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Relationship between regulatory T cells subsets and lipid profile in dyslipidemic patients: a longitudinal study during atorvastatin treatment

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Abstract

Background: The CD4+ T-lymphocytes and their subtype CD4 + CD25^{high}FoxP3+ regulatory T cells are receiving growing interest as major regulators of atherogenesis. We sought to investigate 1) whether the CD4 + cell subsets were expressed differently in dyslipidemic patients (Pts) and healthy subjects (HS) and 2) whether atorvastatin treatment could be associated in-vivo and in-vitro with cell changes in expression and functional response.

Methods: CD4+ subsets frequency (CD4 + CD25^{high}FoxP3+, CD4 + CD25-FoxP3+) and mRNA expression for FoxP3, IL-10 and TGF-β were evaluated in 30 consecutive Pts at baseline and after a 3-month atorvastatin therapy, and in 17 HS.

Results: The % of CD4 + cells did not differ between HS and Pts. The % of CD4 + CD25^{high}FoxP3+ was higher in Pts than HS and did not change during treatment. The CD4 + CD25-FoxP3+ cells were similar between the two groups and were lower in Pts at visit 2. Cytokine expression and FoxP3 did not differ in HS and Pts and no substantial change was observed during treatment. At visit 1, CD4 + CD25^{high}FoxP3+ cells were significantly correlated with both total-cholesterol ($r = 0.570$, $P = 0.0002$), LDL-cholesterol ($r = 0.715$, $P = 0.0001$), Apolipoprotein B ($r = 0.590$, $P = 0.0001$). In-vitro atorvastatin (up to 5 μM) failed to induce any significant modulation of cell functions.

Conclusion: CD4 + CD25^{high}FoxP3+ regulatory cells seem to be over-stimulated in the early pre-clinical phase of atherosclerosis and a relationship exists between their frequency and circulating lipids. A potential immuno-modulation by statin treatment is not achieved through a normalization in peripheral CD4 + cell subsets.

Keywords: CD4+, CD4 + CD25^{high}FoxP3+, CD4 + CD25-FoxP3+, Regulatory T cells, Atorvastatin, Dyslipidemic patients

Background

Atherosclerosis is now considered an inflammatory disease and it is well established that plaque initiation and destabilization are strictly linked with mechanisms involving both circulating and vascular immune cells and mediators of inflammation [1–3].

Atherosclerotic lesions initiate with endothelial damage and the proatherogenic potential of T cells is crucial in the progression from fatty streaks to mature plaques [4, 5]. Among the major classes of T lymphocytes, the CD4+ cells are viewed as major regulators of atherogenesis [1, 6]. Among the CD4+ subsets, specialized lymphocytes play a key regulatory role in the suppression of immune responses against self as well as foreign antigens, thus being pivotal for the suppression of detrimental Th1 immune responses [7].

In animal models the functional interplay between regulatory T cells and CD4+ effectors T lymphocytes may be critical in the control of atherosclerotic plaque development [8–11]. Regulatory T cells are now viewed as the master modulators of the immune system

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possessing the immunosuppressive capacity to prevent unfavorable immune responses and maintain tolerance to self-antigens and therefore as a potential therapeutic target [12]. Moreover, it is of great interest to understand whether pharmacological treatments can modulate the T regulatory control system.

Beyond the cholesterol-reducing effect, statins may interfere with inflammatory mechanisms associated with atherosclerosis and functional tissue responses, and recent evidence points to an interaction between these drugs and both the innate and adaptive immune cell function [13–19].

We sought to investigate in dyslipidemic subjects bearing an increased risk for vascular events whether the CD4+ cell subsets, including specific T cell subsets known to be involved in the immune-response regulation, such as T cells characterized as CD4 + $CD25^{high}$FoxP3+, were differently expressed compared to controls. As a secondary endpoint, we evaluated whether atorvastatin ex vivo and in vitro treatment could be associated with changes in expression and functional response of $CD4^+$ T cell subsets.

Methods

Subject's enrollment

We enrolled 30 consecutive dyslipidemic patients (Pts) evaluated at our Lipid outpatient Clinic who showed a "moderate risk" for vascular events according to the NCEP - Adult Treatment Panel III (ATPIII) guidelines [20]. Subjects were included in the study if a lipid-lowering pharmacological treatment with statin was clinically indicated and they did not assume any pharmacological treatment. Ongoing clinical infection and/or the presence of infections in the previous three months were considered as exclusion criteria. The Pts were studied after 6 weeks of life-style modification including dietary treatment (qualitative counseling) and recommendations for mild physical activity. Pts were studied at the day of institution of atorvastatin (10–20 mg/od) therapy (visit 1) and 3 months later (visit 2). We also enrolled a control group of healthy subjects (HS), showing a low cardiovascular risk according to ATPIII. Blood samplings were obtained between 8.00 and 9.00 A.M., after a fasting night. Laboratory examinations included total cholesterol (Tot-c), low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), Tryglicerides (TG), Apolipoprotein B (ApoB) and apolipoprotein A (ApoA), fasting glycemia, serum C-reactive protein (CRP). Our study complies with the Declaration of Helsinki; the "Circolo and Macchi Foundation Hospital" Ethics Committee has approved the research protocol and written informed consent has been obtained from the subjects.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMC were isolated as previously described [13]. Purity and viability of cells were assessed by means of flow cytometric assay and a typical PBMCs preparations contained about 80 % lymphocytes and 16 % monocytes and cell viability was always >99 %. One samples of 2×10^6 cells were immediately assayed for the study of the frequency of the major CD4+ T cell subsets.

Flow cytometry analysis of CD4+ subsets frequency

The frequency of circulating total CD4+ T cells as well of CD4+ T cell subsets was evaluated by a two-color flow cytometric analysis. Moreover, Forkhead box P3 (FoxP3) intracellular staining was evaluated to study the frequency of CD4 + $CD25^{high}$FoxP3+ T cells, according to the protocol recommended by eBioscience (San Diego, CA, USA). Acquisition was performed on a FACSCanto II flow cytometer (BD Bioscience, Milan, Italy) and data were analyzed with BD FACSDiva software (version 6.1.3; BD Bioscience). Total CD4+ T lymphocytes were identified and gated on the side scatter *vs* CD4-FITC dot plot and regulatory T cells frequency was expressed as percentage (%) of $CD25^{high}$ FoxP3+ cells among CD4+ T cells.

Purification of CD4+, CD4 + CD25+ and CD4 + CD25- T lymphocytes for real time PCR assays

CD4+ were obtained from whole blood by means of immunomagnetic cell sorting using the CD4+ Positive Isolation (Dynal Biotech, Invitrogen). CD4 + CD25+ and CD4 + CD25- subsets were obtained by means of immunomagnetic cell sorting using CD4+ Negative Isolation and subsequently using the anti-CD25 monoclonal antibodies contained in the CD4 + CD25+ T Cell isolation Kit (Miltenyi Biotec). For all the cell subsets, after immunomagnetic cell sorting, the purity and the viability were assessed by flow cytometry and were always >96–99 %; samples were subsequently analyzed by measuring the expression of the mRNA for FoxP3, which is a transcription factor expressed in high amount on CD4 + CD25+ T lymphocytes.

RNA isolation and real-time PCR analysis

Total mRNA was extracted from 1×10^6 cells by Manual PerfectPure^{TM} RNA Cell & Tissue (5PRIME, GmbH). RNA was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) and real time PCR were performed as previously described [21]. Finally, raw data were analyzed by the ABI prism SDS software (Applied Biosystems) and primers (Ct1) mRNA expression data were obtained from Ct values, normalized to 18 s RNA (Ct2) (housekeeping) content and finally expressed as $2^{-\Delta ct}$ for each gene.

Cell viability, CFSE staining and cell proliferation assay

Cell viability tests on PBMC and CD4+ were performed by means of flow cytometry and propidium iodide (PI) staining. Acquisition was performed on a BD FACS-Canto II flow cytometer. Cells were identified on the basis of the forward-scatter (FSC) and side-scatter (SSC) properties, and a minimum of 15.000 cells from each sample was collected in the gate. Finally, cell viability was assessed by calculating the percentage (%) of cells negative at PI staining with respect to total cells included in the gate. For cell proliferation, PBMC, CD4+, and CD4 + CD25- were resuspended in RPMI 1640 without fetal bovine serum (10^7cells/ml) and incubated with 5,6-carboxy fluorescein diacetate succinimidyl ester (CFSE; 1 μM; 5 min, 37 °C). Cells were then resuspended in RPMI supplemented with 10 % of FBS and washed two times, cells were resuspended in culture medium and cultured. Phytohaemagglutinin (PHA; 10 μg/mL)-induced cell proliferation was assessed for PBMC, $CD4^+$ and for coculture of CD4 + CD25+ and CD4 + CD25- (ratio: CD4 + CD25+ /CD4 + CD25- = 1:1). The PHA was selected as stimulus for both PBMC and T reg according to our previous results [22].

Then, cells were incubated for 5 days in a 37 °C in 5 % of CO_2. At the end of culture, cells were centrifuged (300 g, 10 min) washed one time in PBS 1X and then prepared for the cytofluorimetric analysis. Cell proliferation was measured by using 488 nm excitation and emission filters appropriate for fluorescein. A minimum of 20,000 events were analyzed and data were expressed as % of CFSE low cells (% of proliferation).

Ability of atorvastatin to influence in vitro FoxP3 and cytokine expression

Atorvastatin (concentration range: 0.001–10 μM) was added on PBMC or CD4 + 1 h before PHA (10 μg/ml) and incubated for 24 h (for cytokine production) or 48 h (for FoxP3 expression).

At the end of cell culture, cells were harvested and stored (−80 °C) for the subsequent real time assay of cytokine mRNA expression, while PBMC and CD4+ were analyzed with flow cytofluorimetric technique for the evaluation of FoxP3 on CD4+ specific subsets.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine production was measured in cell culture supernatants by using commercialized enzyme-linked immunosorbent assay (ELISA, Amersham, UK).

Statistical analysis

Our study was designed as a pilot study. We also have not found any previous study to calculate sample size and statistical power. Data are presented as mean and standard deviation (SD) or median and 25–75th percentile range (IQR) if continuous and as counts and percent if categorical. Comparisons between dependent measures were performed with the Wilcoxon signed-rank test. The Bonferroni correction was applied for post hoc comparisons. Comparisons between independent measures were performed with the Mann–Whitney U test. The relationships between both the T cell subsets and T cell subsets potential changes during treatment, and both baseline levels and changes occurring during the follow-up in lipid parameters (Tot-c, LDL-c, ApoB) were investigated using univariate analysis by Spearman Rank correlation and then by linear regression analysis. Calculations were performed using commercial software (Stata 11, Stata Corp, College Station, TX, USA). A two-sided P, 0.05 was retained for statistical significance.

Results

Effect of 3-month treatment with atorvastatin on cell subsets of dyslipidemic Pts and comparison with HS

Clinical and laboratory parameters in Pts and HS

Mean age and gender distribution were similar in Pts and HS (49 ± 9 *vs* 45 ± 8 years, $P = 0.21$; 80 % males *vs* 65 %), as well as body mass index (BMI) and waist circumference (WC) [(BMI 26.8 ± 4 *vs* 23.7 ± 3.4 kg/m^2 ($P = 0.07$); WC 97.4 ± 10.6 *vs* 87.3 ± 10.7 cm ($P = 0.052$)]. 19 Pts and 6 HS were habitual smokers (7 ± 5 cigarette/day) and no subject changed the smoking habitus during the study. Laboratory parameters of both Pts and HS are shown in Table 1. Values measured in Pts at visit 1 were significantly different with respect to values measured in HS fot TotC, LDLc, ApoB and TG. In Pts, the Tot-c, LDL-c, ApoB and TG were significantly reduced after atorvastatin treatment (visit 2).

T cells subsets in Pts and HS

The % of CD4+ cells did not differ between HS [35.9 (34.2–47.6)] and untreated Pts at visit 1 [43.5 (34.5–49.0)] ($P = 0.372$). Comparing Pts at the two visit times, no difference was found between the % of CD4+ [(visit 2: 41.8 (36.2–49.1)] ($P = 0.294$). The % of CD4+ cells obtained from Pts at visit 2 was similar to the % of CD4+ cells of HS ($P = 0.315$). A significant difference was observed in the % of CD4 + CD25^{high}FoxP3+ ($P = 0.03$) (Fig. 1, panel a). CD4 + CD25-FoxP3+ were similar between the two groups of subjects ($P = 0.509$) (Fig. 1, panel b). No differences were observed between Pts at the two visit times with regards to CD4 + CD25^{high}FoxP3+ ($P = 0.826$) and at visit 2 the values remained higher when compared to HS ($P = 0.03$). The % of CD4 + CD25-FoxP3+ cells measured at visit 2 was significantly lower than the values measured

Table 1 Laboratory parameters before institution of atorvastatin treatment (visit 1) and after 3 months (visit 2)

	HS ($n = 17$)			Pts ($n = 30$)		
		*P** vs Pts				*P***
		Visit 1	Visit 2	Visit 1	Visit 2	Visit 2 vs 1
Tot-c (mg/dL)	198.0 (188.5–221.5)	<0.0001	0.036	269.0 (226.0–318.0)	166.0 (141.5–204.5)	<0.0001
LDL-c (mg/dL)	115.0 (107.0–147.5)	0.003	n.s.	168.0 (119.3–263.5)	135.0 (94.0–196.5)	0.0013
HDL-c (mg/dL)	53.0 (44.0–67.5)	n.s.	n.s.	49.5 (42.8–56.3)	47.0 (39.5–54.5)	n.s.
TG (mg/dL)	132.0 (102.5–163.5)	0.002	0.0004	181.5 (137.7–220.6)	87.0 (61.2–111.9)	<0.0001
ApoA (mg/dL)	144.0 (121.5–161.0)	n.s.	n.s.	126.0 (114.3–139.5)	130.0 (112.0–143.5)	n.s.
ApoB (mg/dL)	98.0 (85.0–108.5)	<0.0001	0.038	139.0 (120.0–155.0)	80.0 (64.0–.98.0)	<0.0001
hs-CRP (mg/L)	1.50 (0.60–2.70)	n.s.	n.s.	0.90 (0.45–2.20)	1.1 (0.7–2.4)	n.s.
Glucose (mg/dL)	96 (89–97)	n.s.	n.s.	93.5 (89.75–102)	96 (89.75–100)	n.s.

Values are expressed as median and 25–75th percentile
n.s. not significant
* = Mann Whitney test; ** = Wilcoxon signed rank test

at visit 1 ($P = 0.014$) and these values did not differ from those measured in HS ($P = 0.322$) (Fig. 1).

mRNA expression for FoxP3, IL-10 and TGF-β in CD4 + CD25+ and CD4 + CD25- cells of Pts and HS

As shown in Table 2, mRNA expression for IL-10, TGF-β and FoxP3 did not differ in CD4 + CD25+ and CD4 + CD25- cells of HS and untreated Pts. No change was observed between Pts values during the study (vist 2 *vs* visit 1) for IL-10 and TGF-β in CD4 + CD25+ and CD4 + CD25- cells. FoxP3 mRNA expression was significantly reduced in CD4 + CD25- cells ($P = 0.019$).

Relationships between T cells subsets and both clinical and laboratory parameters

The % of CD4+ cells was not related with LDL-c, HDL-c, TG, ApoB and ApoA whereas tended to be associated (R 0.298, $P = 0.073$) with Tot-c. CD4 + $CD25^{high}$FoxP3+ cells were significantly correlated with both Tot-c, LDL-c, ApoB (Fig. 2). We didn't found significant correlation between HDL-c and CD4 + $CD25^{high}$FoxP3+ cells ($R = 0.172$; $P = 0.303$).

After dividing the sample into healthy and hypercholesterolemic subjects, these correlations remained statistically significant only in Pts (data not shown). The associations remained significant when adjusting for smoking habits.

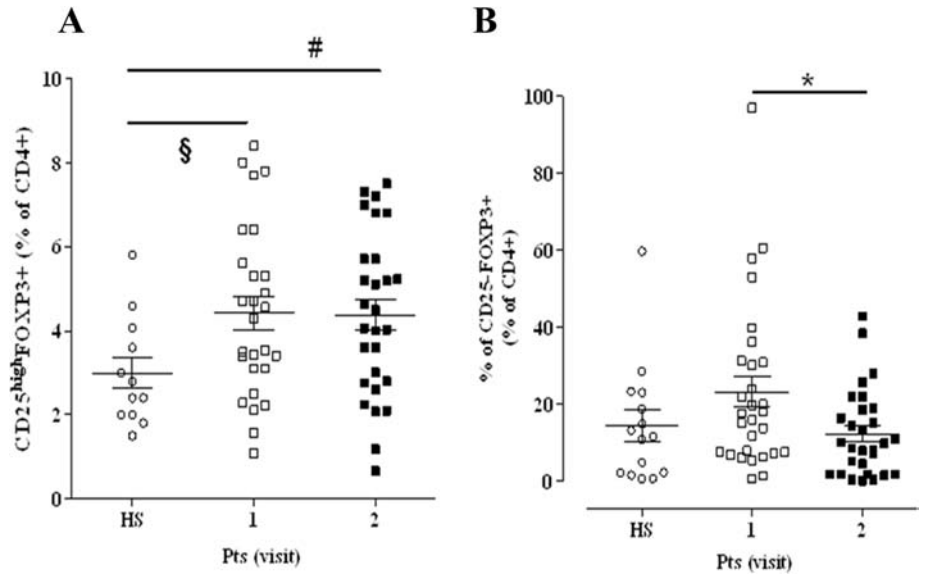

Fig. 1 Percent of FoxP3 expression in CD4 + $CD25^{high}$ (panel **a**) and CD4 + CD25- (panel **b**) subsets analyzed by means of flow cytofluorimetric assay in venous blood from HS (empty circles) and Pts at the two visit times (empty squares: visit 1; filled squares: visit 2). Data are expressed as median and 25 and 75th percentile. § = *P* 0.03 visit 1 vs HS (Mann–Whitney test); * = *P* 0.014 visit 2 *vs* visit 1 (Wilcoxon matched pairs test); # = *P* 0.03 visit 2 vs HS (Mann–Whitney test)

Table 2 mRNA expression for IL-10, TGF-β and FoxP3 before atorvastatin (visit 1) and after 3 months (visit 2)

	HS ($n = 17$)			Pts ($n = 30$)		
		P* vs Pts				P**
		Visit 1	Visit 2	1	2	Visit 2 vs 1
CD4 + CD25+ cells						
IL-10	1.46 (0.202–4.747)	0.455	0.058	1.65 (0.3355–4.100)	1.52 (0.134–3.350)	0.173
TGFβ	18.070 (11.510–37.770)	0.111	0.178	39.850 (14.850–62.850)	32.300 (15.200–56.000)	0.185
FoxP3	252.600 (72.520–389.700)	0.811	0.858	138.000 (56.800–581.00)	274.000 (59.900–534.000)	0.417
CD4 + CD25- cells						
IL-10	0.018 (0.008–0.321)	0.263	0.171	0.328 (0.105–0.447)	0.101 (0.008–0.230)	0.203
TGFβ	0.851 (0.317–2.055)	0.097	0.428	1.780 (0.716–4.575)	1.640 (0.297–13.700)	1.000
FoxP3	0.545 (0.208–0.968)	0.627	0.425	0.574 (0.155–3.090)	0.273 (0.133–0.887)	0.019

Values are expressed as median and 25–75th percentile range
* = Mann Whitney test; ** = Wilcoxon signed rank test

At a univariate analysis we didn't see any correlation between CD4 + $CD25^{high}$FoxP3+ cells and age, body mass index, systolic and diastolic blood pressure, waist circumference, fasting plasma glucose, creatinine levels. So we didn't include these variables in the regression analysis model.

At a univariate analysis we found a significant correlation only between Apo B and waist circumference ($R = 0.412$; $P = 0.014$) . Adding waist circumference to the regression analysis model we confirmed the association between Apo B and CD4 + $CD25^{high}$FoxP3+ cells.

Changes in lipid profile after atorvastatin treatment were not associated with changes in CD4 + $CD25^{high}$-FoxP3+ cells measured between the two visits (data not shown). No association was found between CD4 + CD25-FoxP3+ cells and the lipid profile. No relationship was observed between mRNA levels (FoxP3, IL-10 and TGF-β) and clinical (age, blood pressure, smoking habitus) and laboratory parameters (lipid parameters, CRP levels) both in CD4 + CD25+ and CD4 + CD25- cells (data not shown).

In vitro study: effect of stimulation with atorvastatin on cell subsets

Effect of treatment with atorvastatin on PBMCs and CD4+ T lymphocytes

We tested the ability of atorvastatin to modulate cell viability (24 h – 5 die), proliferation (5 die) and cytokine production (24 h; IFNγ/IL-4) on PBMCs and CD4+ cells (see Table 3). Atorvastatin up to 1 μM, did not affect any parameter investigated while higher concentrations significantly influenced all the parameters investigated with the only exception of resting cell viability in PBMCs and IL-4 mRNA expression both in PBMCs and CD4+ T lymphocytes (Table 3).

Effect of atorvastatin on frequency of CD4 + $CD25^{high}$ and on FoxP3 expression

Treatment of PBMCs with atorvastatin (concentration range: 0.1–10 μM) for 24 h did not affect the frequency of CD4+, CD4 + CD25+, CD4 + CD25-, CD4 + $CD25^{high}$-FoxP3+ and CD4 + CD25-FoxP3+ (data not shown); in addition in the same conditions, no differences were observed in % of FoxP3+ expression in the same cell subsets when cells were treated with atorvastatin for 24 or 48 h (data not shown). As shown in Table 4, incubation for 48 h with atorvastatin did not affect PHA-induced FoxP3 expression up to 5 μM, wheres incubation with 10 μM atorvastin induced a significant reduction of FoxP3 expression on CD4+ ($P < 0.05$ vs PHA) and on CD4 + CD25+ ($P < 0.05$ *vs* PHA). Atorvastatin did not influence FoxP3 expression on CD4 + CD25- ($P > 0.05$ *vs* PHA).

Effect of atorvastatin on coculture of CD4 + CD25- and CD4 + CD25+ cells

Co-culture of CD4 + CD25- cells and CD4 + CD25+ cells (1:1) induced a decrease in PHA-induced CD4 + CD25- cells proliferation (CD4 + CD25-: 63.3 ± 21.1, co-culture: 25.5 ± 22.3; $n = 5$; $P = 0.004$); incubation with atorvastatin before PHA (concentration range: 0.001–1 μM) did not affect the CD4 + CD25+ cells inhibitory effect ($P > 0.05$ for all the concentrations tested).

Discussion

The complex regulatory network of T cells comprises a number of T cell subpopulations with distinct phenotypes and distinct mechanisms of action as CD4 + CD25 + FoxP3+ T cells, possibly influencing the atherosclerotic processes through inhibition of immune responses [23].

Only a few studies investigated in patients the relationship between regulatory T cells and atherosclerosis, and

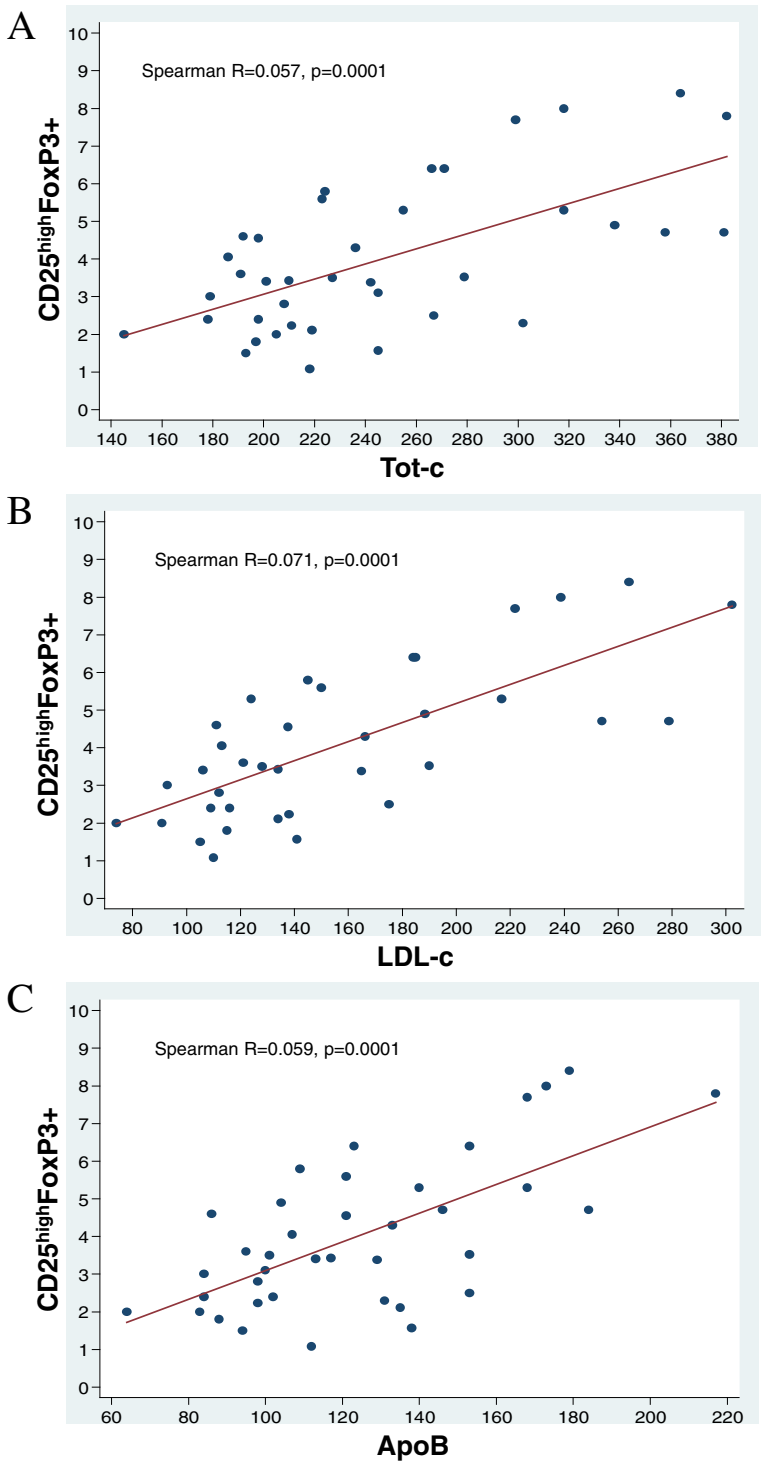

Fig. 2 Correlations between CD4 + CD25^{high} FoxP3+ cells and Tot-c (panel **a**), LDL-c (panel **b**) and ApoB (panel **c**) in untreated Pts and HS. The non parametric Spearman R correlation coefficient was computed together with its *p*-value

very few authors reported longitudinal results on potential changes of these cells frequency and/or function after pharmacological cardiovascular treatments in vascular diseases.

In our study we report that dyslipidemic, otherwise healthy, patients at increased cardiovascular risk show a mild, but significant, increase in CD4 + CD25^{high}-FoxP3+ cells and a similar expression of the other

Table 3 In vitro effect of stimulation with atorvastatin (0.001–10 μM) on PBMC and CD4+

PBMC						
		Atorvastatin (μM)				
	Control	0.001	0.1	1	5	10
Resting cell viability (% of PI negative)[a]	100 ± 2.11	101.41 ± 0.64	96.89 ± 3.63	102.82 ± 1.28	=	101.43 ± 2.19
PHA-induced cell viability[b]	100.00 ± 27.59	106.10 ± 9.81	107.40 ± 14.65	107.50 ± 23.48	50.76 ± 42.44*	33.72 ± 20.61**
PHA-induced cell proliferation[b]	100.00 ± 26.24	98.61 ± 15.79	96.83 ± 35.19	83.47 ± 25.37	62.26 ± 22.45	33.82 ± 22.84**
PHA-induced IL-4 mRNA expression[a]	100.00 ± 38.99	=	=	103.90 ± 35.17	98.97 ± 40.82	83.34 ± 37.06
PHA-induced IFN- mRNA expression[a]	100.00 ± 48.40	=	=	102.50 ± 32.99	56.97 ± 32.36	17.03 ± 10.88*
	CD4+					
		Atorvastatin (μM)				
	PHA (10 μg/ml)	0.001	0.1	1	5	10
Cell viability[a]	100 ± 10.66	98.12 ± 2.92	103.60 ± 7.14	84.88 ± 20.64	53.04 ± 17.73*	34.69 ± 17.28**
Cell proliferation[b]	100.30 ± 34.75	91.92 ± 13.94	105.10 ± 18.60	109.60 ± 42.90	37.38 ± 4.37*	13.03 ± 10.32**
IL-4 mRNA expression[a]	100.00 ± 25.99	=	=	133.20 ± 64.78	98.97 ± 33.77	62.11 ± 6.19
IFN- mRNA expression[a]	100.00 ± 39.62	=	=	127.20 ± 35.17	123.70 ± 66.18	54.17 ± 27.68

Cells were cultured for 1 (a) or 5 (b) days as appropriate. Values are expressed as percentage of resting values (presented in table as control) or of values obtained after stimulation with PHA (10 ng/ml) and finally expressed as mean ± SD of at least 4–6 separate experiments; P values were assessed by ANOVA followed by Dunnett comparison

* = $P < 0.05$; ** = and $P < 0.0001$ *vs* respective control

CD4+ subsets studied when compared to control subjects.

A significant reduction of regulatory CD4+ cells or FoxP3+ cells, together with altered functional activities, was previously reported in patients with acute coronary syndrome or in vulnerable patients who had undergo at least two myocardial infarctions in medical history compared with both normal subjects and patients with stable angina [24–28]. Besides the setting of acute myocardial infarction, peripheral Th17/Treg imbalance has been reported in patients with acute atherosclerotic cerebral infarction [29]. In a prospective cohort, CD4 + FoxP3+ cells and not CD4 + CD25 + FoxP3+ cells were independent predictors of acute myocardial infarction whereas no relation was found with stroke [30].

In patients with vascular lesions, unstable carotid plaques patients showed lower levels of regulatory T cells, regulatory T-related cytokines (IL-10 and TGF-β1), and FoxP3 mRNA [31].

On the other hand, previous observations in chronic stable atherosclerosis showed that the atherosclerotic burden is not related to T regulatory cells expression. Indeed in patients submitted to carotid echography evaluation, the intima-media thickness was not related to regulatory T cell levels in a large patient population and no differences in regulatory T cell levels were observed comparing rapid versus slow intima-media thickness progressors [32]. Moreover, an absence in regulatory T cells level alteration was confirmed in chronic stable angina patients [32].

In our dyslipidemic patients who did not show ouvert vascular diseases and therefore in a pre-clinical stage of atherosclerosis, we found a hightly significant correlation between CD4 + $CD25^{high}$FoxP3+ cells and both Tot-c, LDL-c and ApoB. Hypercholesterolemia was found to induce an accumulation of regulatory T lymphocytes in the atherosclerotic aorta and spleens in mice, but the regulatory T cells content is not maintained over time under sustained hypercholesterolemic conditions [33]. Possibly the characteristics of the patient population of the present study may account for our results.

Adaptive immune responses may change in the progression of the atherosclerotic process and increased levels of $CD4CD25^{high}$FoxP3+ cells may represent an early response to initial changes associated with atherosclerosis.

Table 4 In vitro effect of atorvastatin on FoxP3 expression in CD4+ subsets obtained from healthy subjects

	Atorvastatin (μM)			
	PHA	1	5	10
CD4 + FoxP3+ (%)	78.17 ± 9.03	80.30 ± 4.35	72.33 ± 9.77	60.79 ± 9.39*
CD4 + CD25 + FoxP3+ (%)	78.81 ± 8.23	80.46 ± 1.32	74.17 ± 8.48	64.28 ± 7.63*
CD4 + CD25-FoxP3+ (%)	19.56 ± 12.64	10.20 ± 3.82	17.46 ± 12.07	11.80 ± 9.65

Values are expressed as mean ± SD; *P* values were assessed by ANOVA followed by Dunnett comparison; * = $P < 0.05$ *vs* respective control

It has been shown that inflammed enviroments may modulate FoxP3-driven epigenetic modifications [34]. Systemic inflammation as expressed by serum CRP, was similar in our groups of healthy subjects and dyslipidemic patients, pointing to very early modifications occurring in our patients in the "atherosclerosis continuum" from functional disturbances to ouvert diseases.

Moreover, in this study we showed that a 3-month atorvastatin treatment in dyslipidemic patients is not associated with changes in expression of CD4+ cells and/or in functional properties of both CD4+ subset and CD4 + CD25^{high}FoxP3+ T cells.

The clinical significance of specific newly identified subsets of T cells is still under investigation and cell types such as CD4 + CD25-FoxP3+ are now receiving increasing attention. Although some authors found that these cells phenotypically, and to a certain extent also functionally, resemble conventional T regulatory cells, others point to a dysfunctional nature of the suppressive effect of these cell type [35, 36]. In the present study the MFI of CD4 + CD25-FoxP3+ measured during atorvastatin treatment was significantly lower than values measured at baseline, though this value did not differ from values measured in HS. These observations are derived by evaluation of the phenotype and therefore we are not allowed to have clues on functional parameters of these cells and their potential changes during treatment. Only a few previous studies have investigated the potential influence of statin treatment on regulatory T cells. In normocholesterolemic healthy subjects a short-term treatment with low doses of lovastatin or atorvastatin was associated to increased CD4 + FoxP3+ cells. Conversely to our results, the authors found also a significant correlation between CD4 + FoxP3+ cells and HDL-c levels [37]. When CD4 + CD25+ T cells were investigated in the setting of acute myocardial infarction, the inhibitory activity of CD4 + CD25+ regulatory T cells was found to be modified with atorvastatin treatment [28, 38]. A previous retrospective study reported that simvastatin (20 mg; $n = 7$) and pravastatin (10-40 mg; $n = 5$) treatment were associated with increased CD4 + CD25^{high} in hypercholesterolemic subject [39]. In PBMCs of patients with acute coronary syndrome, in-vitro simvastatin 10 μM was able to increase the percentage of CD4 + CD25 + FoxP3+ regulatory T cells to total CD4+ cells [40]. However it is known that statin levels during clinical treatment do not reach levels comparable to the higher concentrations used for in-vitro experiments. In our in-vitro study, treatment of PBMC and CD4+ susbsets with atorvastatin failed to induce any significant modulation of cell functions in the concentration-range comparable to clinical treatment, while interestingly 10 μM significantly reduced cell viability and affected cell functions. In particular, in the co-colture stimulated for proliferation, the expected inhibitory effect of CD4 + CD25+ cells on CD4 + CD25- was not reversed by pretreatment with atorvastatin, therefore atorvastatin does not influence these cells physiological functioning. Taken together, both our data obtained during chronic statin administration in a prospective study and in-vitro observations point to the absence of significant effects of atorvastatin therapy on CD4+ subsets in dyslipidemic patient except for the CD4 + CD25-FoxP3+ cell type.

Although we did not observe relevant changes in the circulating T cell subpopulation studied, it cannot be ruled out the possibility of different local concentration of cells in inflammed vascular tissues or specific differentiations induced by local mediators or that potential conflicting results could be obtained using other statins.

Moreover, the follow-up time and/or the atorvastatin dose used in our study could be inadequate to observe potential changes/redistribution of the immune cells subsets.

Conclusions

Our results show that the immune system cell types involved in regulatory mechanisms may be over stimulated in the early pre-clinical phase of atherosclerosis and that a relationship exists between CD4 + CD25^{high}-FoxP3+ frequency and circulating lipids. A potential immuno-modulation by statins is not achieved in a short term follow-up period through a normalization in peripheral CD4+ cell subsets. Moreover, in-vitro atorvastatin does not seem to affect the CD4+ subtypes function.

Abbreviations

Pts: dyslipidemic patients; HS: healthy subjects; ATP III: Adult Treatment Panel III; Tot-c: total cholesterol; LDL-c: low density lipoprotein cholesterol; HDL-c: high density lipoprotein cholesterol; TG: tryglicerides; ApoB: apolipoprotein B; ApoA: apolipoprotein A; CRP: serum C-reactive protein; PBMCs: peripheral blood mononuclear cells; FoxP3: forkhead box P3; PI: propidium iodide; FSC: forward-scatter; SSC: side-scatter; CFSE: carboxy fluorescein diacetate succinimidyl ester; RPMI 1640: Rosewell Park Memorial Institute 1640; FBS: fetal bovine serum; PHA: phytohaemagglutinin; PBS: phosphate buffered saline; ELISA: enzyme-linked immunosorbent assay; SD: standard deviation; IQR: 25–75th percentile range; BMI: body mass index; WC: waist circumference; IL-10: Interleukin-10; TGF-β: transforming growth factor β; IL-4: Interleukin-4; MFI: mean fluorescence intensity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LG partecipated in coordination and study design, draft the manuscript; AMM, LRT partecipated in study design, data collection and interpretation and draft the manuscript; LS, ER, FM partecipated in study design, performed experimental analysis and draft the manuscript; FD, AS, CM, LC, WA, AMG, MC partecipated in study design and data interpretation; CK partecipated in data interpretation and performed statistical analysis. All authors read and approved the final manuscript.

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