ThGM Cells as a Prognostic and Predictive Marker for Coronary Heart Disease

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Abstract

Background Coronary heart disease (CHD) is caused by the aberrant aggregation of immune cells in plaque. This study aimed to identify abnormal T cell subtypes and inflammatory factors in CHD patients.

Methods and results T cell subsets from 187 CHD patients were analyzed using flow cytometry. Plasma concentration of cytokines were analyzed by Luminex. Flow cytometric analysis revealed that the number of ThGM cells was higher in CHD patients. The proportion of Th17 and Th1 cells were also increased in CHD patients. Levels of IL-4, IL-5, IL-6, and IL-10 were significantly higher in CHD patients ($P < 0.05$). However, levels of GM-CSF were slightly lower in CHD patients.

Conclusions ThGM can be considered as a diagnostic marker of CHD.

Introduction

Coronary heart disease (CHD) is a kind of chronic inflammatory disorder involving a variety of immune cells and inflammatory mediators[1–3]. The pathogenesis of CHD is complex and heterogeneous, and its etiology remains unclear. Detection of early-stage CHD is important for maximizing the benefits of medical intervention. CD4⁺T cells, macrophages, neutrophil, High-sensitivity C-reactive protein, IL-6, and GM-CSF have been reported to play roles in the occurrence and development of CHD[4–7].

Atherosclerosis is a prerequisite for CHD. The main components of atherosclerotic plaques are immune cells, including T lymphoid cells, macrophages, and mast cells are from blood. Among them, T lymphoid cells play an important role. T helper (Th)1 and T helper (Th)17 cells presented an activated pro-inflammatory state in the local atherosclerotic lesions[8]. Th1 cells predominantly produce interferon-gamma (IFN- γ), a strong pro-inflammatory cytokine, and have been observed in atherosclerotic plaques both in humans and animals[9]. Th17 cells play critical roles in the development of atherosclerotic lesions by producing IL-17 and, to a lesser extent, tumor necrosis factor (TNF)- α and Interleukin-6 (IL-6)[10]. Recently, the role of Granulocyte macrophage colony-stimulating factor (GM-CSF) producing Th cells (ThGM) cells in the initial and progression of some autoimmune diseases have been widely accepted, although its mechanism remains unclear. GM-CSF expression in ThGM was promoted by the IL-7-STAT5 pathway. GM-CSF are pro-inflammatory cytokines that participate in the pathogenesis of many kinds of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE)[11], multiple sclerosis (MS)[12], and Myasthenia gravis (MG)[13]. The frequency of GM-CSF-producing T cells were reduced in blood but enriched in the inflamed thymus of MG patients. Besides, the frequencies of thymic GM-CSF-producing T cells were correlated with the clinical disease severity of MG patients[13]. The effector molecules, including IL6, GM-CSF, and IL-17, released by these immune cells accelerate the enlarged and rupture of plaques[14, 15]. The activation of inflammatory factors in the plaque is manifested as a marker of the acute coronary syndrome. The rupture of the plaque releases phospholipids, tissue factor, and platelet adhesion factor to the blood, which accelerate the formation of thrombus and lead to myocardial infarction[16–18]. Plaque rupture can be detected in 60~70% of patients with acute myocardial infarction[19, 20]. Current research confirmed that CHD is a systemic disease caused by

abnormal immune cell function, dysregulated inflammatory factors, and the dysfunctional metabolic system[21, 22]. This study evaluated the role of T cells (Th1, Th17, and ThGM) and cytokines (GM-CSF, IFN- γ , IL-10, IL-17A, IL-1 β , IL-2, IL-4, IL-23, IL-5, IL-6, IL-7, and TNF- α) in the development of CHD. We found a strong correlation between ThGM and CHD, this result may provide a new target in CHD drug development.

Material And Methods

Patients

This study was approved by the Ethics Committee of West China Hospital (2019-332), and all patients signed informed consent. All samples were obtained from West China Hospital. Peripheral blood samples were collected from 141 CHD patients (29 women and 112 men, aged 62.2 ± 11.9 years) and 46 healthy control (8 women and 38 men, aged 61.24 ± 10.12 years) between June 2020 and August 2020. Patients were divided into 5 groups according to the coronary angiography score and the clinical manifestations: (1) Coronary atherosclerosis negative control group (n=46) including patients whose coronary artery examination showed no obvious stenosis; (2) Asymptomatic coronary heart disease group (N=17) including patients with coronary artery stenosis, but no clinical symptoms; (3) Stable angina pectoris group (n=20) including patients with coronary artery stenosis, clinical manifestations of patients with stable angina pectoris; (4) Unstable angina pectoris group (n= 69) including patients with coronary artery stenosis, clinical manifestations of unstable angina; (5) Acute myocardial infarction group (n=35) including patients with acute myocardial infarction. Patients with any one of the following diseases were excluded: tumor, severe infectious disease, and severe inflammation.

Preparation Of Peripheral Blood Mononuclear Cells (Pbmc) And Plasma

Blood samples were collected from patients with coronary heart disease and healthy controls. 10 mL of peripheral blood were collected from each patient and put in an EDTA anticoagulation vacuum tube (BD, 9036857). 2ml of whole blood were transferred into 15ml centrifuge tube, centrifuge at $400 \times g$ for 5min. The plasma was stored at -80°C . The remaining whole blood was transferred to a centrifuge tube (Greiner bio-one, 163288) containing 3mL ficoll solution (Solarbio, P8610), and centrifuged at $800 \times g$ for 15 minutes at room temperature (minimum acceleration and deceleration). After centrifugation, the middle buffy coat layer was collected and washed for twice with PBS to obtain PBMC.

Flow Cytometry For Detecting Th Cells

T cell subtypes in PBMCs were quantified by flow cytometry (BD, FACSAria SORP). $\text{CD4}^{+}\text{IFN-}\gamma^{+}\text{T}$ cells were considered to be Th1 cells. $\text{CD4}^{+}\text{IFN-}\gamma^{-}\text{GM-CSF}^{+}\text{T}$ cells were considered to be ThGM cells. $\text{CD4}^{+}\text{IL-}$

17A⁺ T cells were considered to be Th17 cells. 1×10^6 cells were transferred to a 1.5ml centrifuge tube containing 100ul FACS buffer (PBS solution containing 1% FBS). Anti-human CD4-PerCP-Cy5.5 flow cytometry antibody (BD, 552838) was added and incubated for 20min in the dark at room temperature. After washing with FACS buffer, 200ul of IC Fixation Buffer (invitrogen, 2235872) and Permeabilization Buffer (invitrogen, 1961659) were added into the tube, followed by incubating for 20 minutes at room temperature in the dark. After that, cells were resuspend using 100ul FACS buffer, add 5ul each of IFN- γ -APC (BD, 554702), GM-CSF-PE (BD, 554507) and IL17A-BV421 (BD, 562933) into the tube and incubate for 30 minutes at room temperature. Then, resuspending cells with 300ul FACS buffer and detected using flow cytometry. Data were analyzed using Aria 2 software (BD, Inc.).

Luminex Assay

levels of GM-CSF, IFN- γ , IL-10, IL-17A, IL-1 β , IL-2, IL-4, IL-23, IL-5, IL-6, IL-7, and TNF- α in plasma was detected using Luminex method. High throughput analysis was performed using the Milliplex Map Kit (Merck, HSTCMAG-28SK) according to the manufacturer's instructions. The operation method was shown in the instructions. Simply, setting up blank control wells (2), standard wells (14), QC wells (4) and sample wells (76) in the 96-well plate. Add 50ul of Serum Matrix to the blank well, 50ul of standards with different dilution concentrations into the standard wells, 50ul of quality control products into the QC wells, and 25ul of plasma and 25uL of Assay buffer into the sample wells. Add human monoclonal antibodies coupled with magnetic beads to 96 wells, 25uL per well and incubate with shaking for 16-18h at 4°C. Washing 3 times with wash buffer, and then add 50uL Detection Antibody to each well. After incubating for 1 h at room temperature, add 50uL Streptavidin-Phycoerythrin (R-PE) into each well. Then, incubating for 30min at room temperature, wash 3 times in wash buffer, adding 150uL of Sheath Fluid and run plate on LuminexR 200TM with Xponent software. Finally, calculating the amounts of cytokines based on the fluorescence intensity.

Statistical analysis

Using ANOVA for CHD clinical score studies. Using the student's t test to assess between groups. The correlation was analyzed by Pearson correlation analysis. All statistical analyses were performed using the Statistical Package for the Social Sciences version 20.0 (SPSS, USA). P<0.05 was considered significant.

Results

Clinical laboratory data of CHD patients

As shown in *Table 1*, this study enrolled 187 patients in west china hospital, all patients were detected by coronary angiography and were evaluated with Gencini and SYNTAX scores by two highly trained physicians. Based on the clinical conditions, patients were divided into healthy control group (Control)

(n=46), asymptomatic coronary heart disease group (SCHD) (n=17), stable angina group (SAP) (n=20), unstable angina group (UAP) (n=69) and acute myocardial Infarct group (AMI) (n=35). The proportion of male patients is no different in Control, SAP, UAP, and AMI, except for SCHD (Table 1). Patients with autoimmune disease, tumor, severe infection, and infectious diseases were excluded.

Frequency of ThGM and other GM-CSF⁺ T cell in PBMCs of CHD patients

ThGM is a special subtype of T cells. The IL-7-STAT5 axis promotes the generation of GM-CSF/IL-3-producing Th cells, which were designated as ThGM[11]. The proportion of ThGM is positively correlated with the severity of inflammation. To clarify the relationship between ThGM and CHD, we detected the T cell subsets using flow cytometry. ThGM is represented by CD4⁺IFN- γ ⁻GM-CSF⁺T cells (Fig. 1a).

Interestingly, we found the proportion of GM-CSF positive CD4⁺T cells was higher in CHD patients ($P<0.001$) (Fig. 1b). Besides, the number of ThGM was very high in SCHD ($P=0.001$), SAP ($P=0.002$), UAP ($P=0.001$), and AMI ($P<0.001$) patients (Fig. 1c).

The frequency of Th1 and Th17 cells are increased in PBMCs of CHD patients

Th1 and Th17 are two important T cell subsets. We found the number of the Th1 cells was slightly higher in SCHD ($P=0.0027$), SAP ($P=0.0147$), and UAP ($P=0.0021$) patients, but not in AMI ($P=0.0558$) (Fig. 2a). Furthermore, we found an increased ratio of Th17 cells in all disease stages ($P<0.001$) (Fig. 2b).

Cytokine Levels Were Increased In Plasma Of Chd Patients

Cytokines secreted by T cells play an important role in CHD development. To test the relationship between cytokine level and CHD severity, we detected GM-CSF, IFN- γ , IL-10, IL-17A, IL-1 β , IL-2, IL-4, IL-23, IL-5, IL-6, IL-7 and TNF- α in patient blood using Luminex. Concentrations of IL-4, IL-5, IL-6, and IL-10 were higher in CHD patients. Levels of IL-4 and IL-5 increased significantly in SAP and UAP patients ($P<0.05$) (Fig. 3a,b). It is worth noting that levels of IL6 increased in concomitant with the severity of the disease ($P<0.05$) (Fig. 3c). levels of IL-10 gained significantly in patients with serious CHD patients ($P<0.0001$) (Fig. 3d). However, levels of GM-CSF, IFN- γ , IL-17A, IL-2, TNF- α were slightly lower in CHD patients ($P<0.05$) (Fig. 4a-e). Levels of IL-1 β , IL-7, and IL-23 were normal in CHD patients (Fig. 4f-h).

Discussion

Number of ThGM cells are increased in CHD patients, but the plasmatic GM-CSF is not elevated. These data indicated that ThGM cells may be in an inactivated state in CHD patients. The proliferation of ThGM cells in vitro could be activated by anti-CD3 and anti-CD28. The in vivo mechanisms under ThGM proliferation and differentiation are more complex. The IL-7-STAT5 pathway activates the transcription of GM-CSF in ThGM cells[11]. Under specific circumstances, ThGM cells secretes GM-CSF to the plasma. This process must be precisely regulated to maintain the level of GM-CSF in plasma. The GM-CSF in plasma may in turn inhibit the excessive proliferation of ThGM cells to maintain homeostasis in the cell

number. However, this feedback was destroyed in CHD patients, which leads to the paradox that the number of cells increases but the content of cytokines decreases. Further studies are needed to test this hypothesis.

Proportion of CD3⁺IFN⁺ cells is elevated in SAP and UAP patients[23]. Furthermore, the proportion of Th1 cells increased abnormally in the peripheral blood of CHD patients[24]. We found that the proportion of Th1 cells (CD4 IFN- γ ⁺ T cells) was significantly higher in CHD patients. It is worth noting that in AMI patients, the proportion of Th1 cells is normal. These results indicated that Th1 cells only take part in the early steps of atheromatous plaque formation.

IL-6 is an important inflammatory factor and promotes the development of CHD[14]. Levels of IL-6 were higher in CHD patients[25–27]. IL-6 promotes cell proliferation through JAK-STAT pathway[28]. The high levels of Th2 related cytokines, such as IL-4, IL-5, and IL-10, suggest that in addition to T cell-mediated inflammation, B cells may also participate in immune regulation in CHD patients. Besides, the proportion of Th17 cells (CD4⁺IL-17A⁺T cells) was significantly higher in the peripheral blood of CHD patients. Th17 cells are involved in the formation of atherosclerosis[29]. We found an increased Th17 cell level in SA and ACS patients. IL-17A binds to IL-17 receptor (IL-17R), activates NF- κ B and JNK pathway by IL-17R-Act1-TRAF6 signaling axis. Although Th17 cells were increased in CHD patients, the plasmatic IL-17A remain unchanged. Unexpectedly, levels of IL-17A were decreased in SCHD, SAP, AMI patients. These results indicated that those Th17 cells, like ThGM cells, were in an inactivated state in CHD patients. The role of these silencing Th17 or ThGM cells needs to be discovered by further studies. Besides, unlike Th1, we found in this study that ThGM was in an inactivated state in the peripheral blood of CHD patients.

Conclusion

ThGM was a newly found Th subset, there was a strong connection between ThGM cell number and CHD seriousness. These results can provide new markers for CHD inspection and evaluation.

Declarations

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Author contributions Conception and design: X Bai and Y Zhou. Data analysis and interpretation: D Mou. Collection and assembly of data: S Wu and L Jiao. Manuscript writing: All authors. Final approval of manuscript: All authors.

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Data availability All data are included in this article and datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors declared that they have no conflict of interest.

Ethical approval Ethics approval was obtained from the Ethics Committee of West China Hospital (2019-332).

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Tables

Table 1. Clinical characteristics of the CHD patients and healthy controls.

Characteristics	Control (n=46)	SCHD (n=17)	SAP (n=20)	UAP (n=69)	AMI (n=35)
Clinical characteristics					
Male, n (%)	38 (82.6)	9 (52.94)*	18 (90)	55 (79.71)	30(85.71)
Age (years)	61.24±10.12	68.63±9.15	60.65±11.39	62.27±11.86	60.71±12.82
BMI	23.82±4.37	22.62±14.61	24.61±2.33	24.32±3.35	24.3±3.38
Hypertension, n (%)	27 (58.7)	8 (47.06)	13 (65)	38 (55.07)	12 (34.29)
Diabetes, n (%)	4 (8.7)	4 (23.52)	4 (20)	19 (27.54)	9 (25.71)
Hyperlipaemia, n (%)	5 (10.9)	1 (5.89)	1(5)	4 (5.8)	6(17.14)
Laboratory characteristics					
Hematocrit (L/mL)	40.33±6.12	35.86±5.84	43.8±3.37	40.1±4.54	40.54±4.97
CK-MB (ng/mL)	4.95±10.46	2.87±2.57	2.01±1.21	1.65±0.96	45.97±81.85*
Total cholesterol (mmol/L)	3.78±1.22	3.56±0.81	3.38±1	4.42±0.93	4.15±1.29
HDL cholesterol (mmol/L)	1.34±0.45	1.48±0.36	1.509±0.61	1.32±1.07	1.2±0.38
LDL cholesterol (mmol/L)	1.89±1.06	1.86±0.67	5.2±15.5*	1.81±0.73	2.51±1.22
Coronary angiographic score					
gencini score	0	8.06±10.92*	44.9±32.34*	75.12±49.72*	72.03±47.73*
syntax1 score	0	1.58±4.23*	14.8±9.57*	22.14±12.35*	18.1±12.15*

The values are expressed as proportions or the mean ± standard deviations. CHD, coronary heart disease; SCHD, asymptomatic coronary heart disease; SAP, stable angina pectoris; UAP, unstable angina pectoris; AMI, acute myocardial infarction. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein. *P<0.05, all kinds of CHD vs control group.

Figures

Figure 1

ThGM is represented by CD4+IFN- γ -GM-CSF+T cells (Fig. 1a). Interestingly, we found the proportion of GM-CSF positive CD4+T cells was higher in CHD patients ($P < 0.001$) (Fig. 1b). Besides, the number of ThGM was very high in SCHD ($P < 0.001$), SAP ($P = 0.002$), UAP ($P < 0.001$), and AMI ($P < 0.001$) patients (Fig. 1c).

Figure 2

Th1 and Th17 are two important T cell subsets. We found the number of the Th1 cells was slightly higher in SCHD ($P=0.0027$), SAP ($P=0.0147$), and UAP ($P=0.0021$) patients, but not in AMI ($P<0.0558$) (Fig. 2a). Furthermore, we found an increased ratio of Th17 cells in all disease stages ($P<0.001$) (Fig. 2b).

Figure 3

Levels of IL-4 and IL-5 increased significantly in SAP and UAP patients ($P < 0.05$) (Fig. 3a,b). It is worth noting that levels of IL6 increased in concomitant with the severity of the disease ($P < 0.05$) (Fig. 3c). levels of IL-10 gained significantly in patients with serious CHD patients ($P < 0.0001$) (Fig. 3d).

Figure 4

However, levels of GM-CSF, IFN-γ, IL-17A, IL-2, TNF-α were slightly lower in CHD patients ($P < 0.05$) (Fig. 4a-e). Levels of IL-1β, IL-7, and IL-23 were normal in CHD patients (Fig. 4f-h).