Research article

**Cytokine mRNA and protein expression in primary-culture and repeated-passage synovial fibroblasts from patients with rheumatoid arthritis**

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**Abstract**

Constitutive mRNA expression and secretion of proinflammatory and anti-inflammatory cytokines was comparatively analyzed in rheumatoid arthritis (RA) synovial fibroblasts (SFB), isolated from primary culture or derived by repeated passage; normal-skin fibroblasts were used as controls. First-passage RA-SFB \( n = 3 \) secreted large amounts of IL-6 \( (15,800 \pm 2,110 \, \text{pg/ml}) \), but only limited amounts of tumor necrosis factor (TNF)-\( \alpha \) \( (22.1 \pm 1.1 \, \text{pg/ml}) \) or IL-10 \( (35.7 \pm 34.2 \, \text{pg/ml}) \) (only one of three samples was positive). IL-1\( \beta \), IL-15, and IL-18 were not detectable at the protein level and showed very low mRNA levels by semiquantitative RT-PCR. In repeated-passage RA-SFB (tenth passage), protein secretion was significantly lower for IL-6 (one-twentieth of the initial level) and TNF-\( \alpha \) (two-thirds), and markedly reduced for IL-10 (one-quarter, with only one of three samples positive). While the decrease of IL-10 protein from first to tenth passage was paralleled by a corresponding decrease of mRNA, the relative mRNA levels for IL-6 and TNF-\( \alpha \) were actually increased (20-fold and 300-fold, respectively), indicating post-transcriptional and/or post-translational regulation of these cytokines. Due to highly variable levels among individual patients, however, no significant differences were observed for any cytokine mRNA between primary-culture and repeated-passage RA-SFB (ninth passage). Likewise, no significant differences were detectable between RA-SFB and normal-skin fibroblasts (primary-culture and repeated-passage). By producing high amounts of IL-6 and limited amounts of TNF-\( \alpha \), RA-SFB may contribute to the (im)balance of proinflammatory and anti-inflammatory cytokines in the inflamed joint.

**Keywords:** cytokines, inflammation, mRNA, rheumatoid arthritis, synovial fibroblasts

**Introduction**

Activated synovial fibroblasts (SFB) appear to play a major role in the pathogenesis of rheumatoid arthritis (RA) \([1,2]\). It has been speculated that their ability to induce and perpetuate inflammation, as demonstrated in transfer experiments in mice with SCID (severe combined immunodeficiency). DMEM = Dulbecco’s modified Eagle’s medium; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescence-activated cell sorter; FB = fibroblasts; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IL = interleukin; PBMC = peripheral blood mononuclear cells; PBS = phosphate-buffered saline (solution); PCR = polymerase chain reaction; RA = rheumatoid arthritis; RPMI = Roswell Park Memorial Institute (medium); RT-PCR = reverse transcriptase polymerase chain reaction; SCID = severe combined immunodeficiency; SD = standard deviation; SEM = standard error of the mean; SFB = synovial fibroblasts; TNF = tumor necrosis factor.
immunodeficiency) [3–5], is associated with the production of proinflammatory cytokines. Exuberant concentrations of various cytokines have been detected within the arthritic joint, such as tumor necrosis factor (TNF-α [6]), interleukin (IL)-1β [7], IL-6 [8], and IL-8 [9]. Other cytokines have also been detected, including IL-10 [10], IL-13 [11], IL-15 [12], and IL-18 [13,14], although these have appeared to be less prominent. The production of some of those cytokines in RA joints, in particular IL-6 [14], IL-15 [15], and IL-18 [13,14], has been at least partially attributed to SFB. This cytokine production may drive or control many of the characteristic features of rheumatoid inflammation and may also contribute to the arthritogenic potential of RA-SFB in SCID transfer models [4,5].

However, conflicting data exist about the inherent activation of RA-SFB in culture, which may be in part related to differing isolation methods [16–18]. In contrast to conventional procedures (collagenase digest or tissue outgrowth), which require repeated passages in order to isolate sufficient numbers of pure SFB [17,18], we have recently published a protocol for obtaining pure fibroblasts from primary culture using anti-CD14 Dynabeads® to deplete contaminating macrophages [16]. In these studies, primary-culture RA-SFB clearly differed from fourth-passage SFB in phenotypic and functional features, indicating considerable alteration in culture. To investigate whether similar problems apply to their expression of cytokines, this functional feature was comparatively analyzed in RA-SFB isolated by different methods. Normal-skin fibroblasts were used as noninflammatory controls.

### Materials and methods

Synovial tissue was obtained after joint surgery from patients with RA (n = 18) who had given their informed consent. The patients met the American College of Rheumatology 1987 revised criteria for the classification of RA [19]. Foreskin samples were obtained from six healthy individuals. The study was approved by the Institutional Review Board of the University of Leipzig. RA-SFB were isolated either by trypsin/collagenase (Roche, Mannheim, Germany) digestion, short-term in vitro adherence (7 days) to remove nonadherent cells, and negative isolation using magnetobead-coupled anti-CD14 monoclonal antibodies (primary-culture RA-SFB) [16] or by outgrowth from tissue pieces (repeated-passage RA-SFB; Table 1), a conventional isolation procedure previously used in a number of studies.

Skin fibroblasts (FB) were obtained either by Dispase II/collagenase (Roche) digestion of normal skin samples (primary-culture normal-skin FB) or by tissue outgrowth from normal skin (repeated-passage normal-skin FB) (see Table 1). The phenotype and composition of isolated/passed FB were characterized by flow cytometry in a fluorescence-activated cell sorter (FACS), using saturating amounts of the monoclonal antibodies listed in Table 2 and a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) for analysis.

Cytokine concentrations in supernatants of primary-culture and repeated-passage RA-SFB (n = 3 each) were assessed using commercial ELISA kits for IL-1β, IL-6, TNF-α (DPC, Bad Nauheim, Germany), IL-10, IL-13, IL-15 (R&D, Wiesbaden, Germany), and IL-18 (Diaclone, Besançon, France), following the manufacturer’s protocols. Cytokine mRNA was quantified by isolating the total RNA [20], converting it to cDNA by standard procedures [21], and amplifying the DNA by PCR using specific primers for the cytokines TNF-α, IL-1β, IL-6, IL-10, IL-13, IL-15, and IL-18. Quantification of relative units of cDNA was performed using 32P-end-labeled oligonucleotides as probes [22], normalizing the relative concentrations of cytokine cDNA to those of cyclophilin. Lipopolysaccharide-stimulated peripheral blood mononuclear cells (PBMC) were used as positive controls for cDNA quantification of IL-1β, IL-10, TNF-α, and IL-13; TNF-α- and IL-1β-stimulated primary-culture normal-skin FB were used as controls for IL-6, IL-15, and IL-18. Controls were designed to yield maximum expression of the relevant cytokine mRNA.

For statistical analysis, only PCR experiments with a coefficient of determination $r^2 > 0.9$ in linear regression were used. The mRNA levels of different FB were compared using the Kruskal–Wallis test followed by the Mann–Whitney rank sum test. Correlations between clinical

### Table 1

**Source and characteristics of fibroblasts used for mRNA quantification**

| Source of isolated cells                                      | Number of patients | Method of cell isolation     | Median no. of passages (range) |
|---------------------------------------------------------------|--------------------|-------------------------------|--------------------------------|
| Rheumatoid arthritis (synovial membrane)                      |                    |                               |                                |
| Primary culture                                              | 5                  | Collagenase digest            | 0                              |
| Passage ≥5                                                    | 7                  | Tissue outgrowth              | 9 (5–14)                       |
| Foreskin (normal skin)                                        |                    |                               |                                |
| Primary culture                                              | 6                  | Collagenase digest            | 0                              |
| Passage ≥5                                                    | 6                  | Tissue outgrowth              | 5.5 (5–13)                     |

This table shows the source and characteristics of fibroblasts used for mRNA quantification. The table includes information on the source of isolated cells, number of patients, method of cell isolation, and median number of passages (range).
parameters of each patient (age, rheumatoid factor, disease duration) and cytokine mRNA levels were calculated using the Spearman rank correlation. In all cases, differences were considered statistically significant at the level $P \leq 0.05$.

**Results**

**Composition of isolated/passaged fibroblasts**

All cell preparations were highly enriched for FB, as determined by positivity for prolyl-4-hydroxylase and for CD90 (mAb [monoclonal antibody] AS02 [23]; Fig. 1 and Tables 2 and 3). Contamination with CD3+ T cells, CD19+ B cells, CD14+ and/or CD68+ macrophages, and vWF+ and CD31+ endothelial cells was routinely below 2% with the exception of primary-culture normal-skin FB, which contained approximately 6% vWF+ (von Willebrand’s factor-positive) and CD31+ cells (see Fig. 1 and Tables 2 and 3).

**Quantification of cytokine protein**

First-passage RA-SFB secreted large amounts of IL-6 but only small amounts of TNF-α or IL-10 (one of three samples positive) (Table 4). IL-1β, IL-15, and IL-18 protein was not detectable (see Table 4). In repeated-passage RA-SFB (tenth-passage), cytokine secretion was significantly lower for IL-6 and TNF-α and was markedly lower for IL-10 (one of three samples positive) (see Table 4).

### Table 2

Monoclonal antibodies used in the present study

| Cellular target                        | Antigen            | Clone       | Labeling | Source                                      |
|----------------------------------------|--------------------|-------------|----------|---------------------------------------------|
| All human cells                        | HLA-ABC           | W6/32       | None     | DAKO, Hamburg, Germany                      |
| Fibroblasts                            | CD90 (Thy-1)      | FibAS02     | None     | Dianova, Hamburg, Germany                   |
| Collagen-producing cells               | Prolyl-4-hydroxylase | 3-2B12     | None     | Calbiochem, Bad Soden, Germany              |
| Antigen-presenting cells (MHC class II)| HLA-DP, DQ, DR    | CR3/43      | None     | DAKO                                        |
| Monocytes, macrophages                 | CD68              | KP1         | FITC     | DAKO                                        |
| Monocytes                              | CD14              | MoP9        | FITC     | Becton Dickinson, Heidelberg, Germany       |
| B cells                                | CD19              | HD37        | None     | DAKO                                        |
| T cells                                | CD3               | UCHT1       | None     | DAKO                                        |
| Endothelial cells, some leukocytes     | CD31 (glycoprotein IIa) | JC/70A  | None     | DAKO                                        |
| Endothelial cells                      | von Willebrand’s factor | 4F9       | None     | Immunotech, Marseilles, France              |

### Table 3

Characterization of synovial and skin fibroblasts by flow cytometry

| Antigen                                      | Rheumatoid arthritis synovial fibroblasts | Primary-culture (n = 4) | Repeated-passage (n = 4) | Primary-culture normal-skin FB (n = 4) |
|----------------------------------------------|-------------------------------------------|-------------------------|--------------------------|----------------------------------------|
| MHC class I (HLA-ABC)                       | 98.3 ± 0.8                                 | 97.6 ± 1.1              | 98.5 ± 0.5               |
| Prolyl-4-hydroxylase (intracellular)         | 96.5 ± 2.0                                 | 98.5 ± 0.5              | 98.1 ± 0.3               |
| CD90 (Thy-1)                                | 92.7 ± 4.4                                 | 98.9 ± 0.3              | 88.8 ± 3.8               |
| MHC class II (HLA-DP, DQ, DR)               | 3.8 ± 3.1                                  | 0.3 ± 0.4               | 3.1 ± 2.3               |
| CD14                                         | 0.1 ± 0.1                                  | 0.2 ± 0.2               | 0.1 ± 0.1               |
| CD68                                         | 0.6 ± 0.7                                  | 0.2 ± 0.1               | 0.3 ± 0.2               |
| CD3                                          | 1.5 ± 1.0                                  | 0.3 ± 0.1               | 0.2 ± 0.4               |
| CD19                                         | 0.5 ± 0.9                                  | 0.1 ± 0.1               | 0.3 ± 0.4               |
| von Willebrand’s factor (intracellular)      | 1.6 ± 1.6                                  | 0.1 ± 0.1               | 6.0 ± 3.1               |
| CD31                                         | 0.1 ± 0.1                                  | 0.2 ± 0.1               | 6.1 ± 3.2               |

Values are means ± SD of positive cells. Intracellular antigens detected following permeabilization of the cells; the remaining antigens were detected by surface staining. FB = fibroblasts.
Expression of surface and intracellular molecules in primary-culture or repeated-passage rheumatoid arthritis synovial fibroblasts (RA-SFB) and in primary-culture normal-skin fibroblasts (FB). All preparations were highly enriched for FB, as demonstrated by expression of prolyl-4-hydroxylase and CD90 (Thy-1). Contamination with endothelial cells (indicated by von Willebrand’s factor), T cells (indicated by CD3), B cells (indicated by CD19), and macrophages (indicated by CD14) was below 2%, with the exception of contaminating endothelial cells in primary-culture normal-skin FB, which averaged approximately 6% (see Table 3). ○, controls; ●, cell markers; M1, gate set in order to exclude 99% of the cells stained with the respective isotype control; percentages indicate the percentage of cells positive for each marker, numbers on the x axis indicate fluorescence channels.
Quantification of cytokine mRNA
The lack of IL-1β, IL-15, and IL-18 protein secretion by first-passage RA-SFB was reflected by very low levels of mRNA in primary-culture cells (Fig. 2). Whereas the decrease of IL-10 protein secretion in RA-SFB from the first to the tenth passage was paralleled by a corresponding decrease in mRNA levels (only one positive sample in the ninth passage), the levels of mRNA for IL-6 and TNF-α increased with time (20-fold and 300-fold increase of the median, respectively), suggesting post-transcriptional or post-translational regulation (see Fig. 2). There were large variations of the cytokine mRNA levels between individual patients, conceivably preventing significant differences among groups (see Fig. 2).

Interestingly, mRNA for IL-10 was detected only in three of five primary-culture RA-SFB samples and two of six primary-culture normal-skin FB samples. Limited IL-10 expression would be compatible with a minimal contamination of macrophages (approximately 0.7%) demonstrated by FACS. Passaged SFB or skin FB contained only individual positive samples (see Fig. 2).

IL-18 mRNA was present in three of six primary-culture normal-skin FB samples and in two of six repeated-passage normal-skin FB but was not detectable in primary-culture and repeated-passage RA-SFB (see Fig. 2).

IL-13 mRNA was not detectable in any of the FB samples; in contrast, IL-13 mRNA was readily detectable in lipopolysaccharide-stimulated PBMC used as positive control (data not shown).

No significant correlations (Spearman rank correlation) were observed between the age, rheumatoid factor status, or disease duration of individual RA patients and the cytokine mRNA levels in SFB.

Discussion
The present study demonstrates considerable alterations in the cytokine expression of repeated-passage RA-SFB upon extended in vitro culture, as shown by significantly lower protein concentrations for IL-6 and TNF-α and non-significant changes in the mRNA levels for almost all cytokines. These findings are important because most studies have been performed with fibroblasts at higher passage (3–10), which are prone to similar alteration in vitro. They also point to the suitability/usefulness of isolated early-passage cells for studies on the features/functional characteristics of SFB in RA.

Composition of isolated and passaged fibroblasts
A high percentage of RA-SFB positive for FB markers (≥97%) and a contamination of <2% leukocytes and endothelial cells indicated a limited contribution of contaminating cells to the cytokine expression. Primary-culture normal-skin FB, however, showed a contamination of approximately 6% endothelial cells, possibly accounting for the detection of IL-18 mRNA.

Cytokine expression in RA-SFB
IL-6, a pleiotropic cytokine produced predominantly by synovial macrophages and fibroblasts, plays a major role in cellular activation. The detection of high levels of IL-6 mRNA/protein (see Fig. 2 and Table 4) agrees with previous observations in passaged SFB/synoviocytes [24–28]. In those studies, however, stimulation of cells with IL-1β or TNF-α was necessary to induce the production of 80–90 ng/ml of IL-6 [25]. In the present study, in contrast, first-passage RA-SFB constitutively secreted IL-6. This indicates a high intrinsic activation status of the cells, i.e. retention of in vivo features present before removal from the synovial membrane [29]. A clearly decreased IL-6 production in repeated-passage RA-SFB (see Table 4) may represent culture alterations due to the lack of cytokine stimulation.

Table 4
Cytokine concentrations in supernatants of rheumatoid arthritis synovial fibroblast (RA-SFB) cultures (pg/ml)

| Cytokine | Detection limit of the ELISA (pg/ml) | First passage (n = 3) | Tenth passage (n = 3) |
|----------|--------------------------------------|-----------------------|----------------------|
| IL-1β    | 5                                    | Not detectable        | Not detectable       |
| IL-6     | 5                                    | 15,800 ± 2110         | 790.0 ± 35.6*        |
| IL-10    | 1.5                                  | 35.7 ± 34.2b          | 9.0 ± 7.5b           |
| IL-15    | 1                                    | Not detectable        | Not detectable       |
| IL-18    | 94                                   | Not detectable        | Not detectable       |
| TNF-α    | 1.7                                  | 22.1 ± 1.1            | 15.1 ± 0.7*          |

bCytokine detected in only one of three samples. *P ≤ 0.05 in comparison with first-passage RA-SFB.
IL-10 is produced by T cells, B cells, mast cells, and activated monocytes or macrophages. Its primary function appears to limit and terminate inflammatory responses (including those in experimental arthritis). In contrast to recent suggestions [30], the capability of RA-SFB to produce IL-10 remains uncertain. First, synovial macrophages, rather than SFB, are known to produce considerable amounts of IL-10 [31, reviewed in 32]. Second, the low amounts of IL-10 detected in primary-culture RA-SFB in the present study are probably the product of the few remaining macrophages (see Table 3). This hypothesis is supported by the lack of IL-10 protein/mRNA detected in most repeated-passage RA-SFB (only one positive sample each), which lack contaminating macrophages.
Similarly, the proinflammatory cytokine TNF-α is predominantly produced by synovial macrophages [25,33,34], and not by SFB [35–37]. In contrast to IL-10, however, TNF-α protein or mRNA were also detected in repeated-passage RA-SFB (lacking contaminating macrophages). TNF-α may therefore be a genuine product of RA-SFB, allowing these cells to contribute to synovial inflammation. Its significantly decreased production in repeated-passage RA-SFB may reflect either loss of macrophages and/or in vitro alterations of SFB due to withdrawal of cytokine stimulation.

In this study, no IL-1β protein (and only minimal amounts of IL-1β mRNA) was detected in primary-culture and repeated-passage RA-SFB. This result is in contrast to reports showing IL-1β mRNA or protein in nonstimulated RA-SFB [24,38]; however, it confirms findings that showed expression of IL-1β mRNA but lack of secretion of IL-1β protein by RA-SFB [39]. As for TNF-α, the detection of IL-1β mRNA in repeated-passage RA-SFB suggests some IL-1β transcription.

IL-13 is an anti-inflammatory cytokine expressed in a subset of activated T cells. It downregulates macrophage activity and thereby reduces the production of proinflammatory cytokines. IL-13 mRNA was not detectable in any of the FB preparations assayed, in good agreement with the lack of IL-13 protein production by passed RA-SFB, whether nonstimulated or after stimulation with IL-1β, TNF-α, or lipopolysaccharide [40]. Therefore, it appears that RA-SFB do not contribute to the local production of this potent anti-inflammatory cytokine [11,40].

IL-15, an IL-2-like cytokine produced by monocytes/macrophages, dendritic cells, fibroblasts, and bone-marrow stroma cells, activates cell proliferation, cytotoxicity, and cytokine production in natural killer cells and is a potent chemoattractant for T cells. Low levels of IL-15 mRNA were detected in all FB preparations; however, IL-15 protein was absent in the supernatants of first-passage or repeated-passage RA-SFB (see Fig. 2, Table 4). This finding is in contrast to reports showing IL-15 mRNA and protein in repeated-passage SFB cell lines (approximately 50 pg/ml; [15]). However, a lower expression of IL-15 mRNA in SFB cell lines in comparison with freshly isolated synovial tissue cells (containing synovial macrophages, the predominant producers of IL-15 [12,15]) strongly indicates that RA-SFB produce very little, if any, IL-15 [12].

IL-18 is produced by PBMC, dendritic cells, and activated endothelial cells and has pleiotropic effects on T cell functions. The failure to detect IL-18 mRNA or protein in nonstimulated, primary-culture, or repeated-passage RA-SFB confirms previous reports in nonstimulated, passedage RA-SFB lines [13]. The capability of FB to express IL-18 mRNA [13] was confirmed by its detection in nonstimulated RA-SFB. Interestingly, the cytokines IL-1β, IL-6, IL-15, and TNF-α in RA-SFB showed a dissociated pattern of mRNA and protein expression, suggesting post-transcriptional or post-translational regulation. Such regulation (at the level of mRNA stability, translational efficacy, stability of the protein product or other post-translational modifications) has been demonstrated for IL-1β [41–43], IL-6 [44,45], IL-15 [46], and TNF-α [47]. Its importance for RA-SFB functions remains to be analyzed.

Phenotype of RA-SFB
In contrast to our earlier findings [16], which confirmed reports on the expression of MHC-II in the RA/OA synovial membrane [48-51], in the present study only 4% of RA-SFB expressed MHC-II molecules (see Table 3). Possible explanations include differences in isolation procedures, in the patient populations analyzed, and in the epitopes of the MHC molecules recognized by the respective monoclonal antibodies [37,52].

Conclusion
On the basis of the present results, the arthritogenic and tissue-destructive properties of RA-SFB in the inflamed rheumatoid joint may be supported by production of high amounts of IL-6 and limited amounts of TNF-α. Early-passage RA-SFB provide a source of cells with limited alteration from the in vivo phenotype/functional features and allow future studies comparing early- with late-stage RA; RA with normal and inflammatory controls; and in vivo with in vitro cytokine production by RA-SFB. Post-transcriptional and post-translational regulation of cytokine production and large variability among samples must be taken into consideration.

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References
1. Kontoyiannis D, Kollias G: Synovial fibroblasts in rheumatoid arthritis: leading role or chorus line? Arthritis Res 2000, 2:342-343.
2. Kinne RW, Palombo Kinne E, Emmrich F: Activation of synovial fibroblasts in rheumatoid arthritis. Ann Rheum Dis 1995, 54:501-504.
3. Muller Ladner U, Gay RE, Gay S: Cellular pathways of joint destruction. Curr Opin Rheumatol 1997, 9:213-220.

4. Lehmann J, Jungel A, Lehmann I, Busse F, Biskop M, Saalbach A, Emmrich F, Sack U: Grafting of fibroblasts isolated from the synovial membrane of rheumatoid arthritis (RA) patients induces chronic arthritis in SCID mice – a novel model for studying the arthriticogenic role of RA fibroblasts in vivo. J Autoimmun 2000, 15:301-313.

5. Muller Ladner U, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, Gay RE, Gay S: Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafed into SCID mice. Am J Pathol 1996, 149: 1607-1615.

6. Buchan G, Barrett K, Turner M, Chantry D, Maini RN, Feldmann M: Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha. Clin Exp Immunol 1988, 73:449-455.

7. Miossec P: The role of interleukin 1 in the pathogenesis of rheumatoid arthritis. Clin Exp Rheumatol 1987, 5:305-308.

8. Field M, Chu CQ, Feldmann M, Maini RN: Interleukin-6 localisation in the synovial membrane in rheumatoid arthritis. Rheumatol Int 1991, 11:45-50.

9. Koch AE, Kunkel SL, Burrows JC, Evanoff HL, Haines GK, Pope RM, Streiter RM: Synovial tissue macrophage as a source of the chemotactic cytokine IL-8. J Immunol 1991, 147:2187-2195.

10. Cush JJ, Sapliewski JB, Thomas R, McFarlin JE, Schule-Koops H, Davis LS, Fujita K, Lipsky PE: Elevated interleukin-10 levels in patients with rheumatoid arthritis. Arthritis Rheum 1995, 38: 596-604.

11. Isomaki P, Luukkainen R, Toivanen P, Punnonen J: The presence of interleukin-13 in rheumatoid synovium and its antinflammatory effects on synovial fluid macrophages from patients with rheumatoid arthritis. Arthritis Rheum 1996, 39:1693-1702.

12. Collman IB, Hughes J, Field M, Leung BP, Huang FP, Dixon R, Sturrock RD, Wilkinson PC, Liew FY: The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. Nat Med 1996, 2:175-182.

13. Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR, Kennedy K, Carter R, Wei XQ, Xu D, Field M, Foulie A, Liew FY, Mclnnes IB: A proinflammatory role for IL-18 in rheumatoid arthritis. J Clin Invest 1999, 104:1393-1401.

14. Wood NC, Symons JA, Dickens E, Duff GW: In situ hybridization of IL-6 in rheumatoid arthritis. Clin Exp Immunol 1992, 87: 183-189.

15. Harada S, Yamamura M, Okamoto H, Morita Y, Harada S, Makino H, Ota Z: The synovial expression and serum levels of interleukin-6, interleukin-11, leukaemia inhibitory factor, and oncostatin M in rheumatoid arthritis. Arthritis Rheum 1997, 40:1096-1105.

16. Firestein GS, Zvailler NJ: How important are T cells in chronic rheumatoid synovitis? Arthritis Rheum 1990, 33:768-773.

17. Rittchon C, Dwyer E, Bucala R, Winchester R: Sustained and distinctive patterns of gene activation in synovial fibroblasts and whole synovial tissue obtained from inflammatory synovitis. Scand J Immunol 1994, 40:292-298.

18. Guerre PA, Torkeltaub R, Zuraw BL, Lotz M: Inflammatory microcrystals stimulate interleukin-6 production and secretion by human monocytes and monocyte-derived macrophages. Arthritis Rheum 1989, 32:1443-1452.

19. Firestein GS, Alvaro-Gracia JM, Maki R: Quantitative analysis of cytokine expression in rheumatoid arthritis. J Immunol 1990, 144:3347-3353.

20. Rittchon C: Fibroblast biology: Effector signals released by the synovial fibroblasts in arthritius. Arthritis Res 2000, 2:356-360.

21. Danning CL, Illei GG, Hitchen C, Greer MR, Boumpas DT, Mclnnes IB: Macrophage-derived cytokine and nuclear factor kappaB p65 expression in synovial membrane and skin of patients with psoriatic arthritis. Arthritis Rheum 2000, 43:1244-1256.

22. Schanzle-Koops H, Lipsky PE, Daviss LS: Elevated Th1- or Th2-like cytokine mRNA in peripheral circulation of patients with rheumatoid arthritis. Modulation by treatment with anti-ICAM-1 correlates with clinical benefit. J Immunol 1995, 155:5029-5033.

23. Saalbach A, Kraft R, Hermann K, Haustein UF, Anderger U: The monoclonal antibody AS20 recognizes a protein on human fibroblasts being highly homologous to Thy-1. Arch Dermatol Res 1988, 290:360-364.

24. Buclua R, Rittchon C, Winchester R, Cerami A: Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. J Exp Med 1991, 173:569-574.

25. Okamoto H, Yamamura M, Morita Y, Harada S, Makino H, Ota Z: The synovial expression and serum levels of interleukin-6, interleukin-11, leukaemia inhibitory factor, and oncostatin M in rheumatoid arthritis. Arthritis Rheum 1997, 40:1096-1105.

26. Firestein GS, Zvailler NJ: How important are T cells in chronic rheumatoid synovitis? Arthritis Rheum 1990, 33:768-773.

27. Rittchon C, Dwyer E, Bucala R, Winchester R: Sustained and distinctive patterns of gene activation in synovial fibroblasts and whole synovial tissue obtained from inflammatory synovitis. Scand J Immunol 1994, 40:292-298.

28. Guerre PA, Torkeltaub R, Zuraw BL, Lotz M: Inflammatory microcrystals stimulate interleukin-6 production and secretion by human monocytes and monocyte-derived macrophages. Arthritis Rheum 1989, 32:1443-1452.

29. Firestein GS, Alvaro-Gracia JM, Maki R: Quantitative analysis of cytokine expression in rheumatoid arthritis. J Immunol 1990, 144:3347-3353.

30. Rittchon C: Fibroblast biology: Effector signals released by the synovial fibroblasts in arthritis. Arthritis Res 2000, 2:356-360.

31. Danning CL, Illlei GG, Hitchen C, Greer MR, Boumpas DT, Mclnnes IB: Macrophage-derived cytokine and nuclear factor kappaB p65 expression in synovial membrane and skin of patients with psoriatic arthritis. Arthritis Rheum 2000, 43:1244-1256.

32. Kinne RW, Brauer R, Stuhlmuller B, Palombo Kinne E, Burmester GR: Macrophages in rheumatoid arthritis. Arthritis Res 2000, 2:189-202.

33. Chu CQ, Field M, Abney E, Zheng RC, Allard S, Feldmann M, Maini RN: Transforming growth factor beta 1 in rheumatoid synovial membrane and cartilage/pannus junction. Clin Exp Immunol 1991, 86:380-386.

34. Alsalam S, Winter K, Al Ward R, Wendel J, Kalden JR, Kinne RW: Distribution of TNF-alpha, TNF-R55 and TNF-R75 in the rheumatoid synovial membrane; TNF receptors are localized preferentially in the lining layer; TNF-alpha is distributed mainly in the vicinity of TNF receptors in the deeper layers. Scand J Immunol 1999, 49:278-285.

35. Burmester GR, Stuhlmuller B, Keyser G, Kinne RW: Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? Arthritis Rheum 1997, 40:5-18.

36. Bombara MP, Webb DL, Conrad P, Marlor CW, Sarr T, Ranges GE, Aune TM, Greve JM, Blue ML: Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. J Leukoc Biol 1994, 54:399-406.

37. Schwachula A, Riemann D, Kehlen A, Langner J: Characterization of the immunophenotype and functional properties of fibroblast-like synoviocytes in comparison to skin fibroblasts and umbilical vein endothelial cells. Immunobiology 1994, 190: 87-92.

38. Matsuoka N, Eguchi K, Kawakami A, Tsuboi M, Kawabe Y, Aoyagi T, Nagataki S: Characterization of rheumatoid arthritis fibroblast-like cells. Arthritis Rheum 1996, 39:96-104.

39. Schwachula A, Riemann D, Kehlen A, Langner J: Characterization of the immunophenotype and functional properties of fibroblast-like synoviocytes in comparison to skin fibroblasts and umbilical vein endothelial cells. Immunobiology 1994, 190: 87-92.

40. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium-phenol-chloroform extraction. Anal Biochem 1987, 162:156-159.

41. Schanzle-Koops H, Davis LS, Kavaanagh AF, Lipsky PE: Elevated cytokine messenger RNA levels in the peripheral blood of patients with rheumatoid arthritis suggest different degrees of myeloid cell activation. Arthritis Rheum 1997, 40:839-847.
the University of Leipzig. The study was approved by the Institutional Review Board of the University of Leipzig. Written, informed consent was obtained from all patients before specimens were collected. The study was approved by the Institutional Review Board of the University of Leipzig.

**Tissue samples**

Tissue samples were placed in cell-culture medium containing DMEM and RPMI 1640 (1:2), 10% FCS, 25 mM HEPES, penicillin (100 U/ml), and streptomycin (100 µg/ml; all Gibco, Eggenstein, Germany; hereafter called culture medium) at ambient temperature and processed within 2 hours. RA synovial membrane specimens were washed in culture medium, fat and fibrous tissue were removed, and the remaining synovial tissue was used for further preparation.

**Isolation of synovial fibroblasts and skin fibroblasts**

Isolation of SFB from the RA synovial membrane and of skin FB was performed by two different protocols. For the preparation of primary-culture RA-SFB, synovectomy samples were minced and digested for 30 min at 37°C in phosphate buffered saline (PBS; Gibco) containing 0.1% trypsin followed by a digestion step with 0.1% collagenase P (Boehringer Mannheim, Germany) in culture medium at 37°C. To resolve cell aggregates, the suspension was filtered through a sterile sieve (80 mesh, Sigma, Deisenhofen, Germany). The cells were collected by centrifugation after two washing steps with medium and cultured for 7 days in culture medium at 37°C in a humidified atmosphere containing 5% CO₂. The nonadherent cells were depleted by medium exchange every 2–3 days. Macrophages were then depleted by incubation with CD14-coated Dynabeads® (Dynal, Oslo, Norway) as follows: Trypsinized and washed synovial cells from primary culture (10⁷/ml) were incubated with 4 x 10⁷/ml Dynabeads® M-450 CD14 (clone RMO52; Dynal) in PBS/2% FCS for 1 hour at 4°C with bidirectional rotation. Nine ml of PBS/2% FCS was added and the conjugated cells were collected by using the Dynal Magnetic Particle Concentrator®. Unconjugated SFB were collected, washed twice in PBS/2% FCS, and used for mRNA preparation. Primary-culture normal-skin FB were prepared by first incubating skin samples with Dispase II (0.5 U/ml; Boehringer Mannheim) overnight at 4°C, removing the epidermis, and digesting the rest with 0.25% collagenase P (Boehringer Mannheim) in DMEM/1% FCS at 37°C and 5% CO₂ for 4 hours. For the preparation of repeated-passage RA-SFB and repeated-passage skin FB, cells were obtained by outgrowth from tissue pieces. Small samples of synovial membrane or skin (in the latter case, after overnight incubation with 0.5 U/ml Dispase II [Boehringer Mannheim] at 4°C and removal of the epidermis) with a volume of approximately 30 mm³ were cultured in triplicate for 1–2 weeks using 75-cm² culture flasks (Greiner, Solingen, Germany). At confluence, the cells were trypsinized with trypsin–EDTA (0.25% trypsin/0.2% EDTA; Gibco) for 5 min and passaged 1:2 in 150 cm² culture flasks. SFB were used from passages 5 to 14, and skin FB from passages 5 to 13. Passaged SFB and skin FB were tested every 14 days for contamination with *Mycoplasma spp.* by ELISA (Boehringer Mannheim).

**Flow cytometry**

The purity and phenotype of the isolated fibroblasts were characterized by flow cytometry. Cultured cells were harvested with trypsin/EDTA (Gibco) and washed twice in...
PBS. The cells (2 × 10^6) were stained with saturating amounts of one of the monoclonal antibodies listed in Table 2 for 30 min at 4°C. In all cases, except for fluorescein isothiocyanate (FITC)-labeled primary monoclonal antibodies, secondary labeling was performed for 30 min at 4°C with an FITC-labeled goat-anti mouse immunoglobulin (DAKO, Hamburg, Germany). For the detection of intracellular antigens, permeabilization of the cells was performed using 0.5% saponine as previously described [16]. Cells were analyzed with the FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA). A gate was set to exclude 99% of the cells stained with control immunoglobulin.

**Preparation of mRNA and generation of complementary DNA (cDNA)**

Total RNA was isolated by a single step guanidinium thiocyanate-phenol-chloroform method [20] using Tri-Reagent (Sigma, Deisenhofen, Germany) following the manufacturer’s protocol. The RNA-pellet was dissolved in H_2O/0.1% diethyl pyrocarbonate, and the OD_{260} was determined in a UV spectrophotometer. Ten to sixty micrograms of RNA was obtained per sample. For first-strand DNA synthesis, 2 µg of total RNA was converted to cDNA by incubation with 5 U of avian myeloblastosis virus reverse transcriptase (Promega, Mannheim, Germany), 20 µg/ml oligo(dT) primers (pd(T)_{12-18}, Pharmacia LKB, Uppsala, Sweden) and 1 mM dNTPs for 1 hour at 42°C in a total volume of 20 µl. The cDNA was stored at −20°C until analysis.

**Amplification and quantification of target DNA**

Before performing semiquantitative PCR experiments, kinetic experiments were carried out to determine the number of amplification cycles for exponential product accumulation. For PCR, 2 µl of the cDNA preparations were incubated with 0.5 U/ml Taq DNA polymerase (Perkin Elmer, Weiterstadt, Germany) in the presence of 1.5 mM mgCl_2, 0.4 µM oligonucleotide primers, and 0.2 mM dNTPs (Pharmacia). Amplification was performed with a Gene Amp PCR-System 9700 (Applied Biosystems, Weiterstadt, Germany) using the following conditions: denaturation at 94°C for 5 min, followed by repetitive cycles of denaturation at 94°C (45 s), annealing at 60°C (45 s), and polymerization at 72°C (45 s). The primers and the hybridization probes specific for IL-1β, IL-6, and TNF-α were purchased from Clontech Laboratories (Palo Alto, CA). The primers and the probes specific for IL-13 [11], IL-10 [10], IL-15 [46], IL-18 [13], and the constitutively expressed cyclophilin mRNA [51] were deduced from the published sequences and controlled for cross-reactivity by gene-bank search (IL-13: sense 5’-ACA GCT GGC ATG TAC TGT GC-3’; antisense 5’-GTG GCC TGT GTG TGA AGT GG-3’; hybridization probe 5’-TAG CTT AGA CTT CAG CCT GTG C-3’; IL-10: sense 5’-ATG CCC CAA GCT GAG AAC CAA GAC CCA-3’, anti-sense 5’-TCT CAA GGG GCT GGG TCA GCT CCA-3’, hybridization probe 5’-CAT TCT CCA CCT GCT CCA CG-3’; IL-15: sense 5’-CAG GAC TCG ATG GAG AAT CC-3’, antisense 5’-CAT GAA TGG CAT CTC CGG-3’, hybridization probe 5’-CCA TCA TGT TCC ATG CTG C-3’; and IL-18: sense 5’-ACC TCA GAC CTT CCA GAT CG-3’, antisense 5’-TCA GGC TGG TCT TGA ACA CC-3’, hybridization probe 5’-TTG TCG CAG GAA TAA AGA TGG-3’).

**Quantification of relative amounts of cytokine cDNA**

Quantification of cDNA was performed using a radioactive protocol that has previously been shown to be highly sensitive and reproducible for similar types of experiments [21,22]. In brief, cDNA containing the gene of interest (positive control) was diluted over a five-log scale and samples of each dilution were simultaneously amplified by PCR. For each cytokine, samples from all fibroblasts were simultaneously amplified in duplicates to minimize inter-assay variations. After the amplification, a sample of each PCR product was transferred onto a nylon membrane (Zeta-Probe Blotting membrane, Bio-Rad Laboratories, Richmond, CA, USA) by alkaline transfer using a slot blot apparatus. Blots were incubated for at least 1 hour at 52.5°C in hybridization fluid containing 6 x sodium chloride, sodium citrate (SSC), 4% Denhardt’s solution, 10 mM EDTA, 100 µg/ml denatured, fragmented salmon-sperm DNA, and 1% SDS. Amplified target DNA was detected by incubation of the blots in hybridization buffer containing target-DNA-specific ϒ^{32P}-labeled probes for at least 8 hours at 52.5°C. Probes were end-labeled with ϒ^{32P} using T4 polynucleotide kinase (Promega) as described elsewhere [21,22]. The blots were washed, and hybridized probes were quantified with a radioanalytic imaging system (Bio-Rad). Background counts from areas of the blot containing no amplified sequence (water controls) were subtracted. The amount of hybridized probe was found to be linearly related to the amount of the PCR products in the samples. Using the standard curve obtained by amplification of dilutions of the positive controls (lipopolysaccharide-stimulated PBMC or TNF-α- and IL-1β-stimulated primary-culture normal-skin FB), the relative concentration of initial target DNA in each individual sample could be calculated. The ubiquitously expressed housekeeping gene cyclophilin was used to normalize the amount of cDNA employed in each RT-PCR reaction. The results were expressed as relative units. The sizes of the amplification products were controlled by agarose-gel electrophoresis.

**Cytokine ELISA**

To assess the concentration of secreted cytokines, supernatants of primary-culture RA-SFB and repeated-passage RA-SFB were analyzed using commercially available ELISA kits for IL-1β, IL-6, TNF-α (DPC), IL-10, IL-13, IL-15 (R&D), and IL-18 (Diaclone), in accordance with the manufacturers’ protocols.
Statistical analysis
The standard curve for each PCR experiment was determined by linear regression analysis. Only experiments with a coefficient of determination $r^2 > 0.9$ were used for this study. Protein/mRNA levels of RA-SFB and of normal-skin FB were compared using the Kruskal–Wallis test followed by the Mann–Whitney rank sum test. Correlations between clinical parameters from each patient (age, rheumatoid factor, disease duration) and cytokine mRNA levels were assessed using the Spearman rank correlation test. In all cases, differences were considered statistically significant if $P \leq 0.05$.

Supplementary reference
S1. Haendler B, Hofer-Warbinek R, Hofer E: Complementary DNA for human T-cell cyclophilin. EMBO J 1987, 6:947-950.