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Original Article

In vitro effect of biological and conventional disease-modifying antirheumatic drugs on fibrocyte differentiation in patients with rheumatoid arthritis and healthy controls

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Abstract

Objective: Fibrocytes are circulating bone-marrow-derived cells that migrate to organs with ongoing repair or inflammation. In the target organ, the cells differentiate, become long and spindle-shaped, and are able to produce extracellular matrix components. In fibrotic diseases, the levels of fibrocytes are increased, both in circulation and the diseased tissue. In rheumatoid arthritis (RA), fibrocytes have been proposed to be involved in the spread of the disease and possibly in RA fibrotic manifestations, as can be seen in RA interstitial lung disease (RA-ILD). Therefore, we aimed to investigate a range of current RA treatment modalities (corticosteroids and conventional and biological disease-modifying antirheumatic drugs (DMARDs)) regarding their effect on *in vitro* fibrocyte differentiation.

Methods: A total of 10 participants were included (5 patients with RA and 5 healthy controls). Peripheral blood mononuclear cells (PBMCs) were isolated and cultured for 5 days with prednisolone, conventional DMARDs (methotrexate, sulfasalazine, and hydroxychloroquine), and biological DMARDs (etanercept, tocilizumab, adalimumab, abatacept, and rituximab). The numbers of fibrocytes were counted. Dose-response data for abatacept and tocilizumab were collected.

Results: Abatacept and prednisolone significantly suppressed differentiation of PBMC into fibrocytes compared with control ($P = .02$ and $P < .01$, respectively) (n=10). In overall analysis (n=10), abatacept reduced fibrocyte levels with an average of 44% overall and 71% in the RA group compared with the control wells. Tocilizumab reduced the fibrocyte count by 63% overall and 45% in the RA group, although it was not significant ($P = .07$ and $P = .06$, respectively). Both tocilizumab and abatacept display a dose-response relationship.

Conclusion: Abatacept and prednisolone suppress the differentiation of mononuclear cells to mature fibrocytes *in vitro* in patients with RA, and data indicate a similar effect of tocilizumab; this was further supported by the observed dose-response relationship. Clinical trials are needed to compare the effect of these drugs on fibrotic RA manifestations, for example, RA-ILD.

Keywords: Fibrocytes, rheumatoid arthritis, antirheumatic agents

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Introduction

Fibrocytes are bone-marrow-derived cells, which express the stem-cell marker CD34, the hemopoietic marker CD45, monocyte markers CD14 and CD11, as well as other markers, such as CD80 and CD86.[1] Immature fibrocytes circulate as circular mononuclear cells and migrate to sites of inflammation and tissue damage where they differentiate into long spindle-shaped cells, which are hereafter called mature fibrocytes. The mature fibrocytes are over 40 µm long and are morphologically easily distinguished from macrophages and other circulating cells. They produce extracellular matrix, such as vimentin, collagen, and fibronectin, which strengthen the connective tissues.[1-4] In a physiological setting, the released substances contribute to wound healing and maintenance of tissue integrity. However, fibrocytes are also suspected to be involved in the pathologic processes in fibrotic diseases, such as rheumatoid arthritis (RA), RA interstitial lung disease (RA-ILD), idiopathic pulmonary fibrosis (IPF), and systemic sclerosis (SSc).[1,2,5] Studies have shown the fibrocyte as a possible precursor cell to the lung fibroblast and myofibroblast, which makes the fibrocyte a potential treatment target.[2,5] A fibrocyte differentiation inhibitor has recently been tested in patients with IPF, stabilizing lung function and walking distance.[6] Fibrocytes possess a wide range of surface markers, such as CD80 and CD86, which can be targeted by abatacept, and secrete mediators, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL) 6, which can be

targeted by TNF-α inhibitors and tocilizumab.[7] Several studies have proposed that the number of circulating fibrocytes is a biomarker for poor prognosis in several fibrotic diseases, such as IPF, severe asthma, liver fibrosis, and RA-ILD.[2,8-11] Fibrocytes have also been proposed to be involved in RA disease activity in murine models, migrating to joints with active inflammation, worsening disease activity, and possibly spreading the disease to new joints.[12-15] However, the effect of current available biological or conventional disease-modifying antirheumatic drugs (b/cDMARDs) on fibrocyte differentiation has not been examined. We hypothesize that b/cDMARDs have an inhibitory effect on fibrocyte differentiation as they have an effect on other monocyte-derived cells and cells known to regulate fibrocyte differentiation, for example, T cells.

We present a study of the effect of corticosteroids, cDMARDs, and bDMARDs on *in vitro* differentiation of mononuclear cells to mature fibrocytes.

Methods

Study design, settings, and cohort

We performed a single-center cross-sectional study, including 5 blood donors from the department of clinical immunology and 5 patients with RA from the department of rheumatology. The patients with RA met the 2010 The European League against Rheumatism and the American College of Rheumatology criteria and disease activity score of 28 joints-C-reactive protein as well as positivity for immunoglobulin M rheumatoid factor and anti-citrullinated protein antigen was noted at the time of inclusion. There was no requirement for the duration of disease for patients with RA. All healthy blood donors met the 2017 inclusion criteria set by the Danish society for Clinical Immunology.[16] Age and sex data for all the participants were registered.

The study is approved by the regional ethics review board (S-20140062) and the Danish data protection agency (2008-58-0035). All participants gave written and oral consent.

Main Points

- Abatacept suppresses the differentiation of fibrocytes *in vitro* in a dose-response relationship.
- Prednisolone suppresses the differentiation of fibrocytes *in vitro*.
- Tocilizumab tends to suppress the differentiation of fibrocytes *in vitro* in a dose-response relationship.

Fibrocyte differentiation in culture

Peripheral blood mononuclear cells (PBMCs) were cultured as previously described.[4] Briefly, PBMCs were isolated from 20 mL of peripheral blood with ethylene diamine tetra-acetic acid using Lymphoprep (StemCell, Grenoble, France). Monocyte, lymphocyte, granulocyte, and eosinophil counts in the PBMC suspension were measured using a hematology automated analyzer (Sysmex, Kobe, Japan). The cells were then adjusted to 1×10^6 cells/mL culture media. Flat bottom 96-well plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were used with each well filled with 50 µL of cell suspension and 150 µL of culture media. Culture media (50 mL) consisted of 47 mL of FibroLife (LifeLine Cell Technology, Frederick, USA), 0.5 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM) (All Sigma-Aldrich, St. Louis, USA), 0.5 mL of non-essential amino acids (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.5 mL of sodium pyruvate (1 mM) (All Sigma-Aldrich, St. Louis, USA), 0.5 mL of glutamine (2 mM) (All Sigma-Aldrich, St. Louis, USA), 0.5 mL of Pen-Strep (Penicillin [100 U/mL] (All Sigma-Aldrich, St. Louis, USA)) and streptomycin [100 µg/mL]) (All Sigma-Aldrich, St. Louis, USA), 0.5 mL of ITFS-3 (iron(III)triflouromethanesulfonate) (All Sigma-Aldrich, St. Louis, USA), and 5 ng/mL of IL-4 (Cell Systems, Troisdorf, Germany). A total of 11 different drugs used for RA treatment were added to the medium to make the concentration in the well C_{max}+1 standard deviation (Table 1). There was a drug-free control for each individual. The dose-response relationship was tested using PBMCs from 2 healthy individuals. Findings were confirmed with PBMCs from 1 patient with RA. Dimethyl sulfoxide (DMSO) is a commonly used dissolvent known to affect cell cultures, including cultures of PBMC (17). To avoid confounding by DMSO, we used intravenous or subcutaneous formulas when possible. For drugs in tablet form (sulfasalazine, hydroxychloroquine, and prednisolone), we used lyophilized drug from Selleck Chem (Huston, USA). These were water-insoluble and dissolved in DMSO (WAK-Chemie Medical GmbH, Steinbach, Germany). We tested the independent effect of 1% DMSO on fibrocyte differentiation in 8 wells with a sample from a healthy control and compared it with 8 control wells without DMSO. We also indirectly tested the effect of 0.0002% DMSO, as this was the concentration in the wells with sulfasalazine. Lower concentrations were present in the cultures with hydroxychloroquine and 10-fold lower concentration in cultures with prednisolone.

The plates were placed in a humidified incubator (37°C, 5% CO_2) for 5 days and observed on days 1 and 3. Non-adherent cells (for example, T and B cells) were discarded, and the remaining cells were fixed using ethanol and stained as previously described.[4]

Quantification of mature fibrocytes

Mature fibrocytes were identified as spindle-shaped cells >40 µm in length. Before trial initiation, we stained the cultured cells as described with procollagen type 1 and found the described cells positive (Figure 1). Using this culture method, it has been previously validated that these cells fulfill the criteria for being fibrocytes.[4,18,19] A total of 4 different microscopic fields of view (2 in the central and 2 in the peripheral area of the well) were counted per well, and 4 wells were counted per drug/control per individual similar to the method used in previous studies.[2] We calculated the total number of fibrocytes in the well on day 5 and then calculated the number of fibrocytes per 10^5 monocytes added to the well on day 0, which was referred to as the number of fibrocytes. The senior and first authors counted the fibrocytes. To assess the inter-observer variability, both senior and first authors counted 32 identical vision fields and calculated kappa value. The final cell-counts were then performed by the first author.

Acquiring medications

For the biologic and biosimilar medicine, we collected the residual drugs after patient treatments and stored them under sterile conditions at the advised temperature. For methotrexate, we used fluid suspension in the pen solution (Metex) to avoid the use of DMSO. The cDMARD and bDMARD medications in this study and the applied concentration are presented in Table 1.[20]

Statistical analysis

Categorical variables on patient characteristics were presented as numbers and percentages and continuous variables as means with standard deviation.[21] In Figure 2, we describe the *in vitro* effect of medications as mean±standard error of mean. In multiple comparisons of wells with added medication, statistical significance was determined by analysis of variance in relation to the control well. Thus, each individual served as its own vehicle when calculating the effect of different medical agents. $P < .05$ was considered statistically significant. Statistics were performed in STATA software (version 15) (StataCorp LLC, College Station, Texas USA), and figures were created in GraphPad Prism 8 (GraphPad Software, San Diego, Calefornia, USA).

Results

Population demographics are listed in Table 2. When evaluating the inter-observer variability, the standard error of the square root of differ-

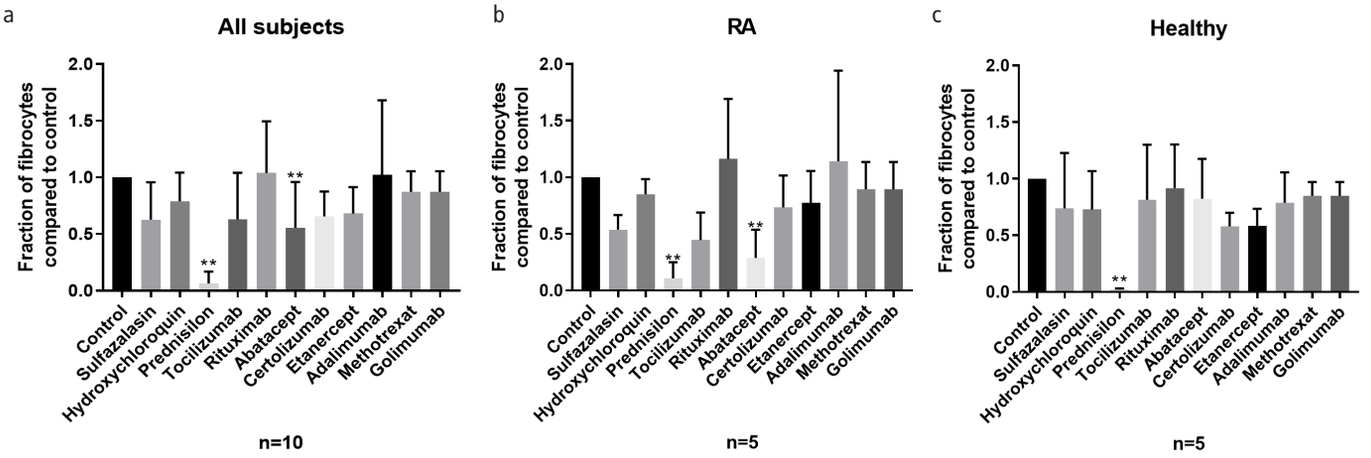

Figure 1. a-c. Effect of conventional disease-modifying antirheumatic drugs (cDMARDs), biologic disease-modifying antirheumatic drugs (bDMARDs), and prednisolone on fibrocyte differentiation. The x-axis represents the different c/b DMARDS/prendnisolone and the y-axis represents the number of fibrocytes per 105 monocytes as a fraction of the number of fibrocytes in the untreated control. Values are mean±standard error of mean. ** indicates statistical significance compared with the control ($P < .05$). Total analysis of all subjects (a). Sub-analysis of individuals with rheumatoid arthritis (b). Sub-analysis of healthy controls (c).

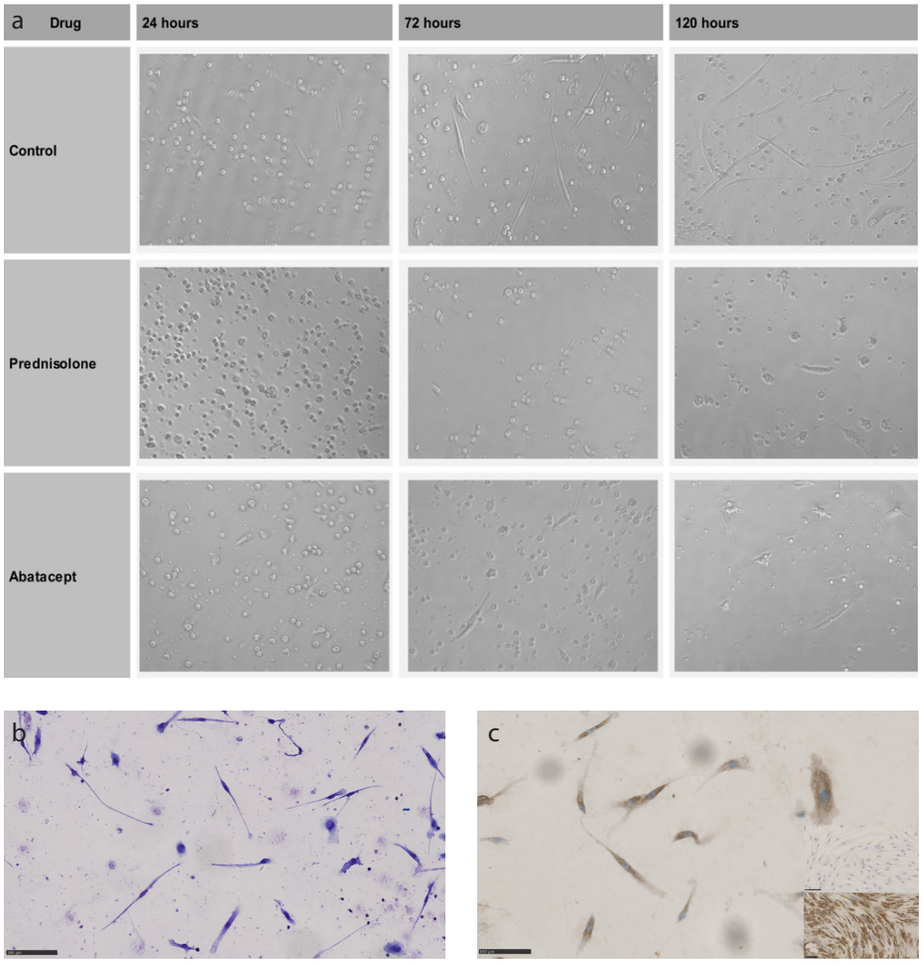

Figure 2. a-c. Fibrocyte differentiation and staining with Giemsa and procollagen type 1. Fibrocyte differentiation with prednisolone and abatacept (a). Representative pictures of cultures with prednisolone or abatacept as well as a control culture from an individual with rheumatoid arthritis. After 24 hours, a few cells begin to elongate into fibrocytes in the control but not in wells with prednisolone or abatacept. At 72 hours, there are several identifiable fibrocytes in the control but none in the wells with prednisolone and only 1 when abatacept is added. The difference between control and prednisolone/abatacept is obvious after 120 hours in the culture. Fibrocytes stained with Giemsa (b). Fibrocytes stained with procollagen type 1 and procollagen type-1-stained fibroblasts for positive control and IgG isotype control on cultured fibroblasts (c).

ence was 0.15 with a correlation coefficient of 0.95 and a 95% confidence interval of 0.65-1.25. After 24 hours, the cells started to differentiate into spindle-shaped cells, and by the 3rd and 5th day, the differentiating cells grew in length and were identifiable as long spindle-shaped fibrocytes (Figure 1a) with Giemsa stain (Figure 1b) and procollagen type 1 (Figure 1c).

Of the 11 different medications tested in our study (Table 1), abatacept and prednisolone significantly reduced the number of fibrocytes compared with the controls. Tocilizumab also reduced the number of fibrocytes but not significantly ($P = .07$) (Figure 2).

Abatacept significantly decreased the number of fibrocytes with an average of 44% in overall analysis (n=10 individuals) and of 71% in a subgroup analysis of the RA group (n=5 individuals) ($P = .02$ and $P = .009$, respectively). The effect of abatacept was not significant in the healthy population (Figure 2). Abatacept demonstrated a dose-response relationship with decreased number of fibrocytes with increasing concentrations of abatacept (Figure 3).

Overall, tocilizumab tended to reduce the number of fibrocytes with an average of 63% overall and 44.6% in the RA group, but the difference was not statistically significant ($P = .07$ overall and $P = .06$ in the RA group). However, tocilizumab had a clear dose-response effect on the number of fibrocytes (Figures 2 and 3).

Adding rituximab, adalimumab, hydroxychloroquine, sulfasalazine, golimumab, and methotrexate did not have any significant effect on the number of fibrocytes.

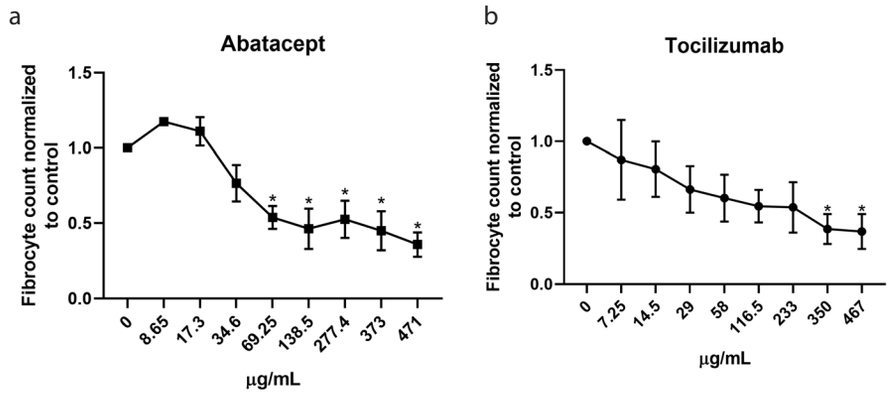

Figure 3. a, b. Dose-response curves for abatacept and tocilizumab. Dose-response effect of tocilizumab (a) and abatacept (b) on the number of fibrocytes per 105 added monocytes as a fraction of the number of fibrocytes in the untreated control. Control peripheral blood mononuclear cells (PBMCs) were cultured without tocilizumab/abatacept. Values are mean±standard error of mean of 8 different cultures of PMBCs from 2 healthy individuals. *$P < .05$ compared with control (analysis of variance).

Table 1. Concentration of the different medical agents in the culture medium (20).

Drug	C_{max}+1 SD	Supplier
Sulfasalazine	10 ug/mL	Salazopyrin
Hydroxychloroquine	362 ng/mL	Plaquenil
Prednisolone	0.51 mg/L	Prednisolone "DAK"
Tocilizumab	350 µg/mL	RoActemra
Rituximab	662 µg/mL	Mabthera
Abatacept	297.4 µg/mL	Orencia
Certolisumab	57.8 µg/mL	Cimzia
Etanercept	3.39 µg/mL	Benepali
Adalimumab	11,1 mg/L	Humira
Methotrexate	754 ng/mL	Metex
Golimumab	9.8 µg/mL	Simponi

SD, standard deviation.

We found that 1% DMSO markedly reduced the number of fibrocytes (Figure 1). DMSO was also indirectly tested at 0.0002%, as this was the concentration in wells with sulfasalazine, but this combination did not significantly decrease the number of fibrocytes (Figure 2).

Discussion

We systematically tested the effect of medication used in RA treatment in the form of corticosteroids, 3 cDMARDs, and 7 different bDMARDs on *in vitro* differentiation of PBMCs into fibrocytes. Our results demonstrated several important findings. First, abatacept and prednisolone significantly reduced the number of fibrocytes in culture. Second, the results indicate that tocilizumab had the same effect.

To the best of our knowledge, this is the first study evaluating the effects of cDMARD and bDMARD on *in vitro* fibrocyte differentiation in healthy individuals and patients with RA. Fibrocytes have been found to be involved in a range of fibrotic diseases, such as RA-ILD, where there is an eminent need of new treatment options.[1,2]

We found that prednisolone strongly inhibited fibrocyte differentiation in culture, similar to previous studies (Figure 2). We did not investigate the dose-response relationship partly because this has been established previously and partly because the clinical relevance is sparse as glucocorticoids are already used for treating fibrotic diseases but have many known side effects, especially when the cumulative dose is high, and thus there is a need to establish new treatment options.[5,22,23]

None of the TNF-α blockers had a significant effect on the number of maturing fibrocytes in the culture (Figure 2). Shao et al[24] have found that TNF-α had no significant effect on cultured fibrocytes, whereas Pilling et al[3] have found that TNF-α stimulated fibroblasts to produce lumican, which promoted fibrocyte differentiation. Neither our study nor the study by Shao et al[24] had fibroblasts in the cultures and might, therefore, have underestimated the effect of TNF-α blockers on fibrocyte levels *in vitro*.

The 3 tested cDMARDs (methotrexate, sulfasalazine, and hydroxychloroquine) did not reduce the number of fibrocytes in the culture (Figure 2) nor did we observe any stimulating effect of methotrexate, the use of which is often debated in fibrotic diseases (for example, RA-ILD).[23] More studies are needed to investigate the *in vitro* and *in vivo* effects of methotrexate in fibrotic diseases.

We found that abatacept significantly inhibited fibrocyte differentiation and demonstrated a dose-response relationship (Figure 1, 2, and 3). Similar findings have been obtained in other diseases, but we are, to the best of our knowledge, the first to investigate this effect in patients with RA.[25] CTLA4-Ig fusion protein (abatacept) binds to CD80 and CD86 with a greater affinity than to CD28 on the T cell, and thus inhibits co-stimulation between the antigen presenting cell (APC) and the T cell.[26] Fibrocytes express class 2 major histocompatibility complex molecules, co-stimulatory molecules CD80 and CD86, as well as adhesion molecules CD11a, CD54, and CD58 that are required for antigen presentation and co-stimulation.[27] Chesney et al[27] had shown that the fibrocytes function as a potent APC and are able to induce T-cell proliferation almost as effectively as dendritic cells. CTLA4-Ig fusion protein downregulate IL-6, TNF-α, IL-1b, and transforming growth factor when cultured with macrophages, both in presence and absence of T cells, indicating a direct effect on APCs.[25] A recent *in vitro* study on fibrocytes in patients with SSc indicated that circulating fibrocytes are responsive to abatacept treatment and abatacept may decrease adhesion/migration molecules on the fibrocytes.[25] If abatacept makes the fibrocyte non-adherent, our study has a source of error as non-adherent cells were discarded before counting. However, there seemed to be fewer differentiating fibrocytes on days 1 and 3 of culture with abatacept compared with the control (Figure 1). In agreement with our study, Cutolo et al[25] found that the effect of abatacept was not significant in the cells from healthy subjects. This observation might be owing to an upregulation of co-stimulatory proteins on fibrocytes in diseases where fibrocytes are involved, making the effect more prominent in patients. However, we do find a dose-response

Table 2. Demographics and clinical characteristics.

Variable	RA	Control	*P*
n (%)	5 (50)	5 (50)	
Female, n (%)	3 (60)	3 (60)	1.00
Age, mean (21)	71.0 (7.8)	42.6 (19.5)	.02
CRP, mean (21)	4.6 (4.2)		
ACPA positive, n (%)	2 (28)		
RF Positive, n (%)	1 (20)		
Smoking			
Never, n (%)	2 (28)		
Former, n (%)	3 (60)		
DAS28-CRP, mean (21)	3.0 (1.3)		
Treatment			
cDMARD, n (%)	1 (20)		
bDMARD, n (%)	0 (28)		
Prednisolone, n (%)	0 (28)		

CRP, C-reactive protein; ACPA, anti-citrullinated protein antibody; RF, rheumatoid factor; DAS28-CRP, disease activity score in 28 joints combined with C-reactive protein; SD, standard deviation; cDMARD, conventional disease-modifying antirheumatic drugs; bDMARD, biologic disease-modifying antirheumatic drugs.

relationship between abatacept and the number of fibrocytes in cultures from healthy individuals (Figure 3). A putative explanation for these different responses could be that the intracellular activation (STAT3 and NF-kB pathways) in fibrocytes is markedly higher in patients with RA than in healthy controls.[13]

In our study, the IL-6 inhibitor tocilizumab tended to decrease the number of fibrocytes. This finding was not statistically significant, but the observed dose-response effect strongly indicates that tocilizumab inhibited fibrocyte differentiation *in vitro*. This is contradictory to a previous study, which found that IL-6 did not significantly influence the number of cultured fibrocytes from PBMC.[24] However, a recent double-blind phase 2 study (faSScinate) showed improvement in skin thickening and evidence of less decline in the lung function in patients with SSc treated with tocilizumab compared with those receiving placebo.[25] Furthermore, a recent case series suggested that tocilizumab could have a positive or stabilizing effect on respiratory function in SSc-ILD.[25]

This study had some limitations. It was an *in vitro* study, and the results should be confirmed *in vivo* in RA clinical studies. Furthermore, the study was not blinded, the number of included individuals was relatively low, and small differences might therefore be masked in this study. We confirmed that DMSO could affect the number of viable cells in PBMC culture, including fibrocytes (supplementary material). We used DMSO in cultures with sulfasalazine, hydroxychloroquine, and prednisolone. However, in the concentrations used in our study (0.0002% and below), DMSO had no inhibitory effect. This was indirectly proven as sulfasalazine did not inhibit the number of fibrocytes cultured and was the culture with the highest concentration of DMSO (0.0002%). There is a theoretical possibility that sulfasalazine and DMSO have opposite effects in fibrocyte differentiation; therefore, no effect is seen. However, this is unlikely, and we do not believe that DMSO was a confounder in our study.

Quantification of total cell death has not been possible as all non-adherent cells were discarded as described previously and in line with other publications with similar culturing technique.[4]

The mean age difference between the case and control populations may have an effect on the number of fibrocytes as fibrocytes seem to be both quantitatively and qualitatively altered in aged individuals; however, because each individual served as their own control, we believe that this effect was minimized.[1]

We did not test if abatacept altered the expression of CD80/CD86; however, this was previously performed in a study by Cutolo et al,[25] who have found that fibrocytes from patients with SSc have a decreased gene expression of CD86, but this was not significant. This indicated that the effect of abatacept on fibrocytes is as expected by regulating CD80/CD86.

In conclusion, we demonstrated that abatacept reduced and tocilizumab tended to reduce fibrocyte differentiation in a dose-dependent manner. Further research is needed to determine if using abatacept or tocilizumab could prevent/delay fibrotic RA manifestations as RA-ILD.

Ethics Committee Approval: Ethics committee approval was received for this study from the Regional Committee on Health Research Ethics for Southern Denmark (Approval Date: June 17, 2014; Approval Number: S 20140062). and the Danish data protection agency (2008-58-0035).

Informed Consent: Written and verbal consent was obtained from the individuals who participated in this study.

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Conflict of Interest: The authors have no conflict of interest to declare.

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