A three-dimensional model to study human synovial pathology

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Abstract
Therapeutic agents that are used by patients with rheumatic and musculoskeletal diseases were originally developed and tested in animal models, and although retrospective studies show a limited predictive value, it could be explained by the fact that there are no good in vitro alternatives. In this study, we developed a 3-dimensional synovial membrane model made of either human primary synovial cell suspensions or a mix of primary fibroblast-like synoviocytes and CD14+ mononuclear cells. We analyzed the composition of the mature micromasses by immunohistochemical staining and flow cytometry and show that the outer surface forms a lining layer consisting out of fibroblast-like and macrophage-like cells, reflecting the in vivo native synovial membrane. To recreate the affected synovial membrane in rheumatoid arthritis (RA), the micromasses were exposed to the pro-inflammatory cytokine Tumor Necrosis Factor Alpha (TNF-α). This led to increased pro-inflammatory cytokine expression and production and to hyperplasia of the membrane. To recreate the synovial membrane in osteoarthritis (OA), the micromasses were exposed to Transforming Growth Factor Beta (TGF-β). This led to fibrosis-like changes of the membrane, including increased Alpha Smooth Muscle Actin and increased expression of fibrosis-related genes PLOD2 and COL1A1. Interestingly, the macrophages in the micromass showed phenotypic plasticity as prolonged TNF-α or TGF-β-stimulation strongly reduced the occurrence of Cluster of Differentiation 163-positive M2-like macrophages. We showed the plasticity of the micromasses as a synovial model for studying RA and OA pathology and propose that the synovial lining micromass system can be a good alternative for testing drugs.

1 Introduction

Rheumatoid arthritis (RA) and osteoarthritis (OA) are the most common rheumatic diseases. The global incidence of RA is estimated to be 0.5%-1% (Gabriel and Michaud, 2009) and an estimated 12.1% of the adult population has clinical symptoms of OA (Lawrence et al., 2008). RA and OA are both viewed as multi-factorial diseases involving multiple risk factors (Alamanos and Drosos, 2005, Blagojevic et al., 2010) but their exact etiology is unknown. Both diseases cannot be cured at present, but disease-modifying drugs (DMARDs) are available for RA and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for OA.

During the abovementioned rheumatic diseases, many processes occur in the synovium. Already in the early phase of RA, hyperplasia of the synovial lining occurs and immune cells infiltrate the sublining (Timmer et al., 2007, Hitchon and El-Gabalawy, 2011). The cells in the synovium can produce several pro-inflammatory cytokines and matrix-degrading enzymes. At the interface of the synovium, cartilage and bone, pannus tissue is formed that contributes to bone destruction. An important mediator of these processes is Tumor Necrosis Factor Alpha (TNFα) (Vasanthy et al., 2007). Specific inhibitors of TNF-α are the most widely used biological therapies and can successfully inhibit the progress of RA in the majority of patients (Mewar and Wilson, 2011).

Synovial pathology is also described for OA. Although OA has traditionally been described as a wear-and-tear disease of the articular cartilage, synovitis is observed in up to 50% of the OA patients and might contribute to the disease process (Scanzello and Goldring, 2012). A second factor that contributes to the synovial thickening in OA is tissue fibrosis (Wenham and Conaghan, 2010). Synovial fibrosis is also associated with joint pain and stiffness (Remst et al., 2015). Although the pathogenesis of OA is poorly understood, the cytokine most critically associated with processes occurring in OA is Transforming Growth Factor Beta (TGF-β) (Fang et al., 2016). TGF-β is pivotal for the development and maintenance of the...
articular cartilage, but excessive signaling can have detrimental results. For example, adenoviral overexpression of TGF-β in mice resulted in osteophyte formation (Blaney Davidson et al., 2007). Moreover, TGF-β stimulates the differentiation of myofibroblasts and the expression of collagen type 1 that contribute to synovial fibrosis (Verrecchia and Mauviel, 2007; Remst et al., 2013).

Many experiments have been performed in animal models of RA and OA to study the initial stages of inflammation, hyperplasia and fibrosis in the synovium. However, the predictive value of animal models for the screening of potential drugs in RA is variable and for OA, many therapies have been successful in treating experimental OA, but no treatment has been sufficiently effective in clinical trials (Malfait and Little, 2015; McNamee et al., 2015). In addition, the translation of these findings to the human synovium is difficult because there is a lack of relevant human in vitro 3D models to simulate synovial pathology, including hyperplasia and synovial fibrosis.

Translational arthritis research with human cells is often performed in monolayer culture with in vitro expanded synovial fibroblasts. Although monolayer culture with fibroblasts is easy to perform, the absence of extracellular matrix and other cell types results in alterations of cell functioning and a rapid loss of phenotype (Zimmermann et al., 2001). One way to maintain the cell composition and matrix interactions of the cells is by using synovial explants instead of cultured monolayer cells. Although synovial explants better represent the original joint environment for the cells, important disease processes including hyperplasia and cartilage damage may have already taken place and can thus not be studied using explants. Moreover, there is considerable variability between biopsies from a single joint, resulting from a variation in lining thickness (Smith et al., 2003), sublining composition and an unequal distribution of synoviocyte types in the synovial membrane (Shikichi et al., 1999, Iwanaga et al., 2000).

To circumvent these challenges and provide a relevant model for the synovial membrane in which cells can interact with cellular matrix, Kiener and co-workers have developed a 3-dimensional (3D) micromass model based on a mixture of fibroblast-like synoviocytes (FLS) and Matrigel solution. When kept on ice, the Matrigel was liquid and could be mixed with cells to obtain a homogenous solution. Single drops were pipetted in a culture well and became solid at 37 °C. Within weeks, the FLS formed a lining layer on the Matrigel-medium surface that was reminiscent of a synovial membrane (Kiener et al., 2006). Reticulin fibers on the surface of the micromasses were arranged in an orientation similar to basement membrane structures in synovial biopsies. The FLS in the micromass lining produced lubricin and showed hyperplasia after 3 weeks stimulation with TNF-α (Kiener et al., 2010). The lining formation was not observed when using dermal fibroblasts.

We have recently applied the synovial micromass using the complete cell suspension after biopsy digestion for micromass formation (Broeren et al., 2016). This was performed without further isolation and purification of the synovial fibroblasts to better mimic their cellular composition and interactions as present in the starting material. In this study, we further characterized the synovial micromasses to study the fate of the different cell types. After lining formation, the micromass mainly consisted of synovial fibroblasts and macrophages. We therefore used micromasses produced from primary FLS and macrophages throughout the rest of the study. TNF-α exposure of the synovial micromasses was used to mimic the inflammatory conditions present in RA and OA. This showed that short-term exposure to TNFα already leads to proinflammatory gene expression and long-term exposure to hyperplasia. Long-term exposure to TGF-β resulted in fibrosis-like changes of the micromass lining. In conclusion, these results provide a detailed analysis of the synovial micromasses and show the suitability as a synovial membrane model for translational research in RA and OA. The micromasses may replace animal experiments in which advanced synovial pathology can be studied. An unpublished survey done by us under 20 leading research groups in the arthritis field revealed that 40% is of the opinion that in vitro models may replace animal experimentation entirely and 60% finds it a useful addition.

2 Materials and methods

Patient material
Synovial biopsies from 5 RA patients were obtained during joint replacement surgery from the Orthopedics department of the Sint Maartenskliniek, Nijmegen, the Netherlands. This material was considered surgery surplus material. Patients gave written informed consent for the use of their material for research. The patient material was pseudonymized. There was no need for the approval by an ethical committee. Procedures were performed in accordance to the code of conduct for responsible use of human tissue in medical research1. The presence of a synovial lining was determined on 7 μm cryosections stained with hematoxylin and eosin staining (H&E) to confirm the synovial origin of the tissue (not shown). All patients adhered to the American College of Rheumatology (ACR) criteria and were end-stage RA.

Human CD14+ cells
Peripheral blood mononuclear cells (PBMCs) were obtained from 3 healthy donors by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA). Cluster of Differentiation (CD) 14+ cells were isolated from the PBMC fraction using the MagniSort Human CD14 Positive Selection Kit (Invitrogen, Carlsbad, CA, USA) (purity >90%) according to the manufacturer’s protocol.

Micromass production
Micromasses were produced from both complete synovial cell suspensions from RA synovium as described previously (Broeren et al., 2016) and from a combination of primary RA fibroblast-like synoviocytes (FLS) after several passages and CD14+ monocytes. In short, for micromasses from complete cell suspensions, the biopsies were digested using Liberase (Roche,

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1 https://www.federa.org/code-goed-gebruik
Basel, Switzerland) for 1h at 37 °C and the cells were filtered through a 70 μm cell strainer (Corning, NY, USA). Red blood cells (RBCs) were lysed for 2 min at RT in 4 ml RBC lysis buffer (155 mM NaCl, 12 mM KHCO₃, 0.1 mM EDTA, pH 7.3). The cells were mixed on ice with liquid Matrigel and 25 μl droplets containing ~5x10³ cells were pipetted on a poly-2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma-Aldrich, Zwijndrecht, The Netherlands) coated 24-wells plate (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Alternatively, micromasses were produced from 2x10⁵ FLS combined with 10⁶ CD14⁺ mononuclear cells. FLS were obtained after biopsy digestion and RBC lysis by culturing the adhering cell fraction and passing the cells at least once. After 30 min gelation at 37 °C, 500 μl Roswell Park Memorial Institute (RPMI) medium (Gibco, Thermofisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (v/v) (Gibco, Thermofisher Scientific), 1 mM pyruvate (Thermofisher Scientific) and 1% penicillin/streptomycin (P/S) (v/v) (Westburg, Leusden, the Netherlands) was added. The micromasses were cultured for the time and conditions as indicated in the separate Figures. In general, medium was replaced twice weekly and micromasses were cultured at 37 °C and 5% CO₂.

Flow cytometry
Micromasses were kept on ice for 2h and the cells were washed to remove the liquid Matrigel. Unspecific binding of antibodies by Fc receptors was blocked by incubation with Fc block (1:100 for 20 minutes at 4 °C) (564220 BD Biosciences, San Jose, CA, USA). For all cell suspensions, CD90 was stained using mouse anti-human CD90-APC (1:20 for 30 minutes at 4 °C); CD68 was stained using mouse anti-human CD68-PE (1:20 for 60 minutes at 4 °C); CD14 was stained using mouse anti-human CD14-APC (1:20 for 60 minutes at 4 °C). The samples were measured on the CyAn ADP analyzer (Beckman Coulter, Woerden, the Netherlands) using the 488 nm laser at the FL7 and FL8 channels for Alexa Fluor 568/PE and APC respectively. Gates were determined using Fluorescence minus one (FMO). A gating strategy and unsatisfied controls are shown in Figure S1. A comprehensive overview of all antibodies used in this study is shown in Table 1.

Immunohistochemistry
Micromasses were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS)/1 mM CaCl₂ for 2h, dehydrated and embedded in paraffin. 7 μm sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) or processed for immunohistochemistry (IHC). For IHC, endogenous peroxidase activity was block with 3% H₂O₂ (Merck Millipore, Amsterdam, the Netherlands) in methanol and antigen retrieval was performed in 10 mM citrate buffer pH 6.0 at 60 °C. Subsequently, sections were stained with primary antibodies: mouse anti-human 11-Fibrau (1:100 for 60 minutes at room temperature (RT)), mouse anti-human CD68 (1:100 for 60 minutes at RT), mouse anti-human CD163 (1:25 for 60 minutes at RT), rabbit anti-human Alpha Smooth Muscle Actin (α-SMA) (1:400 overnight at 4 °C), or rabbit anti-human Ki67 (1:100 for 60 minutes at RT). Isotype control antibodies were used at the same concentrations as the primary antibodies. Subsequently, the primary antibodies for CD68 were stained with HRP-conjugated rabbit-anti-mouse IgA/G/M (1:200 for 60 minutes at RT), antibodies for 11-Fibrau and CD163 were stained with biotinylated anti-mouse IgG H+L (1:100 for 30 minutes at RT and antibodies for α-SMA and Ki67 were stained with biotinylated anti-rabbit (1:400 for 30 minutes at RT) For 11-Fibrau, CD163 and α-SMA, a biotin-streptavidin detection system was used according to manufacturer’s protocol (Vector Laboratories). Peroxidase was developed with diaminobenzidine and counterstained with hematoxylin for 60 seconds. Representative pictures of all control IgGs are shown in Figure S3. A comprehensive overview of all antibodies used in this study is shown in Table 1.

Image analysis
Microscopic images for histology were taken using the VS120 slide scanner (Olympus, Leiderdorp, the Netherlands) using Panoramic Viewer (3DHISTECH, Budapest, Hungary) and for quantification, pictures were taken using the Leica DMR light microscope after randomization and blinding of the samples. For hyperplasia, Ki67 and fibrosis analysis, 3 sections were analyzed per micromass and per section up to 5 pictures were taken from the micromass lining after H&E-staining, Ki67 staining or α-SMA staining respectively. For CD163 analysis, pictures from the complete micromass matrix were taken. Images were analyzed using the Leica Application Suite (LAS) software.

Gene expression analysis
The micromasses were dissolved in 500 μl Tri reagent (Sigma-Aldrich) and total RNA was isolated according to the manufacturer’s protocol. qPCR was performed as previously described (Vermeej et al., 2015). Primer sequences are listed in Table 2. Values are depicted as threshold cycle, corrected for GAPDH expression.

Multiplex ELISA
Cytokines and chemokines in supernatants were measured on a Bio-Plex 200 system using a magnetic bead-based multiplex immunoassay. Data analysis was performed with Bio-Plex Manager software (both Bio-Rad).

Statistical analysis
The results are displayed as box plot (median) with whiskers: min to max. Statistical analysis was performed by Mann-Whitney U test using Graphpad Prism software v5.03. p-values below 0.05 were regarded as significant.

2 doi:10.14573/altex.1804161s
Tab. 1: List of antibodies

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<th>Antibody</th>
<th>Iso-type</th>
<th>Conjugate</th>
<th>Monoclonal/Polyclonal</th>
<th>Stock Concentration</th>
<th>Dilution</th>
<th>Application</th>
<th>Company</th>
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APC = allophycocyanin
FC = flow cytometry
HRP = horseradish peroxidase
IHC = immunohistochemistry
N/A = not applicable
PE = phycoerythrin
* similar concentration (not dilution) to primary antibody directed to antigen of interest

Tab. 2: Primer sequences

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3 Results

3.1 Analysis of micromass composition and formation
The composition of the cell suspension after digestion of a synovial biopsy was determined using cell surface markers. In the cell suspension prior to micromass formation, we observed cells positive for fibroblast marker CD90 (67.6%), monocyte/macrophage marker CD68 (23.1%) and monocyte marker CD14 (CD14low 22.9% and CD14high 4.58%) (Fig. 1A, Fig. S1). This cell composition was comparable in repeat experiments. No T cells (CD3), B cells (CD20) or dendritic cells were detected.
Fig. 1: Flow cytometry and histological analysis of synovial cell suspension and micromasses
(A) A RA synovial biopsy was digested using Liberase. Cells were cultured for <24 hours (Figure 1A, day 0) or mixed with Matrigel and cultured for 7 days, after which the Matrigel was re-liquified on ice (Figure 1A, day 7). The cell suspensions were subsequently stained for fibroblasts (CD90), macrophages (CD68) and monocytes (CD14). Similar profiles were obtained in at least 3 experiments with unique donors. FS = forward scatter, SS = side scatter. The gating strategy and unstained controls are shown in Fig. S1². (B) At different time points (day 0, 4, 5, 7, 14 and 35) micromasses were fixed and embedded in paraffin. 7 µm sections were stained with H&E, anti-CD68 or antibodies against fibroblast marker 11-Fibrau. Pictures were taken at 200x magnification. Negative control IgG stainings are shown in Fig. S3².
A significant increase in expression of IL6 and IL8 with both stimuli (Fig. 3A). TNF and IL1B expression were not significantly increased. On protein level, IL-6 levels in the micromass supernatants were increased after stimulation with both TNF-α and TGF-β (Fig. 3B). IL-8 was only increased after stimulation with TNF-α and TGF-β stimulation could induce the release of TNF-α from the micromasses. These results show that the micromasses are sensitive to inflammatory triggers.
Long-term TNFα exposure of synovial micromasses leads to hyperplasia of the micromass lining and an altered macrophage phenotype

In addition to pro-inflammatory effects, we observed striking differences in the cells residing in the micromass matrix after long-term stimulation with TNF-α. When the micromasses were not treated with TNF-α, some cells positive for CD68 throughout the complete micromass showed a hypertrophic phenotype (Fig. 4A). After stimulation with TNF-α, there was a strong reduction in this phenotype and instead, cells showed a spindle-like morphology. The large round cells were positively stained for CD163, a membrane marker associated with type 2 macrophages (Fig. 4B) (Kowal et al. 2011). The appearance of these cells throughout the complete micromass could be abolished by both TNF-α and TGF-β stimulation (Fig. 4E).

An additional important feature of arthritis is synovial hyperplasia, which was also assessed after 3 weeks of repeated stimulation. Without TNF-α stimulation, the lining thickness was on average 1 cell layer (Fig. 4C). After long-term stimulation with TNF-α, the lining thickness increased. The hyperplasia was assessed by quantifying the amount of hematoxylin staining and by the proliferation marker Ki67 in the lining >9 images per micromass. The cellularity and proliferation of the lining was significantly increased after TNF-α stimulation (Fig. 4D, F, G) whereas this was unaltered by TGF-β stimulation.

TGF-β can induce fibrosis-like changes in the micromass lining

The effects of 3-week stimulation on micromass fibrosis-associated processes were first assessed on gene expression level. TGF-β signaling marker SERPINE1 was significantly induced by recombinant TGF-β, indicating that active TGF-β signaling was induced in the micromasses (Fig. 5A). This resulted in an increased expression of PLOD2, Alpha Smooth Muscle Actin (ACTA2) and collagen type 1 (COL1A1), which are all associated with fibrosis (Remst et al., 2014). Histological staining of α-SMA showed that fibrotic-like changes had occurred in the micromass lining, which was significantly increased after stimulation with TGF-β (Fig. 5B,C). Moreover, TGF-β induced a stellate-cell morphology of α-SMA-positive cells, characteristic for myofibroblasts (Fig. 5B) (Bagalad et al., 2017).

4 Discussion

In this study, we further characterized the 3D synovial micromass model produced from primary human biopsies for application in both RA and OA research as an alternative to animal experiments. The model was first explored by Kiener and co-workers, based on primary synovial fibroblasts (FLS) culture. The experiments were focused on cell-cell and cell-matrix interactions during the formation of the lining and the involvement of Cadherin-11 (Kiener et al., 2006; Lee et al., 2007). We adapted a procedure that was briefly explored in these studies to produce micromasses with both FLS and CD14+ PBMCs, which resulted in a lining that included 11-Fibrau-positive fibroblasts and CD68-positive macrophages (Kiener et al., 2010). These 2 cell types were the predominant lining cell types in the micromasses produced from the complete synovial cell suspension and correspond with the cell types observed in the intimal layer of the synovium, the type B synoviocyte (FLS) and type A synoviocyte (macrophage-like synoviocyte), respectively (Smith, 2011). The survival of the macrophage-like cells has previously been shown to be dependent on the presence of synovial fibroblasts (Kiener et al., 2010). The inclusion of these cells in the micromass model is important, because the macrophages produce large amounts of TNF-α and TGF-β and can activate latent TGF-β (Wahl et al., 1990; Kinne et al., 2000). Moreover, macrophages are pivotal for bone erosion, cartilage damage and ectopic bone formation in RA and OA (Goldring and Gravallese, 2000; Blom et al., 2004, Bondeson et al., 2006).
Fig. 4: Micromass lining hyperplasia and macrophage phenotype after long-term stimulation

Micromasses were generated from primary RA FLS and CD14+ PBMCs and stimulated for 3 weeks with 10 ng/ml recombinant human TNF-α or TGF-β, which was replaced 2 times per week. Control micromasses received culture medium without stimulation. Micromasses were stained for CD68 (A), CD163 (B) with H&E (C) and (D) Ki67. Black arrows indicate cells positive for the marker of interest. Grey arrow indicate a hyperplasic lining. (E) Quantification of CD163-positive cells throughout the entire micromass. Values were corrected for the total surface area of the images analyzed and are depicted as fold change compared to the average of the unstimulated controls. (F) Quantification of H&E staining for lining hyperplasia. (G) Quantification of Ki67 staining for proliferation of the micromass lining. n= 5/6 per group and the average score of >3 images per 3 sections of every micromass was calculated. Statistical analysis was performed by Mann-Whitney U test comparing to medium condition. * =p<0.05, ** =p<0.01. Negative control IgG stainings are shown in Fig. S3.
Fig. 5: Fibrosis-like changes after long-term stimulation of micromasses

Micromasses were generated from primary RA FLS and CD14+ PBMCs and stimulated for 3 weeks with 10 ng/ml recombinant human TNF-α or TGF-β, which was replaced 2 times per week. Control micromasses received culture medium without stimulation.

(A) qPCR analysis of downstream TGF-β signaling genes and fibrosis-related genes. Gene expression values were corrected for GAPDH expression and are depicted as ΔCt.

(B) Staining of Alpha Smooth Muscle Actin (α-SMA) in the micromasses. Magnification 200x.

(C) Digital quantification of α-SMA-positive lining surface. Values are depicted as a fold change compared to unstimulated micromasses. n=5/6 per group. Statistical analysis was performed by Mann-Whitney U test comparing to medium condition. * =p<0.05, ** =p<0.01. Negative control IgG stainings are shown in Fig. S3.

For the micromasses that were generated from primary CD14+ PBMCs and primary FLS, FLS were first cultured in vitro. It has been observed that directly after digestion of the synovium, the adherent fraction also contains adherent synovial macrophages and the FLS have a disease phenotype, which includes the spontaneous production of pro-inflammatory cytokines (Firestein, 1996). However, after prolonged culture the disease imprint is gradually lost (Zimmermann et al., 2001; Hirth et al., 2002; Hardy et al., 2013), making the FLS more suitable for the micromass model in which part of the disease is mimicked by stimulation with TNF-α or TGF-β. The micromass model is therefore not dependent on the original disease phenotype of the fibroblasts. This fits with the observation that without stimulation with exogenous TNF-α, no spontaneous hyperplasia was observed.

The survival of FLS and macrophage-like cells was observed both by immunohistochemistry and flow cytometry. Several results imply changes in the macrophage population. During micromass culture, the amount of CD68+ cells expressing CD14 decreased at day 7 compared to day 0. This can be the result of macrophage differentiation, which is associated with a decrease in CD14 expression (Ohradanova-Repic et al., 2016). During the 3-week micromass culture, we observed large CD68+ and CD163+ cells. These cells represented an M2-like anti-inflammatory macrophage phenotype (Vandooren et al. 2009; Kowal...
et al., 2011). The appearance of these cells was abolished by stimulation with TNF-α, which is known to polarize the macrophages to a pro-inflammatory M1-like phenotype (Kennedy et al., 2011). We also observed a significant down-regulation of CD163 by TGF-β, which has also been described previously (Pliotis et al., 2004). Although additional markers are required for more accurate and reliable identification of specific cell types and cell subsets, the results clearly indicate a plasticity in the macrophage-like cell population in the synovial micromass.

Interestingly, we found that when kept on ice, the Matrigel liquidized and could be washed away from the cells. The resulting cell suspension could be analyzed by flow cytometry without the requirement of enzymatic digestion, which further enhances the applicability of the micromass model.

We evaluated the long-term effects of TNF-α exposure on micromasses. In addition to the well-known role of TNF-α in RA, the TNF-α produced by the osteoarthritic synovium is strongly associated with systemic low-grade inflammation and production of matrix-degrading enzymes by multiple cells in the joint (Bondeson et al., 2006; Ozler et al., 2016). The observation that systemic TNF-α levels are increased prior to the most pronounced cartilage damage supports the hypothesis that inflammation in OA is a cause rather than a consequence. We observed that the pro-inflammatory effects of TNF-α can be studied in the micromasses. In addition, synovial hyperplasia is observed in the synovium of both RA and OA patients (Bondeson et al., 2010). We could re-create this pathogenic process by stimulating the micromasses for 3 weeks with TNF-α, which is a major advantage compared to studies using synovial biopsies, in which hyperplasia has already occurred (Izquierdo et al., 2011).

Although both pro- and anti-inflammatory functions of TGF-β have been described for arthritic diseases, we observed a strong increase in expression of IL-6 and TNF-α after stimulation with TGF-β. IL-8 mRNA was significantly increased by TGF-β, but not IL-8 protein levels. The effects of TGF-β on IL-6 and IL-8 have been described before in PBMCs and synovial fibroblasts (Turner et al., 1990; Cheon et al., 2002). Interestingly, TGF-β stimulation resulted in increased TNF-α secretion, but not on mRNA level. Previous experiments with TGF-β and synovial cell cultures did not result in increased TNF-α production, which indicates that our finding of increased TNF-α might be an indirect effect (Brennan et al., 1990). The most pronounced effect of long-term TGF-β stimulation was the strong increase of an α-SMA-positive lining, which was not observed after stimulation with TNF-α. The increased α-SMA was not caused by hyperplasia, which did not occur after TGF-β stimulation.

The joint is considered a complex organ and joint diseases like RA and OA are thought to involve several cell types and tissues, including the synovium, but also the articular cartilage and (subchondral) bone (Mathiesen and Conaghan, 2017). These tissues are also dependent on the extracellular matrix for proper interactions and several in vitro studies have aimed at recreating 3D matrix mixed with specific cells. For example chondrocytes can be co-suspended with gelatin microspheres in an alginate solution to obtain an articular cartilage-like structure (Su, Lau et al., 2012). This can be cultured in combination with FLS and in vitro differentiated macrophages to mimic OA cartilage pathology (Peck et al., 2018). In addition, FLS have been cultured in several scaffolds to generate 3D structures. In one study, FLS were combined with endothelial cells in a methylcellulose sphere which was subsequently incorporated in a collagen gel (Maracle et al., 2017). This method was used to study angiogenesis into a collagen scaffold. These studies highlight the added value of 3D models for joint tissues and the micromass adds a model to study the synovial membrane.

We propose that the synovial micromass model can be a solution for experiments that require a synovial membrane in which disease processes have not yet occurred, since the availability of healthy synovial tissue is very limited. The production of micromasses will result in a uniform composition of the starting material, which can also be easily manipulated in sense of composition. In future applications, the micromasses may be co-cultured with different cell types or scaffolds, or in co-culture with cartilage explants to study effects on cartilage degeneration. Although no 3D models are available yet that include all cell types and tissues involved, the micromass model can be a good alternative for animal experiments in RA and OA, both for fundamental research, target validation and pre-clinical treatment testing. To our knowledge, this is the first human 3D culture model to study the interplay between macrophages and fibroblast-like synoviocytes in hyperplasia and synovial fibrosis-like pathology by stimulation with TNF-α and TGF-β, respectively.

References


**Conflicts of interest**

There are no conflicts of interest to report.

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