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MicroRNA-98 plays a critical role in experimental myocarditis

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ABSTRACT

Background and aims: Myocarditis is inflammation in the heart; its pathogenesis is to be further investigated. Activities of micro RNAs (miR) are associated with immune inflammation. This study tests a hypothesis that miR-98 is involved in the development of myocarditis.
Methods: BALB/c mice were immunized with cardiac α-myosin heavy chain peptides (MyHC-α) to induce myocarditis. The effects of miR-98 on regulation of interleukin (IL)-10 were assessed by real time RT-PCR.
Results: Mice immunized with MyHC-α showed myocarditis and lower frequency of IL-10$^+$ B cells (B10 cell) in the hearts. Expression of miR-98 was higher, IL-10 was lower, in B cells isolated from the mouse hearts with myocarditis, which was negatively correlated with each other. Exposure to tumor necrosis factor-α up regulated miR-98 expression in B cells. Over-expression of miR-98 suppressed IL-10 expression in B cells. Blocking miR-98 or adoptively transplanting B10 cells attenuated experimental myocarditis.
Conclusions: miR-98 suppresses IL-10 expression in B cells in the heart, which plays an important role in myocarditis. MiR-98 may be a therapeutic target in the treatment of myocarditis.

1. Introduction

Myocarditis is an inflammatory condition in the heart muscle, which can be induced by viral infection, hypersensitivity to certain substances and autoimmune reactions. If it is not treated properly, myocarditis may complicate the dilated cardiomyopathy, the latter may develop to heart failure [1]. Although research in this area advanced rapidly in the recent years, the remedies to cure myocarditis are still limited [2]. Thus, to elucidate the pathogenesis of myocarditis is of significance.

The autoimmune myocarditis has been developed with mouse models. Myocyte-specific antigens (including cardiac troponin-I and myosin heavy chain) [3,4] and coxsackie virus B3 [5] can be used to induce autoimmune myocarditis. The experimental mouse hearts show increases of nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), interleukin (IL)-4, IL-6, transforming growth factor-β (TGF-β), creatine kinase, C-reaction protein (CRP), adhesion molecule-1, monocyte chemoattractant protein-1 and interferon-γ (IFN-γ) [6]. Yet, the underlying mechanism remains to be investigated.

Immune regulatory cells can suppress autoimmunity in the body [7]. Regulatory T cells and regulatory B cells are the two major cell fractions fulfilling the immune regulatory function [8,9]. IL-10 is an immune regulatory molecule that can be released by regulatory T cells or regulatory B cells to inhibit other effector immune cell activities [10]. The IL-10-producing B cells are also called B10 cells, which play an important role in the suppression of autoimmunity [11]. Whether B10 cells suppress myocarditis has not been defined.

Cumulative reports indicate that microRNAs (miR) are associated with the regulation of immune inflammation in the cardiovascular system [12]. MiRs are single non-coding RNA chain with 18–22 nucleotides in length and regulate bio-molecule expression post-transcription. Published data indicate that miR-98 negatively regulates IL-10 production [13]. Whether miR-98 affects B10 cells in the hearts in myocarditis is unclear. Based on the information above, we hypothesize that miR-98 may suppress the expression of IL-10 in B cells of the hearts. Thus, we developed a mouse model of myocarditis and observed that the expression of IL-10 in B cells of the hearts with myocarditis was significantly suppressed, in which miR-98 played a critical role.

2. Materials and methods

2.1. Mice and ethic statement

Male BALB/c mice (6–8 weeks old) were purchased from the Beijing Experimental Animal Center. The mice were maintained in a pathogen-free environment with accessing food and water freely. The experimental procedures were approved by the Animal Ethic Committee at Fuwai Hospital.

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2.2. Collection of human heart tissue

Human hearts were collected from 3 patients (1 female, age: 46 years old; 2 males, age: 37 and 56 respectively) died from myocarditis and 3 persons (1 female, age: 35; 2 males, age: 36 and 44 years old) with normal hearts died from injuries. The using human tissue in the present study was approved by the Human Ethics Committee of Beijing Fuwai Hospital. An informed written consent was obtained from the relatives of each patient.

2.3. Induction of myocarditis in mice

BALB/c mice were subcutaneously injected with cardiac α-myosin heavy chain peptides (MyHC-α, 614–629 [AcRSLKLMATLFSTYASADR-OH]; purity > 95%; GL Biochem, Shanghai, China) at 1 mg/mouse mixed with 0.2 ml complete Freund's adjuvant on day 0 and day 7 respectively.

2.4. Histology of the mouse heart

Upon sacrifice, the hearts were excised from mice and fixed with 4% formalin overnight. The heart tissue was processed for paraffin sections. The sections were stained with hematoxylin and eosin, and observed under a light microscope. Following published data [14], the heart tissue inflammatory condition was graded from 0 to 4; the "0" indicates no inflammation, the "4" indicates severe inflammation. The scoring system is demonstrated in Table 1. The tissue section slides were coded. The observers were not aware of the code to avoid the observer bias.

2.5. Isolation of B cells from the mouse hearts

The hearts were excised from mice immediately after death. The hearts were cut into small pieces (about 2 × 2 × 2 mm in size). The samples were incubated with collagen IV (0.5 mg/ml) at 37 °C with mild stirring for 1 h. The mononuclear cells were filtered through a cell strainer (40 μm). $CD19^+$ B cells were further isolated from the mononuclear cells by magnetic cell sorting with a reagent kit (Santa Cruz Biotech) following the manufacturer's instructions. The purity of the isolated B cells was greater than 98% as checked by flow cytometry.

2.6. Cell culture

The isolated cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 0.1 mg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine. The viability of the cells was greater than 98% before handing over for further experiments as assessed by Trypan blue exclusion assay.

2.7. Assessment of miR-98 and IL-10 mRNA by real time RT-PCR (RT-qPCR)

The total RNA was extracted from B cells with the TRIzol reagents (Invitrogen). The cDNA was synthesized with the RNA and reverse transcription reagents (Invitrogen). The samples were amplified in a real time PCR device (Mini Opticon, Bio-Rad, Hercules, CA) with the SYBR Green Master Mix (Invitrogen). The primers of miR-98 were provided by Enke Biotech (Shenzhen, China). Reference gene RNA U6B (Invitrogen) was analyzed as an internal control. The sequences of IL-10 primers for PCR are gttctttggggagccaacag and gctccctggtttctcttcct. The results were calculated by the $2^{-\Delta\Delta Ct}$ method and presented as fold change against controls. The samples were tested in triplicate.

2.8. Flow cytometry

For the surface staining, cells were stained with fluorochrome-labeled antibodies of interest (or isotype IgG) for 30 min at 4 °C. For the intracellular staining, the cells were fixed with 1% paraformaldehyde for 1 h and exposed to 0.5% saponin for 30 min to increase the permeability of the cell membrane. The cells were then incubated with fluorochrome-labeled antibodies of interest (or isotype IgG) for 30 min at 4 °C. After washing with phosphate-buffered saline (PBS) for 3 times, the cells were analyzed with a flow cytometer (FACSCanto II, BD Bioscinece). Data were analyzed with software FlowJo (TreeStar, Ashland OR). The data of isotype IgG staining were used as a gating reference.

Table 1
Myocarditis inflammatory score system.

Score	Assessment
0	No inflammation
1	1–5 distinct mononuclear inflammatory area, with the involvement of 5% or less of the cross-sectional area of the heart
2	More than 5 distinct mononuclear inflammatory area, or the involvement of over 5% but not over 20% of the cross-sectional area of the heart
3	Profound mononuclear infiltration involving over 20% of the area, without necrosis
4	Diffuse inflammation with necrosis in the heart

2.9. Assessment of serum cytokines by enzyme-linked immunosorbent assay (ELISA)

The blood was collected from each mouse at sacrifice. The sera were isolated from the blood samples by centrifugation at 4 °C. The levels of Th1 and Th2 cytokines in the sera were determined by ELISA with purchased reagent kits (R&D Systems) following the manufacturer's instructions.

2.10. Assessment of the effects of TNF-α or IL-1β on enhancing miR-98 expression in B cells

$CD19^+$ B cells were isolated from the naive mouse spleen by magnetic cell sorting (MACS) with commercial reagent kits (Miltenyi Biotech) following the manufacturer's instructions. The purity of the B cell was greater than 99% as checked by flow cytometry. Some naive B cells were stimulated by LPS (Sigma Aldrich) in the culture as reported by previous studies in order to enhance the expression of IL-10 by B cells [15]. To assess the effects of TNF-α or IL-1β on the expression of B cells, B cells were exposed to LPS with or without the presence of TNF-α or IL-1β at gradient concentrations for 48 h. The levels of IL-10 mRNA in the B cells were determined by RT-qPCR as described above.

2.11. Assessment of the role of miR-98 in the TNF-α-suppressed IL-10 expression in B cells

2.11.1. Preparation of miR-98-over expressing B cells

B cells were prepared as described above and transfected with miR-98-plasmids (or empty control plasmids) provided by the Enke Biotech (Shenzhen, China) following the manufacturer's instructions. The effects of the transfection were assessed by RT-qPCR.

2.11.2. Preparation of miR-98-deficient B cells

B cells were prepared by transducing the miR-98-shRNA-laden lentivirus or control lentivirus (Enke Biotech, Shenzhen, China) following the manufacturer's instructions. The effects of miR-98 knockdown were assessed by RT-qPCR with the procedures described above.

2.12. Attenuating myocarditis in mice with anti-miR-98 or adoptive transfer with B10 cells

2.12.1. Preparation of anti-miR-98 liposome

Following published procedures [16], anti-miR-98 oligonucleotides (0.086 μmol; Beijing Yijie Biotech; Beijing, China) were mixed with a lipid mixture (Sigma Aldrich) in 200 μl of 100% ethanol and 300 μl of 20 mM citrate buffer (pH 4) at 60 °C. The samples were extruded through a polycarbonate membrane (100-nm-diameter) using a LiposoFast basic extruder (Avestin, Toronto, Canada). A Sepharose CL-4B column was prepared and equilibrated with HBS pH 7.4. The samples were passed through the column to remove ethanol and nonencapsulated *anti*-miR-98. The total lipid concentration was assessed by cholesterol quantification using the Liebermann–Burchard test [17].

2.12.2. Generation of B10 cells

$CD19^+$ B cells were isolated from the naïve mouse spleen by MACS (the purity was greater than 99% as checked by flow cytometry). The B cells were cultured at 10^6 cells/ml in the presence of LPS (1 μg/ml) and *anti*-CD40 mAb (20 ng/ml) for 3 days. As checked by flow cytometry, the IL-10^+ B cells were more than 98% (data not shown).

2.12.3. Inhibition of miR-98 in mice with anti-miR-98 liposomes

Mice were immunized with MyHC-α to induce myocarditis as described above. One day prior to each exposure to MyHC-α, the myocarditis mice were treated with one of the following procedures: (A) Intraperitoneal injection with anti-miR-98 liposome (0.1 mg/mouse; or control liposomes). (B) Adoptive transfer with B10 cells or naïve B cells at 10^6 cells/mouse via tail vein injection. After sacrifice, the mouse hearts were excised to be processed for paraffin sections to evaluate the inflammatory condition.

2.13. Statistics

Data are presented as mean ± SD. The difference between two groups was determined by the paired non-paired Student t test or ANOVA if more than two groups. $p < 0.05$ was set as the significant criterion.

3. Results

3.1. Levels of miR-98 negatively correlate with IL-10 expression in B cells of mouse hearts with myocarditis

To elucidate the role of miR-98 in myocarditis, we developed an autoimmunity myocarditis mouse model. Profound infiltration of inflammatory cells was observed in the heart of mice immunized with MyHC-α, indicating the myocarditis was developed in the mice (Fig. 1A–B). A similar phenomenon was found in patients with myocarditis (Fig. 1C). We also found that the frequency of IL-10^+ B cells were much less in the hearts with myocarditis (Fig. 1D–E).

To characterize the cells infiltrated in the heart tissue as shown by Fig. 1, we isolated the mononuclear cells from the hearts. The results

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