherichia coli*; (d) *Enterococcus faecalis*; (e) *Enterococcus faecium*. Data from experiments carried out three times are presented as mean \pm SD. * $P < 0.05$, vs. control

correlated with the level of IL-6 ($P < 0.05$; Figs. 4f and 4g). In contrast, the amounts of *E. coli*, *E. faecalis*, and *E. faecium* were positively correlated with the level of IL-6 ($P < 0.05$; Figs. 4h–4j).

3.6 Correlations of the amount of intestinal flora with the number of CD4⁺ T lymphocytes in peripheral blood

The correlations between the intestinal flora and the number of CD4⁺ T lymphocytes were determined using Spearman correlation analysis. The results showed that the amounts of *Bifidobacterium* and *Lactobacillus* in the AIDS group were negatively correlated with the number of CD4⁺ T lymphocytes ($P < 0.05$; Figs. 5a and 5b). In contrast, the amounts of *E. coli*, *E. faecalis*, and *E. faecium* in the AIDS group were positively correlated with the number of CD4⁺ T lymphocytes ($P < 0.05$; Figs. 5c–5e).

4 Discussion

Intestinal parasitic infections and HIV/AIDS are major public health problems in developing countries, and are the main causes of high mortality rates (Gedle et al., 2017). The progression of AIDS is a diverse

process. Slow or rapid progression could present distinct behaviors of specific cell subpopulations and compartments, and components of the patient's microbiota. HIV/AIDS is characterized by the targeting and destruction of CD4⁺ T lymphocytes in peripheral blood. Importantly, CD4⁺ T cells are the key to host defense and the primary driver of immune-mediated disease (O'Shea and Paul, 2010; Zhang J et al., 2017). In this study, the number of CD4⁺ T lymphocytes in peripheral blood was shown to be very low in AIDS/HIV patients. This was consistent with previous research which revealed that high levels of CD4⁺ T lymphocytes could improve the quality of life of AIDS patients (Taiwo et al., 2009; Ren, 2018).

Immune activation has long been recognized as an important consequence of untreated HIV infection and a predictor of AIDS progression (Utay and Hunt, 2016). In particular, IL-6, a pro-inflammatory cytokine, has a proinflammatory role in the regulation of immune responses (Hirano and Kishimoto, 1989). Elevated levels of IL-6 can indicate a systemic inflammatory status, which is used as a marker of the HIV infection process (de Medeiros et al., 2016). Sobti et al. (2010) reported that suppressing IL-6 might decrease viral replication in the host after infection. TNF- α is an important mediator of inflammation and

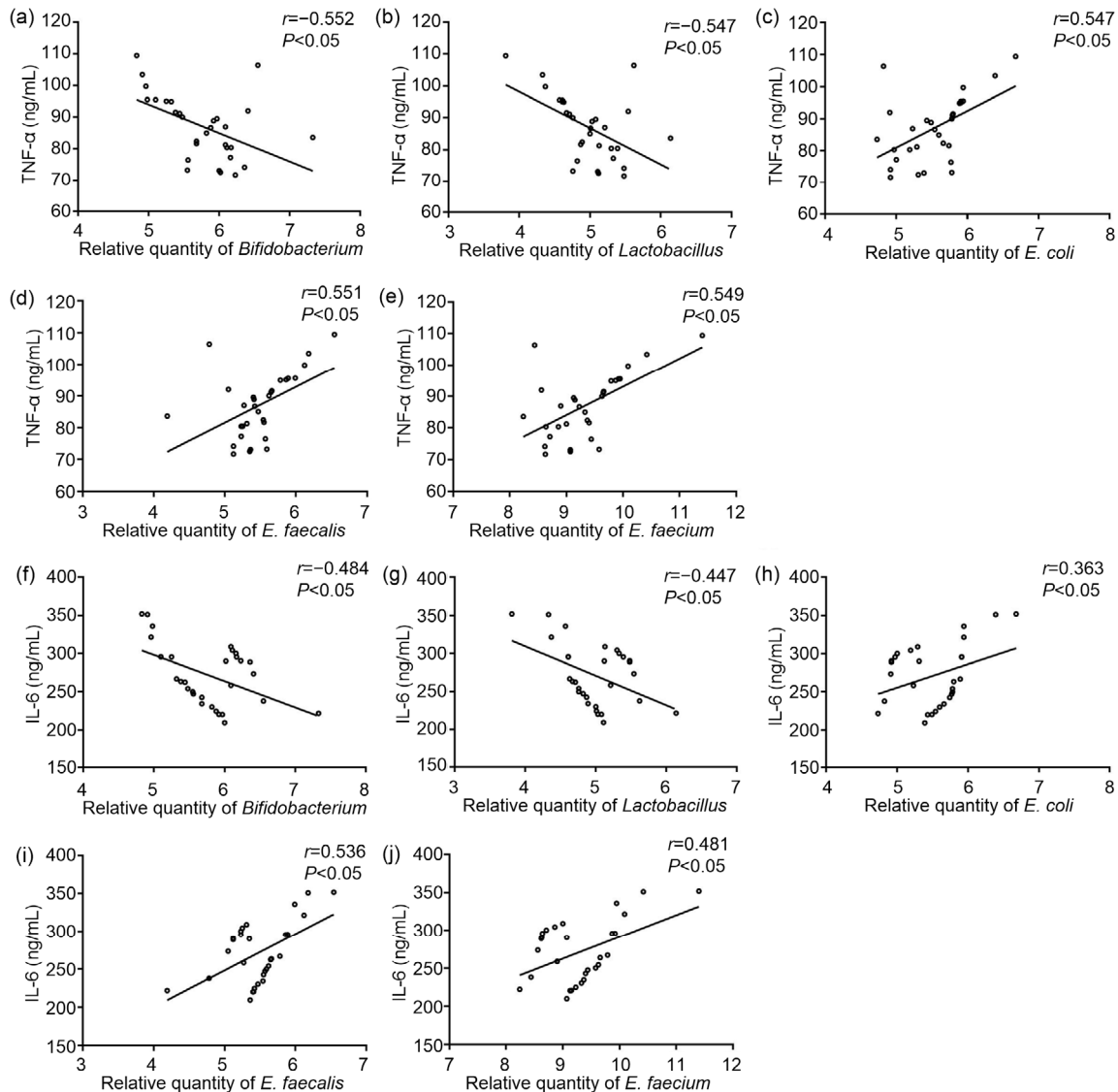


Fig. 4 Correlation analysis between the contents of intestinal flora and inflammatory factors

Correlations between the content of *Bifidobacterium* and the level of TNF- α (a), *Lactobacillus* and the level of TNF- α (b), *Escherichia coli* and the level of TNF- α (c), *Enterococcus faecalis* and the level of TNF- α (d), *Enterococcus faecium* and the level of TNF- α (e), *Bifidobacterium* and the level of IL-6 (f), *Lactobacillus* and the level of IL-6 (g), *E. coli* and the level of IL-6 (h), *E. faecalis* and the level of IL-6 (i), *E. faecium* and the level of IL-6 (j)

immune response (Fragoso et al., 2014). The level of TNF- α is much higher in AIDS/HIV patients than in healthy people, and continuous changes of TNF- α levels might lead to tissue damage and contribute to the development of other diseases (de Pablo-Bernal et al., 2014). In this study, levels of TNF- α and IL-6 were higher in the AIDS group, strongly suggesting that the progression of AIDS might be related to inflammatory response.

Elevated IL-6 in HIV-infected patients was related to impeded recovery of low CD4 levels (Rosado-Sánchez et al., 2017). CD4 is a T cell receptor for major histocompatibility complex class II antigens, a key regulator of immune response, and also a receptor for HIV (Wang et al., 1994). Evidence suggests that poor CD4⁺ T lymphocyte recovery is associated with microbial translocation in HIV-infected patients (Ponte et al., 2016). The enrichment of gut bacteria

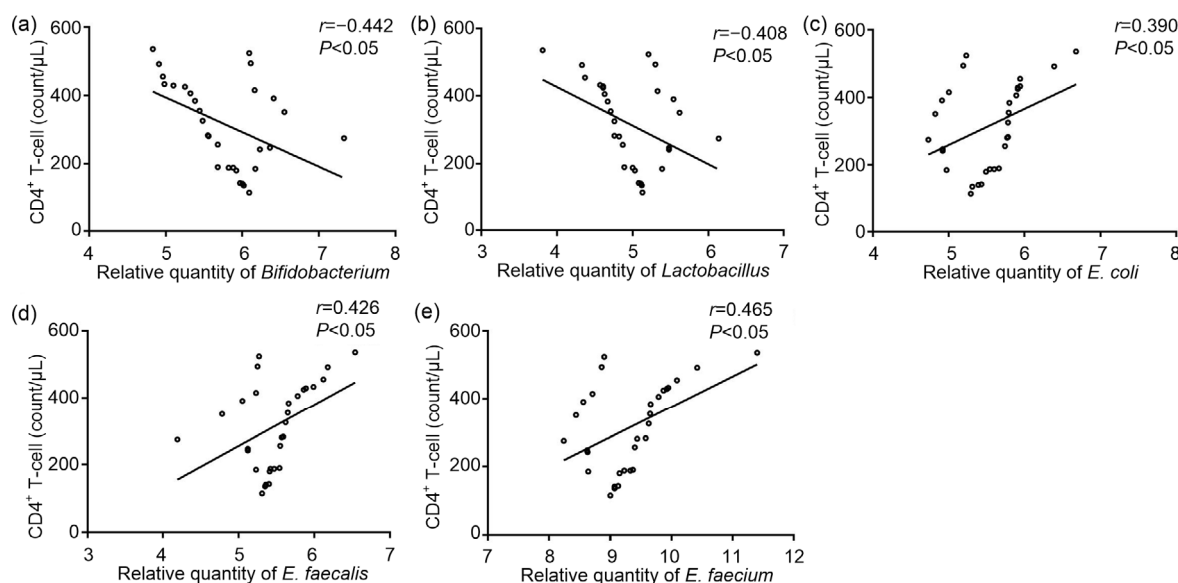


Fig. 5 Correlation analysis between the number of CD4⁺ T lymphocytes and the content of intestinal flora

Correlations between the number of CD4⁺ T lymphocytes and the content of *Bifidobacterium* (a), *Lactobacillus* (b), *Escherichia coli* (c), *Enterococcus faecalis* (d), *Enterococcus faecium* (e)

has been reported to be associated with poor CD4⁺ T cell reconstruction (Lu et al., 2018). Previous studies have indicated that the composition of gut microbiota is related to cancer immunotherapy (Routy et al., 2018) as well as HIV infection (Vujkovic-Cvijin et al., 2013). The human gastrointestinal tract is a complex ecosystem inhabited by the microbiome, including bacteria, viruses, archaea, and eukaryotic organisms. Probiotics can improve intestinal barrier function and even enhance the immunity of HIV-infected individuals by reducing the duration of diarrhea (Monachese et al., 2011). When the intestinal flora of AIDS patients is dysfunctional, the levels of *Bifidobacterium* and *Lactobacillus* are significantly lower, and the contents of *E. coli*, *E. faecalis* and *E. faecium* are higher (Lei et al., 2012). Shah et al. (2016) reported that *E. coli* is the most common bacterium associated with diarrhea in HIV patients. In addition, bacterial microbiome alteration is linked to IBD and inflammation in HIV-1 infection (Bäckhed et al., 2012; Lyte, 2013; Márquez et al., 2016). In the current study, the amounts of *Bifidobacterium* and *Lactobacillus* were low and negatively correlated with TNF- α and IL-6, and the number of CD4⁺ T lymphocytes. The indications and correlations above in *E. coli*, *E. faecalis*, and *E. faecium* were exactly opposite to those of

Bifidobacterium and *Lactobacillus*. Our results were consistent with those of previous studies. For example, He et al. (2014) pointed out that the amount of *Enterococcus* in AIDS/HIV patients increased markedly, and CD4⁺ T lymphocyte counts were strongly and negatively correlated with *Enterococcus* counts. Li and Zhong (2017) reported that the expression of inflammatory cytokines (IL-6 and TNF- α) in peripheral blood of patients with ulcerative colitis was higher than that of healthy people, and was negatively correlated with the contents of *Bifidobacterium* and *Lactobacillus*, but positively correlated with the contents of *Enterobacter* and *Enterococcus*.

There were some limitations in our study. Firstly, our study was based on a small number of Chinese subjects and did not include people from other countries. Next, the effects of ART and antibiotic dosages and treatment regimens on the microbiome of AIDS/HIV patients were not investigated. These limitations will be addressed in our future research.

5 Conclusions

In conclusion, the intestinal microbiota of AIDS/HIV patients were disordered, and the amount

of intestinal flora was correlated with the number of CD4⁺ T lymphocytes and the levels of TNF- α and IL-6, providing strong data to support the clinical treatment of AIDS/HIV patients.

Contributors

Jing LU and Sai-sai MA: conception and design of this study, and analysis of data. Wei-ying ZHANG and Jian-ping DUAN: revising the article critically for important intellectual content. All authors have read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Jing LU, Sai-sai MA, Wei-ying ZHANG, and Jian-ping DUAN declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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中文概要

题目: AIDS 和 HIV 阳性个体外周血炎症因子 (TNF- α 和 IL-6) 和肠道菌群的变化研究

目的: 本研究探究获得性免疫缺陷综合征 (AIDS) 和人类免疫缺陷病毒 (HIV) 阳性个体患者外周血炎症因子和肠道菌群的变化, 同时探讨了肠道菌群、外周血炎症因子和 CD4⁺ T 淋巴细胞之间的关系。

创新点: 首次探讨了 AIDS 和 HIV 阳性个体患者中肠道菌群、外周血炎症因子和 CD4⁺ T 淋巴细胞之间的

关系。

方法: 从艾滋病组和对照组分别收集 30 份血液和粪便样本。使用酶联免疫吸附测定 (ELISA) 法测定肿瘤坏死因子 (TNF- α) 和白细胞介素-6 (IL-6) 的水平; 用流式细胞仪测定 CD4⁺ T 淋巴细胞数目; 采用实时定量聚合酶链式反应 (qRT-PCR) 法检测双歧杆菌、乳酸菌、大肠杆菌、粪肠球菌和屎肠球菌的 mRNA 水平; 采用 Spearman 分析

肠道菌群、炎症因子与 CD4⁺ T 淋巴细胞之间的相关性。

结论: 不但 AIDS/HIV 患者体内肠道菌群紊乱, 而且肠道菌群数量与 CD4⁺ T 淋巴细胞数量以及 TNF- α 、IL-6 水平存在相关性。

关键词: 获得性免疫缺陷综合征; 肿瘤坏死因子 (TNF- α); 白细胞介素-6 (IL-6); 肠道菌群; CD4⁺ T 淋巴细胞