

Immune Modulatory Effects of Hypercholesterolemia: Can Atorvastatin Convert the Detrimental Effect of Hypercholesterolemia on the Immune System?

Zeinab Emruzi¹, Pegah Babaheidarian², Mahmoud Arshad³, Hannes Stockinger⁴, and Ghasem Ahangari¹

¹ Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

² Department of Pathology, Rasul-e Akram Hospital, Iran University of Medical Sciences, Tehran, Iran

³ Department of Internal Medicine and Endocrinology, Minimally Invasive Surgery Research Center, Rasul-e Akram Hospital, Iran University of Medical Sciences, Tehran, Iran

⁴ Institute for Hygiene and Applied Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Molecular Immunology Unit, Vienna, Austria

Received: 7 February 2019; Received in revised form: 28 April 2019; Accepted: 4 May 2019

ABSTRACT

Many observations showed that hypercholesterolemia can disrupt immune response. Statin drugs that were used for the treatment of hypercholesterolemia patients can interfere in the regulation of the immune response and cytokine secretion. The primary aim of the current study was to investigate the immune response among treatment-naïve patients with hypercholesterolemia and healthy subjects. The secondary goal of the study was to determine whether atorvastatin can reverse the detrimental effect of hypercholesterolemia on the immune system.

Peripheral blood mononuclear cells (PBMCs) were isolated from 50 patients afflicted with hypercholesterolemia who were treatment-naïve along with 50 sex/age-matched hypercholesterolemia patients receiving atorvastatin, and 50 sex/age-matched healthy subjects. Quantitative PCR and ELISA methods were used for gene and protein expression analysis of T helper 1 (Th1) and Th2 related cytokines. Additionally, the expression of the cluster of differentiation (CD) markers on T, B, and natural killer (NK) cells was measured by flow cytometry method.

The results showed that hypercholesterolemia and atorvastatin down-regulated the expression of Th1-related cytokines and elevated the levels of Th2-related cytokines. The expression of cell surface markers, CD25 and CD69, was significantly decreased in the treatment-naïve, and atorvastatin groups.

It seems that atorvastatin is not able to repair the deleterious effects of hypercholesterolemia on the immune system. Moreover, elevated levels of cholesterol along with the administration of atorvastatin tilt the Th1/Th2 balance in favor of Th2 and reduce T cell activation.

Keywords: Atorvastatin; Cytokine; Hypercholesterolemia; Immune response

Corresponding Author: Ghasem Ahangari, PhD;
Department of Medical Genetics, National Institute of Genetic
Engineering and Biotechnology (NIGEB), Tehran, Iran. Tel: (+98 21)
4478 7384; Fax: (+98 21) 4478 7399, E-mail: ghah@nigeb.ac.ir

INTRODUCTION

Hypercholesterolemia is principally defined by high fasting cholesterol that stems from the disturbance in

lipid metabolism and genetic defects. It increases the risk of atherosclerosis, cardiovascular diseases, angina, and stroke.¹ Of note, hypercholesterolemia impedes the immune response and cytokine secretion in immune cells. These effects of hypercholesterolemia were due to alteration of cholesterol rates in the cell membrane and cytoplasm of immune cells.² Studies indicated that ApoE^{-/-} mice had increased levels of Th2 cells³ and the decreased number of Th1 cells showing a deficiency in cell-mediated immunity.⁴ However, the research did not come to a consensus about the association between hypercholesterolemia and the risk of developing cancer.^{5,6}

Statins, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, are a class of compounds used for the treatment of patients with hypercholesterolemia, hyperlipidemia, and cardiovascular diseases.⁷ Despite the beneficial effect of atorvastatin on cholesterol lowering, the drug has several side effects including Myalgia,⁸ Rhabdomyolysis, and Myopathy.⁹ Statins also influence the immune system as they are able to halt T cell homing, migration, and adhesion, the latter is mediated by the down-regulation of integrins, adhesins, and matrix metalloprotease proteins (MMPs).¹⁰ It is thought the modulation of T cell response is exerted by the hindrance of antigen presenting cells (APCs) development through a reduction in major histocompatibility complex II (MHC II) leaving the T cells inactive.¹¹ As a result, statins predominantly temper the activity of immune cells monocyte/macrophages and T cells.¹² The ability to avert the activation of Th1 cells upon using statins made them desirable candidates for the alleviation of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus (SLE).¹³ In contrast to animal models, studies performed on human clinical trials showed that atorvastatin did not change any shift in the balance of Th1/Th2 cells.¹⁴ It has been demonstrated that blocking the production of isoprenoids required for prenylation of proteins such as Rho, Rac and Ras superfamily GTPases plays a crucial role in fidelity of statins in amelioration of inflammation.¹⁵

There is a few information about the effect of hypercholesterolemia on the immune system in the literature.^{2,4} All of the researches dealt with the human studies in terms of statin interaction with the T cell functions were exclusively confined to cultured

PBMCs taken from healthy individuals, patients with autoimmune and cardiovascular diseases.^{16,17} Regarding the scarcity of human studies on the role of hypercholesterolemia in T cell activation and differentiation, we aimed to investigate whether 1) hypercholesterolemia can affect the immune system function; 2) atorvastatin can oppress the harmful impact of hypercholesterolemia on the immune system.

MATERIALS AND METHODS

Study Population

In this study, a total of one-hundred-fifty individuals participated that were divided into three groups as the following categorization: 50 patients afflicted with hypercholesterolemia who received no medication for cholesterol lowering (treatment-naïve group, this group takes appropriate medication after blood sampling), 50 patients who were sex- and age-matched taking atorvastatin at a dose of 10 mg/kg for at least 4 months (atorvastatin group), and 50 healthy individuals who were sex- and age-matched either (healthy subjects). The participated subjects were chosen from those referring to Rasul-e Akram Hospital, Tehran, Iran. The diagnosis of hypercholesterolemia was made in accordance with the measurements of cholesterol at the particular time interval confirming that the patients suffer from persistent hypercholesterolemia (Table 1). The exclusion criteria for the volunteers were smoking, neurological disorder, cardiovascular diseases, inflammatory and allergen disorders, and diabetes. The protocol used for the sample collection and patient examinations was confirmed by the ethical committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB) (N. IE-715), and each volunteer was given informed consent. All methods were performed in accordance with the Declaration of Helsinki.

Cell isolation & Serum Separation

Whole blood samples (5 mL) were collected from all individuals and poured in 0.5 M ethylene diamine tetra acetic acid (EDTA)-containing tubes to avoid coagulation. PBMCs were separated based on the gradient density centrifugation technique by Ficoll hypaque (Pharmacia, Uppsala, Sweden).

For the serum separation, blood samples were taken in tubes lacking an anticoagulant, and then they were centrifuged and stored at -80°C until use.

Table 1. Demographic and clinical characteristics of subjects of healthy, treatment-naïve and atorvastatin groups.

Characteristics	Healthy (n=50)	Treatment-naïve (n=50)	Atorvastatin (n=50)	p-value
Sex (male/female)	23/27	25/25	26/24	0.9134
Mean age (range), years	47 (41-55)	49 (44-60)	53 (49-62)	0.3432
Mean BMI (range), kg/m ²	23.2 (20-24)	32.8 (29-35)	28.2 (26-31)	0.0321*
Mean cholesterol level±SD (mg/dL)	160.2±11	300.4±15	172±19.8	0.0061**
Mean LDL level±SD (mg/dL)	102.2±11.8	158.8±14.5	105±15.6	0.0176*
Mean HDL level±SD (mg/dL)	40.2±4.3	42.5±10.3	39.5±5.7	0.8971
Mean Triglyceride level±SD (mg/dL)	129.8±15.8	124.1±30	122.6±24.3	0.7692

p-values have been obtained from the comparison of healthy and treatment-naïve groups

BMI; Body Mass Index, LDL; Low-Density Lipoprotein, HDL; High-Density Lipoproteins

RNA Extraction & cDNA Synthesis

Total RNA was extracted from PBMCs using a High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. The quantity and quality of RNA were determined using NanoDrop 2000 (Wilmington, USA) at 260 nm and agarose gel analysis. For reverse transcription polymerase chain reaction (RT-PCR), 400 ng of total RNA was converted into complementary DNA (cDNA) using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Germany) based on manufacturer's protocols. The produced cDNA was stored at -20°C for spare use.

PCR and Real-Time PCR Analysis

Primers designing were performed by oligo7 software (version 7.56) using the RefSeq sequences of GenBank database as described in Table 2; the specificity of each primer pair was confirmed by Primer-Basic Local Alignment Search Tool (BLAST) online software. A PCR was performed to check for any contaminating DNA and in order to confirm the transcription of interested genes in a final volume of 20 µL with master mix PCR (Cinnagen, Tehran, Iran), gene-specific primers (10 pmol/µL), and cDNA (1 µg) in all samples and then loaded them on gel 1.5%.

Real-time-PCR was performed for relative quantification of the messenger RNA (mRNA) expression of all interested genes in a final volume of 10 µL, using the SYBR Green Real-time PCR Master Mix (Light Cycler Fast Start DNA Master Plus SYBR Green I, Roche, Germany), and 10 pmol of the previously mentioned primers and 1 µg cDNA. The real time PCR protocol was as follows: pre-denaturation at 95°C for 600 seconds, denaturation at

95°C for 15 seconds, annealing at 60°C for 20 seconds, extension at 72°C for 20 seconds, with 40 cycles. Identification of fluorescent intensity in each sample was performed by Rotor gene (Termocycler Rotor-Gene™6000 Corbett Research/ Australia). Melting curve analysis showed only one peak for each reaction and this was also confirmed by electrophoresis of PCR products that showed only one band of the expected size.

Cytokine Measurements

The concentration of Interferon gamma (IFN-γ), Tumor necrosis factor alpha (TNF-α), Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Interleukin-5 (IL-5) cytokines was assayed in the serum of three studied groups. In this section, 60 individuals among of 150 samples were selected randomly that consisted of 20 subjects (11 women+ 9 men; mean age=47 years) in the treatment-naïve group, 20 (10 women, 10 men; average age=49 years) patients treated with atorvastatin, and 20 (12 women + 8 men; mean age=48 years) healthy individuals. The aforementioned factors were all analyzed by the commercial kits (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. The optical density of the samples was read using an ELISA reader (Spectra Max M2e, Molecular Devices, USA) at 450 nm.

Immunophenotyping

Cell surface staining was performed for a total of thirty individual consisted of 10 subjects (5 women+5 men; mean age=50 years) in the treatment-naïve group, 10 (6 women+4 men; mean age=48 years) patients in the atorvastatin group, and 10 (5 women+5 men; mean age=45 years) healthy subjects. Immunophenotyping

Table 2. Primers sequence of cytokines, transcription factors, and internal control genes, which were used for Real-Time PCR in healthy, treatment-naïve and atorvastatin groups to investigation effect of hypercholesterolemia and atorvastatin drug on the expression of interested genes

Genes	Genbank Accession Number	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplicon size (bp)
IL-2	NM_000586	ATTACAAGAATCCCAAACCTACCAG	GTTGTTTCAGATCCCTTTAGTTCC	217
IL-4	NM_172348	CTTCCCCCTCTGTTCTTCCTG	CCTTCTCAGTTGTGTTCTTCTGCTC	130
IL-5	NM_000879	TGCTGATAGCCAATGAGACTCTG	GACTCTCCAGTGTGCCATTCC	102
IL-6	NM_001318095	TCTGGATTCAATGAGGAGACTTGC	CTCACTACTCTCAAATCTGTTCTGG	102
IFN-γ	NM_000619	CGGTAAGTACTGACTTGAATGTCCAAC	ACCATTACTGGGATGCTCTTCGAC	141
TNF-α	NM_000594	CTCCTTCAGACACCCTCAACC	CGAAGTGGTGGTCTTGTGTC	101
T-bet	NM_013351	CCAAGTTTAATCAGCACCAGACAG	CAAGACCACGTCCACAAACATCC	131
GATA-3	NM_001002295	CCCTCATTAAGCCCAAGCGAAG	CAGGGGTCTGTTAATATTGTGAAG	167
β-actin	NM_001101	AGACGCAGGATGGCATGGG	GAGACCTTCAACACCCAGCC	161

(bp; base pair)

was done using antibodies against CD3 on the T cells as T cell diagnostic marker (FITC, Dako Denmark), CD4/CD8 on the T helper cells/cytotoxic T cells as signal transduction markers (FITC/PE, BD Biosciences), CD25 on the T cells as activation marker (PerCP/Cy5.5, Biolegend), CD69 on the T cells as activation marker (PerCP/Cy5.5, Biolegend), CD22 on the mature B cells as regulatory marker (FITC, Dako, Denmark), CD16 on the NK cells as signal transduction marker (FITC, Immunostep), CD56 on the NK cells as archetypal phenotypic marker of NK cells (PE, Immunostep), CD45RO on the T cells as memory T cell marker (PE, Dako Denmark). Appropriate isotype controls were used as negative controls to determine the nonspecific staining and background. In the first stage, 100 μ L of whole blood was poured into the test tube, and the antibodies were added to the recommended dilutions. Then, the tubes were incubated for 30 minutes at 4°C. After the incubation time, 1 mL of RBC lysis solution was added and incubated for 10-15 minutes at room temperature. In the last step, the sample was run in BD Fluorescence-activated cell sorting (FACS) Calibur flow cytometry (BD Biosciences, USA). The obtained data were analyzed by the Flowjo Software (version 7.6.1). Gating was applied to analyze the lymphocytes, and the results were reported as the percentage of CD4- and CD8-positive lymphocytes expressing either CD69 or CD25 surface markers in a total of all T cell population, CD3- and CD22-positive lymphocytes expressing CD45RO marker, and finally, CD16-positive lymphocytes that

expressed CD56 surface marker.

Statistical Analysis

For analyzing the real time PCR data, the PCR efficiency (E) and crossing point deviation (Δ CP) was calculated by LinRegPCR Software (Version 11.0), following the use of Relative Expression Software Tool (REST 2009, Version 2.0.13) for statistical analysis. Normality of other data was determined using the Kolmogorov-Smirnov test. With respect to the data (parametric vs. non-parametric) analysis of variance (ANOVA) and Kruskal-Wallis tests were applied where appropriate followed by post hoc Tukey's test. The data were expressed as the mean \pm standard deviation (SD). The calculation of the comparison was carried out by the Statistical Package for the Social Sciences (SPSS) software, version 16.0 (SPSS Inc., Chicago, Ill., USA). The difference between the variables was statistically significant if the *p*-value was less than 0.05.

RESULTS

Hypercholesterolemia and Atorvastatin Decreased the mRNA Expression of Th1-Related Cytokines

The results showed that the treatment-naïve and atorvastatin groups had significantly (*p*<0.0001) lower the mRNA expression of IL-2, IL-6, IFN- γ , TNF- α cytokines, and T-bet transcription factor in comparison to the healthy individuals. In addition, the mRNA expression of IFN- γ , TNF- α cytokines and T-bet was

lower in the atorvastatin group compared with the treatment-naïve group ($p < 0.01$); however, the expression of IL-2 and IL-6 remained unchanged (Figure 1).

Hypercholesterolemia and Atorvastatin Increased the mRNA Expression of Th2-Related Cytokines

The treatment-naïve group had significantly ($p < 0.05$) higher levels of cholesterol and Low-Density Lipoprotein (LDL), as well as the higher expression of IL-5, and GATA-3 in comparison to the healthy

subjects; however, the expression of IL-4 did not significantly differ between the two groups. Correspondingly, the treatment with atorvastatin caused a significant up-regulation in the mRNA expression of IL-5 and GATA-3. The expression of IL-4 was not affected by the administration of atorvastatin in patients with hypercholesterolemia. On one hand, the expression of GATA-3 and IL-5 was significantly increased in atorvastatin group when compared with the treatment-naïve group; yet, the expression of IL-4 did not significantly differ between two groups (Figure 2).

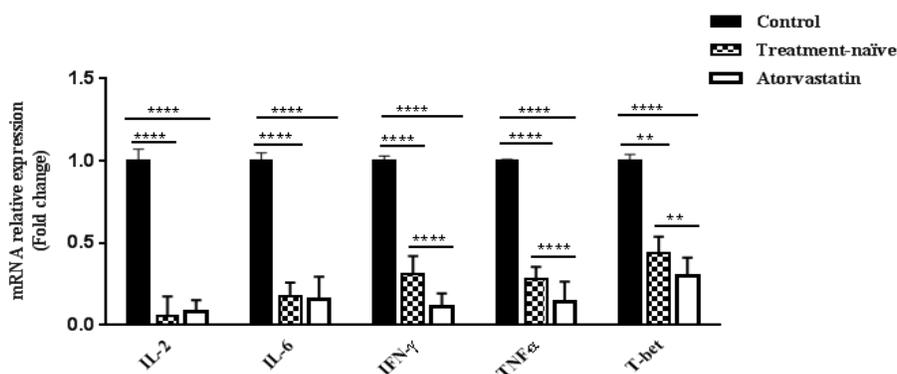


Figure 1. The effect of hypercholesterolemia and atorvastatin drug on the expression of Th1-related genes. The bar plot indicates the mRNA expression of Th1-related genes and T-bet transcription factor in PBMC samples of healthy, treatment-naïve and atorvastatin groups. The expression ratios plotted for IL-2, IL-6, IFN- γ , TNF- α , and T-bet genes were significantly decreased in the treatment-naïve and the atorvastatin group patients in comparison to the healthy individuals. The mRNA expression of IFN- γ , TNF- α cytokines, and T-bet was lower significantly in the atorvastatin group compared with the treatment-naïve group. Post hoc Tukey' test. Error bars represent the standard deviation. The number of subjects in each group= 50. Statistical significance was designated as: **, $p < 0.01$; ****, $p < 0.0001$.

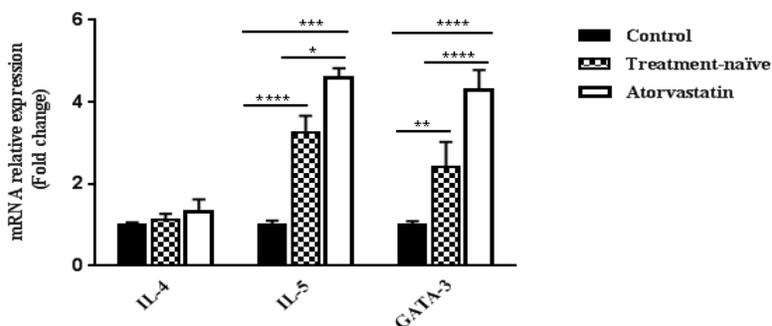


Figure 2. The effect of atorvastatin on the expression of Th2-related genes. The bar plot indicates the mRNA relative expression patterns of Th2-related genes and GATA-3 transcription factor in PBMC samples of healthy, treatment-naïve and atorvastatin groups. The treatment-naïve and atorvastatin groups had significantly higher the mRNA expression of IL-5 and GATA-3 genes in comparison to healthy individuals. The mRNA expression of GATA-3 and IL-5 was increased in the atorvastatin group when compared with the treatment-naïve group significantly. Post hoc Tukey' test. Error bars represent standard deviation. The number of subjects in each group= 50. Statistical significance was designated as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

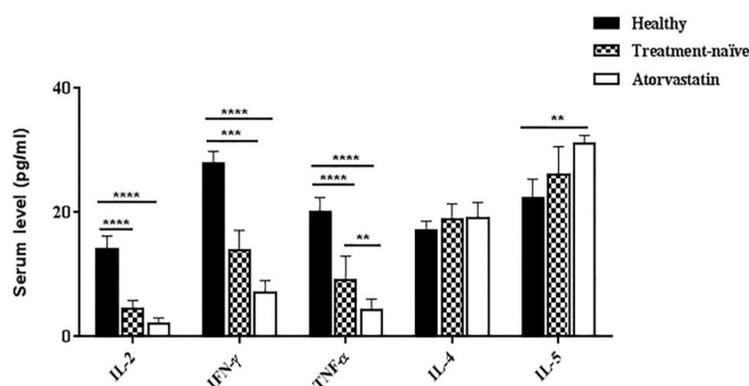


Figure 3. The effect of atorvastatin on the expression of Th1- and Th2-related proteins. The bar plot shows the serum level of IL-2, IFN- γ , TNF- α , IL-4, and IL-5 in healthy, treatment-naïve and atorvastatin groups. The expression ratios plotted for Th1-related proteins were found to be significantly decreased in treatment-naïve and atorvastatin group patients in comparison to a healthy subject. The level of TNF- α was lower in the atorvastatin group compared to the treatment-naïve group. The expression ratios plotted for Th2-related proteins did not differ between three groups except IL-5 were found to be significantly increased in atorvastatin group patients in comparison to a healthy subject. Post hoc Tukey's test. Error bars represent standard deviation. The number of subjects in each group= 20. Statistical significance was designated as: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Effect of Hypercholesterolemia and Atorvastatin on Protein Levels of Th1- and Th2-Related Cytokines

The results showed that hypercholesterolemia leads to a significant decrease ($p < 0.0001$) in the expression of Th1 related cytokines including IL-2, IFN- γ , and TNF- α in the treatment-naïve group in comparison to the healthy subjects. In line with this, it was shown that atorvastatin significantly ($p < 0.0001$) decreased the gene expression of IL-2, IFN- γ , and TNF- α in those patients underwent statin therapy in comparison to the healthy individuals. Individuals treated with atorvastatin had significantly ($p < 0.01$) lower levels of TNF- α than those who were treatment-naïve. On one hand, hypercholesterolemia did not significantly affect in the expression of Th2 related cytokines in the treatment-naïve group in comparison to healthy subjects. Correspondingly, the treatment with atorvastatin caused a significant increase in the expression of IL-5 in comparison to healthy subjects while the expression of IL-4 was not affected by the administration of atorvastatin in patients with hypercholesterolemia (Figure 3).

Correlations of Th1- and Th2-Related Cytokines Protein Expression with Patient's Characteristics

Table 3 shows that no significant correlation between the age, and the expression of all the IL-2,

IFN- γ , TNF- α , IL-4, and IL-5 cytokines. A significant negative correlation was found between the level of IL-2 and the Body Mass Index (BMI) of all individuals. The data also indicated that LDL concentration was significantly negatively correlated with the expression of IL-2, IFN- γ , and TNF- α , while there is no significant correlation with the expression of IL-4 and IL-5. Accordingly, while there are significant correlations between the levels of IL-2, TNF- α , and IL-5 with cholesterol level, no significant correlation was found between the cholesterol levels and the expression of IFN- γ and IL-4.

Hypercholesterolemia and Atorvastatin Decreased Expression of T Cell Activation Markers

Flow cytometry analysis of the surface receptors of the T cells showed that the treatment-naïve group had a significantly lower ($p < 0.05$) number of CD4⁺CD25⁺, CD4⁺CD69⁺, CD8⁺CD25⁺, and CD8⁺CD69⁺ cells over that of the healthy subjects. Similar results were obtained in patients received atorvastatin in comparison to the healthy individuals except for the number of CD4⁺CD25⁺ cells that were not significantly affected in response to atorvastatin treatment. The number of all T cell groups did not significantly differ between the treatment-naïve and atorvastatin groups (Figure 4).

Table 3. Association of Th1- and Th2- related cytokines with characteristics of patients with hypercholesterolemia in the treatment-naïve group.

Proteins	Age (years old)		BMI (kg/m ²)		Cholesterol (mg/dl)		LDL (mg/dl)	
	< 49	≥ 49	<32.8	≥ 32.8	< 248	≥ 248	< 148	≥ 148
IL-2	r=+0.175 p=0.287	r=-0.684 p=0.01*	r=-0.896 p=0.0007***	r=-0.732 p=0.030*				
IFN-γ	r=+0.214 p=0.886	r=-0.196 p=0.392	r=-0.215 p=0.171	r=-0.878 p=0.029*				
TNF-α	r=+0.118 p=0.728	r=-0.216 p=0.534	r=-0.724 p=0.043*	r=-0.927 p=0.004**				
IL-4	r=+0.253 p=0.457	r=+0.186 p=0.856	r=+0.189 p=0.256	r=+0.254 p=0.321				
IL-5	r=+0.196 p=0.792	r=+0.227 p=0.537	r=+0.649 p=0.041*	r=+0.298 p=0.085				

(BMI; Body Mass Index, LDL; Low Density Lipoprotein, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

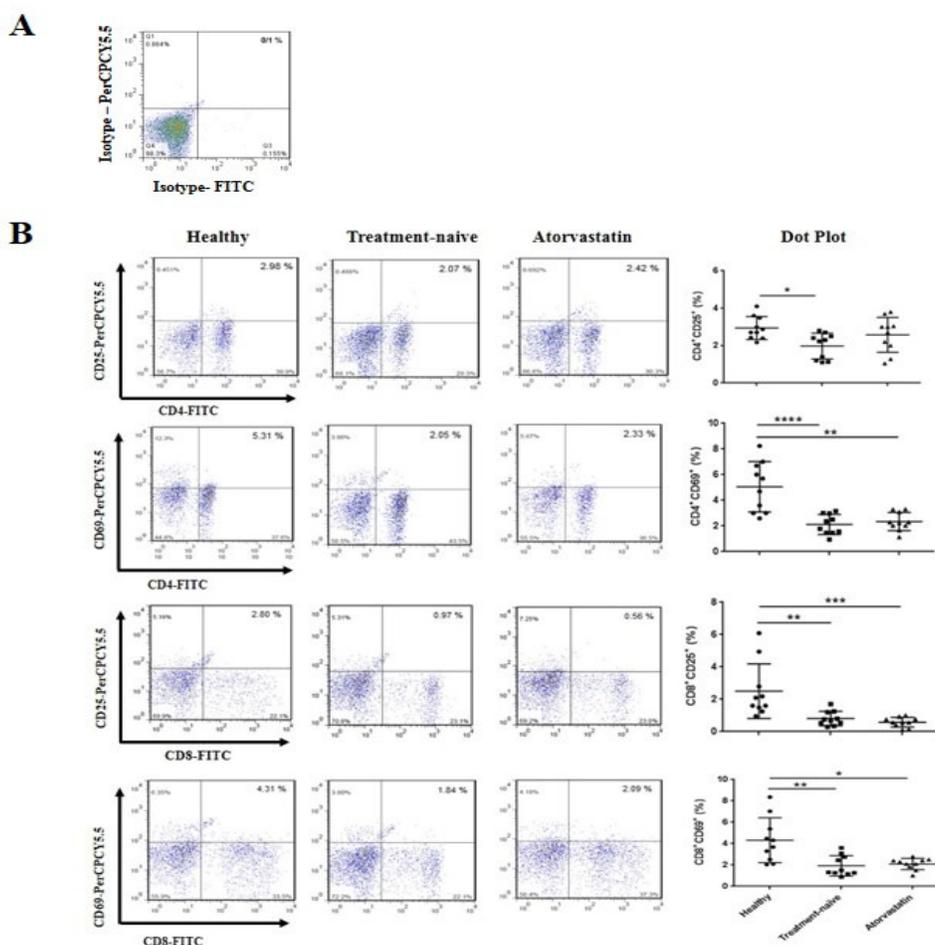


Figure 4. The effect of hypercholesterolemia and atorvastatin on the expression of T cell activation markers. A) Appropriate isotype control. B) The expression level of the cell surface activation markers on T cells was shown in all groups. The number of each T cell surface markers has been determined by flow cytometry analysis. The results of flow cytometry analysis were simplified and expressed as dot plots. Post hoc Tukey's test; n=10 for each group. Statistical significance was designated as: *, $p < 0.05$; **, $p < 0.01$; *, $p < 0.001$; ****, $p < 0.0001$.**

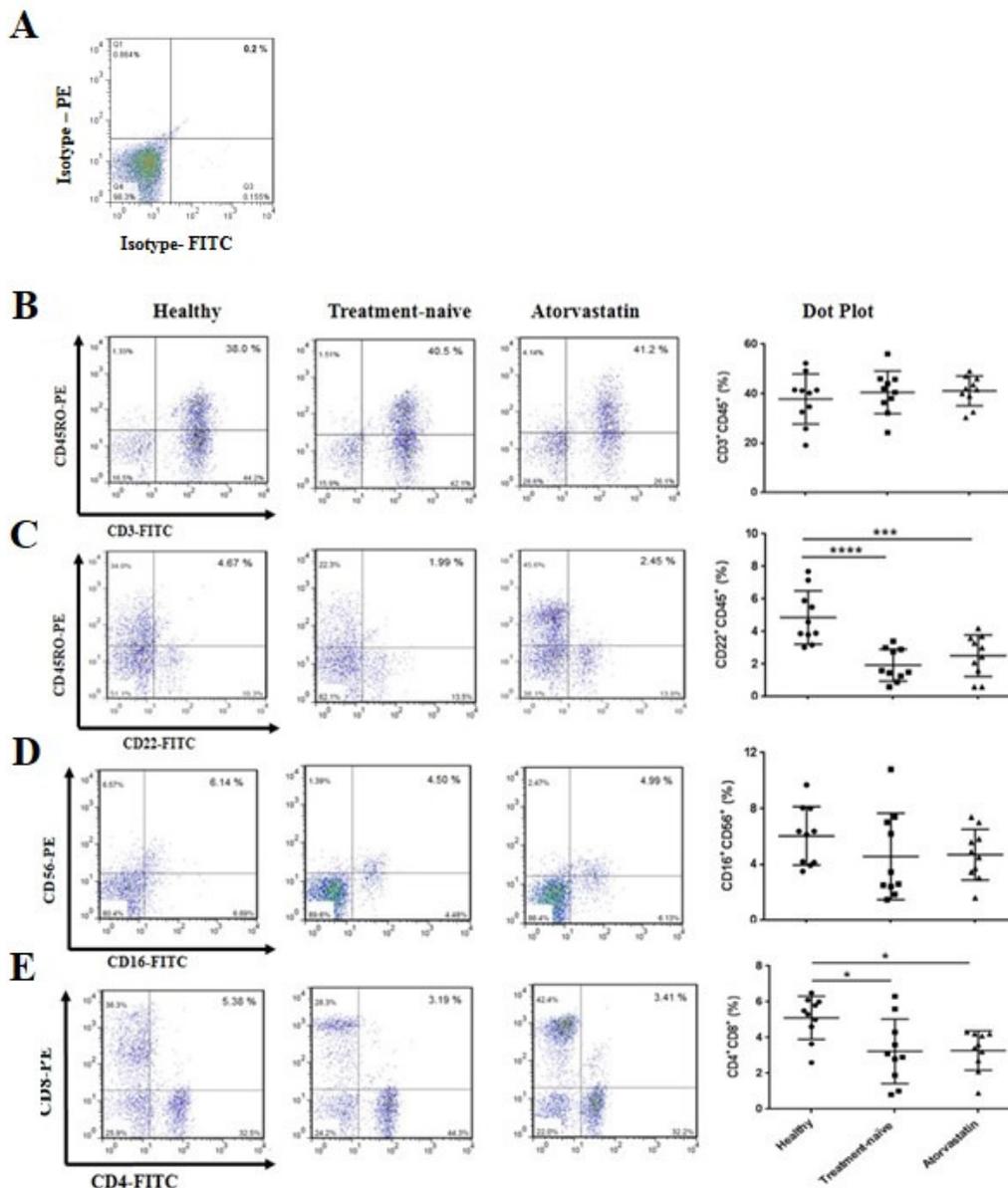


Figure 5. The effect of hypercholesterolemia and atorvastatin on the expression of cell surface marker of T, B memory cells, and NK cells, as well as the CD4/CD8 ratio. Appropriate isotype control (A). Hypercholesterolemia and atorvastatin decreased the percentage of CD22⁺CD45RO⁺ cells (C) and the CD4/CD8 ratio (E) but had no effect on T memory and CD56CD16 NK cells (B, D). The number of each cell types was analyzed by flow cytometry analysis, and the results were simplified and depicted as dot plots. Post hoc Tukey's test; n=10 for each group. Statistical significance was designated as: *, $p < 0.05$; ***, $p < 0.001$.

Effect of Hypercholesterolemia and Atorvastatin on the Phenotype of T, B Memory, and NK Cells as Well as the CD4/CD8 Ratio

It was shown that 1.9% of B cells in the treatment-

naïve group were positive for CD22 and CD45RO that was significantly ($p < 0.0001$) lower than the number of CD22⁺CD45RO⁺ cells in the healthy subjects. However, the number of CD3⁺ T cells and

CD56⁺CD16⁺ NK cells remained unchanged. The results also revealed that the treatment with atorvastatin caused a significant ($p<0.001$) diminish in the number of cells positive for CD22 and CD45RO compared with the healthy individuals; yet, the number of CD3⁺ T cells and CD56⁺CD16⁺ NK cells did not significantly differ between the two groups. The ratio of CD4 to CD8 in the treatment-naïve patients and patients received atorvastatin was 3.23 % and 3.27 %, respectively that shows both hypercholesterolemia and receiving atorvastatin can lead to the decrease ($p<0.05$) in the ratio of those CD markers in comparison to the healthy individuals. The phenotype of T, B memory, and NK cells, as well as the CD4/CD8 ratio, did not

significantly differ between the treatment-naïve and atorvastatin groups (Figure 5).

The comparison between the number of CD69⁺ and CD25⁺ T cells showed that the number of CD69⁺ T cells was significantly elevated in the healthy group compared with the other groups. Besides, when the number of CD4⁺CD25⁺, CD8⁺CD25⁺, CD4⁺CD69⁺, and CD8⁺CD69⁺ was analyzed in all groups, the percentage of cells expressing CD8 and CD25 was more affected in the treatment-naïve and atorvastatin group compared with the healthy control in which a significant decrease in the number of those cells was reported in the treatment-naïve and the atorvastatin groups in comparison to the healthy subjects (Figure 6).

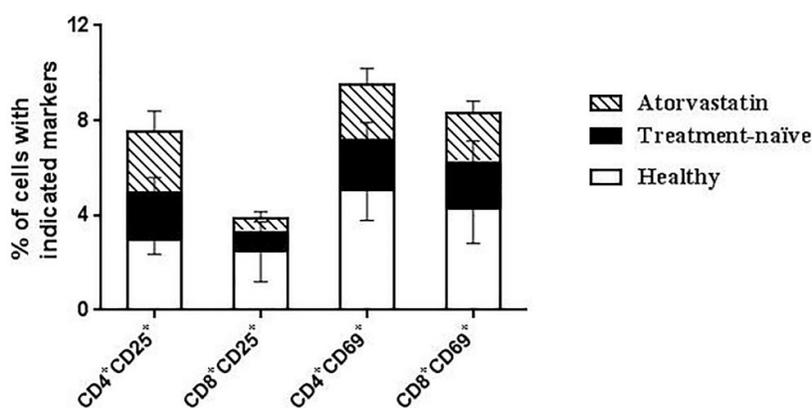


Figure 6. The effect of hypercholesterolemia and atorvastatin on the cell percentage of various CD markers in healthy, treatment-naïve and atorvastatin groups. Means \pm SD; post hoc Tukey's test; n=10.

Table 4. Correlations of CD markers expression in untreated hypercholesterolemia patients with patient characteristics.

CD markers	Age (years old)		BMI (kg/m ²)		Cholesterol (mg/dl)		LDL (mg/dl)	
	< 49	\geq 49	< 32.8	\geq 32.8	< 248	\geq 248	< 148	\geq 148
CD4CD25	r=+0.184	p=0.784	r=-0.134	p=0.763	r=-0.956	p=0.002**	r=-0.868	p=0.009**
CD4CD69	r=+0.213	p=0.897	r=-0.214	p=0.975	r=-0.216	p=0.347	r=-0.674	p=0.023*
CD8CD25	r=+0.182	p=0.533	r=-0.189	p=0.543	r=-0.726	p=0.044*	r=-0.274	p=0.265
CD8CD69	r=+0.138	p=0.461	r=-0.243	p=0.586	r=-0.914	p=0.003**	r=-0.194	p=0.758
CD3CD45	r=+0.245	p=0.364	r=-0.194	p=0.456	r=-0.214	p=0.677	r=-0.169	p=0.885
CD3CD22	r=+0.264	p=0.802	r=-0.149	p=0.939	r=-0.271	p=0.969	r=-0.253	p=0.353
CD16CD56	r=+0.153	p=0.602	r=-0.215	p=0.935	r=-0.213	p=0.478	r=-0.299	p=0.863
CD4CD8	r=+0.231	p=0.593	r=-0.167	p=0.300	r=-0.698	p=0.041*	r=-0.267	p=0.901

(CD; cluster of differentiation, BMI; Body Mass Index, LDL; Low Density Lipoprotein, *, $p<0.05$; **, $p<0.01$)

Correlation of the CD Markers Expression with Patient Characteristics

As was in Table 4, there was no correlation between the age and BMI of individuals and the expressions of all CD markers. There was a significant negative correlation between the cholesterol levels and the number of CD4⁺CD25⁺, CD8⁺CD25⁺, and CD8⁺CD69⁺ cells. Notably, the ratio of CD4 to CD8 was significantly linked to the concentrations of cholesterol in patients who were treatment-naïve. Furthermore, it was shown that the levels of LDL were negatively correlated with the number of CD4 cells positive for CD25 and CD69.

DISCUSSION

Many efforts have been made to illuminate the interaction of hypercholesterolemia and statins on the immune system function. However, those studies were not comprehensive.^{4,18} To get a better insight on the anti-inflammatory effect of hypercholesterolemia and statins on the functionality of the immune system, we investigated the effect of hypercholesterolemia and atorvastatin on T cell differentiation, Th1/Th2 polarization, and T cell activation in patients who were considered either treatment-naïve or atorvastatin users.

In this study, we observed that hypercholesterolemia and atorvastatin shifted the ratio of Th1/Th2 in favor of Th2 resulted in mitigation of the cell-mediated immunity and T cell activity. On one hand, it was shown that hypercholesterolemia and atorvastatin incited the humoral immunity system. Hence, atorvastatin could not reverse the harmful effects of hypercholesterolemia on the immune system. Where the atorvastatin is more safe and well-tolerable amongst statins family, we chose it for the present experiment.¹⁹

Zhou et al suggested that hypercholesterolemia drives Th1/Th2 balance towards Th2-related cytokine release.⁴ Consistent with our results, Lei et al showed that elevated levels of cholesterol and LDL in hyperlipidemia patients resulted in a decrease in Th1-related cytokines such as IFN- γ , TNF- α , IL-2, IL-6, and T-bet transcription factor. Inversely, hypercholesterolemia leads to increasing the expression of Th2-related cytokines such as IL-5 and GATA-3 transcription factor. It is of note that T-bet and GATA-3 are crucial transcription factors in differentiation of Th0 cells into the development of Th1 and Th2 cells,

respectively.¹⁹

In parallel, in vitro studies have displayed that statin drugs, which used for treatment of hypercholesterolemia patients, impair the proper function of the immune cells and consequently weaken their timely response to infectious agents.^{20,21} The inhibitory effects of statins on Th1 cells caused simvastatin is used for the treatment of various inflammatory diseases such as arthritis in rodent models.¹⁶ According to our results, it is suggested that atorvastatin decreases the mRNA expression of IFN- γ , TNF- α , IL-2 and IL-6 and T-bet transcription factor. In addition, it increases the expression of Th2-related cytokine expressions namely IL-5 and GATA3 transcription factor in patients with hypercholesterolemia. One of the possible underlying mechanism in which atorvastatin can drive the Th1/Th2 balance in favor of the overproduction of Th2 is the induction of signal transducer and activator of transcription 6 (STAT6) phosphorylation and up-regulation of the GATA-3 transcription factor.²² It can accordingly inhibit the differentiation of Th1 cells via the preventing STAT4 phosphorylation and down-regulation of T-bet transcription factor.²³ In this context, atorvastatin can down-regulate the expression of IFN- γ through disruption of MHC II transcription activation and finally, resulting in the inhibition of antigen presentation on APCs and damping the generation of proinflammatory Th1 cells.²⁴ We measured the protein levels of Th1- and Th2-related cytokines by the ELISA method in which hypercholesterolemia and atorvastatin both lowered the concentrations of Th1-related cytokines such as IL-2, IFN- γ , and TNF- α in serum and concentrations of IL-5 as Th2-related cytokine increased by atorvastatin. The obtained data from the protein levels corroborated the mRNA expressions of their cognate genes. It should be noted that the correlation of Th1- and Th2-related cytokines was calculated with the demographic characteristics of patients who were treatment-naïve in which the expression of IL-2, TNF- α and IL-5 was associated with the concentration of cholesterol in the serum of hypercholesterolemia patients. In a suitable manner, the level of LDL was also attributed to the levels of IL-2, IFN- γ , and TNF- α in the above patients.

Leung BR and Weber found that atorvastatin can boost the expression of Th2 cells.^{16,25} Link et al showed that rosuvastatin resulted in a rapid reduction in plasma cytokines such as IFN- γ , IL-6, and TNF- α .²⁶ Similar to

our results, Shimada K et al showed that atorvastatin shifted the balance of Th1/Th2 in favor of Th2 in patients with the acute coronary syndrome (ACS).¹⁷ Prevention of protein prenylation is one of the bona fide strategies that atorvastatin employs to dampen the signal transduction of pathways involved in inflammation.²⁷ IL-2 is required for the initiation and activation of T-cell-mediated immunity, which plays an important role in combating the cancer cells.²⁸ Along with IL-2, IFN- γ is able to eliminate tumor tissue by means of elevating Th1 cells and T cell activation.²⁹ TNF- α has been shown a potent tumor killer agent exerting anti-cancer activity both *in vivo* and *in vitro*.³⁰ IL-5 is also an important interleukin which is under the control of GATA-3 transcription factor playing a pivotal role in the pathogenesis of asthma hyper-eosinophilic syndromes and eosinophil-dependent inflammatory diseases.³¹ Collectively, in the present study, we suggested that hypercholesterolemia and atorvastatin through down-regulation of cytokines with anti-tumor effects might impair cell-mediated immunity and probably increase the risk of cancer. Also, these by up-regulation of IL-5 cytokine and GATA-3 transcription factor might induce humoral immunity and probably increase the risk of allergic dependent diseases.

The previous studies showed that increased levels of cholesterol abolish the T cell homeostasis leading to impaired the T cell function.^{32,33} Muldoon MF et al showed that a high cholesterol diet can increase CD4 and CD8 T cells but don't affect B and NK cell number.³⁴ In the present study, it was observed that high level of cholesterol and LDL decreased the expression of CD69 and CD25 activation markers on CD4 and CD8 T cells and reduced memory B cells but had no effect on T memory cells and NK cells. Furthermore, the correlation analysis revealed that the number of CD4⁺CD25⁺ and CD8⁺CD69⁺ cells is directly associated with the levels of cholesterol and a less extent with the LDL concentration in sera of hypercholesterolemia patients. The ratio of CD4 to CD8 was correlated with the levels of cholesterol as well. Guo X et al observed that cholesterol can modify receptor signaling through the elevation of different conformational states in membrane receptors and change the composition of T cell membrane resulting in the altered T cell operation.³⁵

There are contradictory reports about the impact of statins on the expression of CD25 and CD69 on the T

cell surface. A study by Norbert et al they disclosed that atorvastatin lowered the expression of CD25 and CD69 on the T cells,³⁶ whereas Li-Hua WU and colleagues demonstrated that Fluvastatin inhibited CD69 expression *in vitro*, but it did not affect the expression of CD25.³⁷ In this study, we detected decreased expression of CD8⁺CD69⁺, CD8⁺CD25⁺, CD4⁺CD69⁺ cells in response to atorvastatin treatment while the expression of CD4⁺CD25⁺ did not alter. Interestingly, despite a decrease in the number of memory B cells in response to administration of atorvastatin, the number of memory T cells remained unchanged. We also exhibited a decreased number of CD8⁺CD25⁺ cells in the treatment-naïve and the atorvastatin groups compared to the healthy controls.

The preventing effect of atorvastatin on the T cell activation is mediated by the disruption of APCs maturation.²² Additionally, atorvastatin is capable of interfering with the T-cell receptor (TCR) signaling pathways via dysregulation of small Ras-like GTPases and the Rab-dependent pathways that are involved in receptor endocytosis.²² It also blocks Lymphocyte function-associated antigen 1-Intercellular Adhesion Molecule 1(LFA-1-ICAM-1)interaction by binding to LFA-1.³⁸ In agreement on our results, Kanda et al reported that low-dose simvastatin decreased the ratio of CD4 to CD8 T cells when administered in patients with rheumatoid arthritis.³⁹ We observed a significant decrease in the ratio of CD4/CD8 in peripheral blood of the atorvastatin group. Also, high level of cholesterol and LDL obtained similar results about CD4/CD8 ratios in the treatment-naïve group. The low ratio of CD4 to CD8 can raise the risk of cancer because of lacking a sufficient number of Th1 cells.⁴⁰

A limitation of the present study was that, it was difficult to find specimen of patients with only high cholesterol and LDL, but normal triglyceride levels, who additionally did not have any specific disease. Altogether, regarding our study, it seems that hypercholesterolemia can disrupt the immune system through the effect of T cell activation and differentiation. On the other hand, besides of beneficial effect of atorvastatin on cholesterol lowering, it diminished the activation of T cells, made a perturbation in inflammatory cytokines, and is not able to repair the deleterious effects of hypercholesterolemia on the immune system.

ACKNOWLEDGEMENTS

This project was supported by Center for International Scientific Studies & Collaboration (CISSC) [grant number, 715] and National Institute for Genetic Engineering and Biotechnology (NIGEB). We thank Medical University of Vienna, Austria for generous support. The authors also appreciated all patients and healthy individuals who were participated in this study.

REFERENCE

1. Karr S. Epidemiology and management of hyperlipidemia. *The American journal of managed care* 2017; 23(9 Suppl):S139-48.
2. Lei L, Li H, Yan F, Xiao Y. Hyperlipidemia impaired innate immune response to periodontal pathogen *Porphyromonas gingivalis* in apolipoprotein E knockout mice. *PLoS One* 2013;8(8):e71849.
3. Dansky HM, Charlton SA, Harper MM, Smith JD. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci U S A* 1997;94(9):4642-6.
4. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest* 1998;101(8):1717-25.
5. Font-Burgada J, Sun B, Karin M. Obesity and cancer: the oil that feeds the flame. *Cell Metab* 2016;23(1):48-62.
6. Tie G, Yan J, Khair L, Messina JA, Deng A, Kang J, et al. Hypercholesterolemia increases colorectal cancer incidence by reducing production of NKT and $\gamma\delta$ T cells from hematopoietic stem cells. *Cancer res* 2017;77(9):2351-62.
7. Silverstein DM. Indications and outcome of treatment of hyperlipidemia in pediatric allograft recipients. *Pediatr Transplant* 2003;7(1):7-10.
8. Ramkumar S, Raghunath A, Raghunath S. Statin therapy: review of safety and potential side effects. *Acta Cardiologica Sinica* 2016; 32(6):631.
9. Tomaszewski M, Stępień KM, Tomaszewska J, Czuczwar SJ. Statin-induced myopathies. *Pharmacol Rep* 2011;63(4):859-66.
10. Weitz-Schmidt G. Statins as anti-inflammatory agents. *Trends Pharmacol Sci* 2002;23(10):482-7.
11. Yilmaz A, Reiss C, Weng A, Cicha I, Stumpf C, Steinkasserer A, et al. Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. *J Leukoc Biol* 2006;79(3):529-38.
12. Cheng X, Ding Y, Xia C, Tang T, Yu X, Xie J, et al. Atorvastatin modulates Th1/Th2 response in patients with chronic heart failure. *J Card Fail* 2009;15(2):158-62.
13. Mowla K, Rajai E, Ghorbani A, Dargahi-Malamir M, Bahadoram M, Mohammadi S. Effect of atorvastatin on the disease activity and severity of rheumatoid arthritis: double-blind randomized controlled trial. *J Clin Diagn Res* 2016;10(5):OC32-6.
14. Coward W, Chow SC. Effect of atorvastatin on TH 1 and TH 2 cytokine secreting cells during T cell activation and differentiation. *Atherosclerosis* 2006;186(2):302-9.
15. Rando RR. Chemical biology of protein isoprenylation/methylation. *Biochim Biophys Acta* 1996;1300(1):5-16.
16. Leung BP, Sattar N, Crilly A, Prach M, McCarey DW, Payne H, et al. A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J Immunol* 2003;170(3):1524-30.
17. Shimada K, Miyauchi K, Daida H. Early intervention with atorvastatin modulates TH1/TH2 imbalance in patients with acute coronary syndrome: from bedside to bench. *Circulation* 2004;109(18):e213-e4.
18. Földes G, Anker SD, von Haehling S. Atorvastatin modulates Th1/Th2 response in patients with chronic heart failure. *Journal of cardiac failure* 2009; 15(6):551.
19. Youssef S, Stüve O, Patarroyo JC, Ruiz PJ, Radosevich JL, Hur EM, et al. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* 2002;420(6911):78-84.
20. Zeiser R. Immune modulatory effects of statins. *Immunology* 2018;154(1):69-75.
21. Rodríguez-Perea A, Rojas M, Velilla-Hernández P. High concentrations of atorvastatin reduce in-vitro function of conventional T and regulatory T cells. *Clin Exp Immunol* 2019; 196(2):237-48.
22. Ghittoni R, Enea Lazzerini P, Laghi Pasini F, Baldari CT. T lymphocytes as targets of statins: molecular mechanisms and therapeutic perspectives. *Inflamm Allergy Drug Targets* 2007;6(1):3-16.
23. Shimada K, Park JK, Daida H. T helper 1/T helper 2 balance and HMG-CoA reductase inhibitors in acute coronary syndrome: statins as immunomodulatory agents? *Eur Heart J* 2006; 27(24):2916-8.
24. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. *Nat Med*

- 2000;6(12):1399-402.
25. Weber MS, Prod'homme T, Youssef S, Dunn SE, Steinman L, Zamvil SS. Neither T-helper type 2 nor Foxp3+ regulatory T cells are necessary for therapeutic benefit of atorvastatin in treatment of central nervous system autoimmunity. *J Neuroinflammation* 2014;11(1):29.
 26. Link A, Ayadhi T, Böhm M, Nickenig G. Rapid immunomodulation by rosuvastatin in patients with acute coronary syndrome. *Eur Heart J* 2006;27(24):2945-55.
 27. Dunn SE, Youssef S, Goldstein MJ, Prod'homme T, Weber MS, Zamvil SS, et al. Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by atorvastatin. *J Exp Med* 2006;203(2):401-12.
 28. Jiang T, Zhou C, Ren S. Role of IL-2 in cancer immunotherapy. *Oncoimmunology* 2016;5(6):e1163462.
 29. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 2006;6(11):836-48.
 30. Waters JP, Pober JS, Bradley JR. Tumour necrosis factor and cancer. *J Pathol* 2013;230(3):241-8.
 31. Takatsu K, Kouro T, Nagai Y. Interleukin 5 in the link between the innate and acquired immune response. *Adv Immunol* 2009;101:191-236.
 32. Kraakman MJ, Dragoljevic D, Kammoun HL, Murphy AJ. Is the risk of cardiovascular disease altered with anti-inflammatory therapies? Insights from rheumatoid arthritis. *Clin Transl Immunology* 2016;5(5):e84.
 33. Proto JD, Doran AC, Subramanian M, Wang H, Zhang M, Sozen E, et al. Hypercholesterolemia induces T cell expansion in humanized immune mice. *J Clin Invest* 2018;128(6):2370-5.
 34. Muldoon MF, Marsland A, Flory JD, Rabin BS, Whiteside TL, Manuck SB. Immune system differences in men with hypo-or hypercholesterolemia. *Clin Immunol Immunopathol* 1997;84(2):145-9.
 35. Guo X, Yan C, Li H, Huang W, Shi X, Huang M, et al. Lipid-dependent conformational dynamics underlie the functional versatility of T-cell receptor. *Cell Res* 2017;27(4):505-25.
 36. Blank N, Schiller M, Krienke S, Busse F, Schätz B, Ho AD, et al. Atorvastatin inhibits T cell activation through 3-hydroxy-3-methylglutaryl coenzyme A reductase without decreasing cholesterol synthesis. *J Immunol* 2007;179(6):3613-21.
 37. Wu L-H, Wan Y-L, Xie H-Y, Zhang W-J, Zheng S-S. Immunosuppression in human peripheral blood T lymphocytes by fluvastatin. *Acta biochimica et biophysica Sinica. Acta Biochim Biophys Sin (Shanghai)* 2004;36(10):649-55.
 38. Weitz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J, Bruns C, et al. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat Med* 2001;7(6):687-92.
 39. Kanda H, Yokota K, Kohno C, Sawada T, Sato K, Yamaguchi M, et al. Effects of low-dosage simvastatin on rheumatoid arthritis through reduction of Th1/Th2 and CD4/CD8 ratios. *Mod Rheumatol* 2007;17(5):364-8.
 40. McBride JA, Striker R. Imbalance in the game of T cells: What can the CD4/CD8 T-cell ratio tell us about HIV and health? *PLoS Pathol* 2017;13(11):e1006624.