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Statins reduce the expressions of Tim-3 on NK cells and NKT cells in atherosclerosis

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ABSTRACT

3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) have an immuno-regulatory effect in addition to lowing-lipids. Accumulated evidence showed that the expressions of T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) on natural killer (NK) cells increased in atherosclerotic patients and animal models. In this study, 14 patients treated with rosuvastatin and 12 patients with atorvastatin for more than 3 months were included and 20 patients without statins treatment as control. Both statins treatment reduced the expressions of Tim-3 on NK cells and their subtypes, natural killer T (NKT) cells and $CD3^+$ T cells, and increased the proportions of NKT cells among peripheral blood mononuclear cells, accompanied by the decreased levels of total cholesterol, low density lipoprotein, and increased ratios of high density lipoprotein to cholesterol. These may contribute to the functions of statins in the treatment of atherosclerosis.

1. Introduction

Cardiovascular and cerebrovascular diseases are the main causes of death around the world. Their major underlying cause is atherosclerosis. Atherosclerosis is characterized as a chronic inflammatory disease of the arterial wall, which involves both the innate and adaptive immune responses (Chavez-Sanchez et al., 2014).

Natural killer (NK) cells are important components of innate immune system. NK cells in human can be divided into two subtypes: $CD56^{dim}$ NK cells which display potent cytolytic activity and $CD56^{bright}$ NK cells which can produce cytokines (Bonaccorsi et al., 2015). The proportion and cytotoxicity of NK cells decreased in peripheral blood of atherosclerotic patients, which is the same as that in autoimmune diseases (Backteman et al., 2012; Hou et al., 2012; Jonasson et al., 2005). However, the effects of NK cells in atherosclerosis are controversial (Bonaccorsi et al., 2015; Schiller et al., 2002; Selathurai et al., 2014). The natural killer T (NKT) cells own some properties in common with both conventional T cells and NK cells, and they can produce both pro- and anti-inflammatory cytokines upon activation. NKT cells were detected in atherosclerotic plaques and the number of NKT cells decreased in the peripheral blood of patients with cardiovascular diseases (Bondarenko et al., 2014; Rombouts et al., 2016). Once activated, NK cells and NKT cells can produce IFN-γ and many other cytokines (VanderLaan et al., 2007). IFN-γ is regarded as an atherogenic cytokine.

T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) is a member of Tim proteins which are type 1 transmembrane proteins, and is expressed on many immune cells (Foks et al., 2013). The expressions of Tim-3 were up-regulated on peripheral NKT cells in chronic hepatitis B patients (Rong et al., 2014). In many diseases, the expressions of Tim-3 on NK cells were found increased accompanied by the decreased cytotoxicity and cytokines production of NK cells (da Silva et al., 2014; Hou et al., 2014, 2012; Wang et al., 2015; Xu et al., 2015, 2014). The expressions of Tim-3 on NK cells were up-regulated in atherosclerotic patients and $LDLr^{-/-}$ mice (Foks et al., 2013; Hou et al., 2012). So, Tim-3 on NK cells and NKT cells may participate in inflammatory diseases as well as atherosclerosis.

Statins are the inhibitors of HMG-CoA reductase. They can reduce the levels of total cholesterol and low density lipoprotein (LDL) (Park et al., 2016). Statins alleviate atherosclerosis by not only the lipid-lowering activity but also its immunomodulatory effect. Statins can increase the frequency of NK cells, while their effects on the cytotoxicity of NK cells are controversial (Hillyard et al., 2004, 2007; Jonasson et al., 2005; Raemer et al., 2009). Both the negative and positive effects of statins on IFN-γ were reported (Coward et al., 2006; Jameel et al., 2013). Whether statins can influence the expressions of Tim-3 on NK cells and NKT cells remains unclear.

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Table 1
Demographic characteristics of patients included in the study.

	Control (n = 20)	Rosuvastatin (n = 14)	Atorvastatin (n = 12)	*P* Value
Male, n (%)	15 (75)	11 (78)	8 (67)	0.78
Age, y	64.0 ± 1.9	63.3 ± 2.4	67.1 ± 2.3	0.48
Diabetes, n (%)	4 (20)	3 (21)	4 (33)	0.67

Mean value was expressed as mean ± S.E.M. For continuous variables comparison, ANOVA was performed among three groups; χ^2 tests were performed for categorical variables.

In the present study, we explored the effects of statins on the proportions of NK cells and their subtypes, NKT cells and $CD3^+$ T cells, the expressions of Tim-3 and productions of IFN-γ, and analyzed the levels of total cholesterol, LDL, high density lipoprotein (HDL), as well as the ratios of HDL to cholesterol.

2. Materials and methods

2.1. Patients included in the trials

The atherosclerotic patients demonstrated with image or ultrasound, 35–79 years old, were included from Shandong Provincial Qianfoshan Hospital between January and December of 2016. Patients were excluded for any of the following reasons at the time of screening: history of myocardial infarction or cerebral infarction within 3 months, congestive heart failure, renal failure and dialysis treatment, infections (e.g., acute and chronic viral infection, respiratory infection, intestinal infection), dental problems, connective tissue diseases, autoimmune diseases, any anti-inflammatory treatment, malignancy, any types of surgery in the past 3 months, definite hypersensitivity or contraindication to statins, pregnancy, drug or alcohol abuse, inability to walk into the clinic.

In the study, 14 atherosclerotic patients with rosuvastatin treatment (10 mg for 2 patients and 20 mg for the rest patients per day for more than 3 months) (Astrazeneca, England), 12 patients with atorvastatin treatment (5 mg for 1 patient and 10 mg for the rest patients per day for more than 3 months) (Pfizer pharmaceutical company, United States) were included, and 20 atherosclerotic patients without any statin treatment were included as control. The overnight fasting venous blood samples of patients were collected with EDTA-anticoagulant tubes (BD, Franklin Lakes, NJ, USA). Table 1 shows the demographic characteristics of patients included in the study.

The sample sizes were determined according to the formula $n = \psi^2 \frac{\sum_i^k S_i^2 / k}{\sum_i^k (\overline{X}_i - \overline{X})^2 / (k-1)}$ (n is on behalf of the sample size. $\overline{X}_i$ and S_i represent the mean and standard deviation of sample i. $\overline{X} = \sum_i^k \overline{X}_i / k$. k is the number of groups. ψ is got from the corresponding table. $\alpha = 0.05$, $\beta = 0.10$. $\nu_1 = k - 1$, $\nu_2 = \infty$. Get the corresponding ψ from the table to calculate n_1 using the formula. Then get new ψ according to $\nu_1 = k-1$, $\nu_2 = k(n_{(1)}-1)$, and calculate n_2 using the formula. Repeat the calculation until the result is stable. In this study, k equals 3. According to the calculation, n equals 8).

The study was approved by the Ethics Committee of Shandong Provincial Qianfoshan Hospital and each participant was informed about the study.

2.2. The study protocol

1 ml anticoagulant venous blood was mixed with 1 ml phosphate buffered saline (PBS) and the peripheral blood mononuclear cells (PBMCs) of the diluted blood were extracted using Ficoll density gradient centrifugation (Dakewe, Shenzhen, China) according to the instruction. The PBMCs were separated into two parts.

One part of PBMCs was incubated with FITC-conjugated anti-human CD3 antibody (eBioscience, San Diego, CA, USA), PerCP-conjugated

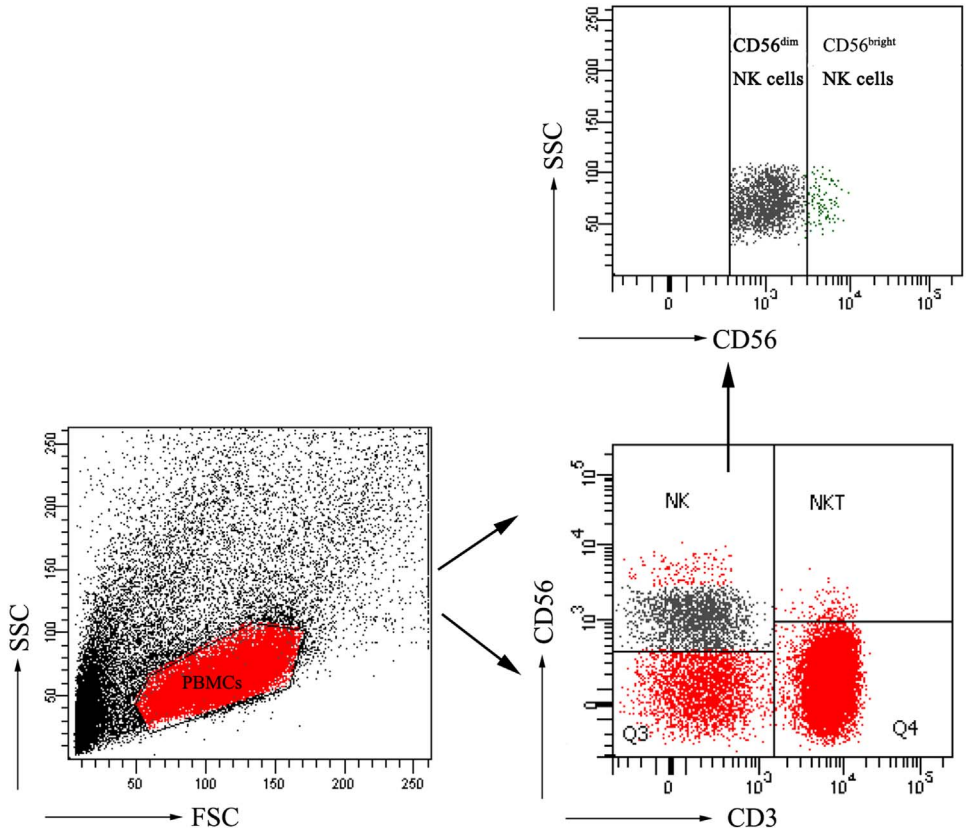

Fig. 1. Representative dot plots of peripheral blood show the gating strategy of different lymphocyte populations. The $CD3^-CD56^+$ cells were gated as NK cells and the $CD3^+CD56^+$ cells were gated as NKT cells. NK cells were divided into $CD56^{dim}$ NK cells and $CD56^{bright}$ NK cells according to the levels of CD56 expressed on them.