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Original Article

Immune-Related Genes Expression in HIV-Infected Patients with Discordance in CD4+T-Cell Levels

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Abstract

Background and Aims: Antiretroviral therapy (ART) should significantly improve the recovery of the immune system in Human immunodeficiency virus (HIV) infected patients. In some patients under ART, it was not possible to increase *CD4+* cells reasonably in spite of effective virological control, which is known as discordant immune response (DIR). The aim of this study is the evaluation of the expression of C-X-C chemokine receptor type 4 (*CXCR4),* C-C chemokine receptor type 5 *(CCR5),* interleukin 12B *(IL12B),* cluster of differentiation 3(*CD3),* tumor necrosis factor alpha (*TNFα),* protein tyrosine kinase 2 beta *(PTK2B),* and t-cell receptor beta *TCR β* genes in patients with DIR.

Materials and Methods: In this case-control study, peripheral blood mononuclear cell (PBMC) specimens from patients of the two groups were isolated, RNA was extracted: patients of the control group who were immunologic responders and patients of the case group, which were non-immunologic responders. Real-time relative quantitative *polymerase chain reaction* (PCR) was performed in duplicate using One Step PrimeScript™ RT-PCR Kit and data analyzed by using GraphPad prism software version 8.0.2.

Results: The expression levels in the patients of the case group in comparison with the patients of the control group were measured for *CXCR4*, *CCR5*, *IL12B*, *TNF-α*, *CD3*, *PTK2B* and *TCR-β*. The Fold change ratio for *CCR5*, *TCR-β*, and *CD3* were (0.225), (0.12), (0.09), respectively, and in all three of them, a significant decrease was observed with confidence values. $p < 0.05$, $p < 0.02$ and $p < 0.01$, respectively. None of the *CXCR4*, *IL12B*, *TNF-α*, and *PTK2B* was statistically significantly different and their fold change ratio was (1.01), (0.6), (1.04), and (0.718) respectively.

Conclusion: we showed significant decrease in the expression of *CCR5*, *TCR-β*, and *CD3* genes in the patients of the case group in comparison with the patients of the control group, but we could not verify this low expression of these genes are the reason of the low *CD4+* T-cell count. Further investigation is necessary, if the suppression of these genes can influence the proliferation or development of *CD4* T cells, in-vitro.

Keywords: HIV-1, discordant immune response, CD4+ cell count, gene expression, cytokine

Introduction

The human immunodeficiency virus type-1 (HIV-1) is a member of the family of *Retroviridae* and the genus *Lentivirus* causing deadly disease known as acquired immunodeficiency syndrome (AIDS) which is pandemic in the world (1).

the main cellular targets of HIV replication (2). One of the marker for the disease progression is decline or destruction of *CD4+* T-cells by HIV ultimately making the patients vulnerable to opportunistic infections leading to AIDS (3, 4). Activation, motility, function of the immune cell is done by critical controllers such as chemokines and cytokines.

Cytokines function to control virus replication during early infection of HIV and disease progression play a role in immunodeficiency and dysregulation of the immune system (5, 6).

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Cytokines are key factors which regulate the differentiation of *CD4+* T-cells and they have important function to induce the naive $CD4^+$ T cells and convert them to T-helper 1 (Th1) or Th2 cells, respectively(7). Antiretroviral therapy in triple drug combination has been displayed to effectively ameliorate symptomatic and asymptomatic human immunodeficiency virus type 1 (HIV-1) pathogenicity like morbidity and mortality in infected persons (8, 9).

Antiretroviral therapy should significantly increase $CD4^+$ T lymphocytes in peripheral blood and suppresses viral load (VL) in plasma to undetectable levels. Despite of the standard treatment, it is possible that some percentage of patients show a paradoxical response showing different *CD4+* T-cell counts and VLs (10, 11).

In 5–27% of ART treated patient's plasma HIV-1 RNA VL is not suppressed to undetectable levels while under ART, since a poor immune response occurs that is determined by meek improvements or absent in *CD4* T-cell counts. This condition involves individuals with VL decline but no immune response improvement, shortly marked as immunologic non responders. The $CD4^+$ T-cell count may stay without any changes from baseline levels or below the critical threshold of 200 cell/mm^3 (12, 13).

It has been proposed that viro-immunological discordant trends may be predicted or associated with immunological factors(14). Hence, the study of regulatory factors of $CD4^+$ T-cells that are associated with HIV infection such as *CD3*, *PTK2B*, *TCR*, *CXCR4*, *CCR5*, *IL12B*, *TNFα* and assessing their impact on the progression or failure of developing HIV to AIDS is an important approach that will greatly help the physician during treatment.

However, there are few studies focusing on immune-related genes profile in HIV-infected patients experiencing DIR worldwide.

Because of that, we aimed to evaluate expression of *CXCR4*, *CCR5*, *IL12B*, *CD3*, *TNFα*, *PTK2B* and *TCR β* genes by real-time PCR technique in two patient groups: a control group and a case group.

Methods

Sample collection: This prospective case-control study was carried out in the Hepatitis and AIDS department of Pasteur Institute of Iran from 2019 to 2020. The data for this study were extracted from the behavioral disease counseling center of Imam Khomeini Hospital in Tehran that involved twenty-eight HIV-1 infected patients who initiated efavirens+ emtricitabine+ tenofovir -based combination triple-drug antiretroviral regimen.

Inclusion criteria for 14 case group patients were: all receiving ART treatment, VL <200 copies/mL and $CD4^+$ cell count did not increase, at least 50 µl in one year, or within two years after treatment, it was below 200 µl. Inclusion criteria for 14 control group patients were: all receiving ART treatment, VL <200 copies/mL and $CD4^+$ cell count increased by at least 50 µl per year, or more than 200 µl per two years after treatment and the other inclusion criteria were as follows: obtaining consent from all cases, no Hepatitis B Virus (HBV) and no Toxoplasma. Exclusion criteria were: refusing to complete the consent form, not receiving ART treatment, and having a viral load above 200 copies/ml.

For first step, all enrolled subjects provided the study with informed written consents and then 10 ml of peripheral blood was collected in Ethylene diamine tetraacetic acid (EDTA) tubes.

The study was approved by the Ethics Committee on Biomedical Research in Pasteur Institute of Iran (ID: IR.PII.REC.1396.49).

Primer designing: In this study, 8 primer pairs were designed manually using Oligo 7 software based on mRNA reference genes and blasted into the National Center for Biotechnology Information (NCBI) database and then synthesized by Sinaclon Company (Tehran, Iran). The sequences for the cytokine genes and for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers used for amplification are listed in Table 1.

PBMC isolation and RNA extraction: Plasma samples were collected in 1.5 ml tubes

Table 1. Primer sets for real-time PCR

target genes	sequence-forward/reverse (5′→3′)	PCR product(nt)	Accession number
cxcr4	Forward: TGCCCTCCTGCTGACTATTC Reverse: TGGGGTAGAAGCGGTCACAG	86	NM_001348060.2
ccr5	Forward: AAGACATGGGGAGGAAGGACAAG Reverse: TTCTGCAACACCAACCAGGATC	104	NM_000579.3
Il12b	Forward: GTTCAGGTCCAGGGCAAGAG Reverse: TGCCCATTCGCTCCAAGATG	147	NM_002187.3
tnf-α	Forward: AACCCCGAGTGACAAGCCTG Reverse: CTCTCAGCTCCACGCCATTG	116	MH180383.1
cd3	Forward: GTGAGTACTGTGTGGAGGTGG Reverse: TCAGCAAGCCCACCTAAGATGATGG	151	NM_007648.5
Ptk2b	Forward: TTCAACACTCTCGCCGGCTTC Reverse: TGAAGTACTGCCTGGCCCTC	212	NM_173174.3
tcr-β	Forward: TTCTACCTGGGAGCTTGGGTG Reverse: CTCCCTCTCCCTTCGATGAGC	121	Z81026.1
gapdh	Forward: TCGTGGAAGGACTCATGACC Reverse: CCATCACGCCACAGTTTCCC	88	NM_001289746.2

and stored at -70°C until use. Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood by the density gradient centrifugation method using Ficoll (Lymphodex) (inno-train, Kronberg im Taunus, Germany).

Almost 10 $\times 10^6$ PBMCs cells were isolated from 10 ml of each patient's blood and 8×10^6 cells were frozen and 2×10^6cells were kept for RNA extraction.

Total RNA was extracted from 2×10^6 PBMCs by RNeasy® Plus Mini Kit (Qiagen, Germany), according to the manufacturer's instruction. Moreover, virus RNA was extracted using QIAamp DSP Virus Spin Kit according to the kit's protocol. RNAs were stocked at -70°C.

Real-time qPCR: Artus HI Virus-1 RG RT-PCR Kit was applied for measuring the viral loads in terms of viral RNA copy numbers from plasma and then real-time absolute quantitative PCR was applied by Rotor Gene 6000 (Corbett research scientific, Australia) following the manufacturer's instruction.

Real-time relative quantitative PCR was performed in duplicate using One Step PrimeScript™ RT-PCR Kit (Takara, Japan) according the manufacturer's instruction for *CXCR4*, *CCR5*, *IL12B*, *CD3*, *TNFα*, *PTK2B* and *TCR β*.

All genes expression underwent normalization to the expression of *GAPDH* that was applied as an endogenous control; and real-time PCR settings were as follows: 42°C for 15 min, then 95°C for 10 s, followed by 40 cycles of 95˚C for 5 s and 60˚C for 45 s. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (fold change ratio).

Table 2. Clinical and biological features of case patients and control patients.		
characteristics	case patients	control patients
males/females, no.	12/2	8/6
age, years Age/years (Mean±SEM) Mean Std. Dev.	 42.78571 9.158255	 39 11.68826
unknown risk factors for HIV acquisition, no		
heterosexual	1	3
injection drug user	1	2
combined	0	2
duration of HIV-1 infection, months	20.8±6.2	20.3±3.5
HBV coinfection, no[a]	0	0
HCV coinfection, no[b]	1	1
PI containing ART regimen	3	3
NNRTI containing ART regimen	11	11
PI and NNRTI containing ART regimen	0	2
plasma viral load (copy/ml)	<50-200	<50-200
$cd4^+$ cell count per µL	266±70.4	516±283

Data are ± SD unless indicated otherwise. HIV-1–infected DIR (n=14) and control (n=14) patients receiving highly active antiretroviral therapy were selected on the basis of having a plasma viral load 200 copies/ml. patients of the case group and patients of the control group were matched by Age, ART, antiretroviral therapy; HBV, hepatitis B virus; HCV, hepatitis C virus; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

Statistical analysis: All Statistical calculations were carried out using GraphPad prism software version 8.0.2 for Windows GraphPad Software, San Diego, CA, USA. One-way ANOVA test was applied for comparing the groups whereas the samples that passed the normality test Shapiro-Wilk; and all samples had normal distribution.

Results

Twenty-eight HIV-1- infected individuals were enrolled in this case control study. Table 2 shows features of the case patients and the control patients at baseline. Fourteen patients were enrolled in each of the two groups in this study; 85.7% were men and 14.2% were women in the case group and 57.1% were men and 42.8% were women in the control group. At baseline, the median age of subjects was 42 years (SD: 33±51). Due to the design of the study, the CD4+ cell counts were lower in case control patients while the HIV-1 plasma loads were VL<200 copies/mL in both groups. The median CD4+ cell counts of case group patients were 214 and the median CD4+ cell counts of patients of the control group were 516. Regarding transmission factors for HIV infection, 21.4% and 7.1%were heterosexual in patients of the control and case groups, respectively, and 14.2% and 7.1% reported being intravenous drug users (IVDUs) in patients of the control and case groups, respectively. 7.1% of subjects had HCV coinfection before enrollment in the present study in both groups. Study patients were first treated with a triple-drug combination therapy with 2 nucleoside reverse transcriptase inhibitors as well as a protease inhibitor 21.4% (3/14) in each group or a nonnucleoside reverse transcriptase inhibitor 78.5% (11/14). 14.2% (2/14) of patients of the control group followed a mixture of PI and NNRTI regimens .

The effects of 7 genes: CCR5, CXCR4, IL12B, TNFα, CD3, PTK2B and TCR β on HIV progression were assessed in this study, and statistical analysis was performed based on the 2-ΔΔCt method as previously described. The fold change ratio of CCR5 (0.225), TCR-β (0.12), and CD3 (0.09) as shown in Fig. 1 in instance PBMCs were significantly decreased in patients of the case group when compared with patients of the control group with confidence value. $p < 0.05$, $p < 0.02$ and $p < 0.01$, respectively. Mutually the fold change ratio for CXCR4 (1.01), PTK2B (0.718), and IL12B

(0.6) and TNF-α (1.04) as shown in Fig. 2 was not statistically significant in the patients of the case group compared to the patients of the control group. Nevertheless, the fold change for IL-12 was not significant but its IL-12 difference was nearly 2-fold and could be object of further discussion (15).

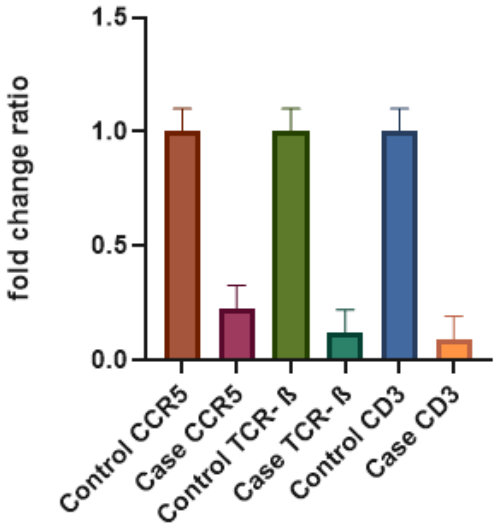

Fig. 1. Expression of CCR5 (0.225), TCR-β (0.12), and CD3 (0.09) genes in case group compared to control group based on fold change. In all three show a significant decrease with confidence value. p <0.05, p <0.02 and p <0.01, respectively.

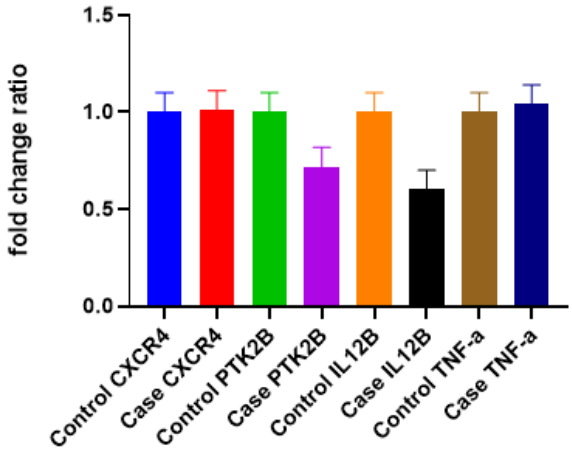

Fig. 2. Expression of CXCR4 (1.01), PTK2B (0.718), IL-12B (0.6) and TNF-α (1.04) genes in case group compared to control group based on fold change. None of them show a significant difference.

Discussion

We showed that the expression of CCR5, TCR-β, and CD3 genes significantly decreased in the patients of the case group. Previous studies have shown that CCR5 is expressed on about 5%–10% of CD4 T lymphocytes in peripheral blood and on some of CD4 T lymphocytes in primary and secondary lymphoid tissue (16). Gp120, the HIV surface envelope protein, interacts with CXCR-4 and CCR-5 as the chemokine receptors which this interaction is mainly stabilized by the presence of the CD4 molecule (16, 17).

The characterization of cytokine disturbances before major CD4 T-cell depletion and progression to AIDS is particularly relevant for understanding HIV immune-pathogenesis (18). Some studies showed Th1 cells due to a higher expression of the CCR5 are more easily infected by R5-tropic HIV-1 and inversely, Th2 cells due to a higher density of CXCR4 are more prone to X4-tropic HIV-1 infection (19, 20). Moreover, several investigations have previously explained that IFN-γ can directly or through increased secretion of its ligands, down-regulate CCR5, while IL-4 can up-regulate the levels of surface expression of CXCR4(21).

Additionally, IL-4 down-regulates CCR5 in primary CD4+ T Lymphocytes and inhibits INF-γ production (22, 23). According to previous studies and result of this study, CCR5 expression was lower in patients of the case group compared to those of the control group, thus we conclude that these patients have a more severe immunodeficiency.

IL-4 expressed by Th2 causes decline of Th1expressing CCR5. Therefore, it is possible that the number of Th1 cells is lower in case group patients in comparison with the control group patients and lower CD4+ cells without a subsetting of Th1 and Th2-cells have been found in patients of this study (Table 2).

HIV-1 and HIV-2 infect CD4+T cells and cause defects in TCR-β/CD3-directed immune response. However, several studies have established an association between T cell dysfunction and disease progression after viral infection (24). The mechanisms responsible for this dysfunction as well as cellular and *molecular processes that trigger the correlation* between of viral gene transcription and TCR/CD3-regulated pathways are still unknown. Previous studies have characterized the virally encoded

proteins gp120, Nef, and Tat can suppress TCR-β/CD3-directed activation. Furthermore, the expression of viral p24 is not dependent on TCR-β/CD3 down-modulation.

Recently, it has been found that the expression levels of CD3 quantitatively decline in the advanced stages of the disease on both activeted and resting CD4+ and CD8+ T cells from HIV-1-infected patients (24, 25). The data in the present study show that the expression level of TCR-β/CD3 may decrease in the course of HIV infection .

The studies showed, some cytokines and microRNA (miRNAs) play roles in activation, differentiation and restoration of CD4+ T-cells. For example, Interferon (IFN) and Transforming growth factor (TGF) cause activation and regulation of lymphocyte activation. MiR-29 limits the differentiation of Th1 cells. MiR-155 and miR-19a were shown to promote Th2 cell differentiation (26-28).

Several methodological limitations should be considered when interpreting the results of the present study. Importantly, the small sample size which limits the precision of estimates and power for detecting differences between the study groups. We would like to investigate the expression of miRNAs related to CD4+ T-cells activation, differentiation and restoration on our case and control groups in the future.

Conclusion

In conclusion, we showed significant decrease in the expression of CCR5, TCR-β, and CD3 genes in the patients of the case group in comparison with the patients of the control group, but we could not verify this low expression of these genes is a reason of declining CD4+ T-cells.

Further investigation is necessary for it, if the suppression of these genes can influence the proliferation or development of CD4 T-cells, in-vitro.

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Conflict of interest

The authors declare no conflict of interests.

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