Association between Interleukin-21, 23 and 27 Expression and Protein Level with Cytomegalovirus Infection in Liver Transplant Recipients

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ABSTRACT

Background: Cytokines have regulatory crosstalk with CMV infection leading to manage of post-liver transplantation virus-related outcomes.

Objective: To investigate the link between IL-21, IL-23 and IL-27 mRNA and protein level with active CMV infection, which was evaluated in reactivated and non-reactivated liver transplant recipients.

Methods: Two groups of liver transplant recipients were enrolled in this study—54 without and 15 with active CMV infection. 3 EDTA-treated blood samples were taken on day 1, 4, and 7 post-liver transplantation. Plasma and buffy coats of all samples were separated. All samples were analyzed for CMV reactivation using antigenemia technique. The separated plasma of positive samples was used for viral DNA extraction and protein evaluation. For evaluating the mRNA expression level by real-time PCR, RNA extraction and cDNA synthesis were done for all samples. Also, the protein level of studied genes was estimated by ELISA.

Results: The expression level of IL-21, IL-23A and IL-27A cytokine genes was increased in CMV reactivated liver transplant recipients in comparison with CMV non-reactivated ones; IL-27A expression pattern was significant (p=0.001) at all sampling times. IL-21 significantly increased on the 2nd and 3rd (p=0.028 and 0.01, respectively) sampling days in CMV reactivated compared with non-reactivated patients. The expression level of IL-23A cytokine significantly increased on the 3rd (p=0.017) sampling day in CMV reactivated compared with non-reactivated liver transplant recipients.

Conclusion: Elevation in the expression level of IL-21, IL-23A and IL-27A mRNA and protein level in CMV reactivated patients emphasized on the antiviral role of these cytokines in CMV reactivated liver transplant recipients.

KEYWORDS: Cytomegalovirus; Liver transplantation; Interleukins

INTRODUCTION

Human cytomegalovirus (HCMV), a member of β-herpes virus subfamily, can cause lifelong infection. This infection is generally controllable in normal hosts and is almost latent before developing clinical symptoms. However, it can cause complications in immunocompromised patients like HIV and organ transplant recipients [1]. This virus can directly or indirectly cause high mortality and morbidity in various transplant recipients [2]. Unfortunately, because CMV infection and transplant rejection are circumstances with similar immunologic mechanisms, it is difficult to distinguish them from one another. For many years, CMV has been a culprit in mortality after liver transplantation and although many progresses have been made in this field, this infection is still one of the most life-threatening conditions

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after liver transplantation [3, 4]. CMV infections can upregulate some of plasma cytokines that have role in inflammatory conditions after transplantation [5].

Cytokines are secretory proteins that interfere in many vital processes like proliferation, differentiation, and survival of hemopoietic and non-hemopoietic cells [6]. Researches have revealed that proinflammatory cytokines can act as antiviral mediators that can suppress the development of CMV pathogenesis [1]. CMV infection can change cytokine levels in transplant recipients [7]. IL-21, a T cell derived cytokine that can act as mediator between innate and adaptive immune responses, has receptors on T cells, NK cells, B cells and dendritic cells (DCs) [6, 8]. IL-21 secreted by antigen-stimulated T cells, TFH cells, and autocrine and/or paracrine supports the development and maintenance of Th17 cells [9]. Some studies have demonstrated that IL-21 may interfere with development of autoimmune diseases; it also has anti-tumor activity. A research shows that IL-21 upregulation occurs in CMV-seropositive patients [8].

IL-23 is a heterodimeric cytokine and predominantly is synthesized by activated DCs and phagocytic cells. IL-23 plays different roles in the immune system; it induces IFN-γ secretion from T cells and then gets involved in Th1 type immune responses. More importantly, this cytokine interferes with the maintenance of Th17 cells effector function and it specifically produces IL-17 [10]. IL-23 has a noticeable role in many autoimmune diseases [11, 12]. For instance, the important role of IL-17/IL-23 mRNA level increase has been demonstrated in acute liver rejection [11]. However, it has been reported that IL-23 plays an important role in patient immunity against CMV [13]. Another study also demonstrates that IL-23 can produce immune responses in HCV-infected patients [14].

IL-27 is a heterodimeric member of the IL-6/IL-12 family of type I cytokines [15, 16]. This cytokine can be detected in response to many inflammatory stimuli, which highlights its role in regulating infection-induced immunity [15]. IL-27 shows anti- and pro-inflammatory properties [16] and has been known to play quite intricate roles in regulating the innate and adaptive immune responses [17]. This cytokine can affect the naïve CD4+ T cells and act as a pro-inflammatory cytokine and cause early initiation of type 1 helper differentiation; it can also play as an anti-inflammatory cytokine and cause decrease in T cell hypersensitivity and pro-inflammatory cytokine production [18].

Considering the obvious important role of inflammatory cytokines, like IL-21, IL-23 and IL-27, in post-transplant outcomes, since CMV is an important complication in liver transplant recipients, we decided to conduct this study to elucidate the role of these cytokines in CMV pathogenesis in liver transplant recipients.

MATERIALS AND METHODS

This study was conducted on liver transplant recipients who were admitted to the Transplant Ward, Namazi Hospital, affiliated to Shiraz University of Medical Sciences, Shiraz, Iran, between 2011 and 2013. Patients were divided into two groups—15 liver transplant recipients with active and 54 without active CMV infection. Three EDTA-treated blood samples were collected from each patient on days 1, 4, and 7 post-transplantation. This study was approved by the Ethics Committee of Shiraz University of Medical Sciences. The protocols used were in conformation with the ethical guidelines of Declaration of Helsinki.

All patients were given the same routine regimen of immunosuppressive drugs which consisted of tacrolimus or cyclosporine with mycophenolate mofetil and steroids. The blood level of 200 mg/mL was considered the therapeutic target for CsA (5 mg/kg/d) or 10 mg/mL for tacrolimus. Donors were selected based on ABO blood group compatibility. HLA matching was not performed as a routine procedure for liver transplant recipients.

Table 1: The primer sequences used for IL-21, IL-23, IL-27 and B-actin transcripts

Gene	Gene ID	Primer Sequence	Product Length
IL-21	NM_021803.2	F:5' TCCAGTCCTGGCAACATGGAGA 3' R:5' GGCGATCTTGACCTTGGGAGC 3'	97bp
IL-23A	NM_016584.2	F:5' AGTGGAAGTGGGCAGAGATTC 3' R:5' CAGCAGCAACAGCAGCATTAC 3'	115bp
IL-27A	NM_145659.3	F:5' GCACTGGGCAGCGCCTTACA 3' R:5' TCCCGCACGGCCCGAGATAA 3'	110bp
B-actin	NM_001199954.1	F:5' GGCGGCACCACCATGTACCC 3' R:5' GACGATGGAGGGGCCCGACT 3'	203bp

Virology

Antigenemia Procedure

Antigenemia test was used to detect active CMV infection. Following the procedure described in CMV Brite Turbo kit (IQ Products, Groningen, The Netherlands) 200,000 cells were extracted from all the EDTA-treated blood samples for cyto-centrifuged preparations (Cytospin 3, Shandon Scientific, Cheshire, England) [19]. Then, a cocktail composed of two fluorescein isothiocyanate-labeled monoclonal antibodies (C10/C11) were used, which was responsible for indirect immunofluorescence staining against CMV lower matrix phosphoprotein pp65. These stained pp65 molecules turned the nucleus of CMV-infected polymorphonuclear cells into bright green under fluorescence microscope, which were reported as infected cells.

Taq-man Real-time PCR

CMV-DNA was extracted using Invisorb Spin Virus RNA Mini kit (Invitek, Birkenfeld, Germany) from plasma of all antigenemia-positive samples according to the manufacturer's instruction. The load of HCMV-DNA was determined by Gensig real-time PCR kit (Primer Design Ltd TM, Advanced kit, United Kingdom). The reaction mix was modified to 20 µL total volume consisting of 5 µL of HCMV-DNA, 10 µL of precision TM Master Mix, 1 µL of primers and probes targeting glycoprotein B (gB), 1 µL of primers and probes targeting the internal control (IC) gene (Applied Biosystems, Grand Island, NY, USA), and finally 3 µL nuclease-free water. The thermal cycling program used for Step One Plus Real-Time PCR thermocycler (Applied Biosystems, Grand Island, NY, USA) included one cycle at 95°C for 10 min, followed by one cycle at 95 °C for 5 sec, and then 50 cycles at 60 °C for 60 sec. The assay was sensitive enough to detect as few as 10 copies of CMV genome/mL of samples.

Assessments

RNA Isolation and cDNA Synthesis

Total RNA of all samples was extracted from buffy coats using RNX plus (CinnaGen, Iran), with a modified protocol. Purity and integrity of the extracted RNA was determined by reading 260/280 nm optical density and electrophoresis of RNA on 1% agarose gel.

cDNA synthesis was performed using 1 µL of each RNA sample using M-mlv reverse transcriptase enzyme and tandem hexamer (Vivantis, Subang Jaya, Malaysia). First, RNA (10 µg/µL), dNTPs (1 µL/10 mm) and random hexamer (0.2 µg/µL) were added, mixed and incubated at 65 °C for 7 min followed by 2 min on ice. Next, M-mlv enzyme (1 µL/200 U), RT buffer (2 µL/10×), RNase inhibitor (1.3 µL/60 U, Vivantis, Subang Jaya, Malaysia), and nuclease-free water were added, mixed and added to the previous mixture. The mixture was then incubated at 45 °C for 90 min and then at 85 °C for 5 min.

SYBR Green Real-time PCR

The mRNA expression level of IL-21, IL-23A, IL-27A and β-actin gene as the internal control, was measured using newly designed primers (Table 1). GAPDH gene was evaluated for internal control but it showed more fluctuations than β-actin. The real-time mixed for each sample included 10 µL of SYBR Green Premix (Ex Taq, Takara, Otsa, Shiga, Japan),

Table 2: The frequency (%) of the underlying diseases in CMV⁺ and CMV⁻ liver transplant recipients

Underlying diseases	CMV^-		CMV^+	
	Male	Female	Male	Female
Cryptogenic	5 (9)	3 (6)	2 (13)	1 (7)
HBV^+	13 (24)	2 (4)	1 (7)	1 (7)
HCV^+	2 (4)	0 (0)	1 (7)	0 (0)
PSC	4 (7)	4 (7)	0 (0)	1 (7)
AIH	4 (7)	5 (9)	0 (0)	2 (13)
Wilson	1 (2)	1 (2)	1 (7)	2 (13)
Hypertyrosinemia	1 (2)	1 (2)	1 (7)	1 (7)
Biliary atresia	3 (6)	0 (0)	0 (0)	1 (7)
Other diseases	4 (7)	1 (2)	0 (0)	0 (0)
Total	37 (68)	17 (32)	6 (40)	9 (60)

0.4 µL of ROX dye, forward and reverse primers (each 3 pM) and 2 µL of cDNA. The thermal program used for this reaction was one cycle at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 sec and then at 65 °C for 20 sec. The melt curve was analyzed for each reaction to verify the specificity of the reaction. All data were normalized using the result of β-actin gene amplification.

Cytokines Protein Level Determination

For estimating the protein level of studied cytokines, plasma of all samples was separated and stored at -80 °C. The protein level of each sample was detected using ELISA kits according to the manufacturer (Mabtech, Sweden for IL-21 and IL-23 and Bioassay technology kit, Korea, for IL-27).

Statistical Analysis

Livak method ($2^{-\Delta\Delta Ct}$) was applied to evaluate the expression level of understudy genes in all study participants. Statistical analysis was performed with SPSS® ver. 19 (SPSS, Chicago, IL, USA). The analyses included Student's t test, ANOVA, and Mann-Whitney U test. A p value <0.05 was considered statistically significant.

RESULTS

The studied groups of patients were named CMV^- (CMV non-reactivated) and CMV^+ (CMV reactivated). CMV^- group was consisted of 54 liver transplant recipients, with 37 males (mean age of 37.5, range: 1–74 years) and 17 females (mean age of 29.5, range: 1–59 years); CMV^+ group included 15 CMV^+ liver transplant patients with six males (mean age of 31.5, range: 1.5–61 years) and nine females (mean age of 30.5, range: 3–62 years). The blood group distribution of CMV^- and CMV^+ recipients were 18 (33%) and 8 (53%) A^+, 14 (26%) and 2 (13%) B^+, and 17 (32%) and 2 (13%) O^-, respectively; blood groups A^-, B^-, and AB^-, there were 1 (2%) in CMV^- group. The underling diseases are shown in Table 2.

Comparing IL-21 Gene Expression in CMV^- and CMV^+ Liver Transplant Recipients

IL-21 expression level was higher in CMV^+ group compared with that in CMV^- group in In all of the sampling days (Fig 1a); this increase was significant in the 2nd ($p=0.028$, 95% CI: 0.00–0.053) and 3rd ($p=0.01$, 95% CI: 0.00–0.055) days. Elevation in the mRNA level of IL-21 in the 1st sampling day was also higher in CMV^+ compared with that in CMV^- recipients, which was not significant ($p=0.869$, 95% CI: 0.784–0.954).

Comparing IL-23A Gene Expression in CMV^- and CMV^+ Liver Transplant Recipients

The mRNA level of IL-23A increased in CMV^+ group compared with that in CMV^- group in all sampling days (Fig 1b); this in-

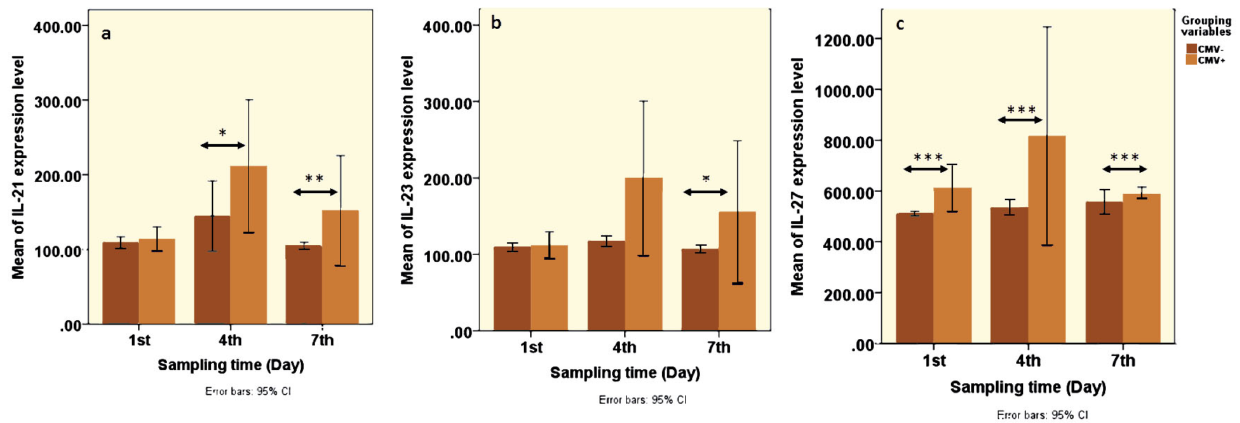

Figure 1: mRNA expression level in CMV⁻ and CMV⁺ recipients: (a) IL-21, (b) IL-23, and (c) IL-27. The fold changes were calculated using $2^{-\Delta\Delta CT}$. *p<0.05, **p<0.01, and ***p<0.001.

crease was only significant in the 3rd (day 7, p=0.017, 95% CI: 0.00–0.063) sampling day; it was not significant in the 1st (day 1, p=0.179, 95% CI: 0.087–0.271) and 2nd day of sampling (day 4, p=0.339, 95% CI: 0.218–0.460).

Comparing IL-27A Gene Expression in CMV⁻ and CMV⁺ Liver Transplant Recipients

The mRNA level of IL-27A increased in CMV⁺ group compared with that in CMV⁻ group in all sampling days (Fig 1c); it was significant in all days of sampling (p=0.001, 95% CI: 0.0–0.049).

Comparing IL-21 Protein Level in CMV⁻ and CMV⁺ Liver Transplant Recipients

In all sampling days, The protein level of IL-21 increased in CMV⁺ group compared with that in CMV⁻ group in all sampling days (Fig 2a); it was significant in the 4th day of sampling (p=0.01, 95% CI: -0.043 to -0.007).

Comparing IL-23 Protein Level in CMV⁻ and CMV⁺ Liver Transplant Recipients

The protein level of IL-23 increased in CMV⁺ group compared with that in CMV⁻ group in all sampling days (Fig 2b); it was significant in 4th and 7th days of sampling (p=0.005, 95% CI:-0.069 to -0.013; and p=0.002, 95% CI: –0.059 to -0.014).

Comparing IL-27A Protein Level in CMV⁻ and CMV⁺ Liver Transplant Recipients

The protein level of IL-27 increased in CMV⁺ group compared with that in CMV⁻ group in all sampling days (Fig 2c); it was significant in 4th and 7th day of sampling (p=0.05, 95% CI: -0.716 to -0.004; and p=0.01, 95% CI: -0.975 to -0.154).

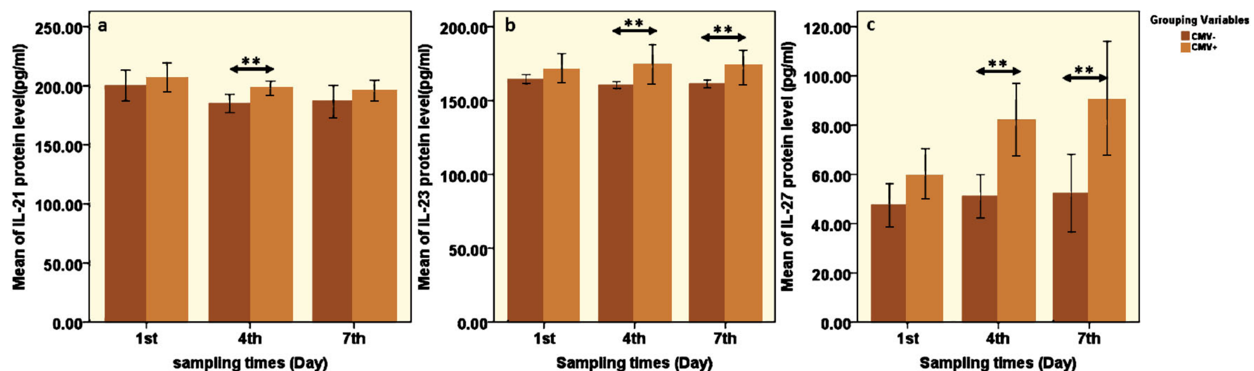

Figure 2: IL-21 (a), IL-23 (b), and IL-27 (c) protein level in CMV⁻ and CMV⁺ recipients. *p<0.05, **p<0.01, and ***p<0.001.

DISCUSSION

Even though there have been many advances in the diagnosis and treatment of CMV infection, it is the most prevalent viral infection which causes morbidity, longer hospital stays, and increases the risk of graft loss and mortality in transplant recipients [20]. Evidence shows that this infection can directly and indirectly affect transplant outcomes. It can also cause allograft injuries like acute or chronic rejections, suppression of the immune responses, and still apt the immune system of transplant recipients for other opportunistic infections [2, 3, 20]. Even though antiviral prophylaxis can decrease CMV infection rate, this infection is still a major risk factor during post-transplantation phase [21]. Another condition that directly affects the immune system is the cytokine secretion. Therefore, in this study the IL-21, IL-23 and IL-27 cytokines expressions were monitored in two groups of CMV reactivated and non-reactivated liver transplant recipients.

IL-21 derives from T cells and changes NK and T cells behaviors via proliferation of naïve human T cells and activation of STAT3 molecule [22, 23]. It is a well-known fact that NK cells are important agents of innate immunity, especially in clearing viral infections by exhibiting cytotoxicity to infected cells [6]. Also, another important fact about IL-21 is that, IL-21 is selectively produced by Th17 cells, which are important in immunity against CMV infection in liver transplant recipients [7, 9]. It is documented that the production of IL-21 is dependent on STAT3; this molecule, itself, binds directly to the promoter region of IL-21 that can be a direct regulator for this gene [9]. Another function of IL-21 is its role in transition of B cells into plasma cells, which renders the growth of $CD8^+$ T cells [8, 9].

Comparing the expression level of IL-21 between CMV reactivated and non-reactivated liver transplant recipients revealed increase in IL-21 mRNA level in all three sampling days; it was significant in the 2nd and 3rd sampling day (days 4 and 7 post-transplantation).

In 2009, Agrawal, *et al*., demonstrate that increase in IL-21 production has a positive correlation with CMV seropositivity. They also believed that increase in IL-21 cytokine during viral infection is due to its ability to control the infection through its effects on $CD8^+$ T cells; they also proposed that the increase in IL-21 in chronic viral infections is as a result of increase in the differentiation of $CD4^+$ T cells towards the Th phenotype during viral manifestation [8]. The importance of IL-21 mRNA changes was also evaluated in other viral diseases [24–26]; our findings were consistent with their findings.

Another cytokine that is involved in the immune responses against viral infections is IL-23, which has an important role in stimulation of Th17 cells for secreting IL-17. Our research group had previously worked on IL-17, a pro-inflammatory cytokine in liver transplantation [7, 27]. The role of IL-17/IL-23 pathway in various autoimmune diseases like MS, CD, psoriasis, EAE, *etc.* was mentioned previously [11, 28]. This cytokine conveys its role through the development and expression of previously activated $CD4^+$ T cells that secrete IL-17 [29]. Hamza, in 2010, reported that IL-23 knockout mice prepared a susceptible environment for bacterial, parasitic, and fungal infections [10]. Our results in this study, comparing IL-23A mRNA in CMV^+ and CMV^- groups in all sampling days showed an increase in CMV^+ group, which was significant in the 3rd sampling day. These data were similar to other reports showing the importance of IL-23A in controlling viral pathogens like CMV.

The last studied cytokine in the current project was IL-27, which after secretion from macrophages and DCs activates Jak/STAT signaling cascade [30]. IL-27 can act either as a pro- or anti-inflammatory cytokine [16]. It can exert its role as a key regulatory cytokine that can bound inflammatory responses, Th1, Th2, and Th17 cells; it can even control neutrophil migration and oxidative bursts [17, 18]. Another study has revealed that neutralization of IL-27 may have potential for curing sepsis; by using a soluble IL-27ra fusion protein to block IL-27 we may cause bacterial clearance and

noteworthy rise in the endurance of animals in a sepsis mouse model; however, it should be mentioned that this kind of treatment can be helpful for a limited time because longterm neutralization of IL-27 can facilitate progress of autoimmunity [31]. Reports suggest that IL-27 can serve as an antiviral cytokine in peripheral blood mononuclear cells; it was also demonstrated that IL-27 can inhibit HIV-1 replication [32]. Studies have also shown that IL-27 has an impact on HCV replication [33, 34] by significant induction of IFN-inducible antiviral genes in macrophages, which results in reduced replication of HIV, influenza virus, and HCV through IFN-like responses [18].

In conclusion, the results of our study showed that elevation in the expression of IL-21, IL-23A, and IL-27A genes and their antiviral immunologic role can be beneficial in designing preventive and therapeutic protocols for liver transplant recipients with active CMV infection.

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