Increased expression of CXCR5 and CXCL13 in mice with experimental autoimmune myocarditis

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Abstract. - OBJECTIVE: Myocarditis is an inflammatory heart muscle disease associated with cardiac dysfunction, and autoimmunity is considered to play an important role in the pathogenesis of myocarditis. CXCL13 and its receptor CXCR5 have been reported to be associated with many diseases including some cancers and inflammatory diseases, but so far there has been no report on CXCR5/CXCL13 expression in myocarditis.

MATERIALS AND METHODS: With a mouse experimental autoimmune myocarditis (EAM) model, it was found that the mRNA and protein expression of both CXCR5 and CXCL13 were increased in myocardial tissue in the EAM mice. This revealed certain connection between CX-CR5/CXCL13 with autoimmune myocarditis, so CXCR5 and CXCL13 may be used as a biomarker for autoimmune myocarditis diagnosis.

RESULTS: The results also demonstrated increased expression of pro-inflammatory cytokines IL-1 β , IL-6, IL-17 and TNF- α in the serum of myocardial tissue in the EAM mice. These pro-inflammatory cytokines may be important targets for developing new drugs in treating myocarditis.

CONCLUSIONS: The current study established an association between CXCR5/CXCL13, autoimmune myocarditis and pro-inflammatory cytokines, and provided sound basis for further studies on mechanism and treatment of autoimmune myocarditis.

Key Words:

CXCR5, CXCL13, Myocarditis, Autoimmune myocarditis, Pro-inflammatory cytokines.

Introduction

Myocarditis, defined as inflammation of the heart with severe ventricular dysfunction, is a major cause of dilated cardiomyopathy and heart failure in individuals younger than 40 years old, which often leads to life-threatening arrhythmia and sudden cardiac death^{1,2}. The pathogenesis of myocarditis remains undefined, but elevated circulating autoantibodies are commonly found in patients with myocarditis or dilated cardiomyopathy, and autoantibody removal improves cardiac function^{3,4}. Autoimmunity is considered to play an important role in the pathogenesis of myocarditis, and cardiac myosin is one of the main autoantigens in virus-induced myocarditis in mice^{5,6}. CXCL13, an inflammatory factor in the microenvironment, plays a vital role in the progression of inflammatory diseases and has been proposed as a biomarker in a variety of conditions. CXCL13 is a member of CXC chemokine family and originally named B-cell-attracting chemokine 1 (BCA-1)⁷. CXCL13 is the unique ligand for CXCR5, which is a member of G-protein coupled receptors (GPCR) family⁸. CXCL13-CXCR5 axis is proved to be involved in regulating lymphocyte migration and promoting inflammation9,10. Besides, the CXCR5-CXCL13 axis is proposed to participate in tumor development. Several studies^{11,12} have demonstrated that CXCL13 and its receptor CXCR5 act as new targets for the detection and treatment of lymphoma. It is also reported that CXCL13 is overexpressed in the tumor tissue and the peripheral blood of breast cancer patients¹³, and the CXCL13-CXCR5 axis is closely related to the poor prognosis of breast cancer¹⁴. However, so far there has been no report on CXCL13-CXCR5 expression in myocarditis.

Some other inflammatory factors are cytokines, which are small proteins that are produced in many diverse cells of the immune system by three different populations of CD4+ T lymphocytes: Th1, Th2, and Th1¹⁵. The Th1/Th2 regulation has been the cornerstone of the mechanistic and therapeutic aspects of autoimmune diseases over the past 2 decades until the discovery of Th17 cells¹⁶. Th1 cells produce pro-inflammatory cytokines IL- 2, IFN- γ , TNF, IL-1 β , which lead to the activation of macrophages and cytotoxic effects. Th2 cells produce IL-4, IL-5, IL-6, IL-10, IL-13, which can inhibit the production of Th1 cytokines, but primarily stimulate B-cells to produce antibodies and activation of anti-apoptotic molecules¹⁷. Th17 cells, which have been described recently, secrete pro-inflammatory cytokines (IL-17, IL-17F, IL-21, IL-22) and play an important role in chronic inflammatory diseases such as asthma and systemic lupus erythematosus¹⁸. In recent years, it has been reported that cytokines play a pivotal role in the pathogenesis of autoimmune diseases¹⁶.

To understand the mechanism of autoimmune myocarditis and gain insight of potential treatment strategies, a mouse experimental autoimmune myocarditis (EAM) was constructed, and RT-CPR and Western Blot were used to examine the mRNA and protein expression of CXCR5 and CXCL13 in mouse myocardial tissue. It was found that mRNA and protein expression of both CXCR5 and CXCL13 were significantly increased in the EAM mice, which indicated certain connection between CXCR5/CXCL13 with autoimmune myocarditis. CXCR5 and CXCL13 may be used as biomarkers for autoimmune myocarditis diagnosis. The results also demonstrated increased expression of pro-inflammatory cytokines IL-1 β , IL-6, IL-17 and TNF- α in the serum of myocardial tissue in the EAM mice. These pro-inflammatory cytokines may be important targets in designing new drugs to treat autoimmune myocarditis. The current exploratory studies have established a connection between CXCR5/ CXCL13 and autoimmune myocarditis, and provided a solid basis for future studies on the mechanism and treatment of autoimmune myocarditis.

Materials and Methods

Animals

Twenty specific-pathogen-free (SPF) male BALB/c mice weighing 18-20 g were purchased from the Experimental Animal Center of Wuhan University (Wuhan, Hubei, China) and maintained in a pathogen-free animal facility, which was maintained at 22-24°C with 50% to 60% humidity. The animals had easy access to food and water before being used in experiments. The study protocol was approved by Animal Care and Use Committee (ACUC) of China-Japan Union Hospital of Jilin University (Changchun, Jilin, China).

Animal Grouping and EAM Model Construction

The 20 male BALB/c mice were randomly divided into two groups, 10 as the control group, and 10 as the EAM model group. For EAM model construction, porcine cardiac a-myosin dissolved in phosphate-buffered saline (PBS) (10 mg/ ml) was emulsified 1:1 with complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 mg/mL heat-killed Mycobacterium tuberculosis strain H37Ra. Each mouse was subcutaneously injected with 200 µl of emulsion (containing 100 µg of porcine cardiac α -myosin) by right side inguinal injection or subaxillary injection on days 0 and 7 to induce EAM¹⁹. The control group mice were treated with CFA mixed with PBS following the same procedure as for the EAM group mice. The assessment and analysis of acute EAM were conducted on days 14 and 21. No mice died during the experiments.

ELISA measurement of Serum IL-1 β , IL-6, IL-17 and TNF- α

On day 14 after the first immunization, 5 mice were taken from each group and anesthetized by intraperitoneal injection of pentobarbital sodium. After eyeball removal, 1 mL of blood was collected from each mouse around eye and kept at 4°C overnight to let the serum separated from the blood cells. The serum was then collected for enzyme-linked immunosorbent assay (ELISA) assay. Serum IL-1 β , IL-6, IL-17 and TNF- α levels were measured using ELISA kit purchased from Boster company (Boster, Wuhan, China), following the manufacture's instruction. On day 21, the same procedure was carried out for the rest 5 mice in each group.

Tissue Processing and Staining

After the blood sample has been collected on day 14, the 5 mice in each group were sacrificed and the hearts were taken out. Hearts were cut into halves, one-half for tissue staining and the rest half for RT-PCR and Western blotting. For staining, the hearts were washed with ice-cold PBS, and blotted with filter paper. Each heart was sectioned into coronal slices of 2 mm thickness, then cassetted and fixed directly in 10% neutral formalin for 24 h, which was followed by dehydration

Table I. Primers used in RT-PCR experiments.

Gene	Primers	Length (bp)
CXCR5	5'-TGGCCTTCTACAGTAACAGCA-3'	21
	5'-GCATGAATACCGCCTTAAAGGAC-3'	23
CXCL13	5'-GGCCACGGTATTCTGGAAGC-3'	20
	5'-ACCGACAACAGTTGAAATCACTC-3'	23
β-actin	5'-CTTCCTGGGCATGGAGTCCTG-3'	21
	5'-GGAGCAATGATCTTGATCTTC-3'	21

in increasing concentrations of ethanol, clearing with xylene and embedding with paraffin. 3-µm sections were prepared from paraffin blocks and stained with Hematoxylin and eosin (H&E) according to the manufacturer's instructions using the Hematoxylin and Eosin Staining Kit (Baihao Biological Technology Co., Ltd., Tianjin, China). The stained sections were evaluated using Olympus light microscopy (Tokyo, Japan). The same procedure was repeated on day 21 after the first immunization.

Ouantitative RT-PCR

To measure the mRNA levels of CXCR5 and CXCL13, the total RNA was extracted from mouse myocardial tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The concentration of total RNA was quantified by UV 260 nm observance. 1000 ng total RNA was used as a template for reverse transcription using M-MuLV and the MasetrMix Reverse Transcription Kit (Takara, Otsu, Shiga, Japan) following the manufacturer's instruction. β -actin was selected as the internal reference. The sequences of primers used in PCR were listed in Table I. The PCR products were amplified using the following cycling parameters: for CXCR5 and CXCL13, 95°C for 3 min, followed by 30 cycles of 94°C for 60 s, 56°C for 40 s, and 72°C for 60 s, and finally a single cycle at 72°C for 10 min; for β-actin, 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 40 s, and finally a single cycle at 72°C for 10 min. 10 μ L PRC product was mixed with 2 μ L loading buffer and applied to 1.5% agarose gel containing 0.05 µg/mL ethidium bromide for 50 min under 70V. $2^{-\Delta\Delta Ct}$ method²⁰ was used to calculate the relative mRNA levels of CXCR5 and CXCL13 mRNA using β -actin mRNA as an internal reference. The measure was repeated for three times and the average was used.

For Western blot analysis, mouse myocardial tissue was lysed with 20 mM Tris-HCl buffer, pH 8.0, containing 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% β -mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was determined by the bicinchoninic acid assay (BCA) protein assay method using bovine serum albumin as standard (Pierce Biotechnology, Appleton, WI, USA). β-actin served as an internal control. 20 µg protein samples were loaded per lane, separated by SDS-PAGE (8% polyacrylamide gels) and then were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dry milk in Tris-Buffered Saline with Tween (TBS-T) 20 (50 mM Tris, 150 mM NaCl, and 0.1% Tween 20 vol/vol, pH 7.4) for 1.5 h or at 4°C overnight, and then incubated overnight at 4°C with polyclonal rabbit anti-mouse CXCR5 and polyclonal goat anti-mouse CXCL13 antibodies as well as β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500). The PVDF membrane was washed 3 times with Tween 20, each time 6 min, after which the horseradish peroxidase-labeled secondary antibodies (rabbit anti-goat and goat anti-rabbit) were added (Boster, Wuhan, China, 1:5000) and then incubated for 2 h. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA). The relative expression of CXCR5 and CXCL13 was quantified densitometrically using the software Image-Pro Plus, and calculated according to the reference bands of β -actin.

Statistical Analysis

Statistical analysis was performed with the program SPSS statistical software, version 19.0 (IBM Corp., Armonk, NY, USA). Data were expressed as the mean \pm standard deviation (SD). Statistical analyses were performed with two-tailed student t-test for two groups comparison. Statistical significance was assumed at p < 0.05, and significant difference was assumed at p < 0.01.

Results

H&E Tissue Staining

Mice were immunized on days 0 and 7, and at sacrifice on days 14 and 21, hearts were removed



Figure 1. H&E staining of mouse myocardial tissue under $200 \times$ microscope. (A) control group mouse on day 14 after first immunization; (B) EAM group mouse on day 14; (C) control group mouse on day 21; (D) EAM group mouse on day 21.

for histological examination with H&E staining (Figure 1). In the control group mice, there are no visible inflammatory infiltration lesions in the myocardial tissue (Figure 1A and 1C). In the EAM group mice, focal inflammatory infiltration lesions could be observed on day 14 after first immunization, mainly under epicardium but not in the interstitial space (Figure 1B). There was some cytoexudation of inflammatory cells, mainly lymphocytes. Inflammatory infiltration lesions became more obvious on day 21 in the EAM group mice, between cardiac muscle cells and also in the There was more cytoexudation of inflammatory cells, mainly lymphocytes, neutrophil granulocytes, and plasmocytes, etc., with occasional zones of myocyte necrosis (Figure 1D). This indicated that the EAM model construction was successful.

mRNA levels of CXCR5 and CXCL13

RT-PCR was used to measure the mRNA level of CXCR5 and CXCL13 in mouse myocardial tissue. The results were shown in Figure 2. In the control group mice, there was small amount mR-NAs of CXCR5 and CXCL13, which is normal. The levels of both CXCR5 and CXCL13 mRNAs on day 14 and day 21 had no significant difference (p > 0.05). However, in the EAM group mice, the mRNA levels of CXCR5 and CXCL13 were both significantly increased on day 14 compared to the control group mice (p < 0.05). On day 21, the mRNA levels of CXCR5 and CXCL13 were further increased, and were significantly higher than that in the control group (p < 0.01).

Protein Expression of CXCR5 and CXCL13

The protein expression of CXCR5 and CXCL13 mouse myocardial tissue was measured by Western Blot. As shown in Figure 3, in the control group mice, there was small amount of expression for both CXCR5 and CXCL13, which is normal. The levels of both CXCR5 and CXCL13 expression on day 14 and day 21 had no significant difference (*p*



Figure 2. The mRNA levels of CXCR5 (left) and CXCL13 (right) in mouse myocardial tissue as detected by RT-PCR. The relative mRNA level of CXCR5 and CXCL13 was calculated based on the mRNA level of β -actin. *and **represent significant difference compared to the control group mice at *p* <0.05 and *p* <0.01 respectively.

> 0.05). However, in the EAM group mice, the protein expression levels of CXCR5 and CXCL13 were both significantly increased on day 14 compared to that in the control group mice (p < 0.05). On day 21, the expression levels of CXCR5 and CXCL13 were further increased, and were significantly higher than that in the control group mice (p < 0.01).

Concentration of IL-1 β , IL-6, IL-17 and TNF- α in Serum

The concentration of some inflammatory factors in serum including IL-1 β , IL-6, IL-17 and TNF- α

was measured by ELISA assay (Figure 4). In the control group mice, the concentration of all these four inflammatory factors was at normal levels at both day 14 and day 21, although the concentration on day 21 was slightly higher than that on day 14. In the EAM group mice, however, the concentration of all the four inflammatory factors was significantly higher than that in the control group mice (p < 0.05) on day 14. On day 21, the concentration of all four inflammatory factors was slightly higher than day 14, significantly higher than that in the control group mice than day 14, significantly higher than that in the control group mice (p < 0.05).



Figure 3. The protein expression level of CXCR5 (left) and CXCL13 (right) in mouse myocardial tissue as detected by Western-Blot. The relative expression of CXCR5 and CXCL13 was calculated according to the reference bands of β -actin. * and ** represent significant difference compared to the control group mice at p<0.05 and p<0.01 respectively.



Figure 4. Concentration of the proinflammatory cytokines IL-1 β (upper left), IL-6 (upper right), IL-17 (bottom left) and TNF- α (bottom right) in the serum of mouse myocardial tissue detected by ELISA. * and ** represent significant difference compared to the control group mice at *p*<0.05 and *p*<0.01 respectively.

Discussion

By constructing an EAM mice model, the experiments reported here have demonstrated that the mRNA and protein expression of both CXCR5 and CXCL13 were increased in EAM mice. Therefore, CXCR5 and CXCL13 can be used as biomarkers of autoimmune myocarditis. This is the first time to establish the connection of CXCR5/CXCL13 with autoimmune myocarditis. It has been shown that neutralization of CXCL13 by anti-CXCL13 can modify disease in a mouse model of arthritis by disrupting tertiary follicles and modulating B-cell responses²¹. Therefore, it will be interesting to test if neutralizing CXCL13 in the EAM mice will have similar therapeutic effect or not. The current studies also identified four critical pro-inflammatory cytokines involved in the autoimmune myocarditis, namely IL-1 β , IL-6, IL-17 and TNF- α . In recent years, some cytokines have been shown to be effective targets

for treating many autoimmune diseases²². For example, anti-TNF-a mAb has been used for treating rheumatoid arthritis (RA)^{23,24}. Tocilizumab, a mAb targeting IL-6Ra which binds IL-6, was reported to attenuate joint inflammation, bone erosion, and systemic inflammation in RA²⁵, juvenile RA (Still's disease) and Castleman's disease²⁶. Anakinra is a recombinant human form of IL-1RA, the natural antagonist of IL-1. Anakinra was found highly effective in modulating the Cryopyrin-associated periodic syndromes, including neonatalonset multisystem inflammatory disease, Muckle-Wells syndrome, acute and chronic gout, and juvenile RA²⁷. Secukinumb is an inhibitory anti-IL-17A mAb and is effective in psoriasis, psoriatic arthritis, and ankylosing spondylitis²⁸. Therefore, targeting the pro-inflammatory cytokines identified in this study may be an effective treatment strategy to autoimmune myocarditis, which is worthy of further study. Overall, as an exploratory work, the currently reported work has been successful in demonstrating increased CXCR5 and CXCL13 expression in EAM mice, as well as identified the pro-inflammatory cytokines involved in the EAM model. The next steps to follow would be to test compounds, reagents and antibodies targeting CXCR5/CXCL13 and the four pro-inflammatory cytokines, IL-1 β , IL-6, IL-17 and TNF- α , to evaluate their therapeutic potential for treating autoimmune myocarditis, and to aid the search for effective treatments of autoimmune myocarditis.

Conclusions

We showed that in a mouse EAM model, the mRNA levels and protein expression of both CXCR5 and CXCL13 were significantly increased in mouse myocardial tissue of EAM mice, which revealed the connection between CXCR5/CXCL13 with autoimmune myocarditis. The results also demonstrated increased expression of four pro-inflammatory cytokines, IL-1 β , IL-6, IL-17 and TNF- α , in the serum of myocardial tissue in the EAM mice. These researches provided sound basis for future studies on the mechanism and treatment of autoimmune myocarditis.

Conflict of interest

The authors declare no conflicts of interest.

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