

The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles

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Rheumatoid arthritis is a chronic inflammatory disease characterized by destruction of cartilage and bone that is mediated by synovial fibroblasts. To determine the mechanisms by which these cells are activated to produce matrix metalloproteinases (MMPs), the effects of microparticles were investigated. Microparticles are small membrane-bound vesicles whose release from immune cells is increased during activation and apoptosis. Because microparticles occur abundantly in the synovial fluid in rheumatoid arthritis, they could represent novel stimulatory agents. Microparticles derived from T cells and monocytes strongly induced the synthesis of MMP-1, MMP-3, MMP-9, and MMP-13 in fibroblasts. The induction was time-dependent, with effects primarily observed after 36 h; under these conditions, MMP-2, MMP-14, and tissue inhibitor of MMP-1 (TIMP-1), TIMP-2, and TIMP-3 were not induced. Microparticles also increased the synthesis of inflammatory mediators including IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), and MCP-2. In α -B-transfected synovial fibroblasts, MMPs were less inducible by microparticles compared with wild-type fibroblasts. Blocking of TNF α and IL-1 β with antibodies against TNF α and with IL-1 receptor antagonist did not abrogate stimulation by microparticles. These data provide evidence for a novel mechanism by which vesicles derived from activated or apoptotic immune cells can promote the destructive activity of synovial fibroblasts in rheumatoid arthritis.

rheumatoid arthritis | arthritis | T cells | macrophages

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammation and destruction of articular structures in association with extraarticular manifestations. Joint destruction in RA results from the invasion of cartilage and subchondral bone by the hyperplastic synovium (1), with synovial fibroblasts and inflammatory cells such as macrophages and T cells playing key roles in this process (1, 2). An important feature of rheumatoid arthritis synovial fibroblasts (RASFs) is an increased production of matrix metalloproteinases (MMPs), likely the most important matrix-degrading enzymes in RA (3). RASF also produce proinflammatory cytokines, which promote critical events in both inflammation and destruction (1). Although various cytokines can affect RASF, the detailed mechanisms leading to the invasive phenotype of RASF are still unknown.

Among molecular structures that could impact on RA pathogenesis, microparticles have recently attracted interest as novel signaling structures. Microparticles are small, membrane-bound structures that are released from cells by exocytic budding of the membrane during activation or apoptosis (4). During this process, the membrane asymmetry is lost, and phosphatidylserine, which is normally confined to the inner leaflet, appears on the outer leaflet of the microparticle membrane (5). In addition to altered surface lipids, microparticles display cell surface markers from the parental cell from which they originate. As such, they

provide a surface in which regulatory and triggering molecules are arrayed with potentially enhanced activity (6).

As shown by flow cytometric analysis of blood and other fluids, microparticle numbers are increased during a variety of inflammatory conditions (6, 7), with high levels of leukocyte-derived microparticles found in synovial fluid of inflamed joints of patients with RA (7). Because microparticles can display immunological activity (8, 9), we have investigated the possibility that these structures stimulate RASF and promote synovitis. The findings presented herein indicate that microparticles from lymphocytes and monocytes potently induce MMP and cytokine expression by RASF and could represent a novel signaling structure to promote the invasive and destructive phenotype of these cells in RA.

Materials and Methods

Cell Culture and Patients. All RA patients fulfilled the American College of Rheumatology criteria for RA. Synovial fibroblasts from patients with RA and osteoarthritis (OASF) as well as dermal fibroblasts were obtained and prepared as described (10). Normal synovial fibroblasts were obtained from trauma patients. Fibroblasts from passages 4–6 were used for the experiments. The number of cells was determined by using the CASY-1 cell-counter (Schärfe System, Reutlingen, Germany). All experiments were performed with protocols approved by the local ethics committee.

Isolation of Primary T Cells and Monocytes. For the isolation of primary T cells and monocytes, buffy coats from healthy donors were obtained from the local blood bank. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Hypaque (Pharmacia Biotech) density gradient centrifugation. After incubation with anti-human CD3-FITC or CD14-FITC antibodies (Becton Dickinson), primary T cells or monocytes were isolated by positive selection by using a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson).

Stimulation of Microparticle Release by Apoptosis and Activation. For the induction of apoptosis, U937 and Jurkat cells were stimulated with Fas ligand (50 μ g/ml, Alexis, Lausen, Switzerland), actinomycin-D (5 μ g/ml), or staurosporine (10 μ M, all Sigma).

The release of microparticles was also induced by stimulation of Jurkat cells with IL-2 (10 ng/ml, R & D Systems), Con A (1 μ g/ml), and a combination of phorbol 12-myristate 13-acetate

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Abbreviations: RA, rheumatoid arthritis; RASF, RA synovial fibroblast; MMP, matrix metalloproteinase; OASF, synovial fibroblasts from patients with RA and osteoarthritis; TIMP, tissue inhibitor of MMP; MCP, monocyte chemoattractant protein; NSF, normal synovial fibroblast; HDF, healthy dermal fibroblast.

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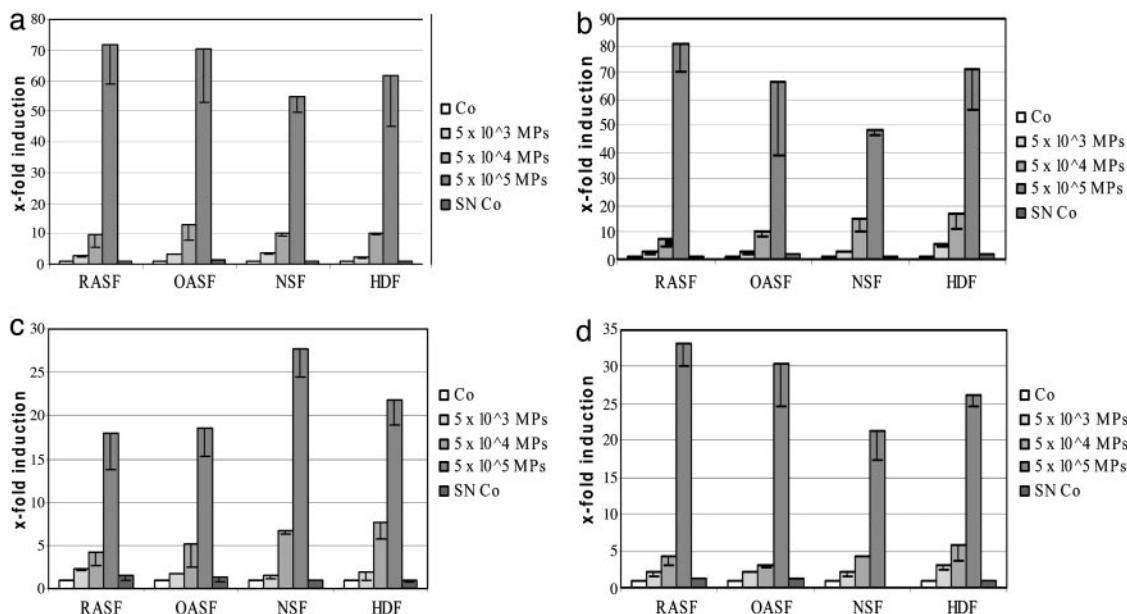


Fig. 2. Up-regulation of MMP-1 (a), MMP-3 (b), MMP-9 (c), and MMP-13 (d) mRNA in fibroblasts by microparticles from Jurkat cells. Cultured RASf, OASf, NSF, and HDFs were stimulated with increasing amounts of microparticles for 36 h. A strong induction of MMP-1, MMP-3, MMP-9, and MMP-13 was observed in all types of fibroblasts. Co, control; SN Co, supernatant control (see *The Effects of Microparticles from Apoptotic and Activated Jurkat Cells on the Induction of MMP mRNA in Synovial Fibroblasts*).

The Effects of Microparticles from Apoptotic and Activated Jurkat Cells on the Induction of MMP mRNA in Synovial Fibroblasts. A strong induction of MMP-1 mRNA was observed in RASf after stimulation with microparticles derived from staurosporine-stimulated Jurkat cells. As compared with buffer controls, incubation with microparticles increased the levels of MMP-1 mRNA up to 71.5 ± 12.5 -fold (Fig. 2a) with the strongest effects seen with doses of 5.0×10^4 and 5.0×10^5 microparticles, whereas smaller effects were seen with lower doses. Similar results were obtained with microparticles from Jurkat cells, which were treated with other stimuli for apoptosis, including TNF α , Fas ligand, and actinomycin-D.

As an additional control, supernatants of the first ultracentrifugation step were processed in the same way as the microparticle-pellets with two washing steps. Thus, although not containing microparticles, these control supernatants had the same concentration of soluble mediators as the microparticle suspensions. However, no induction of MMP-1 was observed with these controls (SN-Co in Fig. 2a).

The effects of microparticles from activated Jurkat cells on RASf were also investigated. Similar to the results with microparticles from apoptotic cells, an induction of MMP-1 mRNA of 55.2 ± 10.2 -fold was observed after incubation with 5.0×10^5 microparticles from Jurkat cells activated with Con A. MMP-3, MMP-9, and MMP-13 mRNAs were also strongly up-regulated by microparticles from both apoptotic and activated Jurkat cells (Fig. 2b–d). With 5.0×10^5 microparticles from staurosporine-treated Jurkat cells, inductions of 80.3 ± 10.0 -fold for MMP-3, 17.9 ± 4.2 -fold for MMP-9, and 36.8 ± 2.4 -fold for MMP-13 were detected (Figs. 2b–d). The same number of microparticles derived from Con A-stimulated Jurkat cells up-regulated MMP-3 by 52.1 ± 7.6 -fold, MMP-9 by 24.8 ± 3.6 -fold, and MMP-13 by 24.1 ± 3.5 -fold.

Of note, the induction was selective for the aforementioned MMPs, because no induction was seen for other matrix-degrading enzymes including MMP-2, MMP-14, and cathepsin K. Likewise, microparticles failed to activate the expression of tissue inhibitor of MMP-1 (TIMP-1), TIMP-2, and TIMP-3 (data

not shown). Furthermore, the effects of microparticles on the increase of MMPs were independent of the origin of the fibroblasts. When OASf, NSF, and healthy dermal fibroblasts (HDF) were stimulated with microparticles from apoptotic or activated Jurkat cells, MMP-1, MMP-3, MMP-9, and MMP-13 were induced in the same range as found for RASf (Fig. 2). However, the induction of MMPs seems to be specific for fibroblasts, because no significant effects were observed with MCF-7 and BT 549 breast cancer cells.

The Effect of Microparticles on Levels of Active MMP Proteins. To measure the biologically active form of MMP proteins, MMP Biotrak Activity Assays were used. Consistent with the findings on the mRNA levels, strong increases of active MMP-1 and MMP-3 protein were observed (Fig. 6, which is published as supporting information on the PNAS web site).

Stimulation of Cytokines by Microparticles. Microparticles derived from Jurkat cells stimulated with Con A dose-dependently induced the synthesis of IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, and MCP-2 (Fig. 3). Microparticles from apoptotic Jurkat cells treated with staurosporine, TNF α , Fas ligand, or actinomycin-D induced IL-6, IL-8, MCP-1, and MCP-2 in RASf to a similar extent. Consistent with the findings for MMPs, there were no differences between RASf, OASf, NSF, and dermal fibroblasts (Figs. 3).

Stimulation of MMPs and Cytokines by Monocyte-Derived Microparticles. Because monocytes are a major component of the synovium, we tested whether monocyte microparticles can activate synovial fibroblasts. The induction of MMPs in fibroblasts was also observed with microparticles derived from the U937 monocytic cell line. Similar to results with T cell-derived microparticles, the induction of MMP-1 reached a 44.5 ± 6.4 -fold induction (Fig. 7a, which is published as supporting information on the PNAS web site). Microparticles from U937 monocytes also strongly induced MMP-3, MMP-9, and MMP-13 (Fig. 7

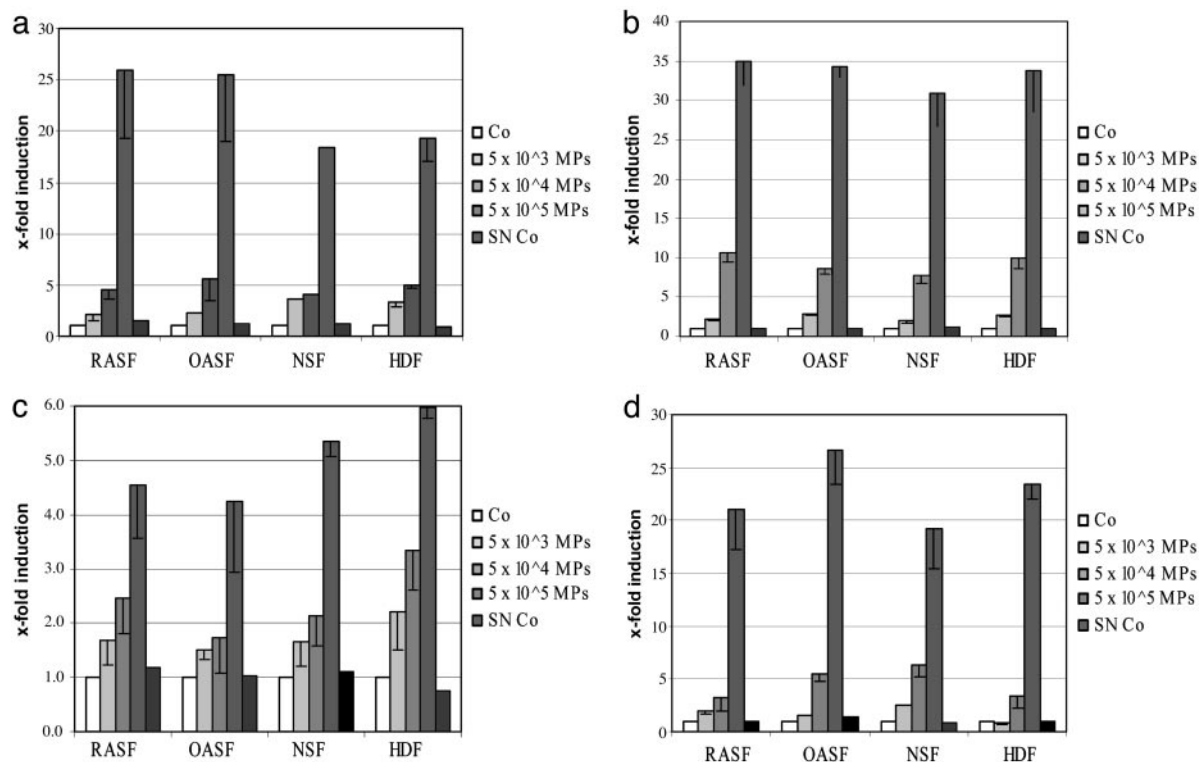


Fig. 3. Induction of IL-6 (a), IL-8 (b), MCP-1 (c), and MCP-2 (d) by microparticles derived from Jurkat cells. These proinflammatory cytokines were up-regulated by microparticles. Comparable results were obtained with RASf, OASf, NSF, and HDF. Co, control; SN Co, supernatant control.

b–d), whereas the levels of TIMP-1, TIMP-2, and TIMP-3 remained unchanged.

Like T cell microparticles, microparticles derived from U937 monocytes up-regulated IL-6, IL-8, MCP-1, and MCP-2 (Fig. 7 *e–h*). As observed with microparticles derived from Jurkat cells, similar results were obtained when OASf, NSF, and dermal fibroblasts were incubated with U937 microparticles. The induction of cytokines by microparticles from stimulated monocytes in synovial fibroblasts was confirmed on the protein level by ELISA (data not shown).

Stimulation of MMPs and Cytokines by Microparticles from Primary CD3- and CD14-Positive Cells. Microparticles from primary T cells and monocytes isolated from peripheral blood induced MMP-1, MMP-3, MMP-9 and MMP-13 was observed. IL-6, IL-8, MCP-1, and MCP-2 were also up-regulated by microparticles from primary cells (Fig. 8, which is published as supporting information on the PNAS web site).

Time Dependence of Stimulation of Cytokines and MMPs with Microparticles. After cocultivation for 6 h, there was only a weak increase of MMPs in synovial fibroblasts compared with the inductions observed after 36 h (Fig. 9*a*, which is published as supporting information on the PNAS web site). Similar results were obtained for MMP-3, MMP-9, and MMP-13. In contrast, the induction of cytokines after 6 h was comparable with that after 36 h (Fig. 9*b*).

The Effects of Antibodies Against TNF α and IL-1 Receptor Antagonist on the Activation of Fibroblasts by Microparticles. Neutralizing anti-TNF α did not reduce the induction of MMPs by microparticles. These antibodies abrogated completely the stimulatory effects of recombinant TNF α on MMPs in synovial fibroblasts, however, confirming their ability to block TNF signaling in our experimental setting.

In coinubation experiments of fibroblasts with microparticles and IL-1Ra, we observed a lower induction of MMP-1 than with microparticles alone (Fig. 10*a*, which is published as supporting information on the PNAS web site). The mean induction in the presence of IL-1Ra was 55.4 ± 7.3 -fold compared with 82.7 ± 12.3 -fold (mean reduction of 33%). This reduction could be explained by an effect on endogenous IL-1, produced by cultured RA synovial fibroblasts, on the synthesis of MMP-1. When IL-1Ra was added to cultured fibroblasts in the absence of any recombinant cytokines or microparticles, the production of MMP-1 was decreased by $39.3 \pm 12.2\%$ compared with unstimulated samples. Similar results were obtained for other MMPs and for inflammatory cytokines such as IL-6 (Fig. 10*b*).

The Role of NF- κ B-Dependent Pathways in the Induction of MMPs in Synovial Fibroblasts by Microparticles. The role of NF- κ B in the induction of MMPs by microparticles was analyzed by EMSA (Fig. 4). No signal for the NF- κ B/probe complex was detectable in unstimulated RASf after serum starvation. Sixty minutes after addition of the microparticles, a strong signal for the NF- κ B/probe complex was observed in nuclear extracts from RASf. The specificity of the strong shift for NF- κ B after stimulation was confirmed by the addition of an excess of unlabeled probe (cold probe), which inhibited completely the formation of the NF- κ B/probe complex (Fig. 4).

Because the NF- κ B/probe complex detected by EMSA could be either repressive p50/p50 homodimers or stimulatory p50/p65 heterodimers, we next performed functional experiments by transfecting synovial fibroblasts with an I κ B α M pCMV expression vector. As assessed by transfection with pEGFP-C1, the transfection efficiency of synovial fibroblasts with the nucleofection protocol was $64 \pm 6\%$. In I κ B α M-transfected fibroblasts, the induction of MMP-1 was $64.1 \pm 20.3\%$ lower compared with mock-transfected fibroblasts (Fig. 11, which is published as supporting information on the PNAS web site). The levels of

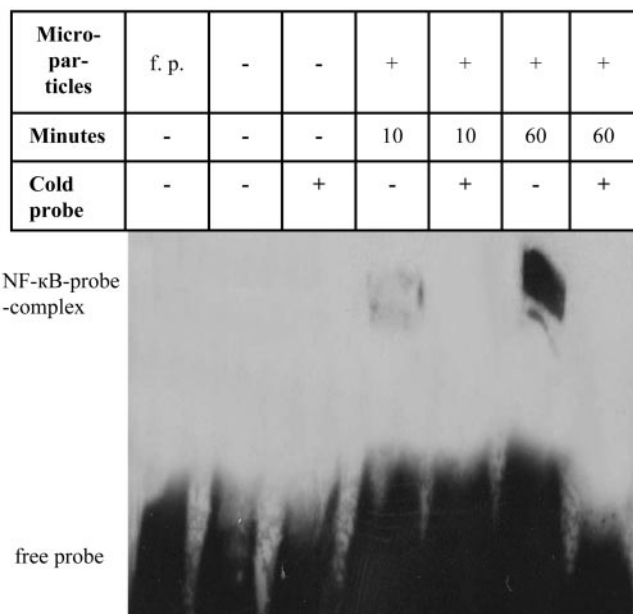


Fig. 4. EMSA demonstrates the activation of NF- κ B in RASF after stimulation with microparticles. Lane 1, free probe without nuclear extracts; lane 2, nuclear extract from unstimulated RASF, with NF- κ B probe; lane 3, nuclear extract from unstimulated RASF, with NF- κ B probe and cold NF- κ B probe; lane 4, nuclear extract from RASF stimulated with microparticles for 10 min, with NF- κ B probe; lane 5, nuclear extract from RASF stimulated with microparticles for 10 min, with NF- κ B probe and cold NF- κ B probe; lane 6, nuclear extract from RASF stimulated with microparticles for 60 min, with NF- κ B probe; lane 7, nuclear extract from RASF stimulated with microparticles for 60 min, with NF- κ B probe and cold NF- κ B probe. Signals for NF- κ B–DNA complexes in lane 4 and especially lane 6 confirm an activation of the transcription factor NF- κ B. No activation of NF- κ B signaling was observed in nonstimulated, serum-starved fibroblasts (lanes 2 and 3). The signal of the NF- κ B–DNA complexes disappeared completely after adding an excess of unlabeled cold NF- κ B probe (lanes 5 and 7).

MMP-3, MMP-9, and MMP-13 were also significantly reduced in I κ B α M-transfected fibroblasts (Fig. 11). These results suggest that NF- κ B accumulates in the nucleus of fibroblasts stimulated with microparticles and activates the transcription of MMP genes.

Discussion

The results of these studies demonstrate a pathway by which immune cell activation or death can modulate production of MMPs by RASF and promote the destruction of cartilage and bone in RA. MMPs are secreted in high levels by RASF and are thought to be key players in this process (2). Although almost all members of the MMP family have been detected in the RA synovium, MMP-1, MMP-3, and MMP-13 seem to be the most important for joint destruction (3, 14). In studies on RA and other fibroblast types presented herein, we show an induction of MMPs by T cell and monocyte microparticles, with massive up-regulation as high as 80-fold compared with controls. Importantly, the induced MMP proteins were biologically active as indicated by assays of enzymatic activity. In view of the dramatic up-regulation of MMP expression induced by the microparticles, these results suggest that microparticles released from immune cells could represent an important signaling element to trigger the destructive phenotype of RASF.

Immune cells, including T cells and macrophages, occur abundantly in synovium and synovial fluid of patients with RA and therefore could serve as a source of microparticles, whether induced by apoptosis or activation. In view of the

abundance of these cells and their capacity to release microparticles, microparticles could serve as important triggering elements in RA to promote RASF activation. Evidence supporting this hypothesis comes from observations that activated T cells and macrophages release microparticles and that these microparticles induce the release of cytokines and chemokines, namely IL-6, IL-8, MCP-1, and MCP-2, from synovial fibroblasts. Importantly, these cytokines, which have all been linked to RA, have been demonstrated to attract leukocytes, thereby increasing the number of inflammatory cells in the joint (15, 16). Furthermore, results from the inhibition studies indicate that the effects of microparticles on synovial fibroblasts are independent of TNF α and IL-1 and may thus not be targeted by therapies directed against these cytokines.

To assess the pathways by which microparticles can activate RASF, we explored the role of NF- κ B. NF- κ B is a key regulator of proinflammatory mediator expression and plays a critical role in the induction of MMPs (17). NF- κ B is also overexpressed in the inflamed synovium, especially in the intimal lining layer of RA patients, where it colocalizes with the expression of MMPs. Our results with the I κ B expression vector and EMSA suggest that NF- κ B also plays an important role in the induction of MMPs by microparticles in synovial fibroblasts. Activation of NF- κ B is also consistent with the pattern of cytokine induction observed.

As shown by this and other studies (8, 9), microparticles may amplify as well as induce pathways of activation and destruction, occupying a place between direct cell–cell interaction and triggering by protein and small molecule mediators. Extensive work has been done during the past years on the identification and characterization of soluble mediators such as proinflammatory cytokines in rheumatoid arthritis (18). Similar to microparticles, resting T cells induce stromelysin, IL-6, and IL-8 in coculture with synovial fibroblasts (19), and plasma membrane preparations from stimulated T cells can induce the production of MMP-1 in synovial fibroblasts (20). The factors involved in these cell contact-mediated activation of synovial fibroblasts are not well defined (21), but membrane-bound surface IL-15 on synovial fibroblasts seems to be crucial for the initiation of the synovial fibroblast–T lymphocyte cross-talk by up-regulating CD69, CD25, IL-17, and TNF α in T cells that in turn induces secretion of IL-6 and IL-8 in synovial fibroblasts (22). However, in contrast to microparticles, this concept requires an intact T cell metabolism. Moreover, the cell contact-mediated effects are strongly dependent on cell membrane-associated IL-1 α and TNF α (20). Other differences between microparticles and membrane preparations include for instance the morphology of microparticles as circular round vesicles. Because microparticles occur in blood, they could act at distant sites as well as locally. Notably, microparticles may also function as a transporter system for surface receptors, rendering the target cells susceptible for new, receptor-specific stimuli (23).

The specific factors that mediate the strong effects of microparticles on synovial fibroblasts are at this point speculative. The lack of effects in breast cancer tumor cells indicates that specific features of the target cells play an important role. Microparticles display surface molecules from their parental cell that could bind to mesenchymal cells and trigger activation. In addition, microparticle contents such as histones and DNA could contribute to stimulation, with the outcome depending on the cell of origin and the specific content of both internal and external molecules. In this regard, microparticles derived from T cells as well as macrophages, two key cell types in RA, were both active, although microparticles from other cell types may differ in properties.

In conclusion, our study provides evidence for a mechanism for the interaction of inflammatory cells and synovial fibro-

