Anti-fibrotic characteristics of Vγ9+ γδ T cells in systemic sclerosis

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ABSTRACT

Objective. *γδ T cells of the Vγ9Vδ2 subtype secrete anti-fibrotic cytokines upon isopentenyl pyrophosphate (IPP) stimulation. In this study, we sought to compare IPP and Zoledronate, an up-regulator of IPP, effects on proliferation and cytokine secretion of Vγ9+ T cells from systemic sclerosis (SSc) patients and healthy controls (HCs). We also examined the effect of IPP-triggered peripheral blood mononuclear cells (PBMC) on fibroblast procollagen secretion.*

Methods. *PBMC from SSc patients and HCs were stimulated by increasing concentrations of Zoledronate, with or without IPP, and Vγ9+ T cell percentages were calculated using FACScan analysis. Subsequently, PBMC were cultured with IPP or toxic shock syndrome toxin-1 (TSST-1), and contents of the anti-fibrotic cytokines tumour necrosis factor (TNF)-α and interferon (IFN)-γ were measured by ELISA kits. Finally, supernatants of IPP-triggered Vγ9+ T cells from SSc patients were added to fibroblast cultures, and relative intensities of procollagen α1 chains were determined by densinometry.*

Results. *Higher concentrations of Zoledronate were required for maximal proliferation of Vγ9+ T cells in 9 SSc patients compared to 9 HCs, irrespective of exogenous IPP. When compared to stimulation by TSST-1, a non-Vγ9+ selective reagent, secretion of the anti-fibrotic cytokines TNF-α and IFN-γ in response to IPP was relatively diminished in SSc but not in HCs. Reduction of procollagen secretion by fibroblasts cultured with supernatants of IPP-stimulated PBMC was observed only in some SSc patients.*

Conclusion. *Activated Vγ9+ T cells could act as anti- fibrotic mediators in SSc, although decreased responsiveness to IPP may play a role in the pathological fibrosis of this disease.*

Introduction

The pathogenesis of systemic sclerosis (SSc), an autoimmune fibroproliferative disorder, is complex and characterised by overproduction of pro-fibrotic cytokines by T helper type 2 lymphocytes (Th2) and underproduction of pro-inflammatory cytokines by Th1 cells (1, 2). The pro-fibrotic cytokines (such as interleukin (IL)-4, IL-13 and transforming growth factor (TGF)-β) activate fibroblast secretion of the extracellular matrix (ECM), while the pro-inflammatory cytokines (such as tumour necrosis factor (TNF)-α and interferon (IFN)-γ) suppress fibroblast proliferation and activation (1).

γδ T cells are a subset of CD4 and CD8 negative thymus derived lymphocytes (3). Their T cell receptor (TCR), first described in 1986, is composed of γ and δ polypeptides, and recognises antigens directly, independent of the major histocompatibility complex (MHC) molecules (4-6). The most common human variant of γδ T cells in peripheral blood, expressing the Vγ9 and Vδ2 genes in the TCR (Vγ9Vδ2 T cells also termed herein Vγ9+ T cells), is activated by phosphorylated low-molecular weight molecules (phosphoantigens), which are intermediates in the ubiquitous mevalonate pathway (7, 8). Phosphoantigens, such as IPP (isopentenyl pyrophosphate), presented by antigen-presenting cells (APC), lead to IL-2 dependent Vγ9+ T cell proliferation and secretion, primarily, of IFN-γ and TNF-α (9).

Our previous study showed that Vγ9+ T cells may be preserved in SSc patients, and when exposed in vitro to IPP and IL-2, proliferate and become activated (measured by TNF-α secretion and CD25 expression) (10).

Aminobisphosphonates (ABP), such as zoledronate (Zol) and Pamidronate, inhibit farnesyl diphosphate (FPP) synthase, an interim enzyme in the meva-

lonate pathway, resulting in intracellular accumulation of the intermediate IPP (7, 8, 11). Both intracellular and extracellular IPP appear to bind the butyrophilin-3A1 (BTN3A1) protein expressed on APC, inducing conformational changes that are sensed by the Vγ9+ TCR, and leading to activation of the Vγ9+ T cell (12, 13). Statins, on the other hand, inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the initiating enzyme of the mevalonate pathway, thus preventing intracellular accumulation of IPP (7). In a previous study, a single 600 mg dose of intravenous Pamidronate induced prolonged production of IFN-γ and TNF-α by unstimulated peripheral blood mononuclear cells (PBMC) of 18 SSc patients, suggesting an important functional role of ABP-induced IPP accumulation in SSc Vγ9+ T cells *in vivo* (14). Together, these data suggest that Vγ9+ T cells may play an important immunomodulatory role in SSc.

The objectives of this study were to further evaluate the *in vitro* proliferation and cytokine-production capacities of Vγ9+ T cells from SSc patients and healthy individuals in response to IPP and to Zoledronate, a potent ABP. We also asked whether these responses may impact fibrosis - a major manifestation of this disease.

Materials and methods

Patients

The study was approved by the Institutional Review Board (Helsinki Committee) of the Chaim Sheba Medical Center, Ramat Gan, Israel. All 9 female patients fulfilled the criteria of the American College of Rheumatology for SSc. Healthy controls (HCs) were recruited from the hospital's medical staff.

Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were drawn from peripheral veins into a heparin washed syringe. PBMC were isolated by Ficoll Hypaque (Sigma, St. Louis, MO, USA) density centrifugation as previously described, washed x 3 with RPMI 1640 medium (Gibco, Invitrogen) and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, UK), 2mM glutamine and 100 μg/ml penicillin-strepromycin solution (Ta'assiot Biologiot, Beit Haemek, Israel) (Final medium, FM) (10, 15).

In vitro *stimulation*

$0.2x10^6$ PBMC were cultured in 96 round bottom well tissue culture plates (Costar) in 200μl FM and 100 IU/ml recombinant human interleukin-2 (rhIL-2) (Boeheringer - Mannheim). Three different interventions were performed simultaneously: first, Zol (Novartis) at 7, 3.5, 1.75, 0.7 or 0.07 μM was added; Then, Zol at these 5 concentrations was added in combination with 7mM of IPP; Last, Zol at these 5 concentrations was added in combination with 25 μM of Mevastatin. Medium was replaced on days 3 and 7, and cells were collected on day 10 of culture.

Characterisation of Vγ9+ T cells by flow cytometry

Staining was performed as previously described (15). Fluoresceine iso-thiocyanate (FITC) conjugated monoclonal antibody for Vγ9 and phycoerythrin (PE) conjugated monoclonal antibody for CD3 (BD Biosciences Pharmigen) were added to a resuspended pellet of 10^5 PBMC (after 10 days in culture, as described above) for 20 minutes in the dark. Cells were then washed in phosphate buffered saline (PBS) pH 7.4 (Gibco, Invitrogen), and viable lymphocytes were gated on a FACScan flow cytometer according to typical forward and side scattergrams (Becton Dickinson, Mountain View, CA, USA). Analysis of gated cells was performed, and percentages of Vγ9+/CD3+ cells out of total CD3+ lymphocytes were calculated.

Cytokine production

PBMC were cultured in FM alone, or FM with 2μg/ml IPP, or FM with 1 ng/ml of toxic shock syndrome toxin -1 (TSST-1), all with added 100 IU/ml rhIL-2 (10). Day 3 supernatants from the in vitro-activated lymphocytes were analysed by commercial ELISA kits (Bender MedSystems) for their content of IFN-γ and TNF-α, as previously described (15). Briefly, high binding (Costar) 96 microtiter plates were coated with anti-IFN-γ or TNF-α capture antibody and incubated overnight at 2–8°C. Plates were washed ×3 with washing buffer and blocked (1% BSA in PBS) for 2h at room temperature (RT). Standard curve reagents and tested samples were added, followed by biotin-conjugated detection Ab, and incubated at RT for 2h. Streptavidin–horse radish peroxidase (HRP) was added to plates for 1h at RT after washing (×3). Colour reaction was obtained by the addition of substrate solution for 30 min at RT and terminated by adding 4 N sulfuric acid. Colour absorbance was read at 450 nm in a spectrophotometer.

Procollagen production

PBMC were cultured in FM with IPP (2ug/ml) and rhIL-2 (100 IU/ml) for 10 days to expand Vγ9+ T cells. A sample of cells was removed for FACS analysis, and PBMC were then washed and replaced in the microtiter wells in FM alone or with IPP (2 ug/ml). After 24 hours supernatants (conditioned medium) were collected. Human skin fibroblasts (CRL1121, American Type Culture Collection) were seeded in 96 wells plates (7,500 cells/well) and grown to confluence in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Biological Industries, Bet Ha'emek, Israel) supplemented with 10% FBS, 2 mM glutamine and antibiotics. At confluence, the cells were placed on fresh medium containing sodium ascorbate (75 ug/ml) for 24h, at which time the medium was replaced by conditioned media from PBMC cultures, mixed with DMEM at a 2:1 ratio, and supplemented with sodium ascorbate. Cells were then incubated for additional 24h and the medium was collected, clarified by mild centrifugation, and stored at -70°C until use. Samples of these media were subjected to SDS-PAGE in 6% polyacrylamide gels, followed by immunoblotting with a rabbit antibody to the C-telopeptide of the collagen type I α1 chain (LF67; a gift from Dr. L. Fisher) (16) and ECL detection. The relative intensities of the proα1 bands were determined by

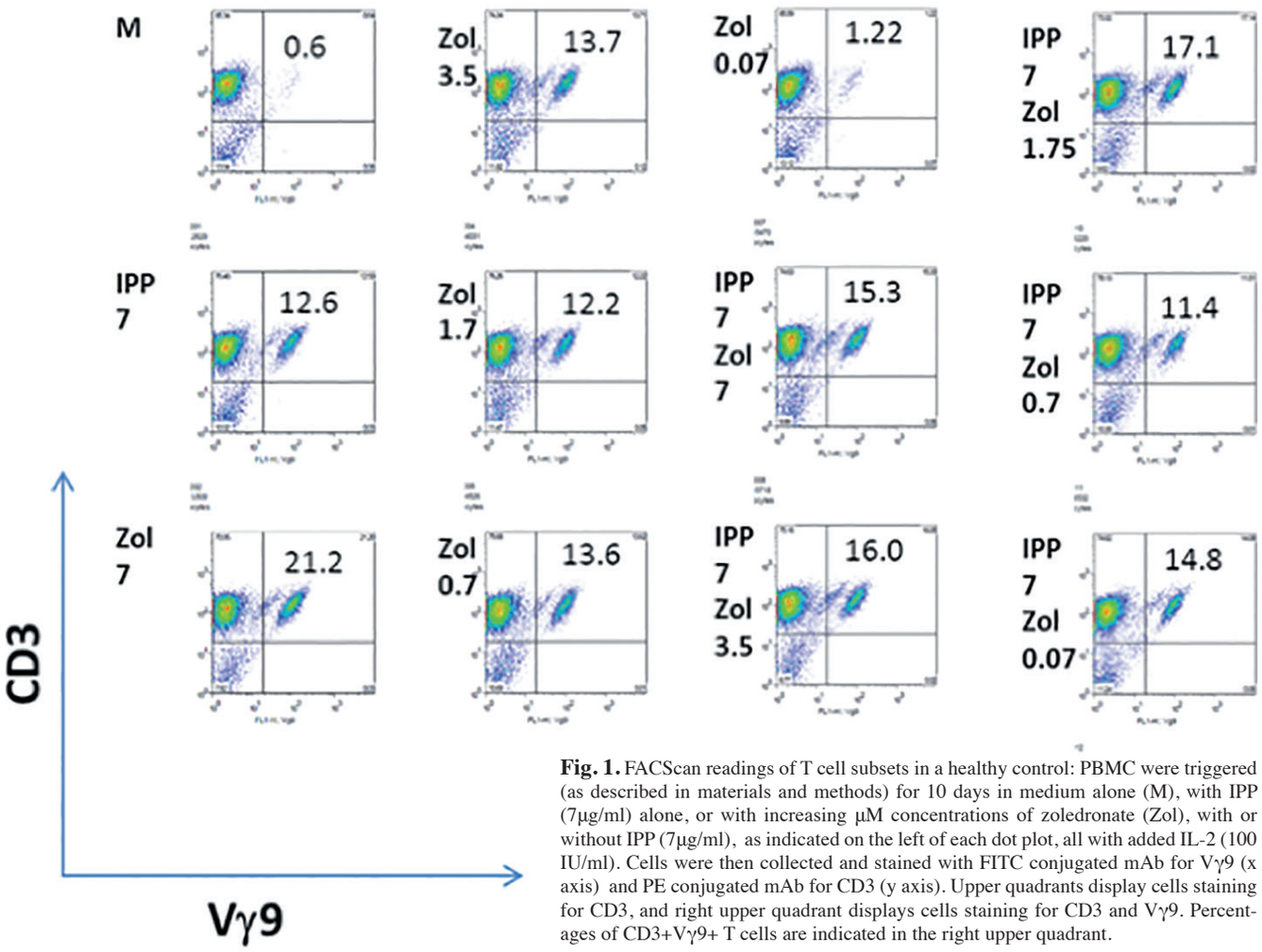

Fig. 1. FACScan readings of T cell subsets in a healthy control: PBMC were triggered (as described in materials and methods) for 10 days in medium alone (M), with IPP (7μg/ml) alone, or with increasing μM concentrations of zoledronate (Zol), with or without IPP (7μg/ml), as indicated on the left of each dot plot, all with added IL-2 (100 IU/ml). Cells were then collected and stained with FITC conjugated mAb for Vγ9 (x axis) and PE conjugated mAb for CD3 (y axis). Upper quadrants display cells staining for CD3, and right upper quadrant displays cells staining for CD3 and Vγ9. Percentages of CD3+Vγ9+ T cells are indicated in the right upper quadrant.

densitometry using the TINA (v. 2.07) software. Preliminary experiments revealed no direct effect of addition of IPP alone to fibroblast medium at the concentrations used to activate the PBMC.

Statistical analysis

The effect of Zol concentration on Vγ9+ cells in SSc patients and HCs, either with or without IPP/Mevastatin, was compared using repeated measures ANOVA. The baseline percentages of Vγ9+ cells in Zol-free medium and the effects of IPP on Vγ9+ cells in Zol-free medium, as well as the effects of IPP and TSST-1 on cytokine production were all tested using Wilcoxon signed-rank test (paired or unpaired, as indicated). Statistical analyses were performed using SPSS statistical software (v. 19.0; SPSS Inc, Chicago, Illinois). The GraphPad Prism software (v. 6.05, GraphPad Software Inc, San Diego, California) was used to fit concentration-response curves.

Results

Vγ9+ T cell proliferative response to zoledronate in systemic sclerosis patients and healthy individuals

To evaluate how Zol affects Vγ9+ T cells in SSc in comparison to healthy donors, we recorded the percentages of Vγ9+ T cells within the CD3+ population of PBMC after culture with increasing concentrations of Zol, as shown in representative Fig. 1. In all, proliferative responses of 9 SSc and 9 HCs Vγ9+ T cells were evaluated.

Although baseline percentage (prior to stimulation with Zol or with IPP) of Vγ9+ T cells was lower among SSc patients (median, 1.65; IQR 0.83-5.25) than among HCs (median, 4.50; IQR 2.58-11.64), this difference did not reach statistical significance, probably due to the rather small sample size and to the large inter-subject variability.

In repeated measures analysis, Vγ9+ T cell percentage in cultures derived from SSc patients' PBMC was significantly associated with Zol concentration ($p<0.001$, Fig. 2a). There was a trend of concentration-related proliferation, peaking at Zol concentration of 3.5μM, with a subsequent decrease in the response, although large inter-subject variability in Vγ9+ percentage was observed. When clustering data from all patients (n=9), the relative response to Zol (calculated as the subtraction of Vγ9+ percentage at baseline, without Zol, from their percentage in response to different Zol concentrations) fit into a concentration-response curve (Fig. 2c), with a maximal response (Emax) of 36.4% [95% CI, 24.6-48.2], and an EC50 (half maximal effective concentration) of 0.7 μM [95% CI, 0.3-1.7]. Large inter-subject variability in the relative response of Vγ9+ cells to

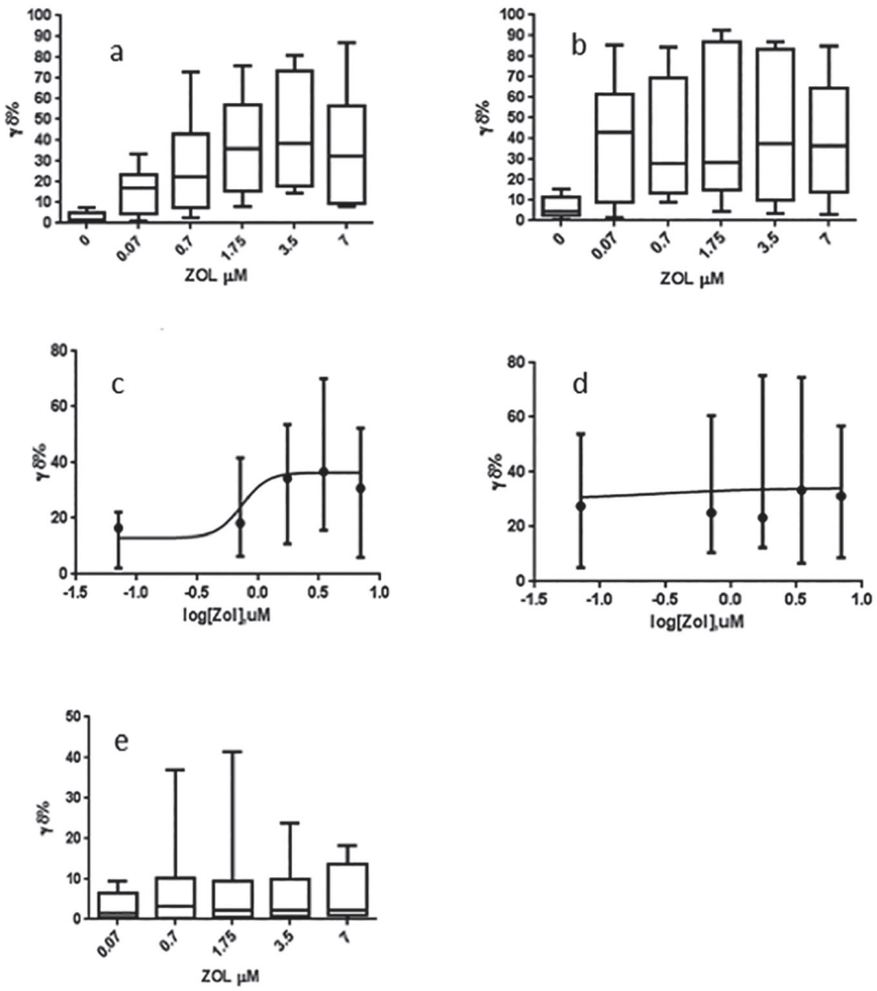

Fig. 2. Differential response of SSc Vγ9+ T cells to zoledronate: PBMC were triggered as described in materials and methods, in the presence of increasing concentrations of Zol (x axis). At day 10 of culture, cells were collected and stained with FITC conjugated mAb for Vγ9 and PE conjugated mAb for CD3. Plotted are the median, interquartile range (IQR) and total range of % Vγ9+ out of CD3+ cells (y axis) in cultures of SSc patients (a) and HCs (b). Also plotted are the concentration-response curves of the overall response to Zol concentration (logarithmic scale): Each of the 5 dots represents the median (IQR) Vγ9+ percentage relative to baseline (*i.e.* enrichment medium without Zol), while the continuous line displays the calculated best fit of proliferation *vs*. Zol concentration, with a maximal response (Emax) of 36.4% [95% CI, 24.6-48.2] and an EC50 of 0.7 µM [95% CI, 0.3-1.7] in SSc patients (c) and no dose response in HCs (d). PBMC of SSc patients were also triggered with increasing concentrations of Zol, in the presence of a fixed concentration of mevastatin (25uM). Plotted (e) are the median, interquartile range (IQR) and total range of % Vγ9+ out of CD3+ cells in cultures.

Zol was also observed among HCs, in whom proliferation was not dependent on Zol concentration (p=0.86, Fig. 2b) nor did it fit into a concentration-response curve, as a plateau was achieved at the lowest concentration of 0.07 µM used in these experiments (Fig. 2d). To examine how inhibition of the mevalonate pathway affects proliferation of Vγ9+ T cells in SSc patients, mevastatin was added, at a constant concentration previously shown to inhibit proliferation in HCs cultures, to SSc-derived PBMC cultured with Zol. As seen in Fig. 2e, the Vγ9+ T cell proliferative response to Zol in SSc patients was markedly diminished by the presence of mevastatin to a maximum median of less than 3.5%.

Effect of zoledronate in combination with IPP on proliferation of Vγ9+ T cells

The addition of 7 µg/ml IPP, a concentration previously shown to be optimal for proliferation in both HCs and SSc patients, (10) to Zol-free medium significantly increased Vγ9+ T cells percentage in both SSc-derived PBMC (median proliferation 1.65% in FM alone, 11.66% in FM+IPP; p=0.008) and HCs (median proliferation 4.5% in FM alone, 26.8% in FM+IPP; p=0.02). To evaluate whether exogenously added IPP would enhance the diminished proliferative response of Vγ9+ T cells from SSc patients to Zol, proliferation experiments were repeated, except that 7µg/ml IPP was added along with the increasing concentrations of Zol. In repeated measures analysis, using the presence or absence of IPP as a grouping factor, Zol concentration remained significantly associated with the relative increase in Vγ9+ T cell percentage in SSc patients (p<0.001, Fig. 3a), whereas neither the presence or absence of IPP in culture plates nor the interaction between Zol concentration and IPP (p=0.28 and p=0.78, respectively) were associated with this increase. In HCs, neither Zol concentration (p=0.57) nor the interaction between Zol concentration and IPP (p=0.27) were associated with the relative increase in Vγ9+ T cell percentage, whereas the presence or absence of IPP was significantly associated with the increase (p=0.02, Fig. 3b).

IFN-γ and TNF-α secretion in response to IPP in systemic sclerosis patients and healthy individuals

To evaluate the capacity of Vγ9+ T cells to secrete anti-fibrotic cytokines in SSc patients and HCs, PBMC were cultured with IPP to activate the Vγ9+ T cell population, and with TSST-1 to activate the αβ T cell population.

As shown in Fig. 4, IPP, a Vγ9+ T cell specific reagent, induced the production of both TNF-α and IFN-γ in the supernatants of SSc patients (n=7, p=0.04 and 0.03, respectively) and HCs (n=7, p=0.02 for both cytokines). The increase in cytokine production in the presence of IPP relative to baseline (medium only) was higher among HCs compared to SSc patients, although this difference did not reach statistical significance (p=0.13 and 0.17 for TNF-α and IFN-γ, respectively). The production of TNF-α and IFN-γ in response to TSST-1, a non- Vγ9+ T cell specific super antigen that triggers αβ T cells, was also significantly increased in both SSc patients (p=0.02 for both cytokines) and HCs (p=0.02 for both cytokines).

Although the increase in cytokine production in the presence of TSST-1 relative to medium was similar between the two groups (p=1.00 for both cytokines), it was significantly higher in SSc patients compared to cytokine production in the presence of IPP (p=0.02 and 0.03 for TNF-α and IFN-γ respectively), while there was no difference in the effects of IPP and TSST-1 in HCs (p=0.31 and 0.4 for TNF-α and IFN-γ, respectively).

Effect of supernatant of IPP-activated systemic sclerosis PBMC on fibroblast procollagen secretion

We evaluated whether IPP-induced perturbation of Vγ9+ T cells in PBMC of SSc patients (n=4) affects the balance of pro and anti-fibrotic factors secreted by the PBMC. Supernatants from PBMC that had been activated with IPP to expand the Vγ9+ T cells, then rested and retriggered with IPP, were collected and added to adherent fibroblast cultures for 24h. In 3 of the SSc patients (SSc 1-3), a reduction of procollagen production by fibroblasts cultured with supernatants of PBMC that were re-triggered with IPP was observed, compared to fibroblasts cultured with PBMC that were not re-triggered (Fig. 5). However, we observed no reduction of procollagen when fibroblasts were cultured with supernatants of IPP-retriggered PBMC from SSc patient 4. Furthermore, we found no clear correlation between the percent of Vγ9+ T cells in the re-triggered PBMC and the degree of fibroblast inhibition, although the effect was maximal for PBMC of patient SSc 2, with a preponderance of Vγ9+ T cells. Importantly, there was no direct effect of the addition of IPP to fibroblast medium at the concentrations used to activate the PBMC (data not shown).

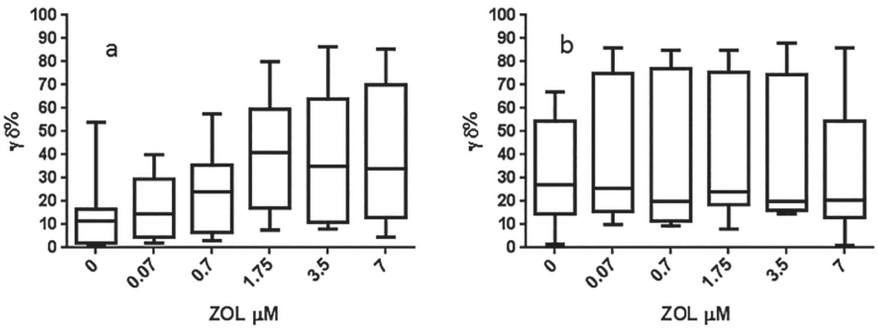

Fig. 3. Differential response of SSc Vγ9+ T cells to zoledronate in the presence of IPP: PBMC were triggered as described in legend to Figure 2, in the presence of increasing concentrations of Zol and a constant concentration of IPP (7μg/ml). Plotted are the median, interquartile range (IQR) and total range of % Vγ9+ out of CD3+ cells in cultures of SSc patients (a) and HCs (b).

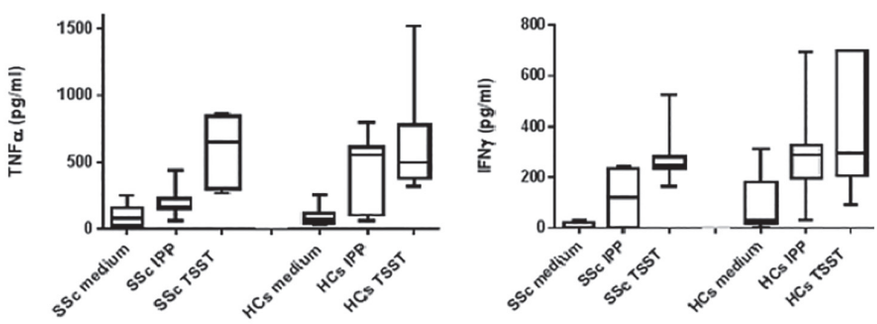

Fig. 4. Cytokines secreted by IPP-triggered SSc and HCs PBMC: PBMC were cultured in medium alone, medium with IPP (2μg/ml), or medium with TSST-1 (1 ng/ml). Day 3 supernatants were analysed for their content of TNF-α and IFN-γ. Plotted are the median, interquartile range (IQR) and total range of TNF-α and IFN-γ concentrations in pg/ml. IPP and TSST-1 significantly increased the production of both cytokines in SSc patients and in HCs. In SSc patients, the increase of cytokine production in the presence of TSST-1 was significantly higher when compared to cytokine production in the presence of IPP. In HCs, IPP and TSST-1 induced a similar increase in cytokine production.

Discussion

In SSc, complex interactions between fibrosis, inflammation and vascular damage lead to significant morbidity and mortality through internal organ damage. Recognition of key mediators that play a role in the disease may give rise to innovative therapies, targeting specific organs or pathological pathways (17).

The involvement of γδ T cells in SSc has been known for more than two decades (18), but their precise role in the pathophysiology of the disease has only recently attracted renewed attention. γδ T cells of the Vγ9Vδ2 subtype have been shown to secrete anti-fibrotic cytokines upon stimulation by phosphoantigens, such as IPP. IPP is thought to drive Vγ9Vδ2 T cell proliferation via its binding to butyrophilin 3A1 (also known as CD277), which is expressed on the cell membrane of APC. Intracellular IPP binds to the intracellular portion of BTN3A1 and induces conformational alterations within its extracellular portion. These alterations are recognised by the Vγ9+ TCR, leading to activation of Vγ9+ T cells. IPP may also activate Vγ9+ T cells by directly binding to the extracellular portion of BTN3A1 upon the APC (12, 13). The mechanism of Zol-mediated activation of peripheral blood Vγ9Vδ2 T cells is through augmentation of intracellular levels of IPP in CD14+ monocytes (19).

Here we showed that proliferative responses to Zol in SSc are retained, but require a higher concentration of Zol compared to HCs for maximal proliferation. Interestingly, even exogenously applied IPP at saturating concentrations could not correct this diminished response to resemble that of HCs. In stark contrast, HC-derived Vγ9+ T cells were highly sensitive to the effects of exogenously applied IPP, and required far lower concentrations of Zol to reach plateau proliferative responses. Moreover, although SSc-derived PBMC were induced to release TNF-α and IFN-γ in response to IPP-triggering of their Vγ9Vδ2 cells, this production of anti-fibrotic cytokines was of lower magnitude when compared to SSc-derived αβ T cells or to Vγ9Vδ2 cells from HCs. Last, IPP-

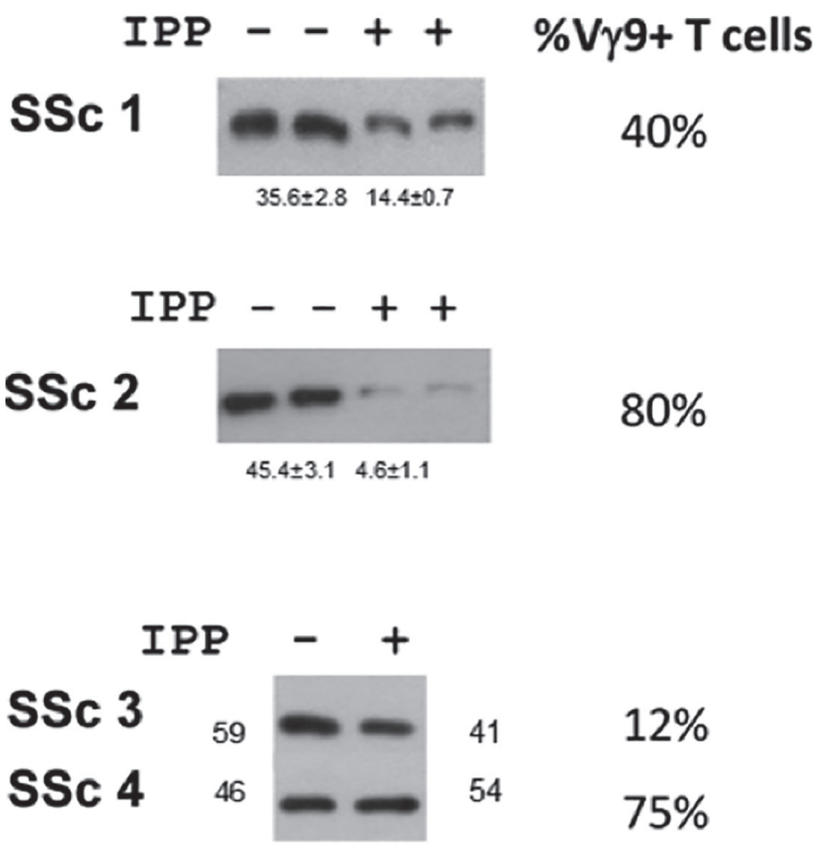

Fig. 5. Effect of IPP-activated Vγ9+ T cell enriched supernatants on procollagen production: Monolayers of fibroblasts were cultured for 24 hours in presence of supernatants of PBMC from 4 SSc patients, which had been triggered for 10 days with IL-2 (100 IU/ml) and IPP (2 μg/ml), then washed, rested for 24 hours in medium alone, and re-triggered with IPP (+) or medium alone (-). Indicated on the right are the percentages of Vγ9+ out of total CD3+ T cells in the IPP-retriggered PBMC. The Immunoblots represent procollagen produced by the fibroblasts. Procollagen content was assessed with an antibody recognising the pro α1 chain of type I collagen. Numbers adjacent to or below the blots represent results of densitometric measurements, expressed as relative optical density units (materials and methods) and, where relevant, presented as the mean ± standard deviation.

triggering of SSc-derived PBMC released factors that inhibited procollagen production by human fibroblasts in 3 SSc patients, while a paradoxical increase in procollagen was measured in the fourth. These diminished responses of Vγ9+ T in cells in SSc patients to directly added IPP or to Zol, which indirectly elevates IPP, may partially elucidate the fibrotic nature of this disease.

Although our data suggest a diminished role for IPP in activation of Vγ9Vδ2 cells in SSc patients, mevastatin blocked the response of these cells to Zol, suggesting that intermediates of the mevalonate pathway, which include IPP, remain crucial for SSc-derived Vγ9+ T cell proliferation in response to Zol. Also, the IPP-induced secretion of TNF-α and IFN-γ suggests that activation of these cells by APC harbors a potential to release anti-fibrotic cytokines in SSc patients. Together, our results could be interpreted such that IPP is essential in SSc-derived Vγ9Vδ2 T cell activation, and that, as exogenous IPP could not enhance the decreased responsiveness of these cells to Zol, this decrease response is not primarily due to lower levels of IPP in SSc patients, but rather to upstream events following its upregulation by Zol. These could include inefficient presentation of IPP to the Vγ9+ T cell by decreased binding to BTN3A1 in APC, alterations in the physiology of BTN3A1 in SSc, or an inherent diminished ability of Vγ9+ T cells from SSc patients to become activated and proliferate. In our previous study, the proliferative response of Vγ9+ T cells in 11 SSc patients was also reduced, though non-significantly, when compared to 12 HCs (10). As both studies are limited by a small sample size and large inter-subject variability in SSc patients and HCs, further experiments are clearly warranted to clarify this issue, which could be critical for the understanding of the immunopathology of SSc.

Pathological fibrosis is a cardinal manifestation of SSc, leading to significant morbidity and also mortality when affecting the lungs. Procollagen is a precursor that is processed to collagen to create overt fibrosis. Factors known to be secreted by IPP-triggered human Vγ9+ T cells include the anti-fibrotic TNF-α and IFN-γ, as well as the pro-fibrotic connective tissue growth factor (CTGF) (20). We and others have previously shown that SSc-derived Vγ9Vδ2+ T cells are able to secrete both TNF-α and IFN-γ, whereas Vδ1+ T cells secrete IL-4, a pro-fibrotic cytokine, in the presence of Zol stimulation (10, 21). Moreover, Vδ1+ T cells accumulate in the skin early in SSc (18), whereas Vγ9Vδ2 T cells, rather than Vδ1 T cells, accumulate in the skin of systemic lupus erythematosus patients, in whom skin fibrosis is not a manifestation of the disease (22). Finally, CD161+Vδ1+ T cells were shown to be increased in peripheral blood in some SSc patients and to augment fibroblast proliferation (23). All these experiments suggest a pro-fibrotic function that resides primarily within the Vδ1+ T cells of SSc patients.

In contrast, our current results suggest that the balance of factors secreted by SSc-derived PBMC that are triggered with IPP, which activates Vγ9Vδ2+ T cells only, favours reduction of procollagen secretion by myofibroblasts at least in some patients. In a preliminary experiment, not shown here, we determined that supernatants of highly purified Vγ9Vδ2+ T cells triggered with IPP also reduced procollagen production, suggesting that the Vγ9Vδ2+ T cells themselves are the cellular source of the anti-fibrotic factors. However, the addition of a mAb to TNF-α did not abrogate this effect. Thus, further experiments will be required to determine the nature of the factors, secreted by Vγ9Vδ2 T cells, which are respon-

sible for the down-regulation of procollagen observed in our preliminary and current experiments. Interestingly, one study has linked reduction of γδ T cells with Scl70 positivity, suggesting that lack of anti-fibrotic Vγ9Vδ2+ T cells is associated with the diffuse rather than the limited form of SSc (24). A recent paper described an increase of procollagen in fibroblasts co-cultured with non-IPP activated peripheral blood γδ T cells (25). It is likely that this finding is due to the preponderance of pathogenic Vδ1+ rather than Vγ9Vδ2+ T cells in SSc, and to the tendency of Vδ1+ T cells from SSc patients to secrete pro-fibrotic factors such as IL-4 (21).

To conclude, the results of the current study reinforce the role of IPP in Vγ9Vδ2 T cells activation, either directly or indirectly via incubation with Zol. Vγ9Vδ2 cells from SSc patients proliferated and were activated to secrete pro-inflammatory, anti-fibrotic cytokines upon stimulation with Zol or IPP, although in lower magnitude when compared to HCs. This study and previous studies suggest that Vγ9+ T cells harbor the potential to reduce fibrosis in SSc. Thus, further evaluation of their anti-fibrotic properties and the mechanism responsible for their reduced response to IPP may be of importance for understanding the immunopathogenesis and treatment modalities of this disease.

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