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LETTER TO THE EDITOR

Statins Inhibit Cytokines in a Dose-Dependent Response in Patients with Systemic Sclerosis

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Abstract— Although statins have been successfully administered in the treatment of hypercholesterolemia and cardiovascular disease due to their lipid-lowering and anti-atherosclerotic action, they have shown immunomodulatory effects in several studies with immune-mediated diseases. The aim of this study was to investigate the effects of statins treatment on Th1, Th2, and Th17 cytokines production from stimulated peripheral blood mononuclear cells (PBMCs) obtained from Systemic Sclerosis (SSc) patients. We recruited 21 patients classified according to the American College of Rheumatology criteria for SSc for PBMCs culture analysis. Cytokine levels (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, IL-17A, and IL-17F) were quantified by ELISA or CBA, and patients were assessed for clinical and exam's variables. Simvastatin and atorvastatin at 50 μM promoted reduction in all cytokine levels with statistical significance, except for IL-6, which had its reduction only induced by the use of simvastatin. Statins, particularly simvastatin, appear to have an immunosuppressive effect in reducing all cytokine secretion levels from PBMCs of SSc in a dose-dependent manner.

KEY WORDS: scleroderma; simvastatin; atorvastatin; Th cells.

INTRODUCTION

Th1 and Th2 pathway cytokines have been implicated in Systemic Sclerosis (SSc) pathogenesis. Th1 cells appear to inhibit collagen deposition and increase metalloproteinase production, either by release of IFN-γ (interferon gamma) or by direct contact with fibroblasts, and thus have an antifibrotic effect [1]. On the other hand, Th2 cells stimulate the production of collagen and inhibit matrix metalloproteinases from the release of IL-4 and IL-13, presenting profibrotic and pro-angiogenic properties [2]. Interestingly, Th2 cytokine levels tend to decrease as cutaneous sclerosis regresses, and a study suggests that a shift in Th2 response to Th1 correlates with improved cutaneous fibrosis in SSc [2].

Another cell pathway that is increasingly being studied in SSc is Th17. Th17 cells produce mainly IL-17, IL-21, and IL-22 cytokines [3]. In general, IL-17 is considered a proinflammatory cytokine capable of inducing production of other cytokines such as IL-6, IL-1, IL-8, and TNF [4]. In addition, it inhibits angiogenesis of endothelial cells and promotes proliferation of fibroblasts; however, no known action on collagen has been observed. Elevated serum levels of IL-17 have been described in patients with SSc, suggesting their participation in the pathophysiology of the disease [5].

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Statins have been successfully administered in the treatment of hypercholesterolemia and cardiovascular disease due to its lipid-lowering and anti-atherosclerotic action. Although the relationship between macro and microvascular involvement in SSc has not been well elucidated, several clinical trials have demonstrated the benefit of statins in the treatment of vasculopathy, evidencing a decrease in levels of endothelin-1, ICAM-1, and CCL-2 and reduction in the risk of development of digital ulcers [6]. Statins also promoted decreased expression of transforming growth factor (TGFβ1) and connective tissue growth factor (CTGF) by renal mesangial cells, renal fibroblasts, and pulmonary fibroblasts [7, 8], suggesting a potential antifibrotic effect of HMG-CoA reductase inhibitors. However, additional studies are needed to prove this effect and to define the mechanism of action involved.

In this context, the possibility of the use of statins as immunomodulatory [9] and antifibrotic agents in the treatment of SSc is suggested. Therefore, the aims of this study were to investigate the effects of statins treatment on Th1, Th2, and Th17 cytokines production from stimulated peripheral blood mononuclear cells (PBMCs) obtained from SSc patients.

MATERIALS AND METHODS

Study Subjects

We recruited 21 patients (all female) for PBMCs culture analysis, classified according to the American College of Rheumatology criteria for SSc [10]. Patients were classified as limited cutaneous SSc (lcSSc; $n = 13$) or diffuse cutaneous SSc (dcSSc; $n = 8$). The characteristics of SSc patients are summarized in Table 1. The mean disease duration, calculated from the time of onset of the first non-Raynaud phenomenon event, was 156.9 ± 113.2 months. All subjects gave their written consent to participate. The patients were not using statins, and this was an exclusion criteria; besides, patients younger than 18 years old, or who had overlap diseases, or had a known diagnosis of cancer or chronic infection, were also excluded.

The study protocol was approved by the ethics committee of Universidade Federal de Pernambuco (CAAE: 63515916.1.0000.5208), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

Table 1. Clinical Parameters and Image's Data of SSc Patients

Characteristics	Diffuse cutaneous ($n = 8$)	Limited cutaneous ($n = 13$)
Age ± SD (yrs)	47.0 ± 13.4	47.4 ± 13.1
Gender female %	100	100
Raynaud phenomenon duration (months) ± SD	157 ± 117.8	144.6 ± 115.4
Diseases duration (months) ± SD	104.3 ± 97.3	114.5 ± 98.9
Initial symptoms %		
Raynaud phenomenon	12.5	76.9
No-Raynaud phenomenon	87.5	23.1
Clinical manifestation %		
Leucomelanodermia	62.5	69.2
Telangiectasia	75	69.2
Microstomia	75	53.8
Objective Raynaud phenomenon	87.5	84.6
Digital pittings	37.5	53.8
Digital ulcer	–	15.4
Dysphagia	75	76.0
Dyspepsia	87.5	84.6
Esophagitis	25	23
Rodnan score (median)	13.9	14.2
Pulmonary arterial hypertension	25	7.7
Lung fibrosis on thorax CT %	62.5	38.5
sHAQ (median)	1.0	1.005

SD standard deviation, yrs years; *CT* computed tomography; *sHAQ* scleroderma Health Assessment Questionnaire

PBMC Purification and Culture

We followed the methods of Dantas et al. [11]. PBMCs were obtained from the heparinized blood of patients and controls. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. PBMCs (1 × 106 cells/ml) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA), and penicillin (10.000 U/ml)/streptomycin (10.000 μg/ml) (Gibco, Carlsbad, CA, USA).

Cells were stimulated with anti-CD3/CD28 (Ebioscience, San Diego, CA, USA) and were treated with simvastatin at 1, 10, and 50 μM doses (Sigma) and atorvastatin at 1, 10, and 50 μM doses (Sigma). The metilprednisolone (Pfizer) at 10 and 50 μM was used as the standard drug. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator. Culture supernatant was collected after 48 h for cytokines quantification.

Evaluation of Cytotoxicity

The simvastatin and atorvastatin cytotoxicity were evaluated in healthy volunteers to verify if there is any toxic concentration *in vitro* in healthy cells and to identify the concentrations that will be used in the cells isolated from SScs. Different concentrations for simvastatin were tested (1, 10, 50 μM) and were founded that at all concentrations, the average viability was > 95% (data not shown) [12].

Cell cytotoxicity for atorvastatin was evaluated using the 3-(4,5-dimeth-ylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT, Molecular Probes, Invitrogen) assay following the manufacturer's instructions. Briefly, PBMCs from healthy volunteers (1.9 × 104/well) were seeded in 96-well plates and were treated with different concentrations of the Atorvastatin (1, 10, 50, and 100 μM) over a 72 h time period. At the end of incubation, the absorbance was read at 570 nm.

Cytokines Quantification

Cytokine (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10, IL-17A, and IL-17F) levels were quantified in PBMC culture supernatants. Concentrations of cytokines in culture supernatants were determined by cytometric bead array (CBA), according to the manufacturer's protocol (CBA, BD Biosciences) for TNF, IL-2, IL-4, IL-10, and IL-17A. Briefly, 50 μl serum samples were subjected to analysis in duplicate using the cytometric bead array kit on Accuri C6 Flow Cytometer (BD, Biosciences). The concentration of cytokines was quantified using FCAP Array software v3.0.1. The detection limits were TNF 3.8 pg/ml, IL-2 2.6 pg/ml, IL-4 4.9 pg/ml, IL-10 4.5 pg/ml, and IL-17A 18.9 pg/ml.

For IL-17F, IL-6 and IFN-γ cytokine levels were used an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). The lower detection limits for the ELISA analyses were, IFN-γ 4.68, IL-17F 31.25, and IL-6 4.68.

Statistical Analysis

Statistical analyses of the data were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) statistical program. The D'Agostino test verified the normality of samples and they were not in Gaussian distribution. Therefore, the Wilcoxon's signed rank test was used to compare differences in the cytokine production of PBMCs. A probability value of $p < 0.05$ was considered significant.

RESULTS

Effects of Statins Treatment on Cytokines Production by PBMC from SSc Patients After Stimulation with Anti-CD3/CD28

We analyzed the effect of the *in vitro* treatment of statins (simvastatin and atorvastatin) on secretion of cytokines in PBMCs from SSc patients, and with metilprednisolone (that was used as the standard drug). When compared to the stimulated condition, simvastatin and atorvastatin 50 μM promoted a reduction in all cytokine levels with statistical significance, except for IL-6, which had its reduction only induced by the use of simvastatin (showed in Fig. 1).

Methylprednisolone was used as a control, and also induced reduction of cytokine secretion with statistical significance. We also observed that simvastatin dose of 50 μM had a better effect on the reduction of all cytokines secretion when compared with metilprednisolone at 50 μM, with statistical significance (data not shown).

DISCUSSION

We demonstrate that simvastatin has immunosuppressive effect dose-dependent on all cytokines, secreted by PBMCs of SSc, tested in this study (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, IL17F, and IL-17A). Atorvastatin also

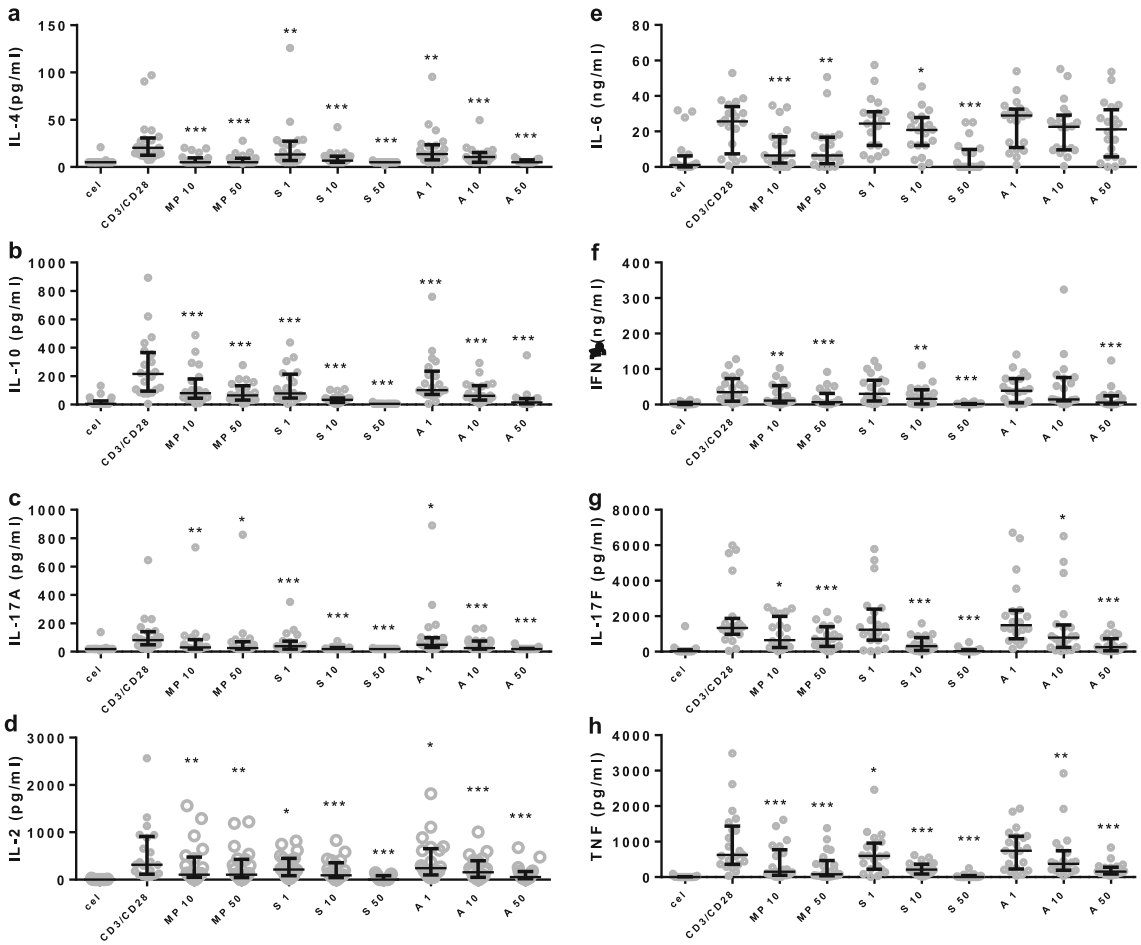

Fig. 1. Evaluation of the response to treatment of statins in PBMC cultures of patients with SSc. (**a**) Simvastatin significantly reduced TNF levels at concentrations of 1 μM, 10 μM, and 50 μM; and atorvastatin significantly reduced levels at concentrations of 10 μM and 50 μM (**b**) simvastatin and atorvastatin significantly reduced IL-4 levels at concentrations of 1 μM, 10 μM, and 50 μM; (**c**) simvastatin and atorvastatin significantly reduced IL-10 levels at concentrations of 1 μM, 10 μM, and 50 μM; (**d**) simvastatin and atorvastatin significantly reduced IL-17A levels at concentrations of 1 μM, 10 μM, and 50 μM; (**e**) simvastatin significantly reduced IL-6 levels at concentrations of 10 μM and 50 μM; and atorvastatin was not able to reduce IL-6 levels; (**f**) simvastatin significantly reduced IL-17F levels at concentrations of 10 μM and 50 μM; and atorvastatin was able to promote reduction of 50 μM; (**g**) simvastatin significantly reduced IFN-γ levels at concentrations of 10 μM and 50 μM; and atorvastatin was able to promote reduction of 50 μM; (**h**) simvastatin and atorvastatin significantly reduced IL-2 levels at concentrations of 1 μM, 10 μM, and 50 μM. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$. Cel cells, CD3/CD28 monoclonal antibodies CD3 and CD28, S simvastatin, A atorvastatin, MP methylprednisolone, ng nanogram, pg picogram. The Wilcoxon's signed rank test was used to compare differences in the cytokine production of PBMCs.

modulated almost all cytokines with dose-dependency, except for IL-6, where no modulation was observed. To our knowledge, no study has evaluated the effect of statins on the cytokines modulation in secretions of SSc's PBMCs *in vitro*.

Studies made to evaluate direct effects of statins on cytokine production in with human peripheral blood mononuclear cells (PBMCs) of healthy people *in vitro* showed decrease of IL-2, IFN-γ, as our study, in a dose-dependent manner, but not effect in IL-6, which was different from our result [13]. Cherfan et al. evaluate the effect of simvastatin on human healthy T cells (only with mild hypercholesterolemia) *in vivo* and were not able to demonstrate any significant influence on the Th1 and Th2 response by simvastatin [14], and in this study, only IL-4 and IFN-γ were analyzed. Although statins appear to have an immunomodulatory effect on healthy cells, some studies evidence different responses between healthy and diseased cells.

Although there are no studies on SSc PBMCs, there are other studies done on immune-mediated diseases, especially in rheumatoid arthritis (RA). In patients with RA, simvastatin

reduced secretion levels of IL-17A, IL-6, IL-22, and IFN-γ by PBMCs in a dose-dependent response [12]. Blaschke et al. observed an effect of atorvastatin in the decreased of IFN-γ levels in secretions by PBMCs from RA patients, but not in IL-6 (as ours results) and IL-4 and IL-10 secretions [15], different from what we observed in our study.

Our results showed a better effect of simvastatin on reducing cytokine secretion when compared to methylprednisolone. However, it is not in agreement with the results of Dantas et al. [11] that only had an effect on the reduction of chemokines secretion, but not on cytokines. Perhaps, this difference is related to the dosage used of methylprednisolone, since the Dantas et al. study used twice the dosage than our study.

We recognize that our study has some important limitations. Our sample size is limited and our findings warrant further study in larger validation cohort. Besides, most of the patients were on specific treatment, what can interfere with cytokine levels studied, but we tried to minimize this bias through statistical analysis adjusted by age and medication used.

We showed that statins, particularly simvastatin, reduced all cytokine secretion levels from PBMCs in a dose-dependent manner. Our findings suggest that the simvastatin therapy does able to modulate Th1, Th2, and Th17 pathways. Further studies will be necessary to elucidate the molecular mechanisms.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

The study protocol was approved by the ethics committee of Universidade Federal de Pernambuco (CAAE: 63515916.1.0000.5208), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

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