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Original Article

Distinct Th17 inductions contribute to the gender bias in CVB3-induced myocarditis

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ABSTRACT

Background: Viral myocarditis is often caused by coxsackievirus B3 (CVB3) infection and occurs more frequently in males. So far, the mechanisms for this sex difference are not fully elucidated. As a new proinflammatory T cell population, Th17 cells are required for the development of CVB3-induced myocarditis, but their impact on the gender bias in viral myocarditis is still unknown.
Methods: Male and female mice were intraperitoneally infected with CVB3; 7 days later, the frequency of splenic Th17 cells and the expression of associated cytokines and transcriptional factors were compared. Meanwhile, the impact of sex hormones on Th17 cell differentiation post CVB3 infection was also evaluated.
Results: In infected male mice, Th17 cell frequency was remarkably increased and significantly higher than that in female mice. Accordingly, the expression of associated cytokines and transcriptional factors was also obviously augmented in males. When neutralizing interleukin-17 by monoclonal antibody, the male prevalence of myocarditis was obviously abolished, further confirming the effect of Th17 cells on gender bias in viral myocarditis. It was also found that estradiol significantly inhibited the Th17 differentiation post CVB3 infection both in vitro and in vivo. However, testosterone showed no such effects.
Conclusions: Th17 cells were predominantly induced in CVB3-infected males than females as the inhibitory effect of estrogen on Th17 differentiation and played an important role in the sex differences in the sensitivity to CVB3-induced myocarditis. This study may help us understand the role of Th17 cells in viral myocarditis and facilitate the development of corresponding therapeutic strategies.

1. Introduction

Viral myocarditis is a cardiac inflammatory and injury disease associated with virus infection. It could further develop into dilated cardiomyopathy, one of the leading causes of heart failure with a 50% motility within 1 to 2 years after diagnosis [1–3]. Although many different infectious agents have been attributed as the cause of viral myocarditis, enteroviruses, in particular, coxsackievirus B3 (CVB3), are consistently among the main etiological pathogens [4].

So far, knowledge about the pathological mechanisms and control strategy for viral myocarditis mainly comes through murine CVB3 infection model [5–8], which remarkably shares many characteristics with human myocarditis including the increased incidence and mortality in male gender [9,10]. Although a line of evidence showed that diverse sex hormones and immune response patterns contribute to the sex differences in the sensitivity to viral myocarditis [11,12], the underlying mechanisms are still not fully clarified.

It is well-accepted that $CD4^+$ T cells play an important pathogenic role in CVB3-induced myocarditis, as the disease severity is significantly attenuated in CD4 knockout mice [13]. Besides the already known interferon (IFN)-γ-secreting Th1 and interleukin (IL)-4-secreting Th2 cells in viral myocarditis [8], the role of IL-17-secreting $CD4^+$ T cells (Th17) in CVB3 infection is receiving more and more attention. Previous studies showed that, in CVB3-infected mice, splenic Th17 cell frequency, as well as serum and cardiac IL-17 expression, was significantly elevated [14] and contributed to the development of myocarditis by promoting virus replication [15] and facilitating anti-adenine nucleotide translocator autoantibody production [16]. These results indicated that Th17 cells were involved in CVB3-induced myocarditis. However, whether Th17 cells could be induced differentially in male and female

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mice and modulate the sex differences in the development of viral myocarditis remains unknown.

In this study, we compared the splenic Th17 cell frequencies in both sexes of CVB3-infected mice and found that more Th17 cells were induced in males, and this increase appeared in time- and virus-dose dependent patterns. Further exploring the underlying mechanism, we found that, in contrast to no obvious influence of testosterone on Th17 cell induction, estrogen significantly inhibited Th17 cell differentiation and led to the alleviated viral myocarditis. Our study may shed light on the role of Th17 cells in gender bias in CVB3-induced myocarditis and provide clues for the development of novel therapeutic strategies against inflammatory heart diseases.

2. Materials and methods

2.1. Animals

Male and female BALB/c (H-2^d) mice, 6 weeks of age, were purchased from Experimental Animal Centre of Chinese Academy of Sciences (Shanghai, P.R. China) and bred in the specific pathogen-free facility. All animal experiments were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Medical Laboratory Animals* (Ministry of Health, P.R. China, 1998). The protocol was approved by the Ethical Committee of Soochow University.

2.2. Virus

CVB3 (Nancy strain) was maintained by passage through Hela cells (ATCC number: CCL-2). Virus titer was determined by a 50% tissue culture infectious dose ($TCID_{50}$) assay of HeLa cell monolayer and calculated by the Reed–Muench method. Mice were infected by an intraperitoneal (ip) injection of 0.1 ml of phosphate-buffered saline (PBS) containing 10^3 $TCID_{50}$ dose of the virus.

2.3. Quantization of viral burden in heart tissues

Seven days after 10^3 $TCID_{50}$ CVB3 infection, hearts were collected and homogenized. To determine the myocardial viral RNA load, total RNA was reversely transcripted to cDNA using CVB3-specific positive-strand RNA primer (5'-CACCGGATGGCCAATCCA-3') or negative-strand RNA primer (5'-GCGAAGAGTCTATTGAGCTA-3') and then subjected to SYBR green real-time polymerase chain reaction (PCR) using CVB3 primers (5'-ATCAAGTTGCGTGCTGTG-3' and 5'-TGCGAAATGAAAGGAGTGT-3'). The expression of virus RNA load was normalized by GAPDH expression.

2.4. Histopathological analysis and myocarditis scoring

Hearts were collected from infected mice at day 7, fixed in 10% buffered formalin solution, and embedded in paraffin. Sections 5 μm thick were cut and stained with hematoxylin and eosin. The extent of myocardial lesions was quantified and scored depending on the severity as follows: 0=no inflammation; 1=one to five distinct mononuclear inflammatory foci with involvement of 5% or less of the cross-sectional area; 2=more than five distinct mononuclear inflammatory foci, or involvement of over 5% but not over 20% of the cross-sectional area; 3=diffuse mononuclear inflammation involving over 20% of the area, without necrosis; and 4=diffuse inflammation with necrosis. Analysis was performed in a double-blinded manner by a trained pathologist.

2.5. Real-time PCR

The total RNA of 10^7 splenocytes was extracted with RNAiso Plus (Takara) and then reverse transcripted into cDNA. Primers for cytokines (IFN-γ, IL-4, IL-17A, IL-6, IL-21, IL-22), transcriptional factors (RORγt, RORα), and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase are designed by Primer Premier 5.0, and sequences were shown in Table 1. Gene expressions were detected by SYBR green real-time PCR, and quantification of data was analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.6. FACS analysis

Single-cell suspension of spleen from CVB3-infected mice was prepared, resuspended at a density of 5×10^6/ml to a 24-well culture plate, and stimulated with 50 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma) plus 5 μg/ml Brefeldin A (eBioscience) at 37°C, 5% CO_2. Five hours later, cells were collected and stained with fluorescein-isothiocyanate-conjugated anti-mouse CD4 (eBioscience); after washing, cells were fixed with Fixation buffer (eBioscience) for 20 min at 4°C, permeabilized with permeabilization solution (eBioscience) at room temperature for 20 min, and then stained with PE-conjugated anti-mouse IL-17A (eBioscience). The percentage of IL-17A-secreting $CD4^+$ T cells was determined by flow cytometry using a FACScalibur instrument (Becton Dickinson) and CellQuest software (Becton Dickinson).

2.7. Intervention with IL-17 neutralizing antibody

Male or female mice were randomly divided into three groups: (a) Control group. Mice were treated with CVB3 and PBS (100 μl per mouse) ip on day 0. (b) Isotype control group. Mice were treated with CVB3 and 100 μg per mouse isotype IgG1 monoclonal antibody (mAb) (eBioscience) ip on day 0. (c) IL-17 neutralizing antibody group. Mice were treated with CVB3 and 100 μg per mouse IL-17A neutralizing mAb (eBioscience) ip on day 0.

2.8. In vitro Th17 cell differentiation

Splenic $CD4^+CD25^-CD62L^{high}CD44^{low}$ T cells were isolated from male BALB/c mice by FACS and stimulated with 2.5 μg/ml anti-CD3 (BioLegend) and 2.5 μg/ml anti-CD28 antibodies (BioLegend) under Th17 cell polarization conditions with 5 ng/ml TGF-β (Prospec) and 30 ng/ml IL-6 (Prospec) for 4 days. For evaluating the effect of estrogen on Th17 cell differentiation, different doses of 17β-estradiol were added at the beginning of T cell differentiation experiments as described [17]. Then, the cells were restimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) for 5 h before intracellular IL-17 staining.

Table 1
Real-time PCR primers.

Genes	Primer sequence (5'-3')
IFN-γ	Sense: ACTGGCAAAAGGATGGTGAC
	Antisense: TGAGCTCATTGAATGCTTGG
IL-4	Sense: GCTAGTTGTCATCCTGCTC
	Antisense: GTGATGTGGACTTGGACTC
IL-17A	Sense: CTCCAGAAGGCCCTCAGACTAC
	Antisense: GGGTCTTCATTGCGGTGG
IL-6	Sense: ACAACCACGGCCTTCCCTACTT
	Antisense: CACGATTTCCCAGAGAACATGTG
IL-21	Sense: GGCCAAACTCAAGCCATCAA
	Antisense: GCCACGAGGTCAATGATGAA
IL-22	Sense: CTTTCCTGACCAAACTCAGCAA
	Antisense: TGGTCGTCACCGCTGATG
RORγt	Sense: CCGCTGAGAGGGCTTCAC
	Antisense: TGCAGGAGTAGGCCACATTACA
RORα	Sense: GCACCTGACCGAAGACGAA
	Antisense: GATCCGCTGACATCAGTACGAA
GAPDH	Sense: CTGCACCACCAACTGCTTAG
	Antisense: GTCTGGGATGGAAATTGTGA