RHEUMATOLOGY

Original article

Targeting of fibroblast activation protein in rheumatoid arthritis patients: imaging and *ex vivo* photodynamic therapy

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Abstract

Objective. Activated synovial fibroblasts are key effector cells in RA. Selectively depleting these based upon their expression of fibroblast activation protein (FAP) is an attractive therapeutic approach. Here we introduce FAP imaging of inflamed joints using ^{68}Ga-FAPI-04 in a RA patient, and aim to assess feasibility of anti-FAP targeted photodynamic therapy (FAP-tPDT) *ex vivo* using 28H1-IRDye700DX on RA synovial explants.

Methods. Remnant synovial tissue from RA patients was processed into 6 mm biopsies and, from several patients, into primary fibroblast cell cultures. Both were treated using FAP-tPDT. Cell viability was measured in fibroblast cultures and biopsies were evaluated for histological markers of cell damage. Selectivity of the effect of FAP-tPDT was assessed using flow cytometry on primary fibroblasts and co-cultured macrophages. Additionally, one RA patient intravenously received ^{68}Ga-FAPI-04 and was scanned using PET/CT imaging.

Results. In the RA patient, FAPI-04 PET imaging showed high accumulation of the tracer in arthritic joints with very low background signal. *In vitro*, FAP-tPDT induced cell death in primary RA synovial fibroblasts in a light dose-dependent manner. An upregulation of cell damage markers was observed in the synovial biopsies after FAP-tPDT. No significant effects of FAP-tPDT were noted on macrophages after FAP-tPDT of neighbouring fibroblasts.

Conclusion. In this study the feasibility of selective FAP-tPDT in synovium of rheumatoid arthritis patients *ex vivo* is demonstrated. Furthermore, this study provides the first indication that FAP-targeted PET/CT can be used to image arthritic joints, an important step towards application of FAP-tPDT as a targeted locoregional therapy for RA.

Key words: synovial fibroblast, fibroblast activation protein, RA, targeted photodynamic therapy

BASIC SCIENCE

Rheumatology key messsages

- Fibroblast activation protein-targeted photodynamic therapy of arthritic synovium increases cell damage markers.
- Activated fibroblast depletion through photodynamic therapy does not change macrophage polarization.

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Introduction

RA is a chronic autoimmune disease affecting the synovial joints in ∼0.5–1% of the general population [1–3]. Sustained inflammation in these joints causes permanent damage to the cartilage and bone, and is a major burden to these patients. Treatment of RA currently focuses on the systemic suppression of the immune system. This is associated with numerous side effects including an increased risk of infection and cytopenia [4]. Further tailoring the therapy of arthritis patients to locally targeting cells of the inflamed synovial joint could reduce the dose of immunosuppressive drugs and thereby their side effects [5, 6].

In addition to the importance of both innate and adaptive immune cells in disease pathogenesis, an increasing role is attributed to the stromal cells present in the synovial joints [7]. In particular, the synovial fibroblasts (SFs) have been identified as key effector cells in RA [7, 8]. SFs have been shown to adopt a disease-associated phenotype that persists even in the absence of inflammation [9]. This has prompted investigation into their potential as a therapeutic target [10].

Disease-associated SFs have recently been divided into subsets with distinct functions in RA pathogenesis [11]. Expression of podoplanin, Thy-1 (or CD90) and fibroblast activation protein (FAP) is characteristic of activated SFs [11]. In particular, the latter is of interest, since it has been shown to be of prognostic value in distinguishing synovium from patients with reactive arthritis from RA patients [12]. Furthermore, FAP is expressed by activated SFs in both the lining and the sublining layers of the synovium, and depletion of cells expressing FAP, using a transgenic mouse model with diphtheria toxin receptor-mediated conditional deletion of FAP, resulted in decreased arthritis in the serum transfer-induced arthritis model in mice [12].

Recently, we have shown the feasibility of depleting activated SFs through photodynamic therapy (PDT) targeting FAP [13]. Using the anti-FAP antibody 28H1 conjugated to the photosensitizer IRDye700DX, FAP$^+$ fibroblasts were selectively depleted, both *in vitro* in 3T3 fibroblasts transfected to stably express FAP, and *in vivo* in experimental arthritis in mice. This resulted in delayed arthritis development in the murine collagen-induced arthritis model. Here, we aim to build on these explorative studies to bring FAP-targeted PDT (FAP-tPDT) a step closer to clinical application in RA patients, by first demonstrating the presence of FAP$^+$ cells in the arthritic joints of an RA patient using PET/CT, and subsequently investigating the therapeutic potential of FAP-tPDT on patient-derived RA synovial tissue *ex vivo*.

Methods

FAPI-PET/CT imaging

The FAP inhibitor FAPI-04 was a gift from Herr Prof. Dr med. Uwe Haberkorn [University Hospital Heidelberg/ German Cancer Research Center (DKFZ), Germany] and was synthesized as previously described [14]. ^{68}Ga-FAPI-04 is an investigational radiopharmaceutical not yet approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA). It was therefore administered under the conditions outlined in §13(2b) of the Arzneimittelgesetz (AMG; German Medicinal Products Act) and in compliance with the Declaration of Helsinki. One RA patient intravenously received 131 MBq of ^{68}Ga-FAPI-04. At 1 h post injection , a low-dose CT with 120 kV ref. using Siemens (Berlin, Germany) CARE Dose kV tube voltage modulation and 40 mAs ref. using CARE Dose4D and 16 × 1.2 mm slice collimation at 0.5 s rotation time and a pitch of 1.0 was acquired. CT was reconstructed with filtered-back-projection (FBP) using B30f and B70f kernels at a slice thickness of 1.5 and 5 mm. Immediately after CT, the whole-body PET scan was commenced. PET data were reconstructed using an ordered subset expectation maximization (OSEM) algorithm with three iterations/24 subsets, modellings of point-spread function (Siemens TrueX) and 5 mm full-width-at-half-maximum Gaussian post-smoothing. Written informed consent for evaluation and publication of the anonymized data was obtained from the patient. The ethics committee of the Universitätsklinikum Erlangen approved this procedure.

28H1-700DX

The anti-FAP antibody 28H1 (Roche, Basel, Switzerland) was conjugated to the photosensitizer (PS) IRDye700DX (Li-Cor Biosciences, Lincoln, NE, USA) with a substitution ratio (number of PS molecules per antibody molecule) of 2.5 using the -NHS ester as described previously [13]. Quality control via HPLC (Agilent Technologies, Santa Clara, CA, USA; using a SEC-3000 column, Phenomenex, Utrecht, The Netherlands) showed that purity of the construct 28H1-700DX was 95%, and binding to FAP was confirmed through a radioactive binding assay on 3T3 cells stably transfected with human FAP as previously described [13, 15].

Human synovium

Synovial tissue was obtained as remnant material from RA patients (*n* = 12) undergoing joint replacement surgery (Supplementary Table S1, available at *Rheumatology* online). Written informed consent was obtained from all patients. The study was approved by the local Ethics Review Board (CMO region Arnhem-Nijmegen, the Netherlands). Tissue was processed into standardized 6 mm biopsies as described previously [16]. From a subset of patients (*n* = 3), additionally synovial tissue was enzymatically digested and processed into single cell suspension [17].

Ex vivo FAP-tPDT on RA synovial biopsies

The 6 mm synovial biopsies were incubated in binding buffer [BB; Dulbecco's modified Eagle's medium (DMEM) with 0.5% BSA (Sigma-Aldrich, St Louis, MO,

USA)] with or without 1 μg/ml 28H1-700DX in 48-well plates for 4 h at 37°C with 5% CO_2. For each experimental condition (28H1-700DX or vehicle control, with/without exposure to 690 nm light), four biopsies per patient were used (16 biopsies per patient in total). After washing, the medium was replaced with BB and the biopsies were subjected to 52.2 J/cm^2 690 nm radiant light exposure. After 1 h, the biopsies were fixed in 4% formalin for 4 h and subsequently stored in 70% ethanol until processed to paraffin. The paraffin-embedded tissue was cut in 5 μm sections and prepared for (immuno)histological evaluation.

Immunohistochemistry

Synovial biopsies were formalin-fixed, paraffin-embedded and stained using haematoxylin and eosin, γH2AX (9718S) cleaved caspase-3 (9661S) (both from Cell Signaling Technology, Danvers, MA, USA) and FAP (LN031634, LabNed, Amstelveen, The Netherlands). Additionally, a terminal uridine nick-end labelling (TUNEL) assay was performed according to the manufacturer's description (ApopTag peroxidase *in situ* apoptosis detection kit, Millipore, Billerica, MA, USA). All sections were scored blinded based on an arbitrary 0–3-point scale of positive staining area. Values depicted in the graphs are the average scores of the four biopsies per condition, normalized to the baseline level of expression as scored from the pre-treatment biopsies.

Primary human fibroblast culture

Primary human synovial fibroblasts from RA patients were cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin for at least five passages prior to use in the PDT assays. Of each passage, 1×10^5 cells were processed into cDNA for qPCR analysis. The StepOnePlus qRT-PCR system (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyse mRNA expression using SYBR green master mix (Thermo Fisher Scientific, Waltham, MA, USA) and specific primers (Sigma-Aldrich; Supplementary Table S2, available at *Rheumatology* online). Relative quantification of the qRT-PCR signals was performed by correcting the C_t value of the genes of interest for glyceraldehyde 3-phosphate dehydrogenase content ($-\Delta C_t$).

In vitro FAP-tPDT

Cells were seeded in 48-well plates and the next day they were incubated in BB with or without 1 μg/well 28H1-700DX, for 4 h at 37°C with 5% CO_2. After washing, BB was added and the cells were subjected to varying doses of 690 nm light. After light exposure, the cells were returned to 37°C with 5% CO_2 for 1 h. Cell viability was subsequently measured using CellTiter-Glo (Promega, Madison, WI, USA) to measure ATP levels. The luminescence signal was normalized to that of the unexposed cells incubated with BB only.

Co-culture of primary fibroblasts with M1- or M2-like macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors using a Ficoll-Paque gradient as previously described [18]. CD14$^+$ cells were positively selected using the magnetic-activated cell sorting (MACS) system (Miltenyi Biotech, Cologne, Germany). Monocytes were then differentiated into M1- or M2-like macrophages by incubation with 50 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN, USA) for M1 differentiation, or 20 ng/ml macrophage-colony stimulating factor (M-CSF; R&D Systems) for M2 differentiation. After differentiation, the macrophages were co-cultured with fibroblasts (ratio 5:1 for macrophages and fibroblasts, respectively) after which the cells were treated with FAP-tPDT as described in the *in vitro* PDT section.

Fluorescence staining and flow cytometry

The co-cultures were analysed for M1 and M2 macrophage marker expression through flow cytometry using the Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and analysed using the Kaluza flow cytometry analysis software (Beckman Coulter). The antibodies and fluorophores used are summarized in Supplementary Table S3 (available at *Rheumatology* online). The gating strategy was determined based upon fluorescence minus one control staining. First doubles were removed by plotting forward scatter height over area and then live single cells were gated using the live/dead stain. Subsequently, FAP$^-$ cells were gated to investigate the effect of FAP-tPDT on the macrophages.

Statistics

Results are presented as mean (s.d.). Statistical significance was tested using unpaired Student's *t*-test or one-way ANOVA in GraphPad Prism software (Version 5.03; GraphPad Software Inc., La Jolla, CA, USA). A *P*-value of <0.05 was considered significant.

Results

Inflamed joints of an RA patient show robust FAPI-04 uptake

The expression of FAP in inflamed joints of RA patients is essential for the concept of FAP-tPDT to be a feasible novel therapeutic strategy. This has previously been demonstrated in human synovium by immunohistochemistry, but not yet *in vivo* [12]. We therefore first collected exploratory evidence for FAP expression in the human inflamed synovium *in vivo* by employing PET-CT imaging with the ^{68}Ga-labelled FAP inhibitor FAPI-04 in a 55-year-old patient with newly diagnosed RA. The patient was treated with corticosteroids only and reported pain in the left shoulder joint and the left knee. The patient presented with elevated CCP and MCV antibodies. While no FAP-specific tracer accumulation was observed in the healthy

Fɪɢ. 1 PET-CT image acquired 1 h post-injection of ^{68}Ga-FAPI-04 in an RA patient
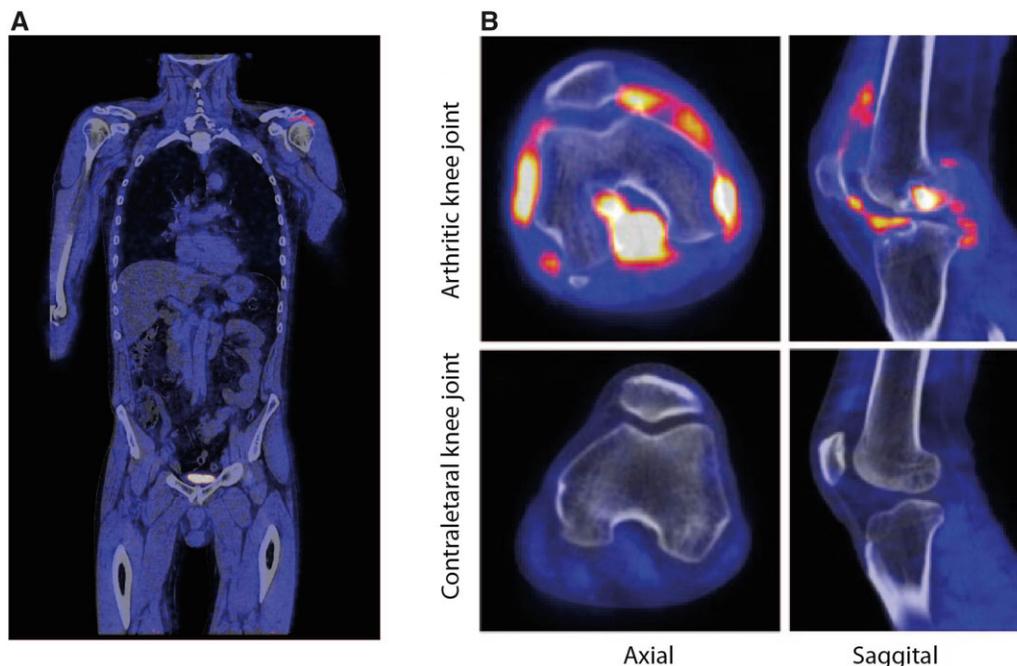
Uptake of the FAPI-04 tracer is clearly visible in the synovium of the inflamed joints of an RA patient. (**A**) Tracer accumulation in the left, arthritic shoulder while no uptake is visible in the contralateral, healthy shoulder joint or the thoracic and abdominal organs. (**B**) Knee joints showing uptake in the synovium of the arthritic knee while there is no visible FAPI-04 uptake in the healthy contralateral joint.

joints, the arthritic joints of the RA patient showed clear tracer accumulation along structures of the synovial membrane indicating that FAP is specifically upregulated in the diseased joints (Fig. 1).

Human synovial fibroblasts have stable FAP gene expression *in vitro*

After this illustrative FAP image *in vivo*, we proceeded *in vitro* to show proof of concept of our FAP-targeted PDT strategy. Since FAP expression on isolated RA synovial fibroblasts is a prerequisite for demonstrating the potential of FAP-based tPDT to induce cell death in these cells, we first determined whether the cells stably expressed FAP over multiple culture passages after isolation from the arthritic joint.

Passaging of cells decreased the expression of CD14, a marker for myeloid cells (Supplementary Fig. S1, available at *Rheumatology* online), reflecting the loss of myeloid cells during prolonged culture. The expression of FAP, collagen 1A1 (COL1A1), podoplanin (PDPN), THY-1, and CD55 remained stable over time in all donors, with minimal variation between the passages (Supplementary Fig. S1, available at *Rheumatology* online).

FAP-tPDT of RA synovial fibroblasts causes light-dose-dependent cytotoxicity

The quantification of cytotoxicity in whole tissue is challenging and the effects on surrounding cell types like macrophages are difficult to assess. Therefore, we first performed *in vitro* FAP-tPDT on primary synovial fibroblasts from RA synovial tissue, and subsequently FAP-tPDT on co-cultures of macrophages and fibroblasts. Fibroblasts from three different RA donors were used in this experiment. After incubation with the anti-FAP-700DX construct or vehicle control, the cells were exposed to different exposures of 690 nm light. This resulted in a linear decrease in cell viability upon increasing radiant light exposure (Fig. 2A). In contrast, cell viability was not significantly different in cells exposed to light without prior incubation with the antibody (Fig. 2B). This potent therapeutic effect of FAP-tPDT could be blocked by pre-incubation with an excess of 100 µg anti-FAP antibody without the photosensitizer 700DX (Fig. 2B), demonstrating the specificity of our targeted therapy. This group with excess unlabelled antibody was not significantly different from the group incubated with the binding buffer control ($P = 0.58$). These results indicate that FAP-tPDT efficiently induces cell death in human RA synovial fibroblasts in a light-dose-dependent manner.

FAP-tPDT on synovial fibroblasts does not alter the phenotype of neighbouring macrophages

Since in synovial tissue fibroblasts are interspersed with macrophages, the potential bystander effect of FAP-tPDT on the viability and phenotype of neighbouring

Fig. 2 FAP-based photodynamic therapy performed on human synovial fibroblasts cultured from three separate donors
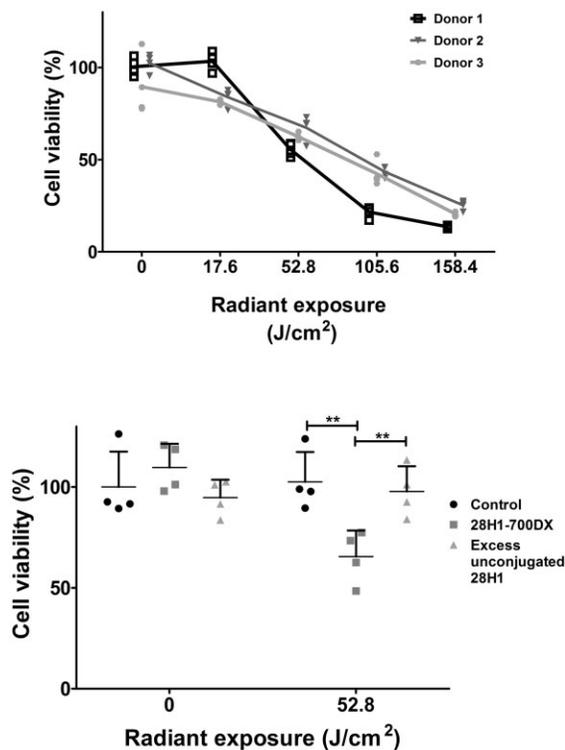
(**A**) Cells were incubated with either binding buffer or the anti-FAP antibody-IRDye700DX construct (28H1-700DX). Cell death is induced in a light-dose-dependent way for all donors ($n = 4$/donor). (**B**) Cells in the control condition were incubated with binding buffer medium only. Blocking of specific binding using an excess unlabelled anti-FAP antibody prevents tPDT-induced cell death. **$P < 0.01$, $n = 4$, one-way ANOVA with Bonferroni correction. FAP: fibroblast activation protein; tPDT: targeted photodynamic therapy.

macrophages was studied. Using flow cytometry, the viability and expression of FAP and M1 and M2 markers were analysed in macrophages isolated from co-cultures with fibroblasts, 24 h after treatment with FAP-tPDT. The gating strategy is depicted in Fig. 3. Macrophage viability was not affected 24 h after FAP-tPDT (Fig. 3C). The M1-like macrophage phenotype was not affected by FAP-tPDT, since expression of the M1 markers, as indicated by the percentage of cells positive for CD206 and CD86, was not altered (Fig. 4A and B). However, a trend towards a decrease in M2-like macrophages, reflected by reduced cell levels positive for the M2 markers CD163 and CD14, was observed ($P = 0.06$ and $P = 0.06$ for CD163 and CD14, respectively; Fig. 4C and D). A similar pattern in the expression of CD markers was observed when studying the mean fluorescence intensity measurements (Supplementary Fig. S2, available at *Rheumatology* online).

Histological markers of cell damage and death are upregulated in RA synovial biopsies following FAP-tPDT

To study the effect of FAP-tPDT on RA synovial biopsies *ex vivo*, a panel of three cell damage and death markers was selected for immunohistochemical analysis (Fig. 5). Baseline expression of the apoptosis marker cleaved caspase-3, and the markers of DNA damage γH2AX and the TUNEL assay varied already considerably between RA donors. To accurately describe the changes as a consequence of FAP-tPDT, results were therefore expressed as the change compared with baseline levels. Synovial biopsies treated with FAP-tPDT showed enhanced expression of cleaved caspase-3, γH2AX and the TUNEL staining in the synovial lining compared with baseline (Fig. 6). The histological score of these three markers in the control groups without 28H1-700DX and/ or light exposure was not significantly different compared with pre-treatment (summarized in Supplementary Table S4, available at *Rheumatology* online). An interesting observation was that positive staining reflecting cell damage in the FAP-based treatment group seemed especially increased in the synovial lining of the biopsies, as can be seen in the representative images of Fig. 5.

Discussion

In this study, we demonstrate that FAP-tPDT kills FAP$^+$ fibroblasts in primary synovial fibroblasts and biopsies from RA patients. Additionally, we show the first evidence that inflamed arthritic synovium of an RA patient can be visualized using a FAP-targeting tracer. This expands on work previously performed in our group in mice models and further indicates the feasibility of this approach in humans [13, 15, 19].

The therapy proposed in this study has important advantages compared with the current treatment approach for RA, which focuses on systemic immunosuppression. With FAP-tPDT the aberrant synovial cells can be selectively targeted, preventing side effects such as increased susceptibility to infections associated with systemic immunosuppression [20]. Despite being administered systemically, the antibody–PS construct is only activated locally through illumination, preventing damage to neighbouring, FAP-negative cells and healthy tissues. It also allows for the local depletion of cell types that, if depleted systemically, could cause serious side effects, e.g. systemic depletion of FAP$^+$ cells resulted in cachexia and anaemia in mice [21]. From previous studies in murine models of arthritis it has been shown that both PET/CT and SPECT/CT radionuclide imaging using FAP targeting tracers can be used to monitor treatment response [15, 19, 22]. Here we show for the first time in an RA patient that FAPI PET/CT images clearly identify the arthritic lesions. Together these results may indicate that this technique could potentially be used theranostically for treatment planning, response monitoring and

FIG. 3 Gating strategy and viability of macrophages 24 h after co-culture with primary human synovial fibroblasts that were subjected to FAP-tPDT
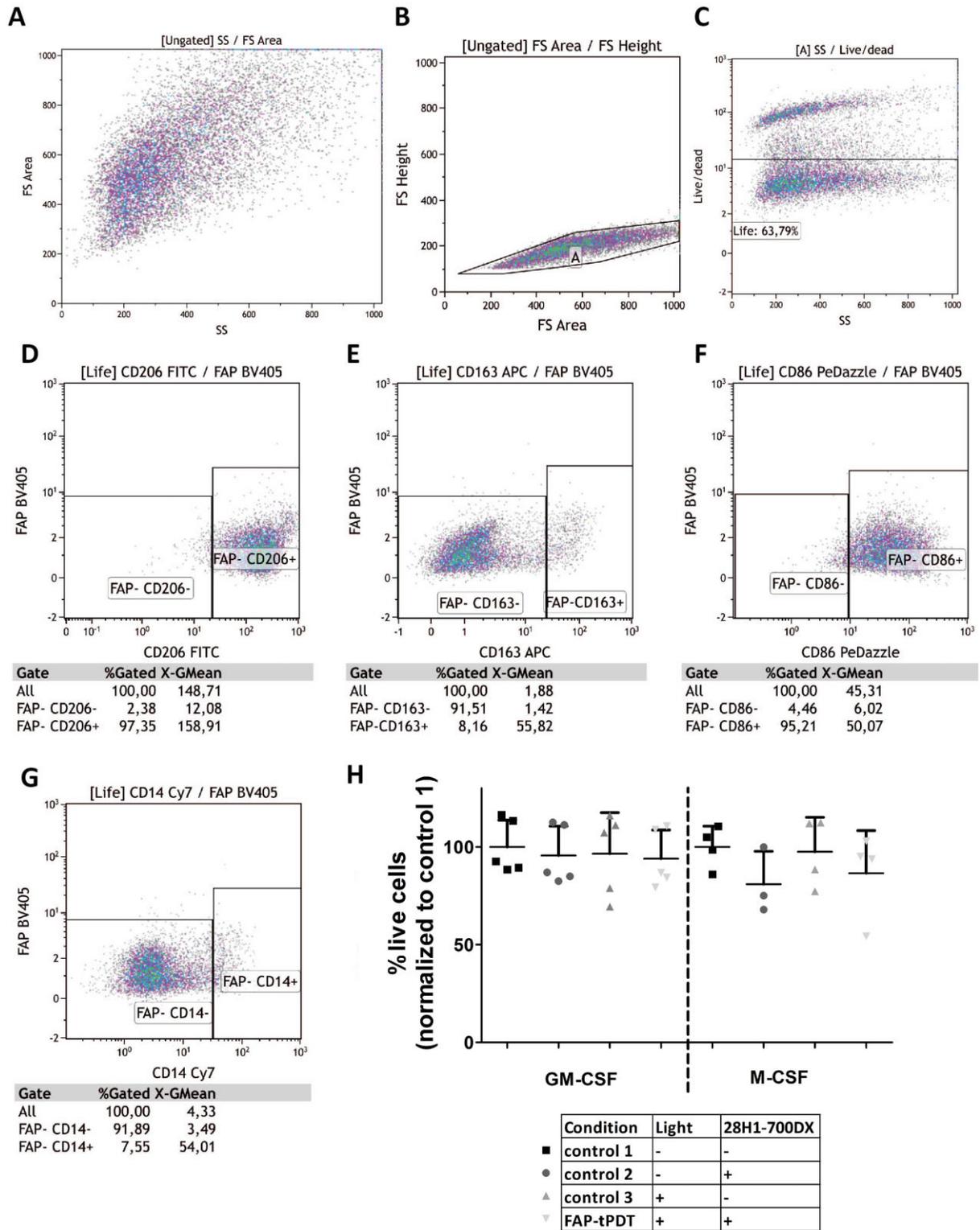
The gating strategy was based upon fluorescence minus one control staining. Viability of the macrophages was not affected in any of the experimental conditions compared with baseline viability of macrophages incubated with buffer and not exposed to light. $n = 5$ and 4 for GM-CSF or M-CSF differentiated macrophages, respectively. FAP: fibroblast activation protein; tPDT: targeted photodynamic therapy; FS: forward scatter; SS: side scatter.

Fig. 4 Expression of macrophage markers as determined by flowcytometry for M1 differentiated (GM-CSF) and M2 differentiated (M-CSF) macrophages

	Condition	Light	28H1-700DX
■	control 1	-	-
●	control 2	-	+
▲	control 3	+	-
▼	FAP-tPDT	+	+
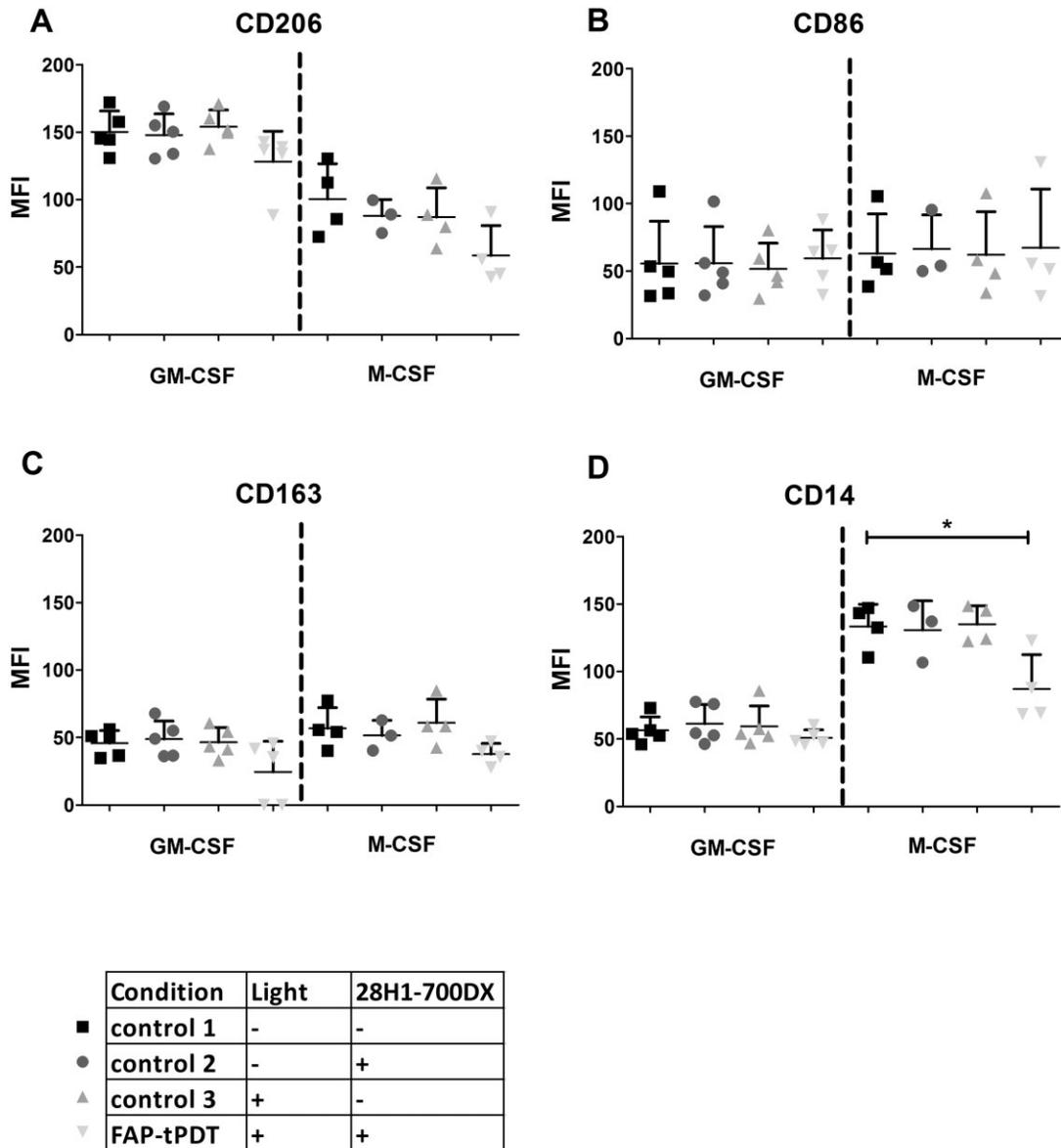
CD206 (**A**) and CD86 (**B**) were used as markers for M1 and CD163 (**C**) and CD14 (**D**) were used as markers for M2-like macrophages. $n = 5$ and 4 for GM-CSF and M-CSF differentiated macrophages, respectively. No significant differences were observed using one-way ANOVA with Bonferroni correction. MFI: mean fluorescent intensity.

efficacy evaluation of this and other treatments (in clinical trials).

The ability of FAPI PET/CT to distinguish rheumatic lesions from more metabolically active lesions (visualized using ^{18}F-fluorodeoxyglucose PET/CT) in the fibro-inflammatory IgG$_4$-related disorder underscores the therapeutic value of this approach also in RA [23]. Specific and locoregional depletion has previously been successfully employed pre-clinically in tPDT of, for example, insulinomas and prostate cancer and clinically in

a phase 3 clinical trial evaluating tPDT using an epidermal growth factor targeting antibody in head and neck squamous cell carcinoma (https://clinicaltrials.gov/ct2/show/NCT03769506) [24, 25]. Another important consideration in PDT is light penetration, which is estimated to be up to 5 mm, sufficient for efficient illumination of the smaller joints [26]. For effective treatment of larger joints, endoscopic light delivery methods may be employed. Several such systems already exist for clinical and pre-clinical use [27].

Fɪɢ. 5 Representative images of immunohistochemical staining of RA synovial biopsies after FAP-tPDT
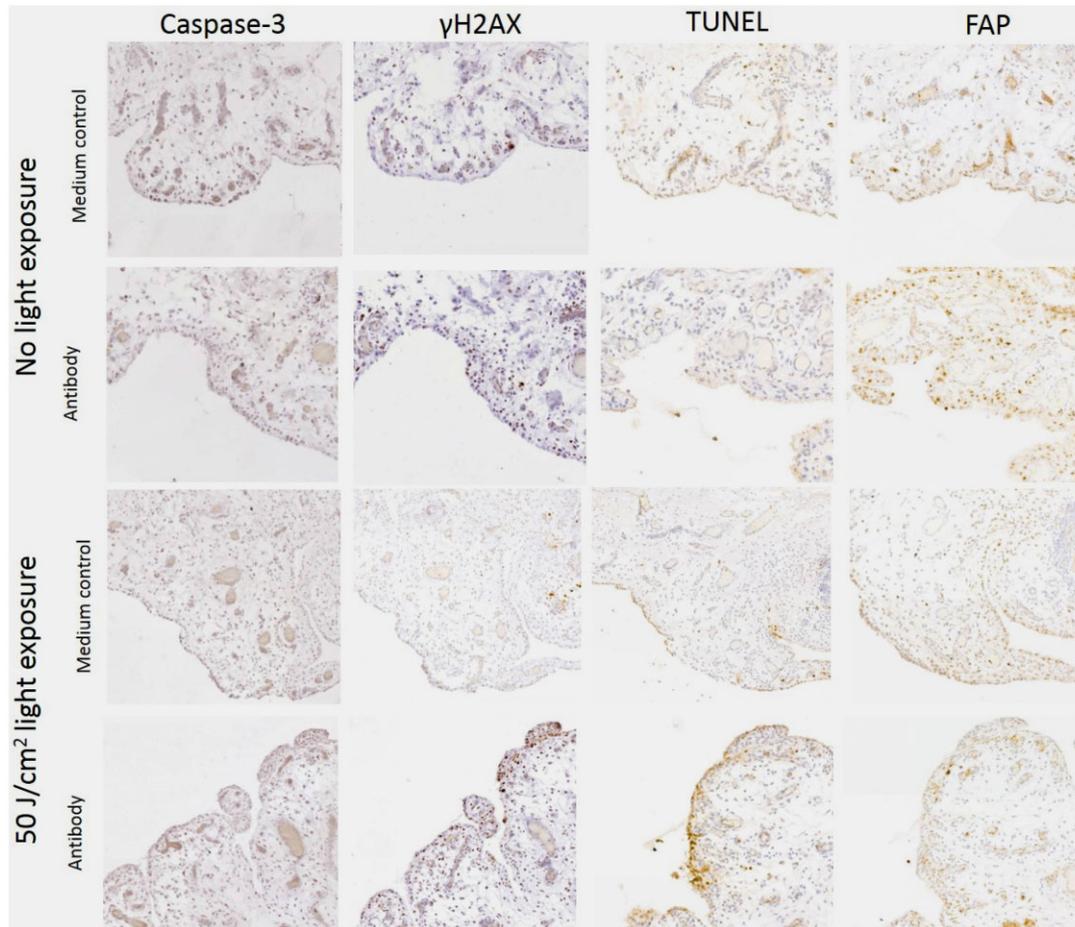
Consecutive slides were stained for FAP, cleaved caspase-3, γH2AX and the TUNEL assay. Biopsies were incubated in medium containing 28H1-700DX. Control biopsies were incubated with only medium. The biopsies were subsequently either exposed or not exposed to light. Staining of FAP is present in all experimental groups while staining of the markers of cell damage increase in the antibody-incubated, light-exposed group relative to the controls. FAP: fibroblast activation protein; tPDT: targeted photodynamic therapy; TUNEL: terminal uridine nick-end labelling.

Characterization of the type of cell death induced by FAP-tPDT is a crucial step in assessing its potential as a therapeutic strategy in RA. If FAP-tPDT would cause widespread necrosis of the FAP$^+$ fibroblasts, it might be an efficient way to deplete these cells from the arthritic joint, but it would also likely lead to a massive inflammatory response [28]. If, instead, the cells predominantly die from apoptosis, the uptake of the apoptotic bodies by phagocytic cells may work anti-inflammatorily due to the activation of Tyro3, Axl, and Mer (TAM) receptors [29]. Here, we investigated the upregulation of two markers of DNA damage (TUNEL and γH2AX expression) as well as a late marker of apoptosis (cleaved caspase-3). The increased expression of cleaved caspase-3 staining after FAP-tPDT indicates that FAP-tPDT results in apoptosis induction already 1 h after treatment. Additionally, more longitudinal studies using for instance immunocompromised mice engrafted with the biopsies should be conducted to further characterize

the type of cell damage induced by FAP-tPDT [30]. Furthermore, by varying the dose of antibody–PS construct or of 690 nm light this balance of apoptosis/necrosis induction could potentially be influenced [31]. To further characterize this, co-staining with FAP and other fibroblast markers can be performed.

The effect that FAP-tPDT may have on off-target cells, the so-called bystander effect, is another important determinant of its therapeutic applicability. By co-culturing RA synovial fibroblasts with M1- and M2-like macrophages and exposing these co-cultures to FAP-tPDT, we explored the effect that this therapy has on the expression of several macrophage differentiation and activation markers. The percentage of macrophages differentiated under M1 conditions positive for CD206 and CD86 did not change after FAP-tPDT. This may be due to the already high expression of those markers at baseline. Based upon this finding we expect no significant impact of FAP-tPDT on the differentiation of this

Fig. 6 Histological score of cleaved caspase-3 (**A**), γH2AX (**B**), TUNEL (**C**) and FAP (**D**) staining of synovial biopsies after FAP-tPDT
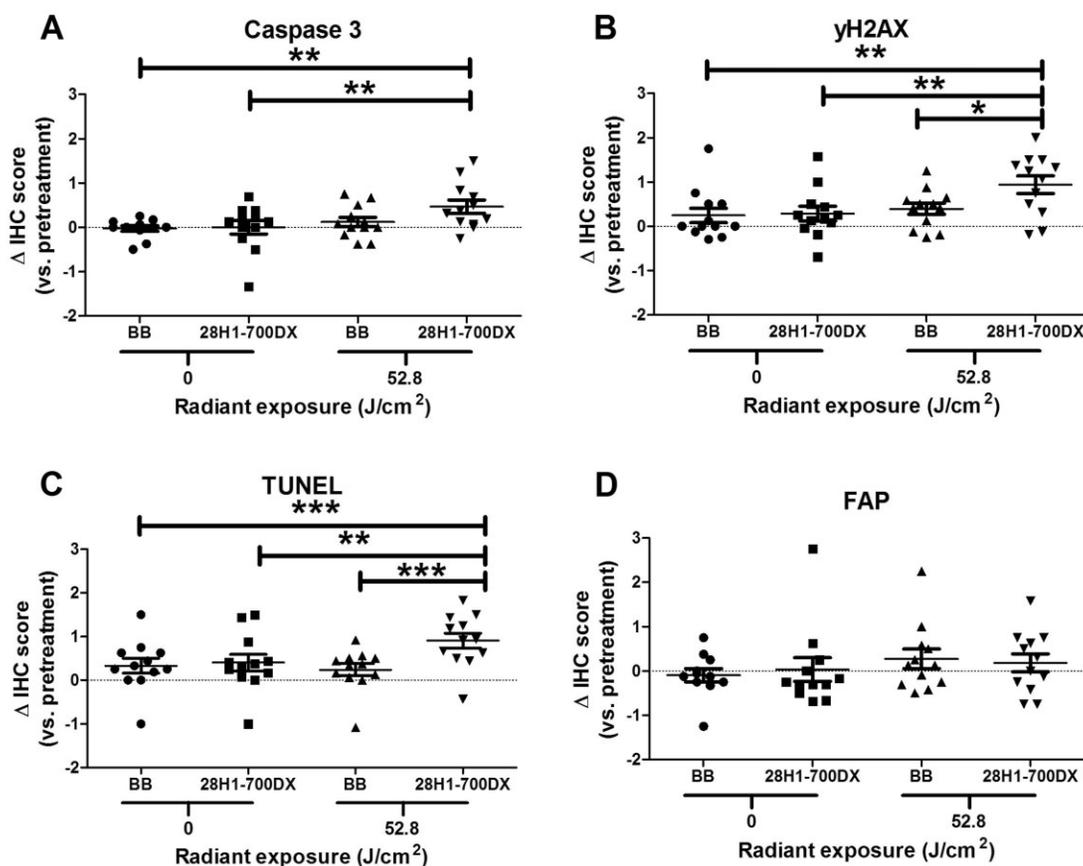
Biopsies were incubated with either binding buffer (BB) or the anti-FAP antibody–IRDye700DX construct (28H1-700DX) and exposed to 0 or 52.8 J/cm^2 690 nm light. Each data point is the average histological score of four biopsies from the same patient. Score is depicted as the difference in staining compared with the pre-treatment condition. $n = 12$ donors with each data point representing the mean of four biopsies included in each condition per donor. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, repeated measures ANOVA. FAP: fibroblast activation protein; IHC: immunohistochemistry; tPDT: targeted photodynamic therapy; TUNEL: terminal uridine nick-end labelling.

pro-inflammatory M1-like subset of macrophages. No significant effect of FAP-tPDT was observed on the M2-like macrophages either. Increasing the sample size and (functional) readout parameters in future co-culture studies with respect to M1/M2 bystander response will give more insight into the consequences of FAP-tPDT on neighbouring macrophages. The effect of FAP-tPDT on other joint resident cells (like endothelial cells, chondrocytes and osteoclasts), as well as immune cells important in RA pathogenesis, should also be investigated. More elaborate co-culture studies, potentially using more complex organoids or organ-on-a-chip models, could be performed in the near future to fill this knowledge gap.

The synovial tissue in this study was obtained from RA patients suffering from secondary osteoarthritis undergoing joint replacement surgery, presenting with highly variable inflammation grades. This was observed by histological analysis, with some of the synovial

samples presenting with an active lining, while others showed very little viable synovium, with fibrous and largely acellular tissue. Despite this variability, we found striking therapeutic effects of FAP-tPDT. As FAP expression specifically reflects areas of active inflammation, using synovial biopsies from very active refractory RA or even early RA patients with active inflammation could more accurately predict the therapeutic efficacy of FAP-tPDT [12, 15]. This material is, however, scarce and obtaining sufficient biopsies from one patient to include all experimental conditions is challenging.

Our study convincingly demonstrated that FAP-based tPDT potently induces cell death in RA synovial fibroblasts *in vitro*, as well as causing FAP-tPDT-specific cell death in RA synovial biopsies *ex vivo*. Importantly, this novel treatment approach seems to have only minimal impact on surrounding macrophages with key regulatory functions in inflammation and the resolution thereof as demonstrated by our co-culture experiments [32].

Together with the illustrative FAPI PET/CT images, with clear tracer uptake in the inflamed joints of an RA patient, a first step towards clinical translation of this novel technique has been made.

Data availability statement

All data will be made available upon reasonable request.

Supplementary data

Supplementary data are available at *Rheumatology* online.

References

1   Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet 2016;388:2023–38.

2   Silman AJ, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. Arthritis Res 2002;4(Suppl 3): S265–72.

3   McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365:2205–19.

4   Burmester GR, Pope JE. Novel treatment strategies in rheumatoid arthritis. Lancet 2017;389:2338–48.

5   Dakin SG, Coles M, Sherlock JP *et al.* Pathogenic stromal cells as therapeutic targets in joint inflammation. Nat Rev Rheumatol 2018;14:714–26.

6   Buckley CD, Filer A, Haworth O *et al.* Defining a role for fibroblasts in the persistence of chronic inflammatory joint disease. Ann Rheum Dis 2004;63(Suppl 2):ii92–5.

7   Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 2010; 233:233–55.

8   Huber LC, Distler O, Tarner I *et al.* Synovial fibroblasts: key players in rheumatoid arthritis. Rheumatology (Oxford) 2006;45:669–75.

9   Bottini N, Firestein GS. Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. Nat Rev Rheumatol 2013;9:24–33.

10 Filer A. The fibroblast as a therapeutic target in rheumatoid arthritis. Curr Opin Pharmacol 2013;13: 413–9.

11 Mizoguchi F, Slowikowski K, Wei K *et al.* Functionally distinct disease-associated fibroblast subsets in rheumatoid arthritis. Nat Commun 2018;9:789.

12 Croft AP, Campos J, Jansen K *et al.* Distinct fibroblast subsets drive inflammation and damage in arthritis. Nature 2019;570:246–51.

13 Dorst DN, Rijpkema M, Boss M *et al.* Targeted photodynamic therapy selectively kills activated fibroblasts in experimental arthritis. Rheumatology 2020;59:3952–60.

14 Lindner T, Loktev A, Altmann A *et al.* Development of quinoline-based theranostic ligands for the targeting of fibroblast activation protein. J Nucl Med 2018;59:1415–22.

15 Laverman P, van der Geest T, Terry SYA *et al.* Immuno-PET and immuno-SPECT of rheumatoid arthritis with radiolabeled anti-fibroblast activation protein antibody correlates with severity of arthritis. J Nucl Med 2015;56: 778–83.

16 Broeren MGA, de Vries M, Bennink MB *et al.* Functional tissue analysis reveals successful cryopreservation of human osteoarthritic synovium. PLoS One 2016;11: e0167076.

17 Broeren MGA, Waterborg CEJ, Wiegertjes R *et al.* A three-dimensional model to study human synovial pathology. ALTEX 2019;36:18–28.

18 van den Bosch MH, Blom AB, Schelbergen RF *et al.* Alarmin S100A9 induces proinflammatory and catabolic effects predominantly in the M1 macrophages of human osteoarthritic synovium. J Rheumatol 2016;43:1874–84.

19 van der Geest T, Laverman P, Gerrits D *et al.* Liposomal treatment of experimental arthritis can be monitored noninvasively with a radiolabeled anti-fibroblast activation protein antibody. J Nucl Med 2017;58:151–5.

20 Listing J, Gerhold K, Zink A. The risk of infections associated with rheumatoid arthritis, with its comorbidity and treatment. Rheumatology (Oxford) 2013;52:53–61.

21 Roberts EW, Deonarine A, Jones JO *et al.* Depletion of stromal cells expressing fibroblast activation protein-α from skeletal muscle and bone marrow results in cachexia and anemia. J Exp Med 2013;210:1137–51.

22 van der Geest T, Roeleveld DM, Walgreen B *et al.* Imaging fibroblast activation protein to monitor therapeutic effects of neutralizing interleukin-22 in collagen-induced arthritis. Rheumatology (Oxford) 2018;57:737–47.

23 Schmidkonz C, Rauber S, Atzinger A *et al.* Disentangling inflammatory from fibrotic disease activity by fibroblast activation protein imaging. Ann Rheum Dis 2020;79: 1485–91.

24 Boss M, Bos D, Frielink C *et al.* Receptor-targeted photodynamic therapy of glucagon-like peptide 1 receptor positive lesions. J Nucl Med 2020;61:1588–93.

25 Lutje S *et al.* Development and characterization of a theranostic multimodal anti-PSMA targeting agent for imaging, surgical guidance, and targeted photodynamic therapy of PSMA-expressing tumors. Theranostics 2019; 9:2924–38.

26 Ash C, Dubec M, Donne K *et al.* Effect of wavelength and beam width on penetration in light-tissue interaction using computational methods. Lasers Med Sci 2017;32: 1909–18.

27 Kim MM, Darafsheh A. Light sources and dosimetry techniques for photodynamic therapy. Photochem Photobiol 2020;96:280–94.

28 Wallach D, Kang T-B, Dillon CP *et al.* Programmed necrosis in inflammation: toward identification of the effector molecules. Science 2016;352:aaf2154.

29 Nagata S. Apoptosis and clearance of apoptotic cells. Annu Rev Immunol 2018;36:489–517.

30 Koenders MI, Marijnissen RJ, Joosten LAB *et al.* T cell lessons from the rheumatoid arthritis synovium SCID mouse model: CD3-rich synovium lacks response to CTLA-4Ig but is successfully treated by interleukin-17 neutralization. Arthritis Rheum 2012;64:1762–70.

31 Mroz P, Yaroslavsky A, Kharkwal GB *et al.* Cell death pathways in photodynamic therapy of cancer. Cancers (Basel) 2011;3:2516–39.

32 Culemann S, Grüneboom A, Nicolás-Ávila JÁ *et al.* Locally renewing resident synovial macrophages provide a protective barrier for the joint. Nature 2019; 572:670–5.