

T cell responses to an HLA-A2-restricted adipophilin peptide correlate with BMI in patients with atherosclerosis

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ABSTRACT

Introduction: Atherosclerosis is an inflammatory disease causing a vast array of cardiovascular diseases. Adipophilin has been reported to be highly expressed in atherosclerotic lesions. This study investigated the possible existence of auto-reactive T cells against an HLA-A02-restricted adipophilin-derived peptide as well as peptides from Epstein-barr virus (EBV), Cytomegalovirus (CMV) and influenza (Flu) virus in patients with atherosclerosis. **Methods:** HLA-A02 expression on peripheral blood mononuclear cells (PBMCs) was examined by flow cytometry. PBMCs from HLA-A02 individuals were stimulated with adipophilin, CMV, EBV, and Flu peptides at a concentration of 10 μ M. Interferon (IFN)- γ production was evaluated in the culture supernatant using a commercial ELISA test. **Results:** The levels of IFN- γ production against an HLA-A02-restricted adipophilin peptide and peptides from CMV, EBV, and Flu revealed no statistically significant differences between patients and healthy controls. However, we found a positive correlation between IFN- γ production against adipophilin and Body mass index (BMI) of patients ($R = 0.8$, $P = 0.003$), whereas no significant correlation was found in healthy controls ($R = -0.267$, $P = 0.378$). No correlation between BMI and IFN- γ production

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against CMV, EBV, or Flu peptides was found. *Discussion:* Atherosclerotic patients with higher BMIs might have greater numbers of T cells against adipophilin that is highly expressed in atherosclerotic plaques. Therefore, autoimmune reactions may have a greater role in the development of atherosclerosis in individuals with higher BMI.

KEYWORDS

atherosclerosis, adipophilin, HLA-A02, BMI

INTRODUCTION

Atherosclerosis is a chronic immune inflammatory disease of the arterial walls characterized by formation of atherosclerotic plaques [1]. The major clinical manifestations of the disease are peripheral arterial disease, ischemic heart pain, myocardial infarction and ischemic stroke which lead to premature death worldwide [2]. While the exact etiology of atherosclerosis is unknown, it is widely accepted that it is a multifactorial disease; for example with contribution of infections and the immune system [2-4].

The contribution of immune cells in the disease process is well acknowledged, and a large body of evidence suggests the underlying importance of the immune system in the development and progression of atherosclerosis [1, 5]. Various infiltrating immune cell types, including monocytes/foam cell macrophages and lymphocytes with their secreted cytokines and chemokines constitute key components of atherosclerotic plaque inflammation [1]. Monocytes can be recruited to the inflamed endothelium where they differentiate into macrophage phenotypes with highly phagocytic ability. They can scavenge modified *low-density lipoprotein* (mLDL) and eventually form foam cells, which along with other immune cells can increase the risk of the development of the unstable atherosclerotic plaque phenotype [5].

In addition, other immune cells including CD4+ T helper and CD8+ cytotoxic T lymphocytes (CTLs) play a pivotal role in the development or instability of the plaque [6, 7]. It is suggested that CD8+ T cells represent about 29% of all lymphocytes in early lesions, increasing up to 50% in advanced plaques [6]. They play critical roles in the development of vulnerable atherosclerotic plaques by recognition of oxidized low-density lipoprotein (Ox-LDL)-derived peptides and other probable auto-antigens presented via MHC class I molecules [8]. The cytotoxic effects of CD8+ T cells happen via granule exocytosis and release of key effector molecules, perforin and granzymes by which they induce apoptosis in different target cells including smooth muscle cells, endothelial cells and macrophages, and eventually lead to necrotic core and vulnerable atherosclerotic plaques formation [7]. Moreover, several cytokines produced by CD8+ T cells, like tumor-necrosis factor (TNF)- α and interferon (IFN)- γ can exert cytotoxic action when secreted in the proximity of target cells [9]. The central role of IFN- γ in pathogenesis of atherosclerosis is shown in IFN- γ -deficient mice which develop smaller and more stable plaques [10].

This cytokine is highly expressed in atherosclerotic lesions and exerts its detrimental roles in atherosclerosis via different mechanisms; IFN- γ activates macrophage and



endothelial cells to produce chemokines and adhesion molecules, respectively, by which it provokes monocyte/lymphocyte recruitment and infiltration into the sub-endothelium, thus accelerating plaque growth. IFN- γ promotes foam cell formation by increasing the uptake of mLDL by scavenger receptors on macrophages. IFN- γ can also help formation of vulnerable plaques by inducing the formation and release of reactive oxygen species and matrix metalloproteinases (MMPs) [11].

Numerous atherosclerosis-related T cell antigens are reported which include both microbial and self-antigens. Among bacterial and viral pathogens, *Chlamydia pneumoniae*, *Helicobacter pylori*, *Porphyromonas gingivalis*, Cytomegalovirus (CMV), Epstein-barr virus (EBV) and influenza (Flu) virus [12] are documented. The proposed mechanisms in microbial pathogenesis in atherosclerosis include direct infection of cells in the arterial wall, molecular mimicry, activation of pattern recognition receptors (PRR; e.g. Toll-like receptor; TLR) and subsequent bystander activation of auto-reactive T-cells and epitope spreading. Another mechanism is based on “danger hypothesis” in which damage-associated molecular patterns (DAMPs) released during cell death act as endogenous self-adjuvants and induce inflammation and auto-reactive T cell responses [13].

Potential candidate T cell auto-antigens in atherosclerosis lie within altered self-structures, such as modified lipoproteins and other proteins including β 2-glycoprotein I and heat shock proteins [14].

Adipophilin, or adipose differentiation-related protein (ADRP) is a 50 kDa protein expressed in adipocytes, which controls the structure and formation of lipid droplets. It is also expressed in macrophage foam cells and its expression is up-regulated by mLDL [15, 16]. This protein is expressed at higher levels in atherosclerotic lesions compared to healthy arterial intima, and the relative expression of adipophilin is higher in symptomatic compared to asymptomatic carotid plaques [15]. In addition, studies have shown that adipophilin is highly overexpressed in some tumors and is a target of CD8+ T cell responses [17]. In a study conducted by Weinschenk et al. a HLA-A*0201-binding peptide, SVASTITGV, derived from the adipophilin protein was identified in renal cell carcinoma (RCC) tumor samples [18] which was further confirmed by other studies [17]. As adipophilin is highly expressed in atherosclerotic lesions, and previous experimental data have revealed the “SVASTITGV” peptide as a CD8+ T cell target, we examined the possible potential of this peptide as a CD8+ T-cell epitope that can induce antigen-specific CD8+ T cells in HLA-A2 atherosclerosis patients.

The existence of CD8+ T-cells specific to adipophilin-derived peptides in patients with cancers has already been shown [17]. Therefore, we hypothesized that adipophilin could be a potential target for T cells in atherosclerosis [16, 19]. Thus the purpose of the present study was to investigate the possible existence of auto-reactive T cells against an HLA-A02-restricted adipophilin-derived peptide as well as peptides from EBV, CMV and Flu virus in patients with atherosclerosis.

MATERIALS AND METHODS

Patients and controls

Thirty-two patients with atherosclerosis from the Diabetes Clinic of Shiraz University of Medical Sciences, Iran entered the study. Forty age- and sex-matched healthy blood donors



were also enrolled in the study. Informed consent was obtained from all the subjects and the study was approved by the relevant Ethics Committees. Coronary angiography (CAG) was performed by a team of expert interventional cardiologists to estimate the coronary lesions. The inclusion and exclusion criteria were as follows: patients who were diagnosed based on the results of coronary angiography were included in the present study. We excluded patients with chronic diseases, including diabetes, hepatic or renal diseases or active infection. Patients with autoimmune diseases, malignancies and histories of taking any immunosuppressive drugs during last 3 months were also excluded from this study. For healthy subjects, the exclusion criteria were current smoking, hypertension, hypercholesterolemia, diabetes, obesity, active infection, autoimmune disease. The patients were grouped into single vessel disease (SVD), double vessel disease (DVD), and triple vessel disease (TVD) according to the number of major epicardial coronary arteries involved. Blood samples from both patients and controls were collected in heparin tubes and kept at room temperature (RT) until processing.

Peptides

HLA-A2-restricted peptides used in this study were >80% pure (China Peptides, Shanghai, China) (Table 1). Peptides were added at a final concentration of 10 μ M.

Isolation of peripheral blood mononuclear cells (PBMCs)

Ficoll-Hypaque solution (Lymphodex, Germany) was placed into a 50-mL conical centrifuge tube using a sterile pipette. Heparinized blood was mixed with an equal volume of phosphate buffered saline (PBS) and the diluted blood was slowly layered over the Ficoll-Hypaque solution. The tube was centrifuged for 20 min at 400 \times g, 22 $^{\circ}$ C. The ring of mononuclear cells was carefully collected and transferred into a 15-mL conical tube, 10 mL complete Roswell Park Memorial Institute (RPMI) medium was added and mixed thoroughly. The tube was centrifuged for 10 min at 400 \times g, 4 $^{\circ}$ C. The supernatant was discarded and peripheral blood mononuclear cells (PBMCs) were used for further experiments.

HLA-A02 typing of PBMCs by flow cytometry

10⁶ PBMCs were re-suspended in 1 mL ice-cold PBS containing 10% fetal calf serum (FCS) and 1% sodium azide to prevent the modulation and internalization of surface antigens which can

Table 1. HLA-A02-restricted peptides used in the stimulation of cultured PBMCs

HLA-A2-restricted peptides	Sequences	SYFPEITHI score	References
adipophilin ₁₂₉₋₁₃₇	SVASTITGV	25	[17]
Flu MP ₅₈₋₆₆	GILGFVFTL	30	[20]
EBV ₂₈₀₋₂₈₈	GLCTLVAML	28	[20]
CMV ₄₉₅₋₅₀₃	NLVPMVATV	30	[20]

Flu MP, Influenza Matrix protein; EBV, Epstein-Barr virus; CMV, Cytomegalovirus; SYFPEITHI (a database for MHC ligands and peptide motifs).



decrease the fluorescence intensity. 100 μL of cell suspension was added to each eppendorf tube, 2 μg of anti HLA-A02 monoclonal antibody (mouse IgG2b, Abcam, USA) was added to the cell suspension (final concentration 20 $\mu\text{g}/\text{mL}$), and cells were incubated for at least 30 min at 4 $^{\circ}\text{C}$ in the dark. Anti HLA-Class I antibody (IgG2a, Abcam, USA) was used as positive control. Cells were washed three times by centrifugation for 5 min at $400\times g$ and re-suspended in ice-cold PBS. 10 μL of fluorescent isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (IgG2a, Abcam, USA) was added and incubation was continued for 30 min at 4 $^{\circ}\text{C}$ in the dark. Cells stained only with the secondary antibody were used as negative control. In the next step, cells were washed 3 times by centrifugation for 5 min at $400\times g$ and re-suspended in ice-cold PBS containing 3% bovine serum albumin (BSA) and 1% sodium azide. The cell suspension was analyzed in a BD FACSCalibur flow cytometer. Analysis was performed by FlowJo version 7 software (TreeStar, USA).

PBMCs culture and stimulation with specific peptides

PBMCs from HLA-A02 individuals ($n = 14$) were seeded in 96-well plates (Nunc, Denmark) at a concentration of 2×10^5 cells/well. PBMCs cultured alone were used as negative control. Phytohemagglutinin (PHA) (Invitrogen, USA) at a final concentration of 2.5 $\mu\text{g}/\text{mL}$ was used as positive control of the kit. Flu, CMV, and EBV peptides at a concentration of 10 $\mu\text{M}/\text{well}/200 \mu\text{L}$ were used as positive controls for the subjects [20]. Adipophilin peptide was also used at the mentioned concentration to determine the responsiveness of PBMCs [17]. Plates were incubated at 5% CO_2 in a humidified incubator for 24 h. Cell culture media were next collected and centrifuged (1,500 rpm, 4 $^{\circ}\text{C}$ for 10 min). Supernatants were kept at -80°C until use for further processing. The sequences of selected peptides used in the present study were listed in Table 1.

IFN- γ detection in the supernatant of cultured PBMCs using ELISA

IFN- γ in the culture supernatant was assessed by using a commercial ELISA kit (Human IFN gamma ELISA Ready-SET-Go, ebioscience, USA) (sensitivity: 4 pg/mL) based on the manufacturer's instructions. Briefly, the ELISA plate was coated with 100 $\mu\text{L}/\text{well}$ of capture antibody in coating buffer. The plate was sealed and incubated overnight at 4 $^{\circ}\text{C}$. Wells were aspirated and washed 5 times with $>250 \mu\text{L}/\text{well}$ wash buffer (1 \times PBS, 0.05% Tween-20) and allowed to soak for about 1 minute during each wash step to increase the effectiveness of the washes. The plate was blotted on absorbent paper to remove any residual buffer. 1 part of 5X concentrated assay diluent was diluted with 4 parts Deionized (DI) water and blocking was done using 200 $\mu\text{L}/\text{well}$ of fetal bovine serum (FBS) 2%. The plate was incubated at room temperature for 1 hour. Wells were aspirated/washed as in step 3 (5 washes). Standards were diluted using 1X assay diluent. 100 $\mu\text{L}/\text{well}$ of standard was added to the appropriate wells. Two-fold serial dilutions of the top standards were made to make the standard curve. 100 $\mu\text{L}/\text{well}$ of the cell culture supernatant was added to the appropriate wells, and the plate was covered or sealed and incubated overnight at 4 $^{\circ}\text{C}$ for maximal sensitivity. The next day, wells were aspirated/washed as in step 3 (5 washes). 100 $\mu\text{L}/\text{well}$ of detection antibody diluted in 1X assay diluent was added, and the plate was sealed and incubated at room temperature for 1 hour. Wells were aspirated/washed as in step 3 (5 washes). 100 $\mu\text{L}/\text{well}$ of Avidin-HRP diluted in 1X assay diluent was added, and



the plate was sealed and incubated at room temperature for 30 minutes. Wells were aspirated, washed, and soaked (7 washes). 100 μ L/well of substrate solution was added to each well, and the plate was incubated at room temperature for 15 min. 50 μ L of stop solution (2N H₂SO₄) was added to each well. The plate was read at 450 nm and a 630 nm filter as a reference wavelength. The levels of IFN- γ within the sample were determined by setting up a standard curve of known target protein concentrations which was provided in the kit.

Statistical analysis

Data presented in the text and Figures are mean \pm SEM of at least three independent experiments. Unpaired Mann-Whitney U test and Kruskal-Wallis test were used to test the probability of significant differences between groups. For testing the correlation between the levels of IFN- γ and clinical manifestation, Pearson’s correlation test was used. A P_{value} less than 0.05 was considered statistically significant.

RESULTS

HLA typing of patients and healthy individuals

HLA typing using flow cytometry revealed that 14 out of the 32 recruited patients with atherosclerosis and 16 out of the 40 healthy individuals were positive for HLA-A02 (Fig. 1). Angiography indicated that five out of the 14 HLA-A02 patients had SVD, three had DVD and six had three-vessel disease (3VD). All HLA-A02 individuals were used for

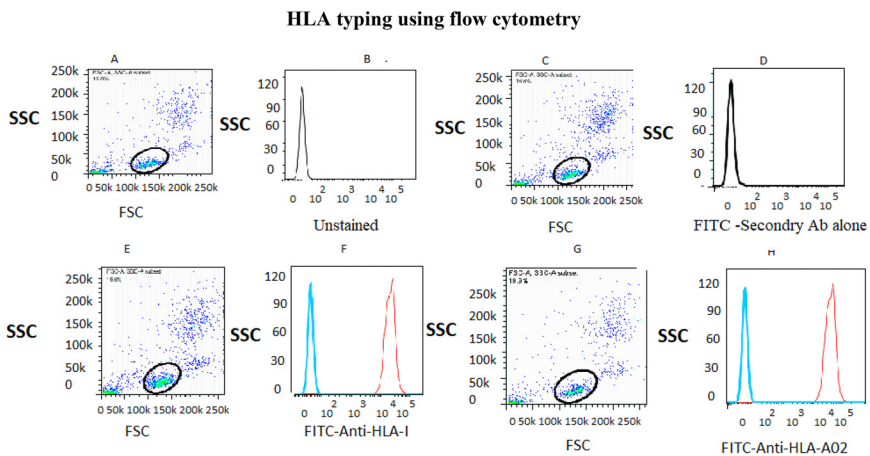


Fig. 1. HLA typing using flow cytometry. Dot plot and histogram plot obtained from flow cytometry analysis of PBMCs isolated from patients and healthy individuals. (A) Unstained; (B) Negative control; (C) Positive control; (D) HLA-A02-positive individual.



Table 2. Demographic characteristics of atherosclerosis patients and healthy controls

Demographic characteristics	Atherosclerosis patients	Healthy subjects
Number	32	40
HLA-A2+	14 (43%)	16 (40%)
Age (year)	41.2 ± 1.03	36.6 ± 0.9
BMI (body mass index)	24.8 ± 0.7	22.95 ± 2.20
Sex: F/M	10/4	10/6

Data represent mean ± SEM of age and BMI.

further experiments. The demographic characteristics of the two groups are listed in Table 2.

ELISA on PBMCs supernatant

The levels of IFN- γ (pg/mL) for HLA-A2-positive patients and controls after stimulation with CMV, EBV, Flu- and adipophilin-derived peptides are shown in Table 3. PBMCs stimulation with the mentioned peptides revealed no statistically significant differences in the levels of IFN- γ production between HLA-A2-positive patients and healthy controls (Fig. 2).

Correlation between IFN- γ and clinicopathologic manifestations of atherosclerosis in patients group

The results of the correlation analysis are shown in Table 4. Interestingly, a positive correlation was found between levels of IFN- γ production against adipophilin and Body Mass Index (BMI) of patients ($R = 0.8$, $P = 0.003$). No significant differences were found between the levels of IFN- γ against adipophilin and other clinicopathologic manifestations. In respect to the other selected peptides, no significant correlations were found between IFN- γ production and clinical manifestations. In addition, the same analysis was done in healthy controls to predict the correlation between IFN- γ productions against adipophilin and BMI. No statistically significant correlation was seen ($R = -0.267$, $P = 0.378$). The same results

Table 3. The levels of IFN- γ (pg/mL) for HLA-A2-positive patients and healthy controls after PBMCs stimulation with CMV-, EBV-, Flu- and adipophilin-derived peptides

Peptides	Patients ($n = 14$)	Controls ($n = 16$)	P value
CMV ₄₉₅₋₅₀₃	83.3 ± 27.6	70.6 ± 14.8	0.68
EBV ₂₈₀₋₂₈₈	39.0 ± 3.3	46.1 ± 9.7	0.5
Flu MP ₅₈₋₆₆	47.8 ± 8.9	49.2 ± 9.5	0.92
adipophilin ₁₂₉₋₁₃₇	36.9 ± 2.4	42.7 ± 8.8	0.53

Flu MP, Influenza (matrix protein); EBV, Epstein-Barr virus; CMV, Cytomegalovirus.



IFN- γ ELISA results in PBMCs cultured with CMV-, EBV-, Flu- and Adipo-derived peptides

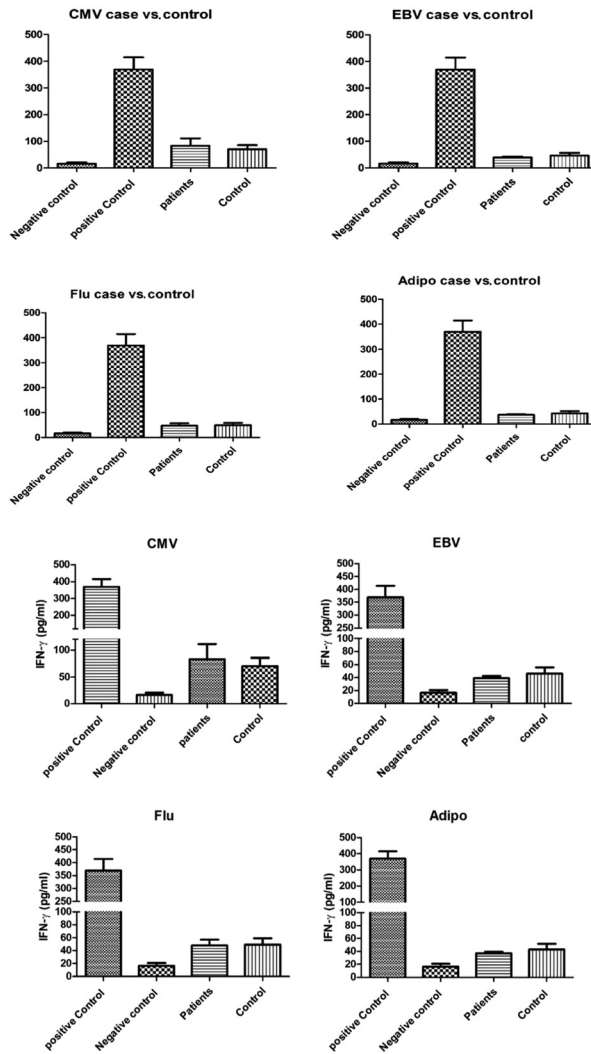


Fig. 2. IFN- γ ELISA results in PBMCs cultured with CMV-, EBV-, Flu- and Adipo-derived peptides. As shown, the levels of IFN- γ between HLA-A2-positive patients and healthy blood donors exhibit no significant differences

were obtained when we pooled the results of IFN- γ of healthy subjects and atherosclerosis patients together. In this case no correlation was seen between IFN- γ and BMI ($R = -0.140$, $P = 0.514$).





Table 4. Correlations between amounts of IFN- γ production against selected peptides and demographic and clinical manifestations of patients

Cytokines	Age correlation/ <i>P</i> value	Weight correlation/ <i>P</i> value	BMI correlation/ <i>P</i> value	Hyperlipidemia <i>P</i> value	Diabetes <i>P</i> value	Smoking <i>P</i> value	BP <i>P</i> value	Angiography <i>P</i> value
IFN- Adipo	0.276 0.319	0.355 0.235	0.800 ^{**} 0.003	0.724	1.000	0.713	0.865	0.252
IFN- CMV	0.454 0.089	-0.191 0.532	0.109 0.750	0.814	0.151	0.903	0.610	0.520
IFN-EBV	0.414 0.125	0.116 0.705	0.482 0.133	0.195	0.296	0.391	0.174	0.354
IFN-Flu	0.259 0.500	0.443 0.272	0.257 0.623	0.806	1.000	0.606	1.000	0.946

BMI, Body mass index; BP, Blood pressure; IFN, Interferon-gamma; Adipo, adipophilin; CMV, Cytomegalovirus; EBV, Epstein-Barr virus; Flu, Influenza
The bold values show the statistically significant correlation between IFN gamma levels against adipophilin and the BMI of the patients.

DISCUSSION

In the current study we found that HLA-A2-restricted adipophilin₁₂₉₋₁₃₇ is recognized by T cells from patients with atherosclerosis as well as control individuals. The magnitude of IFN- γ response was comparable to that of EBV₂₈₀₋₂₈₈ and Flu_{MP58-66} (Fig. 2). Both patients and controls produced notable amounts of IFN- γ in response to CMV₄₉₅₋₅₀₃.

Adipophilin is expressed in a broad variety of human malignancies and the HLA-A*02-binding peptide derived from this protein was identified in tumors of RCC patients [17]. A previous study reported that CTLs can lyse different cancer cells which endogenously express the adipophilin-derived peptides in a HLA-A*0201-restricted manner [16]. CTLs in RCC, malignant melanoma, breast cancer, and multiple myeloma cells can recognize and lyse tumor cells which endogenously express adipophilin in an antigen-specific and HLA-A2-restricted manner [17]. Adipophilin is also shown to be highly expressed in the atherosclerotic plaque [15]. Since the T cells in the circulation home to different tissues as well as pathologic sites, we asked if T (CD8+) cell responses towards adipophilin₁₂₉₋₁₃₇ exist in our patients and if so, how different these responses would be from that of healthy controls. While such responses in the form of IFN- γ secretion existed in both groups, we did not find any significant difference between the two groups. However, we found a positive correlation between the extent of IFN- γ production upon adipophilin peptide stimulation and BMI only among patients. Interestingly, such a correlation did not exist in healthy controls or when both groups were considered. Our result supports the relationship between inflammation induced by obesity and atherosclerosis. Studies have shown that obesity is associated with the production of inflammatory molecules, including inflammatory cytokines. The inflammatory cytokines, by affecting the artery wall, lead to the production of different mediators and surface proteins, followed by accumulation of inflammatory cells, endothelial dysfunction, substantial structural change in the artery and ultimately to atherosclerosis [21]. Earlier studies have demonstrated that the risk of cardiovascular disease (CVD) significantly increases in obese individuals in comparison to those with normal BMI [22, 23]. As noted above, high adipophilin expression in atherosclerotic plaques has already been reported [15]. Furthermore, association of total plaque area with obesity is demonstrated in a recent study [24]. Hence, we assume that atherosclerotic patients with higher BMI might have a greater number of T cells against this protein. Adipophilin plays a very important role in the formation of fat droplets, and the expression of this protein is very high in the adipose tissue of obese people [25]. Accordingly, we suggest that our study provides new information on the auto-reactive responses in general and in atherosclerosis in particular [17]. In our study both patients and controls responded to peptides derived from CMV, EBV and Flu viruses. As already mentioned, several evidences have indicated the relationship between pathogens and atherosclerosis [4, 12, 26]. The proposed mechanisms include direct presence of pathogens in the plaques, molecular mimicry, activation of PRRs, bystander activation of auto-reactive T-cells and epitope spreading [13].

We found no statistically significant differences in the levels of IFN- γ against the CMV peptide between HLA-A2-positive patients and healthy controls. Several studies indicate that CMV infection may contribute to accelerated atherosclerosis [4, 27-31]. CMV infection has been associated with accelerated heart transplant vasculopathy, cardiac allograft rejection and atherosclerosis [32, 33]. A recent report has shown an association between CVD mortality and CMV antibody titers [34]. Although there have been a large number of reports of the presence of



CMV in human plaques [35–39], a number of studies failed to document the presence of CMV in human lesions [40, 41]. The complex role of CMV in atherogenesis and the complex nature of the disease make it difficult to draw any conclusion at this point.

In addition to the CMV, EBV is another herpesvirus that is related to the pathogenesis of atherosclerosis [30]. A previous study indicated that EBV-specific cytotoxic T-cells and EBV DNA could be frequently observed in human atherosclerotic plaques, therefore T-cell response against EBV could contribute to plaque inflammation [42]. Our findings, however, did not show any differences with respect to CD8+ T-Cell IFN- γ responses against a common HLA-A2-restricted EBV peptide between patients and controls. Also, there have been both positive and negative reports of an association between Flu infection or seropositivity for Flu antibodies and coronary artery disease [43–45]. However, IFN- γ responses to Flu_{MP58-66}, while present, did not differ between our studied groups. Obviously by using only one peptide we cannot rule out the differences between patients and controls; however, the production of comparable amounts of IFN- γ induced by adipophilin, EBV and FLU peptides in our study is noteworthy.

This study set out for the first time to explore the presence of specific CD8+ T-cells against adipophilin as an autoantigen in patients with atherosclerosis compared to healthy individuals. Our results showed a high number of cells producing IFN- γ against adipophilin in patients with higher BMI. However, the results of this investigation show no significant differences between the two groups; i.e. adipophilin₁₂₉₋₁₃₇ is recognized by CD8+ T-cells from similar numbers of patients and healthy blood donors as are EBV and Flu peptides. Therefore, auto-reactive responses may be more common than previously thought. It is possible that studying other adipophilin-derived peptides would clarify the role of this molecule and specifically that of CD8+ T-cells in atherosclerosis and other diseases. In addition, culturing and stimulating plaque-derived CD8+ T-cells instead of PBMCs is a more accurate option for finding the candidate antigenic peptides in atherosclerosis. Finally, using more sensitive techniques such as ELISPOT and flow cytometry to investigate the production and secretion of IFN- γ , or using peptide tetramers could help us to find out the role of self and foreign antigens in the pathogenesis of atherosclerosis.

Conflict of interest: The authors declare no conflicts of interest.

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