Significant reduction of peripheral blood interleukin-35 and CD4+EBI3+ T cells, which are negatively correlated with an increase in the plasma IL-17 and cTnI level, in viral myocarditis patients

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Abstract

Introduction: Viral myocarditis (VMC) has become an increasingly common heart disease that endangers human health. In the present study, the plasma interleukin-35 (IL-35) level and the percentage of CD4+EBI3+ T cells in VMC patients were detected to investigate the significance of changes in these parameters in the plasma of VMC patients and their association with the disease.

Material and methods: ELISA was performed to detect the plasma IL-35 level and the percentage of peripheral blood CD4+EBI3+ T cells in 40 VMC patients and in 20 healthy individuals. Moreover, the plasma IL-17 levels in the VMC patients and in the healthy individuals were detected using an ELISA, and the cardiac Troponin-I (cTnI) levels were detected using a chemiluminescent microparticle immunoassay to compare the differences in the groups.

Results: Plasma IL-35 level and the percentage of CD4+EBI3+ T cells in acute phase VMC patients was lower than that in the healthy control group and the convalescent phase VMC patients. Additionally, the plasma IL-35 level in the VMC patients exhibited a negative correlation with the levels of cTnI and IL-17. The percentage of CD4+EBI3+ T cells also showed a negative correlation with the levels of cTnI and IL-17.

Conclusions: The plasma IL-35 level and the percentage of CD4+EBI3+ T cells in VMC patients was reduced, and the amount of the decrease was associated with the severity of the disease. These results suggest that IL-35 and CD4+EBI3+ T might play important roles in the progression of VMC and could be used as indicators of the disease.

Key words: viral disease, myocarditis, interleukin-35, EBI3.

(Cent Eur J Immunol 2017; 42 (1): 91-96)

Introduction

Viral myocarditis (VMC) is an autoimmune disease and often progresses to chronic myocarditis and dilated cardiomyopathy (DCM). It is currently thought that myocarditis induced by viral infection causes myocardial injury due to an excessive immune response [1]. In the pathogenesis of VMC, immune disorders are important trigger factors. Cytokines play important roles in cellular and humoral immunity [2]. Therefore, cytokine studies will help to better elucidate the pathogenesis of VMC. Interleukin-35 (IL-35) is a newly discovered cytokine with anti-inflammatory functions; this molecule is crucial for the inhibitory functions of T regulatory cells (Tregs) [3]. Scholars including Niedbala et al. [4] and Collison et al. [5] have confirmed that IL-35 expressed by Tregs can increase the differentiation and proliferation of Tregs and inhibit the production of the Th17 cell subpopulation and secretion of its cytokines, such as IL-17. Interleukin 17 is a proinflammatory cytokine and produced by several cell subsets. Th17 cells represent an effector cell type that drives inflammatory responses by virtue of producing IL-17 [6]. Th17 cells have been involved in the pathogenesis of autoimmune diseases including multiple sclerosis (MS), morphea, cancer, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [7-9]. Recent evidence suggests that IL-17-mediated inflammation might play a role in the pathogenesis of VMC [10]. Thus, IL-35 plays
an important role in the maintenance of self-tolerance and prevention of immune diseases. Currently, no domestic or international studies have reported whether abnormal expression and secretion of IL-35 occurs in VMC patients, or if it is associated with the severity of VMC.

This study aimed to detect the plasma IL-35 levels and the percentage of CD4⁺EBI3⁺ T cells in the peripheral blood of VMC patients and healthy individuals to determine whether differences exist in their levels and to investigate their clinical significance.

Material and methods

Study subjects

A total of 40 VMC patients treated at the Second Affiliated Hospital of Soochow University between July 2013 and February 2015 were included. The patients consisted of 22 cases of acute phase VMC, including 12 men and 10 women with a mean age of 33 ± 13 years, and 18 cases of convalescent phase VMC, including 10 men and 8 women with a mean age of 35 ± 11 years. The healthy control group consisted of 20 individuals, including 9 men and 11 women with a mean age of 30 ± 10 years. All patients conformed to the 2011 adult viral myocarditis diagnosis criteria. Other acute infections or rheumatic diseases were excluded in all patients. Individuals in the control group had no history of acute infection or autoimmune disease. The procedures used in the VMC patient study conformed to and were approved by the ethics standards formulated by the Medical Ethics Committee. The collection of specimens was performed after informed consent was obtained from the subjects.

Study methods

Specimen collection and detection of plasma IL-35 and IL-17. A total of 3 ml fasting venous blood was collected from VMC patients and healthy individuals in the morning under sterile conditions. Detection was performed using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA). Whole blood was centrifuged at 3000 rpm for 10 minutes. Plasma in the top layer was aspirated and stored in a −80°C freezer for later use. The IL-35 ELISA reagent kit was purchased from Lianchuang Biological Medicine Co., Ltd (Wuhan, China). The IL-17 ELISA reagent kit was purchased from R&D Systems (USA). The specific manipulations were strictly performed according to the instruction manuals in the reagent kits.

Detection of cardiac Troponin-I (cTnI) levels

Serum cTnI was detected with a chemiluminescent microplate immunoassay using an automatic biochemistry analyser.

Flow cytometry

Cubital vein blood (2 ml) was collected in the morning using EDTA as an anticoagulant. Detection of specimens was performed within one hour after collection. Peripheral blood mononuclear cells (PBMC) from healthy individuals and VMC patients were successfully separated using the Ficoll density gradient centrifugation method. After two washes with phosphate-buffered saline (PBS), 1 × 10⁶ cells were added to 12-well plates (each well had been previously filled with 1.5 ml 1640 medium), and phorbol myristate acetate (PMA) (50 ng/ml), ionomycin (500 ng/ml), and IL-2 (100 µ/ml) were added for stimulation for six hours. During the final four hours, the Golgi complex inhibitor brefeldin A (BFA) was added at a dilution of 1 : 1000. After a total of six hours of stimulation, the samples were processed. Cells were aspirated into a 15 ml centrifuge tube for centrifugation. The supernatant was discarded, and the sample was transferred to a 1.5-ml Eppendorf (EP) tube. After the sample was centrifuged and the supernatant was discarded, 20 µl CD4-Alexa Flour monoclonal antibody was added, and the sample was incubated at 4°C in the dark for 60 minutes. After washing with 1 ml PBS, the cells were fixed in 300 µl fix solution in the dark for 20 minutes. The cells were centrifuged, the supernatant was discarded, and permeabilisation with 1 ml permeabilisation solution was performed twice. EBI3-PE monoclonal antibody (5 µl) was added to some tubes, which were incubated in the dark for 60 minutes. The supernatant was discarded after centrifugation, 500 µl PBS was added, and the sample was transferred to a flow cytometry tube for detection. Tubes with EBI3-PE single labelling and CD4-Alexa Flour + EBI3-PE double labelling controls were set up for use as blanks.

Statistical analysis

Statistical analysis was performed using SPSS17.0 software. For data in all groups that conformed to a normal distribution, quantitative data were described as x ± s. Comparisons between two groups were performed using a group t test or one-way analysis of variance. Analysis of covariance (ANOVA) was used to compare data among the three groups. Data that did not conform to a normal distribution were represented with the median, and comparisons between two groups were performed using the Wilcoxon rank sum test. The correlation analysis between clinical indicators was performed with a non-parametric Spearman’s correlation. A value of P < 0.05 indicated a statistically significant difference.

Results

Detection of serum IL-35 levels in VMC patients using an ELISA

There were significant differences of the serum IL-35 levels among the three groups (16.8 ± 3.9, 26.9 ± 5.6,
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Fig. 1. Serum IL-35 levels in each group, including VMC acute phase patients (n = 22), VMC convalescent phase (n = 18) and healthy controls (HCs) (n = 20) were detected using a specific ELISA. Bars indicate mean values ± SD in each group. Serum IL-35 levels were significantly increased in the VMC acute phase group compared with HCs (p < 0.01) and VMC convalescent phase group (p < 0.05). Serum IL-35 levels were also higher in VMC convalescent phase compared with HCs (p < 0.05).

Fig. 2. Decreased percentages of CD4+EBI3+ T cells in VMC acute phase patients. The percentages of CD4+EBI3+ T cells were significantly decreased in VMC acute phase patients compared with the healthy controls (HCs) and VMC convalescent phase patients. The percentages of CD4+EBI3+ T cells were decreased in VMC convalescent phase patients compared with HCs. Bars represent the mean values ± SD.

Fig. 3. The plasma IL-17 levels in the VMC patients and healthy individuals were detected using an ELISA. The plasma IL-17 level in the acute phase VMC patients was higher than that in the healthy control group (p < 0.01) and the convalescent phase VMC patients (p < 0.05). The level in the convalescent phase patients was higher than that in the healthy control group (p < 0.05). Bars indicate mean values ± SD in each group.

Fig. 4. Bars represent the mean values ± SD. The plasma cTnI level in the acute phase VMC patients was significantly higher than that in the healthy control group (p < 0.01) and the convalescent phase VMC patients (p < 0.05), the plasma cTnI level in the convalescent phase patients was higher than that in the healthy control group.

56.3 ±9.8 pg/ml) (F = 6.542, p = 0.002). The plasma IL-35 level in the acute phase VMC patients was lower than that in the healthy control group (16.8 ±3.9 vs. 56.3 ±9.8 pg/ml, p < 0.01) and the convalescent phase VMC patients (16.8 ±3.9 vs. 26.9 ±5.6 pg/ml, p < 0.05). The level in the convalescent phase patients was lower than that in the healthy control group (26.9 ±5.6 vs. 56.3 ±9.8, p < 0.05) (Fig. 1).
Detection of the percentage of peripheral blood CD4⁺EBI3⁺ T cells in the PBMCs of VMC patients and healthy individuals

There were significant differences of the percentage of CD4⁺EBI3⁺ T cells among the three groups (0.89 ±0.15, 1.31 ±0.32, 1.86 ±0.34%) (F = 8.268, p = 0.001). The percentage of peripheral blood CD4⁺EBI3⁺ T cells in VMC acute phase patients was lower than that in the healthy control group (0.89 ±0.15 vs. 1.86 ±0.34%, p < 0.05) and the VMC convalescent phase patients (0.89 ±0.15 vs. 1.31 ±0.32%, p < 0.05). The percentage in the convalescent phase patients was lower than that in the healthy control group (1.31 ±0.32 vs. 1.86 ±0.34%, p < 0.05) (Fig. 2).

Detection of serum IL-17 levels in VMC patients using an ELISA

There were significant differences of the plasma IL-17 levels among the three groups (158.2 ±19.3, 101.4 ±18.8, 66.3 ±18.7 pg/ml) (F = 7.414, p = 0.002). The plasma IL-17 level in the acute phase VMC patients was higher than that in the healthy control group (158.2 ±19.3 vs. 66.3 ±18.7 pg/ml, p < 0.01) and the convalescent phase VMC patients (158.2 ±19.3 vs. 101.4 ±18.8 pg/ml, p < 0.05). The level in the convalescent phase patients was higher than that in the healthy control group (101.4 ±18.8 vs. 66.3 ±18.7, p < 0.05) (Fig. 3).

Fig. 5. A) Serum IL-35 levels were correlated with IL-17 levels. Spearman’s analysis was used for correlation analysis. B) The percentages of CD4⁺EBI3⁺ T cells were correlated with IL-17 levels. Spearman’s analysis was used for correlation analysis. C) The percentages of CD4⁺EBI3⁺ T cells were correlated with the cTnI levels. Spearman’s analysis was used for correlation analysis. D) Serum IL-35 levels were correlated with the cTnI levels. Spearman’s analysis was used for correlation analysis.
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Detection of cTnI levels in VMC patients and healthy controls

There were significant differences of the plasma cTnI level among the three groups (0.28 ±0.04, 0.13 ±0.02, 0.04 ±0.03 µg/l) (F = 5.683, p = 0.02). The plasma cTnI level in the acute phase VMC patients was significantly higher than that in the healthy control group (0.28 ±0.04 vs. 0.04 ±0.03 µg/l, p < 0.01) and the convalescent phase VMC patients (0.28 ±0.04 vs. 0.13 ±0.02 µg/l, p < 0.05), homoplastically, and the plasma cTnI level in the convalescent phase patients was higher than that in the healthy control group (0.13 ±0.02 vs. 0.04 ±0.03 µg/l, p < 0.05) (Fig. 4).

The correlations of the IL-35 level and the percentage of CD4+EBI3+ T cells with the cTnI and IL-17 levels in the plasma of VMC patients

The IL-35 level and the percentage of CD4+EBI3+ T cells both showed negative correlations with the cTnI level; the r values were –0.30 and –0.46, respectively, and the P values were both < 0.05. The IL-35 level and the percentage of CD4+EBI3+ T cells both showed a negative correlation with the IL-17 level; the r values were –0.39 and –0.46, respectively, and the P values were both < 0.01 (Fig. 5).

Discussion

The pathogenesis of VMC is still not completely understood. Studies have shown that an imbalance of cellular and humoral immunity is associated with the development of VMC, and cytokines play an important role in this process [1].

Recent studies have shown that Tregs are a T-cell subpopulation with immune suppression functions. Through mechanisms such as secretion of anti-inflammatory cytokines or direct cell contact, they can control the intensity of immune responses and relieve tissue damage in the body, thus playing important roles in the maintenance of self-tolerance and prevention of autoimmune diseases [11]. It is generally thought that the development of autoimmune diseases results from differentiation and dysfunction of Th17 cells and Tregs [12].

Tregs can generate a heterodimer formed by covalent bonding between two subunits, EBI3 and IL-12p35. This new type of immunosuppressive and anti-inflammatory factor is called IL-35. IL-35 is a new member of the IL-12 family and is an effector that Tregs use to exert negative immune regulation. The p35 subunit does not have a significant effect on the development of autoimmune diseases, and EBI3 is a key subunit for the negative regulatory function of IL-35 [3-5, 13]. Currently, it is known that the EBI3 gene that encodes the IL-35 dimer is a downstream target molecule of the transcription factor Foxp3 in Tregs. A reduction of the EBI3 level affects the regulation of Treg activity by Foxp3. EBI3 is expressed at high levels in placental trophoblast cells, lymphocytes, and activated dendritic cells [14]. Recombinant Ebi3, the specificity subunit of IL-35, can induce IL-17, IL-22, and RORγt production [16].

Recent studies have shown that IL-35 plays an important role in autoimmune diseases. The in vitro studies of Pope and Shahrara [17] confirmed that IL-35 could effectively inhibit Th17 cells and significantly relieve the arthritis symptoms of mice in a collagen-induced arthritis (CIA) mouse model. Similar results were found in an experimental autoimmune encephalomyelitis (EAE) model and an experimental allergic myositis (EAM) model, suggesting that IL-35 has an important immune regulatory function in inflammation and autoimmune diseases [18]. In previous studies, IL-35 has been confirmed to be associated with a variety of inflammatory and immune diseases. However, the study subjects were mainly limited to animal models, and a correlation between IL-35 and VMC has not been reported.

As a marker for myocardial injury, cTnI has been used in clinical practice for many years. In recent years, due to its high myocardial specificity, high sensitivity to the degree of myocardial injury, and longer diagnostic window, cTnI has replaced the creatine kinase-myocardial band (CK-MB) as a specific marker for the diagnosis of myocardial injury. Previous studies have shown that the number of Th17 cells in peripheral blood in acute phase VMC patients is increased and shows a positive correlation with the severity of myocardial injury [10]. After treatment with IL-17 monoclonal antibodies, the myocarditis symptoms in EAM mice were also significantly relieved, suggesting that IL-17 cells have important functions in inflammatory responses and immune injury in the myocardium [19]. Inhibition of the differentiation or function of Th17 cells could disrupt the positive feedback between the inflammatory and immune responses; therefore, these cells could be tested as a new target for VMC treatment.

In this study, we analyzed changes in the serum IL-35 level and the percentage of CD4+EBI3+ T cells in the peripheral blood to determine their correlations with several clinical indicators that reflect disease activity and severity. The results showed that the plasma IL-35 level in VMC acute phase patients was significantly lower than that in the healthy control group and the VMC convalescent phase patients. In addition, the plasma IL-35 level and the percentage of CD4+EBI3+ T cells in VMC patients both showed a negative correlation with the cTnI and IL-17 levels, suggesting that IL-35 and CD4+EBI3+ T cells in VMC patients might play important roles in the progression of VMC and could be used as indicators of the severity of the disease.

Interleukin-35 could inhibit the differentiation of innate CD4+ T cells into Th17 cells [20]. Recombinant
IL-35 could significantly relieve disease progression of experimental colitis in animals, which was accompanied by a decrease in the secretion level of the IL-17 factor [21]. In arthritic mice, induced by the administration of autoimmune collagen, IL-35 could inhibit the production of IL-17, effectively relieve the arthritis symptoms in experimental mice, and inhibit the progressive destruction of the joint structure [17]. In contrast to the features of other inhibitory factors, which only exert their function at the initial stage of inflammation, IL-35 also acts on existing autoimmune diseases. Therefore, some scholars have proposed that IL-35 is a new type of anti-inflammatory immune cytokine that exerts its function through the enhancement of Tregs and inhibition of Th17 cells in viral myocarditis mice [22].

In summary, the serum IL-35 level and the percentage of peripheral blood CD4+EBI3+ T cells in the acute phase patients in the VMC group were significantly lower than those in the convalescent phase patients and the control group, while the IL-17 level was significantly higher than that in the convalescent phase patients and the control group. The serum IL-35 level and the percentage of peripheral blood CD4+EBI3+ T cells in the VMC group both showed a correlation with the levels of IL-17 and cTnI, suggesting that VMC patients exhibit Th17 cell and Treg differentiation and dysfunction as well as an insufficient Treg number or function. Regarding the immunological process of the development of VMC, we considered that, first, the decrease in the number and function of Tregs can cause a reduction of the IL-35 level, thus reducing its myocardial protection function. The low level of IL-35 would in turn significantly decrease the promotion of proliferation and differentiation in Tregs, thus causing abnormal activation of the immune system in VMC patients and the loss of self-immune tolerance in the body. Second, excessive activation of Th17 cells and the production and release of a large amount of inflammatory mediators, particularly IL-17, further mediate the development of VMC. Whether IL-35 and IL-17 can influence each other through a complex cytokine network in VMC patients should be determined in further in-depth studies.

The authors declare no conflict of interest.
This study was partly supported by the National Natural Science Foundation of China (grant no. 81302584, grant no. 81200507) and the Natural Science Foundation of Jiangsu Province (grant no. SBK201121707).

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