Vitamin D increases the production of IL-10 by regulatory T cells in patients with systemic sclerosis

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ABSTRACT

Objective. *Vitamin D status influences the risk to develop autoimmune diseases affecting the percentage and/or functions of regulatory T cells (Tregs). Since low levels of 25 (OH) D have been decreased in patients with systemic sclerosis (SSc), we aimed to study the effect of Vitamin D3 (cholecalciferol) supplementation on Tregs frequencies and functions.*

Methods. *Peripheral blood and sera samples were obtained from 45 SSc patients and controls (HC). A number of eighteen SSc patients had consumed Cholecalciferol (orally) at the dose of 25.000 UI/month for 6 months at the time of enrollment. 25(OH)D serum levels were measured and VDR polymorphisms, were genotyped by polymerase chain reaction (PCR). Tregs isolated from peripheral blood mononuclear cells were in vitro expanded and a suppression assay was performed. Flow cytometry analysis was then carried out. Finally, IL-10 production was assayed by ELISA.*

Results. *Low serum levels of 25(OH)D were detected in SSc patients. The percentage of Tregs in SSc patients was similar to controls, but, among SSc patients, it was higher in those patients taking cholecalciferol. Tregs capability to suppress T cell proliferation was impaired in SSc patients and not restored after* in vitro *pre-treatment with the active form of Vitamin D (1,25(OH)2D3); but at the same time the production of IL-10 was increased in treated samples obtained from patients. The lack of response of Tregs from SSc patients to 1,25(OH)2D3 treatment in vitro was not due to altered Vitamin D/VDR signalling.*

Conclusion. *Altogether, our results indicate that the increased production of IL-10 by 1,25(OH)2D3 -treated Tregs could provide a "suppressive" cytokine milieu able to modulate immune response but it is not sufficient to restore the immune suppressive functions of Tregs.*

Introduction

Systemic sclerosis (SSc) is an autoimmune disorder characterised by tissue fibrosis of several organs, including the skin. SSc can be classified in two main subtypes, the limited cutaneous form (lcSSc) and the diffuse cutaneous form (dcSSc), according to the skin fibrosis extension (1).

Skin fibrosis, together with reduced sun exposure may be considered a possible cause of hypovitaminosis D, which is commonly observed in SSc patients and in patients with several autoimmune conditions, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and undifferentiated connective tissue diseases (UCTD) (2, 3).

However, the role of vitamin D status in the pathogenesis of SSC is not fully investigated (4-6).

The active form of Vitamin D (1,25(OH)2D3) activates the vitamin D receptor (VDR), a member of the superfamily of nuclear receptors, recognised as a key regulator of calcium homeostasis cell proliferation, differentiation and immunomodulation (7, 8). Upon binding to VDR, the 1,25(OH)2D3 stimulates regulatory T cells, which is a central player in the maintenance of peripheral self-tolerance (9).

In vitro treatment of T cells from healthy individuals with TX527, a low-calcemic analogue of bioactive 1,25(OH)2D3, can promote a $CD4^{+}CD25^{high}CD127^{low}$ regulatory (Treg) profile and imprint a migratory signature specific for homing to sites of inflammation (10, 11). Intriguingly, the 1,25(OH)2D3-induced Tregs exhibited the capacity to suppress proliferation of autologous responder T cells.

In SSc, conflicting data are reported on the frequency and functional activity of Tregs, but there is a general agreement in favour of a decreased functional capacity of circulating Tregs (12, 13).
The aim of our study was to evaluate the effect of a 1,25(OH)2D3 supplementation on Tregs frequency (%Tregs) and functions in SSc patients. We have also analysed a possible association between VDR polymorphisms and SSc (14).

Material and methods

Patients

The patients enrolled in this study, had been diagnosed SSc according to the 2013 classification criteria for systemic sclerosis (15) (inclusion criteria). Peripheral blood and sera samples were obtained following informed consent from 45 patients (F:M 41:4) with SSc (32 patients with limited and 13 with diffuse SSc), and 35 healthy controls (HC). Eighteen SSc patients had consumed Cholecalciferol orally, at the dose of 25.000 UI/month for 6 months at the time of enrollment. Patients with other autoimmune disorders were excluded from this study (exclusion criteria).
Modified Rodnan skin score was calculated in all patients (mean±SD: diffuse SSc 31±5, limited SSc 12±7). Pulmonary involvement was defined as a forced vital capacity <70% of predicted, as measured by a dry spirometer, or by the evidence of amorphous or reticulonodular infiltrates in high resolution computed tomography scans (HRCT) (7 out of 45 patients showed pulmonary involvement). Pulmonary arterial hypertension (PAH) was evaluated indirectly by Doppler echocardiography, according to Denton *et al*. and then confirmed by Right heart catheterisation (2 out of 45 patients showed PAH) (16). Autoantibodies profiles were also evaluated and all the patients were ANA$^+$ (27 SSc patients were also positive for Scl70 and 2 for CENPB) (ANA in indirect immunofluorescence and ENA in immunoblot assay). Patients' characteristics are shown in Table I. This study was approved by the ethical committee of the University Hospital Policlinico Paolo Giaccone in Palermo, Italy; and informed consent was obtained from each patient and controls.

Table I. Main demographic, clinical and laboratory features of patients enrolled at baseline.

Patients' features	Whole series n=45
Sex, female, n (%)	41 (91.1)
Age, years mean (SD)	58±14
Disease duration from first non-Raynaud sign/symptom, years median (range)	12 (2–40)
lcSSc, n (%)	32 (71.1)
Pulmonary involvement(%)	7 (15.5)
Pulmonary arterial hypertension (%)	2 (4.4)
ANA	43 (95.5)
Anti-Scl70	18 (40)

SD*:* standard deviation; lcSSc: limited cutaneous disease.

25(OH)D measurements

25(OH)D serum levels were measured on frozen samples (-70°C) with the Architect i1000SR (Abbott Laboratories, Wiesbaden, Germany), according to the manufacturer's instructions.

Genetic analysis

Genomic DNA was extracted from 200 µl of whole peripheral blood using a commercial Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The DNA quality was evaluated by electrophoresis in a 0.8% agarose gel, quantified by using absorbance spectrophotometric analysis and stored at -20°C for subsequent analysis.
The VDR polymorphisms, Fok-I, Bsm-I, Taq-I and Apa-I, were genotyped by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis. Primers for VDR gene polymorphisms are: Fok-I forward 5′-GATGCCAGCTGGCCCTGGCACTG-3′ and reverse 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′; Bsm-I forward 5′- AGTGTGCAGGCGATTCGTAG-3′ and reverse 5′- ATAGGCAGAACCATCTCTCAG-3′; Apa-I forward 5′- CAGAGCATGGACAGGGAGCAAG-3′ and reverse 5′-GCAACTCCTCATGGCTGAGGTCTCA-3′; Taq-I forward 5′-CAGAGCATGGACAGGGAGCAAG-3′ and reverse 5′-GCAACTCCTCATGGCTGAGGTCTCA-3′. The following conditions were used for both Fok-I and Bsm-I amplification: 94°C for 5′, 35 cycles of 94°C for 1′, 60°C for 1' and 72°C for 1′, followed by 72°C for 10′. The PCR products were separately restricted in a 25 µl reaction volume for 2 h with Fok-I at 55°C and Bsm-I at 37°C, respectively.
Fok-I and Bsm-I genotyping was made as follows FF:272 bp, Ff: 272, 191 and 81 bp, ff: 191 and 81 bp; BB:191 bp, Bb: 191,115 and 76 bp, bb: 115 and 76 bp, respectively.
The amplification for Apa-I and Taq-I was performed according to the following program: 93°C for 10 min and 35 cycles of 93°C for 45 s, 66°C for 30 s, 72°C for 45 s, followed by 72°C for 10 min. PCR products were digested for 2 h with APA I at 25°C and TAQ I at 65°C, respectively. Apa-I and Taq-I digestion revealed genotypes denoted, respectively, as AA (740 bp), aa (520 and 220 bp) and Aa (740, 520 and 220 bp) and TT (245 and 495 bp), tt (290, 245 and 205 bp) and Tt (495, 290, 245 and 205 bp).
All PCR products were separated by electrophoresis on a 2% agarose gel stained with Eurosafe (Euroclone).
Approximately, 20% of the samples were randomly selected and genotyped in duplicate and 20% of the samples were confirmed by DNA sequencing. All results were concordant.

Tregs expansion, suppression assay and flow cytometry

PBMCs were isolated by lymphocyte (PAA, Austria) density gradient centrifugation. CD4$^+$CD25$^+$ T cells were purified by negative selection of CD4$^+$ T cells followed by positive selection of CD25$^+$ T cells using miniMACS CD4$^+$CD25$^+$ T Regulatory Cell Isolation Kit (Miltenyi-Biotec, UK). The purity of CD4$^+$CD25$^+$ T cells was between 90-98%. Aliquots of CD4$^+$CD25$^-$ T cells (effector T cells, Teff) were cryopreserved and used as autologous re-

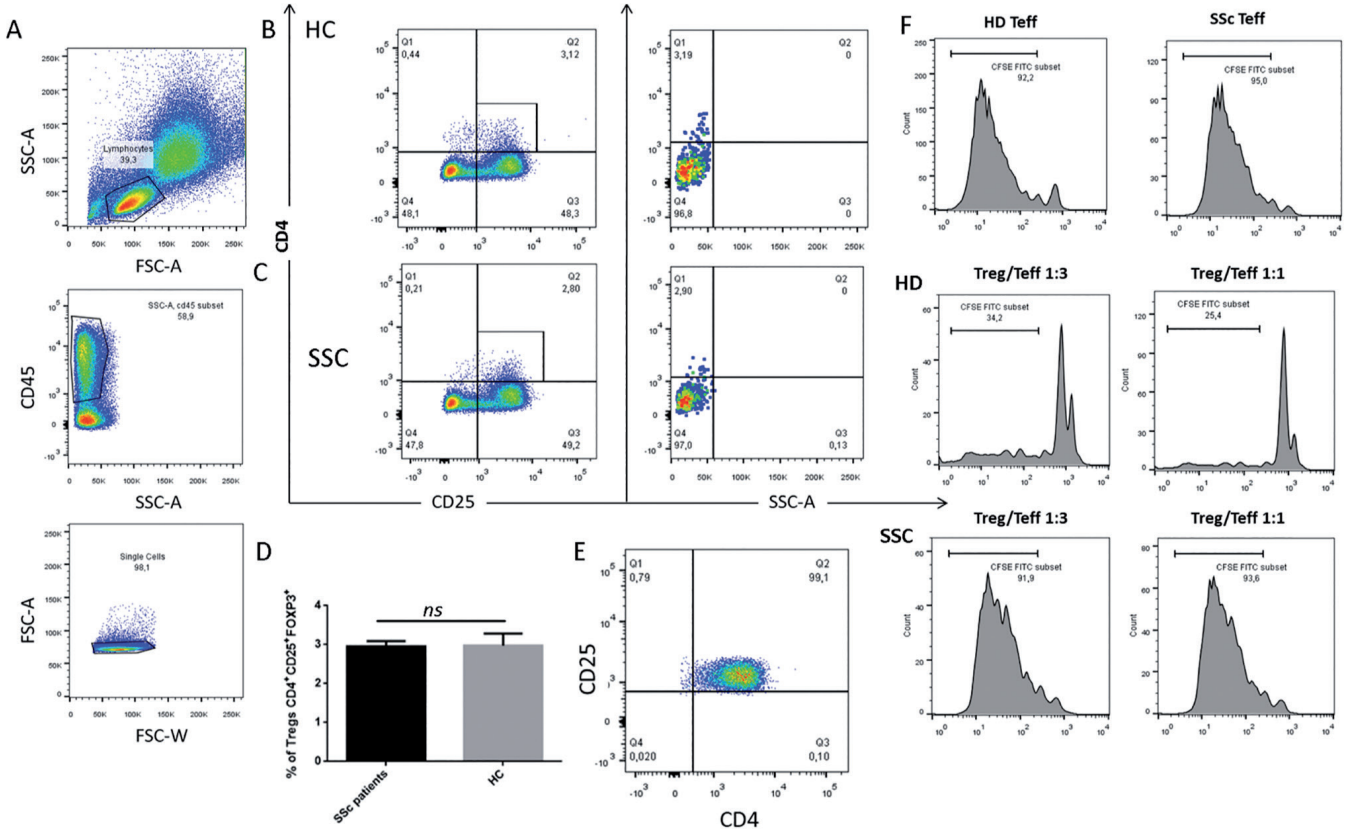

Fig. 1. Representative gating strategy of lymphocytes in SSc patients and controls.
(**A**) Dot plot panels showing mean percentages of Tregs of (**B**) SSc patients and (**C**) controls. (**D**) Histogram showing CD4+ CD25+ FOXP3+ cells percentages detected in healthy controls and SSc patients. (**E**) Dot plot representing purified $CD4^+CD25^+$ cells. (**F**) Representatives histograms of suppression assays of Tregs from SSc patients and healthy controls.

sponder cells in suppression assays after staining with carboxyfluorescein succinimidyl ester (CFSE, eBioscience, San Diego, CA, USA). Human $CD4^+CD25^+$T cells were plated at 1×10^6/mL in X-Vivo15 (Lonza), containing Rapamycin (100nM; LC-Laboratories). Cells were activated with anti-CD3/CD28 beads (ratio bead:cell of 1:2; Invitrogen, UK). IL-2 (1000 IU/mL; Proleukin-Novartis) was added at day 4 post activation and replenished every two days. Cells were re-stimulated every 10-12 days, and used after 36 days from the first activation (3 rounds of stimulation). Cells were also cultured in presence of 1,25(OH)2D3 (10^{-8}M) (Roche) for the suppression assays. As for the characterisation of Tregs, the cells were stained with the following antibodies: anti-human CD3 FITC conjugated, clone HIT3a, (eBioscience Affymetrix INC San Diego, CA, USA), anti-human CD25 PE conjugated (Miltenyi, Biotec), anti-human CD4 PE-Cy7 conjugated, clone OKT4, (BioLegend, San Diego, CA, USA), anti-human FoxP3 APC, clone 259D (BioLegend) and an anti-human CD45 APC/Cy7 conjugated, clone HI30, (BioLegend). For the exclusion of dead cells, a 7-AAD cell viability staining solution was used.

Flow cytometry analysis was performed by using a fluorescence activated cell sorter (FACS)Canto (BD Biosciences, San Jose, CA, USA). At least 50.000 cells (events), gated on the lymphocyte region, were acquired for each sample and data are presented as a percentage of total alive cells in the CD45 channel.

Enzyme-linked immunosorbent assay (ELISA) for IL-10

IL-10 production from cell culture supernatant was assayed by commercial ELISA (Abcam, Cambridge, UK).

Statistical analysis

Parametric and non-parametric statistical analysis was performed by calculating the mean ± standard error of the mean (s.e.m.) and median, respectively. For comparison of parametric and non-parametric data, the t-test and Mann-Whitney rank-sum test were used where appropriate. Spearman's correlation analysis was utilised to quantify the expression associations between the genes of interest. *p*-values less than 0.05 were considered to be significant. The statistical analysis was performed by using GraphPAd software.

Results

Impaired suppressive capability of Tregs in SSc

The mean percentage of $CD4^+CD25^+$ $FOXP3^+$ Treg cells in SSc patients (2.9±0.2) was not different from the control group (3.1±0.52) and data from two groups were not statistically significant (Fig. 1 A-D). No differences were found in patients with PAH and lung involvement (data not shown). In order to evaluate the ability of Tregs from patients and controls to suppress effector cells, purified $CD4^+CD25^+$ T cells (Fig.

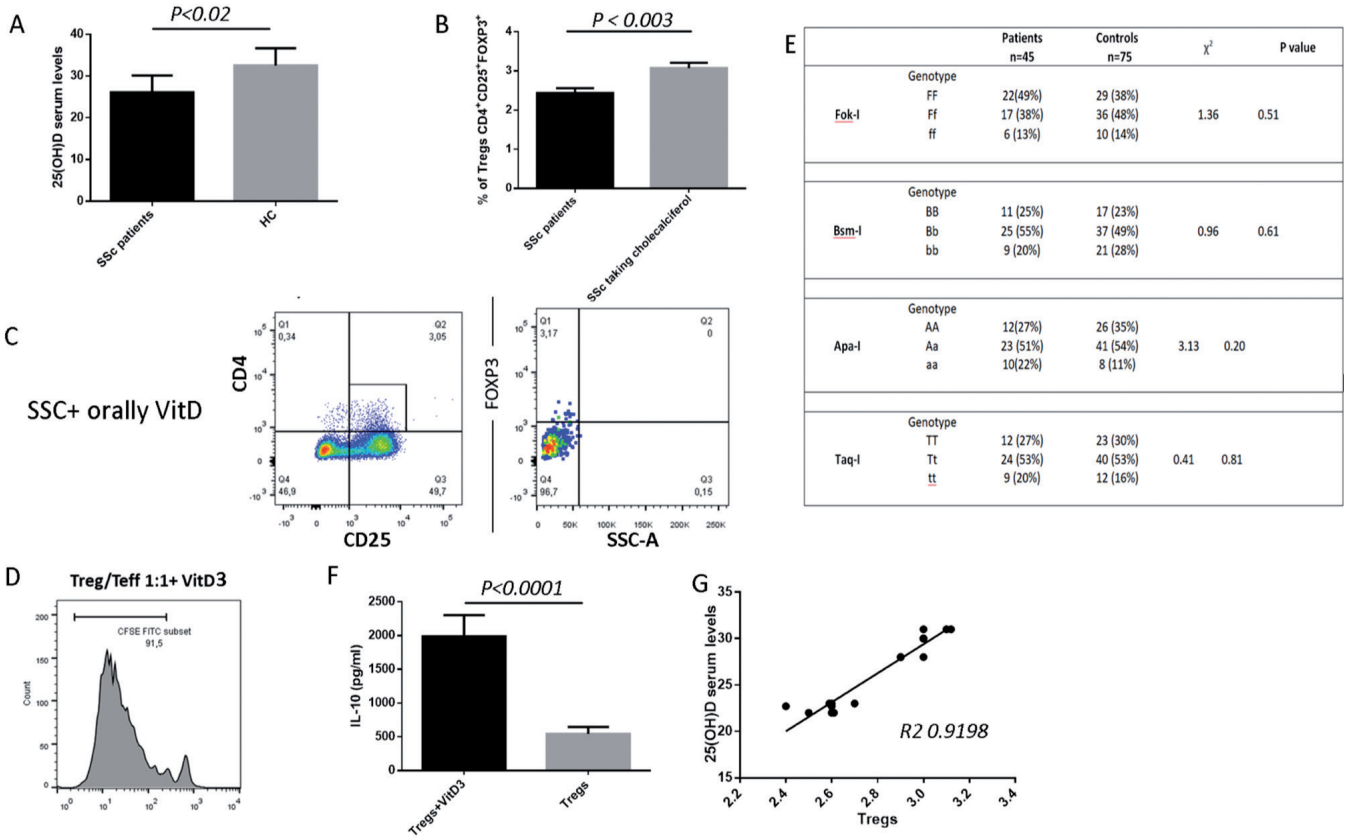

Fig. 2. (**A**) 25(OH)D serum levels in SSc patients and controls. (**B**) Histograms representing percentages of $CD4^+CD25^+FOXP3^+$ cells obtained from SSc assuming cholecalciferol. (**C**) Histogram showing suppression activity of *in vitro* VitD3 Tregs from SSc patients. (**D**) Histogram showing suppression activity displayed by Vitamin D3 *in vitro*-treated Tregs of SSc patients. (**E**) Distribution of genotype frequencies of the VDR gene polymorphisms in SSc and control group. (**F**) IL-10 levels (pg/ml) in the supernatant of Vitamin D3 *in vitro*-treated Tregs.

1E), obtained after 3 rounds of stimulation, were activated with anti-CD3/CD28 beads to perform the suppression assay. Tregs from HC nicely suppressed proliferation of autologous CD4 T cells, but Tregs from SSc patients failed to suppress CD4 T cell proliferation (Fig. 1F). This indicates that Tregs activity is functionally impaired in SSc patients.

Tregs from SSc patients assuming vitamin D are increased in percentages, fail to suppress T cell proliferation but secrete more IL-10

Taking into account the important role of vitamin D in the modulation of Treg functions and the reduced 25(OH) D serum level found in SSc patients (median 22.1±9.7 μg/l, n=45) (Fig. 2A), we evaluated whether treatment with 1,25(OH)2D3 *in vitro* was able to increase the Tregs percentages. As shown in Figure 2B-C, the percentages of Tregs in patients treated with Vitamin D were significantly increased when compared to percentages of Tregs of SSc patients. The suppression assay was performed with Tregs that had been cultured for five days with 1,25(OH)2D3, or with untreated Tregs from SSc patients. As shown in Figure 2D, 1,25(OH)2D3-pretreated Tregs still failed to suppress effector cell proliferation. Furthermore, Tregs from SSc patients who were taking cholecalciferol orally, even if expanded (Fig. 2B) were not able to suppress effector cells proliferation (Treg/Teff 1:3 92.1%, mean percentage of proliferating effector cells). On the contrary, healthy subjects maintained the suppressive ability also when pretreated with 1,25(OH)2D3 (Treg/Teff 1:3 32.3%, mean percentage of proliferating effector cells).

The lack of response of Tregs from SSc patients after *in vitro* treatment was not due to altered Vitamin D/VDR signalling. The analysis of VDR polymorphisms did not reveal significant differences in genotypic and allelic frequencies among the analysed SSc patients and controls (Fig. 2E).

However, despite the treatment with 1,25(OH)2D3 failed to restore the suppressive capability of Tregs, it determined a statistically significant increase of IL-10 production in the supernatant of treated Tregs, when compared to IL-10 secreted by untreated Tregs (Fig. 2F), thus suggesting a possible immunomodulatory function of 1,25(OH)2D3. Moreover, 1,25(OH)2D3 directly correlate with Tregs percentage (Fig. 2G).

Discussion

The reduced numbers, percentages and/or functions of Tregs (mostly $CD4^+CD25^+FOXP3^+$) have been reported in many autoimmune diseases (17). In SSc a big debate still exists on the role of Tregs in the pathogenesis of the disease and the data reported are controversial. Radstalk and Slobodin (18, 19) found increased numbers of Tregs with defective suppressive activities in the peripheral blood of SSc patients. On the contrary, Fenoglio *et*

al. (20) described decreased frequencies of Tregs in SSc patients. In line with other studies, (21) in which the percentages of Tregs did not differ between patients and control subjects, our study shows that Tregs percentages were similar in SSc patients and in controls, but interestingly, the suppressive function of Tregs from SSc patients was abrogated.

It is possible to speculate that peripheral blood Tregs are not able to overcome the inflammatory response challenged by Th1/Th17 cells and/or their cytokines, as demonstrated in many systemic autoimmune diseases. Resistance of T effector cells to Tregs has been described in experimental models of SLE (22), multiple sclerosis (MS) and diabetes (23-27), and might be due to an overwhelming inflammatory response. For this reason, in our model, the use of cholecalciferol could be considered a therapeutic strategy to modify the inflammatory state, given its ability to increase IL-10 production. Vitamin D status modulates immune response by different mechanisms (28), amongst which is the activation of a subset of Tregs expressing IL10 or FOXP3 (29). We speculate that, in our model, 1,25(OH)2D3 could enhance IL10 production and may in turn suppress FOXP3 expression inhibiting their suppressive functions. This mechanism could explain the reason why 1,25(OH)2D3-treated Tregs in SSc patients do not suppress polyclonal proliferation of effector T lymphocytes.

According to our data, different authors (30,31) have described low levels of 25(OH)D in SSc patients and several trials have been conducted using cholecalciferol as a therapeutic agent in different diseases such as MS, RA, SLE, Crohn's disease and type I diabetes. The clinical benefit of cholecalciferol supplementation in autoimmunity was explained by different mechanisms, including upregulation of suppressive functions. Even if we detected a failure of suppression of T effector cells by Tregs *in vitro* exposed to 1,25(OH)2D3 in SSc patients, we found a significantly increased production of IL-10.

Finally, different results from the literature assigned an unclear role to variants of VDR in the pathogenesis of autoimmune diseases (28). In our study, however, the inability of 1,25(OH)2D3 to restore the suppressive potential of Tregs was not correlated to a specific polymorphic variant of VDR because there was no difference in the genomic polymorphic variants expressed by SSc patients and healthy controls.

Conclusions

Altogether, our data suggest that 1,25(OH)2D3 can induce an increase of IL-10 secretion by Tregs from SSc patients, but their inability to suppress T cell proliferation doesn't change with this treatment. In order to better define the pathogenesis of diseases, a deeper analysis is needed of $IL10^+$ Tregs at the site of the disease, the correlation between $IL\text{-}10^+$ cells and $FOXP3^+$ cells in the damaged tissues and a possible resistance to Tregs of T effector cells from SSc patients.

Competing interests

P. Cipriani has received honoraria and research support from Actelion, not related to this paper; P. Ruscitti has received honoraria and/or research support from Sobi, BMS, Ely Lilly, MSD, Pfizer, not related to this paper; R. Giacomelli has received honoraria and/or research support from AbbVie, Celgene, Actelion, BMS, MSD, Roche, Ely Lilly, Novartis, Sandoz, Sobi, not related to this paper. The other co-authors have declared no competing interests.

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