ORIGINAL RESEARCH

The prevalence of Th17 cells in patients with dilated cardiomyopathy

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Abstract

Purpose: Dilated cardiomyopathy (DCM) is a chronic disease characterized by autoimmunity. Th17 cells are a distinct subset from Th1 and Th2 cells and play crucial regulatory functions in inflammatory and autoimmune processes. The current study was designed to investigate the possible involvement of Th17 cells in DCM.

Methods: Th17 cells were detected in blood from DCM subjects and healthy blood donors using several methods including Th17 frequencies by flow cytometric analysis, cytokine (IL-17, IL-6 and IL-23) secretion by enzyme-linked immunosorbent assay and key transcription factor (ROR γ t) by real time-PCR.

Results: Patients with DCM demonstrated increased peripheral Th17 cells ($2.2\pm 1.2\%$ vs $0.4\pm 0.3\%$, $P<0.01$), Th17 related cytokines (IL-17: 62.7 ± 22.8 vs 15.6 ± 8.6 pg/ml, IL-6: 40.7 ± 16.6 vs 10.9 ± 5.3 pg/ml, IL-23: 210.7 ± 89.9 vs 90.6 ± 38.8 pg/ml, $P<0.01$) and ROR γ t (24.6 ± 6.5 vs 3.2 ± 1.1 , $P<0.01$) compared with healthy blood donors (HBD). Furthermore, there was a consistent differential sex-defined cytokine profile. Males showed higher frequencies of IL-17, IL-6 and IL-23 than females (IL-17: 70.7 ± 20.7 vs 54.7 ± 22.2 pg/ml, $P<0.01$; IL-6: 46.0 ± 18.2 vs 35.4 ± 13.0 pg/ml, $P<0.05$; IL-23: 238.1 ± 106.2 vs 183.4 ± 60.2 pg/ml, $P<0.05$) in patients with DCM.

Conclusion: Th17 function is increased in patients with DCM, suggesting a role for Th17 cells in the pathogenesis of DCM.

Dilated cardiomyopathy (DCM) is an often fatal cause of heart failure characterized by ventricular dilatation and impaired systolic function. An annual incidence of 5.4-8.3/100 000 has been reported.¹ Despite improvements in diagnosis and treatment, the incidence and mortality are still high. The mechanism of myocardial damage and related etiologic and prognostic factors are unknown.² Ischemic, metabolic, genetic and infectious causes have all been proposed as contributing factors to the onset and maintenance of DCM. Autoimmunity may play an important role in the pathogenesis of DCM by initiating the disease process or by contributing to the progression of contractile dysfunction.³ There is increasing evidence for the participation of cellular and humoral autoimmunity in the pathologic processes of DCM, such as the presence in patients' sera of autoantibodies directed against heart-specific antigens⁴⁻⁶ or the imbalance between helper and cytotoxic T cells.⁷

T helper 17 (Th17) cells belong to a recently identified subset of T helper cells, in addition to the traditional Th1 and Th2 subsets.⁸⁻⁹ Interleukin (IL)-6, IL-21 and transforming growth factor beta (TGF β), are the differentiating factors of Th17 cells,¹⁰⁻¹¹ whereas IL-23 is required for expansion and pathogenicity of

TABLE 1. Characteristics of patients with DCM and HBD (means±SD)

	DCM(n = 60)	HBD (n = 60)
Age (yr)	51.3±10.4	40.5±9.7
Sex F/M (n)	1	1
HR(beats/min)	84±15	73±13
SBP(mmHg)	123±17	122±16
DBP(mmHg)	75±10	78±11
Functional class (NYHA)		
I	8	60
II	15	0
III	19	0
IV	18	0
Ejection fraction (%)	29±10	Normal

DCM: idiopathic dilated cardiomyopathy, HBD: healthy blood donors, HR: heart rate, SBP: systolic blood pressure, DBP: diastolic blood pressure, NYHA: New York Heart Association.

these cells *in vivo*.¹² ROR γ t is a key regulator of Th17-cell lineage differentiation. Overexpressing ROR γ t induces IL-17 production, whereas ROR γ t-deficient cells produce very little IL-17.¹³ Th17 cells are characterized by production of IL-17 and may have evolved for host protection against microbes that Th1 or Th2 immunity are not well suited for, such as extracellular bacteria and some fungi.¹⁴ They are responsible for, or participate in, the induction of many organ-specific autoimmune diseases. Some of the autoimmune responses formally attributed to Th1 cells, such as experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA), and some forms of inflammatory bowel disease (IBD), have now been shown to be mediated, at least in part, by Th17 cells.¹³ So, Th17 cells play crucial regulatory functions in inflammatory and autoimmune processes.¹⁵⁻¹⁷

Recently, Rangachari et al.¹⁸ found that mice lacking Th1-associated transcription factor, T-bet, were more susceptible than wildtype mice to EAM because these mice had elevated IL-23 and IL-17 production. This suggests that IL-17 is the critical effector cytokine responsible for experimental autoimmune cardiomyopathy (EAM) - a mouse model of postinfectious myocarditis and cardiomyopathy.¹⁹ Furthermore,

DCM, as an autoimmune disease, often results from Cocksackievirus B3 and *Chlamydia*-triggered Myocarditis. We hypothesized that Th17 cells may also play an active role in DCM.

In this study, circulating Th17 frequencies, Th17 related cytokines (IL-17, IL-6 and IL-23) and transcription factor (ROR γ t) levels of DCM were measured. Meanwhile, sex biases are particularly prominent in DCM and men die at twice or three fold greater frequency than women.²⁰ We compared cytokine (IL-17, IL-6 and IL-23) levels between males and females in DCM.

Methods

Patients

The study was approved by the Medical Ethical Committee of Qingdao University, and all subjects provided written informed consent. Sixty patients with DCM (30 male and 30 female, mean age±SD 51.3±10.4 yr) and 60 healthy blood donors (HBD, 30 male and 30 female, mean age±SD 40.5±9.7 yr) were recruited. The characteristics of the study subjects are summarized in Table 1. A diagnosis of DCM was made using the criteria of the World Health Organization definition.²¹ Patients with hypertrophic cardiomyopathy, ischemic heart disease, hypertension, valvular heart disease, diabetes, and other infections were excluded. No patient was treated with anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs, steroids, etc.

Blood samples

Blood samples were obtained from all the subjects in the recumbent position with a 21-gauge needle for clean venipuncture of an antecubital vein. Peripheral blood mononuclear cells (PBMCs) were prepared by standard Ficoll-Hypaque density centrifugation of heparinized peripheral blood from studied subjects for analysis of flow cytometric and real time-polymerase chain reaction (PCR). Serum samples were centri-

fused for 20 min at 10,000 g (5417R microcentrifuge; Eppendorf, Hamburg, Germany). Then, they were subdivided into small aliquots to be stored at -80°C until tested for cytokine levels.

Preparation of PBMCs

PBMCs were washed twice with Hank's balanced salt solution and adjusted to a final concentration of $2 \times 10^6/\text{ml}$ in complete RPMI 1640 medium supplemented with 100U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2mM glutamine and 10% heat-inactivated fetal calf serum (Gibco BRL). The cell suspension was transferred to each well of 24-well plates at 37°C under a 5% CO_2 environment. Cultures were stimulated with phorbol myristate acetate (PMA, 50 ng/ml) plus ionomycin (1 μM) for 4 h, in the presence of monensin (500 ng/ml, all from Alexis Biochemicals, San Diego, CA). 4 h later, the contents of the well were transferred to 5-ml sterile tubes and then centrifuged at 1500 rpm for 5 min.

Flow cytometric analysis of Th17 cells

Cells were aliquoted into tubes and washed twice in phosphate-buffered saline (PBS). Then, the cells were incubated for cell surface analysis with phycoerythrin (PE) anti-human CD4 (eBioscience, San Diego, CA) at 4°C for 20 min. After washing twice with PBS, cells were fixed, permeabilized and stained for the intracellular IL-17A with FITC anti-human IL-17A (eBioscience, San Diego, CA) at 4°C for 30 min. Isotype controls were given to enable correct compensation and confirm antibody specificity. Stained cells were analyzed by flow cytometric analysis using a FACScan cytometer. Flow cytometric data were analyzed by using Cellquest software (BD Biosciences, San Jose, CA).

ROR γ t expression determined by real time-PCR

Total RNA was extracted with TRIzol extraction (Invitrogen) according to the manufacturer's instructions.

cDNA was synthesized using a Revertaid first strand cDNA synthesis kit (Fermentas), following instructions provided by the manufacturer. The cDNA samples were amplified with specific primers and fluorescence-labeled probes for the target genes. Samples were monitored on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Japan). TaqMan primers and probes for human ROR γ t were also purchased from Applied Biosystems. Oligonucleotides used for PCR amplification of ROR γ t were the following: F: 5'-TGAGAAGGACAGGGAGCCAA-3', R: 5'-CCACAGATTTTGCAAGGGA TCA-3'. For each sample, mRNA expression level was normalized to the level of GAPDH house keeping genes.

FIGURE 1. Circulating Th17 frequencies in patients with DCM. A: Representative FACS pictures from a single patient in both patients with DCM and HBD; B: Percentage of positive cells in patients with DCM and HBD. ** $p < 0.01$. DCM: idiopathic dilated cardiomyopathy; HBD: healthy blood donors.

ELISA detection of plasma IL-17, IL-6, IL-23

Solid-phase enzyme-linked immunosorbent assay (ELISA) kits were used to determine the serum levels of IL-17, IL-6, IL-23, according to the manufacturer's instructions. (IL-17 and IL-6 ELISA kits, both from R&D Systems, Minneapolis, MN, USA; IL-23 ELISA kits, from Bender MedSystems, Burlingame, Calif). Results are expressed in picogram per milliliter as mean $\pm$ standard deviation. The minimum detection limits of the assays were: 2 pg/ml for IL-17, 0.7 pg/ml for IL-6, 78 pg/ml for IL-23. Samples and standards were analyzed in duplicates and only variation coefficients $<15\%$ were accepted.

Statistical analysis

Statistical analyses were performed using SigmaStat software. Values are expressed as mean $\pm$ SD in the text and figures. Comparisons between two groups were analyzed by student's t test. When the equal variance test failed, a Mann-Whitney Rank Sum test was used. P -value <0.05 was considered statistically significant.

Results

Th17 frequencies in patients with DCM

The frequencies of peripheral blood Th17 cells (CD4+IL17+/CD4+T cells) were markedly higher in patients with DCM ($2.2 \pm 1.2\%$) than in HBD ($0.4 \pm 0.3\%$, $P < 0.01$) (Figure 1). This indicates that patients with DCM had a predominantly increased population of Th17 cells.

ROR γ t in PBMCs from patients with DCM

Levels of ROR γ t expression were higher in the DCM (24.6 ± 6.5) than in the HBD subjects (3.2 ± 1.1 , $P < 0.01$) (Figure 2).

Serum cytokines in patients with DCM

The IL-17, IL-6 and IL-23 concentrations in patients with DCM ($n=60$, IL-17: 62.7 ± 22.8 pg/ml, IL-6:

FIGURE 2. Ratios of ROR γ t/GAPDH mRNA in patients with DCM and HBD.

** $p < 0.01$.

40.7 ± 16.6 pg/ml, IL-23: 210.7 ± 89.9 pg/ml) were higher than in HBD ($n=60$, IL-17: 15.6 ± 8.6 pg/ml, IL-6: 10.9 ± 5.3 pg/ml, IL-23: 90.6 ± 38.8 pg/ml, $P < 0.01$) (Figure 3). In addition, we observed a consistent differential sex-defined cytokine profile with males showing higher frequencies of IL-17, IL-6 and IL-23 ($n=30$, IL-17: 70.7 ± 20.7 pg/ml, IL-6: 46.0 ± 18.2 pg/ml, IL-23: 238.1 ± 106.2 pg/ml) than females ($n=30$; IL-17: 54.7 ± 22.2 pg/ml, $P < 0.01$; IL-6: 35.4 ± 13.0 pg/ml, $P < 0.05$; IL-23: 183.4 ± 60.2 pg/ml, $P < 0.05$) in patients with DCM. While the plasma concentration of IL-17, IL-6 or IL-23 in males ($n=30$, IL-17: 15.6 ± 9.6 pg/ml, IL-6: 10.6 ± 5.6 pg/ml, IL-23: 88.7 ± 33.0 pg/ml) was similar to that in females ($n=30$, IL-17: 15.5 ± 7.6 pg/ml, IL-6: 11.2 ± 5.1 pg/ml, IL-23: 92.6 ± 44.3 pg/ml, $P > 0.05$) in HBD.

Discussion

IL-17 is a highly inflammatory cytokine with robust effects in many tissues. In humans and in mice Th17 cells play an important role in the induction and propagation of autoimmunity. For example, IL-17 deficient mice or mice treated with an IL-17 receptor

FIGURE 3. Comparison of serum IL-17, IL-6 and IL-23 concentrations in male and female patients with DCM and HBD. * $p < 0.05$, ** $p < 0.01$.

antagonist are resistant to development of CIA and develop EAE.^{22,23} Th17 cells may also be important in the pathogenesis of DCM. To assess the Th17 function in patients with DCM, We examined Th17 cells, including Th17 frequencies, related cytokines (IL-17, IL-6 and IL-23) secretion and key transcription factor(ROR γ t). Flow cytometric analysis was used to detect the Th17 frequencies because it is the most common and accurate method. Patients with DCM revealed increased peripheral Th17 number, Th17 related cytokines (IL-17, IL-6 and IL-23) and ROR γ t levels compared with healthy blood donors (HBD). Furthermore, males showed higher frequencies of IL-17, IL-6 and IL-23 than females in patients with DCM. These results indicate that increased Th17 function exists in patients with DCM, suggesting a potential role for Th17 cells in the pathogenesis of DCM.

Clinical studies suggest that inflammation is a major factor contributing to the pathophysiology of cardiovascular diseases and that Th17 cells are involved in myocardial injury, hypertrophy, and remodeling. EAE is generally attributed to an immune reaction to cardiac myosin following heart infections and is the leading cause of heart failure in young adults.²⁴ EAE may be mediated by IL-17 produced by Th17 cells.²⁵ Sonderegger et al²⁷ targeted IL-17 expression by an

active vaccination approach that breaks B cell tolerance in EAM and found that neutralization of IL-17 effectively reduced heart autoantibody responses and myocardial inflammation. In susceptible individuals, DCM develops from EAE after an initial infection with certain pathogens accompanied by myocardial remodeling. DCM is characterized by increased MMP-1 expression.²⁸ Cortez et al²⁹ found that IL-17 induces primary cardiac fibroblast migration in an MMP-1-dependent manner. Since fibroblast migration and proliferation are two critical steps in cardiac fibrosis, IL-17 may play a role in myocardial remodeling. So, IL-17 may play a causal role in DCM via enhanced expression of MMP-1. Moreover, IL-17 induced NF- κ B, AP-1, and C/EBP- β activation.²⁹ Therefore, IL-17 may upregulate NF- κ B, AP-1, and C/EBP- β -responsive proinflammatory cytokines, chemokines, adhesion molecules, and MMPs in fibroblasts and other myocardial constituent cells. IL-17 may synergize with these mediators and induce myocardial inflammation and injury in DCM.

Sex biases are particularly prominent in autoimmune diseases.³⁰ Consistent with our study, Jane-wit et al³¹ found predominant Th17 lineage responses and sustained self-memory in males using a murine model of DCM. *In vivo* potentiation of IL-17 or IL-2 in females resulted in the male-like severe DCM pheno-

type. Likewise, complementary experiments in males involving inhibition of either IL-17 or IL-2 resulted in anergy and expression of the female-like DCM protective phenotype. Moreover, a variety of immunological conditions contribute to the severe DCM outcome in males including a predominantly Th17 response independent of Th1/Th2 polarization,¹⁸ failure to anergize Th1 and Th17 responses.³¹ We presumed that the increased severity of DCM in males is partly attributable to a consistent increase of IL-17. IL-17 can be effectively targeted using neutralizing antibodies or, alternatively, by auto-vaccination.²⁷ Thus, therapeutic vaccination against IL-17 may be a promising therapy to prevent and treat DCM.

We found a functional increase of Th17 cells in patients with DCM for the first time. Th17 cells may play an important role in DCM, which is helpful for further studies of the immune mechanism of DCM.

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