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Inhibiting phosphorylation of STAT proteins modulates the inflammatory phenotype of osteoarthritic synovium

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ABSTRACT

Synovial inflammation plays an important role in the pathological process of osteoarthritis (OA). This study determines phosphorylation levels of signal transducer and activator of transcription (STAT) proteins in osteoarthritic synovium and investigates whether inhibition of STAT signaling pathways modulates the inflammatory phenotype of OA synovium. To examine this, STAT1, 3, and 6 phosphorylation were determined in OA synovium using Western blot analysis. This was done either directly after harvest or after treatment with 50 μM NSC118-218, 100 μM S3I-201, or 100 nM AS1517499, compounds known to inhibit STAT phosphorylation, for 24 hours with or without the presence of synovial fluid. Different STAT1 and STAT3 phosphorylation levels were observed in OA synovium among the donors. Phosphorylated STAT6 was only detectable when explants were cultured in synovial fluid. NSC118-218 and AS1517499 inhibited STAT1 phosphorylation, although only treatment with AS1517499 also resulted in decreased *IL1B* and *IL6* gene expression. S3I-201 inhibited STAT3 phosphorylation, resulting in less *IL6* expression and increased *TNFA* expression. These data indicate that donor dependent STAT1, 3, and 6 phosphorylation patterns are found in OA synovium. Inhibition of STAT1 phosphorylation had an anti-inflammatory effect, whereas the inhibition of STAT3 phosphorylation enhanced the inflammatory phenotype. Inhibition of STAT1 phosphorylation might be a potential therapy to diminish synovial inflammation eventually to slow down or prevent the pathogenesis of OA.

Key words: Inflammation; modulation; JAK-STAT signalling; osteoarthritis; synovium.

INTRODUCTION

Inflammation of the synovial membrane is a common feature of osteoarthritis (OA) with an accumulation of infiltrating immune cells, such as macrophages[10]. Cells within the inflamed synovial membrane produce factors known to have catabolic effects, such as interleukin (IL) 1β and IL6[29, 35]. The pathophysiological process that occurs in the osteoarthritic joint is largely mediated by pro-inflammatory cytokines and other mediators[35]. However, OA also results in elevated levels of the anti-inflammatory cytokines IL4 and IL10 in the serum or synovial fluid[16, 22, 33]. Therefore, a targeted approach is required to specifically modulate the inflammatory phenotype of the synovial membrane. The intracellular Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathways are a principal signaling mechanism for a wide array of cytokines and growth factors. Signaling through this pathway is mediated by phosphorylation of STAT proteins. It is known that when cells, including fibroblasts and macrophages, are stimulated with interferon (IFN) γ, STAT 1 is phosphorylated[17, 19]. Similarly, IL4 in general results in activation of STAT6, whereas stimulation with IL10 leads to activation of STAT3[1, 25]. Once phosphorylated, STAT proteins form dimers and subsequently regulate gene expression[8]. More specifically, phosphorylated STAT1 regulates transcription of *IL1B* [19], whereas phosphorylated STAT3 targets the *IL6* and *IL10* genes[11, 15]. The anti-inflammatory gene *IL4* is targeted by phosphorylated STAT6[18]. Although previously investigated in rheumatoid arthritis, it is currently unknown which JAK-STAT pathways are activated in OA synovial tissue and whether modulation of JAK-STAT pathways affects the inflammatory phenotype of the synovium.

The main cell types in synovial explants are fibroblasts and macrophages. Macrophages can become activated by environmental cues, resulting in different phenotypes ranging from pro-inflammatory to anti-inflammatory or repair macrophages. *In vitro*, pro-inflammatory or M1-like macrophages can be obtained by stimulation with IFNγ and/or TNFα[4] among others. Macrophages induced by stimuli such as IL4 or IL13 obtain a repair-phenotype and are often referred to as M2-like macrophages. M2-like macrophages induced with IL10 or glucocorticoids obtain a predominant anti-inflammatory phenotype[27]. These subtypes represent the extremes of a spectrum and are a simplified version of the range of phenotypes that can appear *in vivo*. As macrophages are one of the sources for pro-inflammatory cytokines in the knee joint during OA, macrophages were completely depleted from the knee joint prior to OA induction. Indeed, macrophages contributed to the onset and progression of osteoarthritis[31, 32]. However, depleting all synovial macrophages is a non-specific approach and might therefore also abolish some of their beneficial effects. Another approach is to neutralize specific cytokines, but this did not seem to be completely effective in every patient suffering from OA[5]. Targeting proteins involved in intracellular signal transduction pathways might therefore be

an interesting new strategy as it is more specific than depleting cells and it affects the production of multiple cytokines at once.

The aim of the present study was to determine STAT activation in OA synovium and to investigate whether inhibiting STAT signaling pathways modulates the inflammatory phenotype of osteoarthritic synovium. In addition, as macrophages are key role players in inflammation, we determined presence of macrophage phenotypes in OA synovium and their respective STAT activation levels.

METHODS

Modulating synovial tissue

Synovial tissue was obtained from 17 patients with gonarthrosis undergoing total knee replacement at Erasmus MC, University Medical Center, Rotterdam. Consent was given in accordance with the guidelines of the Federation of Biomedical Scientific Societies (www.federa.org) after approval by the local ethical committee (#MEC2004-322). The synovium was washed twice with 0.9% NaCl (Sigma-Aldrich, Saint Louis, Missouri, USA), separated from the surrounding tissue and cut in to $5mm^2$ pieces.

To examine initial activation of STAT proteins, synovial explants were not cultured, but immediately stored at -80°C (n=4, male: 1, mean age: 64). To inhibit STAT phosphorylation, explants were cultured for 24 hours with or without 50μM NSC118-218, 100 μM S3I-201 and 100 nM AS1517499 in medium (Dulbecco's Modified Eagle Medium, low glucose (DMEM; Gibco, Carlsbad, USA) complemented with 10% FCS (n=5, male: 2, mean age: 61) with or without additional 50% synovial fluid from the same donor as the synovial tissue was obtained (n=4, male: 3, mean age: 79). After the culture period, synovial explants were harvested and stored at -80°C until evaluation using Western Blot analysis and gene expression analysis. The medium was harvested and stored at -80°C for cytokine measurements. Doses were chosen based on the current literature. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used as vehicle for all inhibitors with a final DMSO concentration in cultures of <0.01%.

RNA isolation and quantitative RT-PCR

Frozen synovial samples were processed using a Mikro-Dismembrator S (B. Braun Biotech International GmbH, Melsungen, Germany) and consecutively samples were dissolved in 350μl Trizol. A RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands) was used to extract RNA and procedures were performed according to the manufacturer's instructions. RNA was quantified using Nanodrop ND-1000 Spectrophotometer (NanoDrop ND1000 UV-VIS, Isogen Life Science B.V., the Netherlands). A total of 250 ng RNA per sample was reversed transcribed into cDNA using RevertAidTM First strand cDNA synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed on an ABI

Prism 7000 Sequence detection system (Applied Biosystems, Foster City, Ca) using either TaqMan Universal PCR mastermix (Applied Biosystems) or SybrGreen (Eurogentec). Gene expression of *IL1B*, *TNFA*, *CCL18*, Cluster of Differentiation 206 (*CD206*) and *CD163* was evaluated. Glyceraldehyde-3-phosphate Dehydrogenase (*GAPDH*), Hypoxanthine Phosphoribosyltransferase 1 (*HPRT1*) and Beta-2-microglobulin (*B2M*) were all tested as housekeepers, where *GAPDH* was found the most stable (data not shown) and was therefore further used as normalization for the genes of interest. Relative quantification of PCR signals was performed by comparing the threshold cycle value (C_t) for the gene of interest in each sample with the C_t value for the housekeeping gene[23].

Quantification of cytokine production

Quantification of IL6, CCL18 and soluble CD163 (sCD163) in the medium of synovium cultures was performed by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions of human IL6 ELISA Development Kit (PeproTech), human CCL18 DuoSet Development Kit (R&D Systems) and human soluble CD163 DuoSet Development Kit (R&D) systems.

Western blotting

Sample's protein concentrations were measured using a bichinchoninic acid assay (BCA assay) according to the manufacturer's instructions (Thermo Scientific). For immunoblot detection of phosphorylated STAT proteins, 10 – 20 μg of the samples were electrophoresed on 10% SDS-polyacrylamide gels (Thermo Scientific, or Invitrogen, Carlsbad, CA USA), electrotransferred onto nitrocellulose membranes and blocked with 5% non-fat dried milk for two hours. Membranes were incubated overnight at 4°C with primary antibodies against phosphorylated STAT1 (Cell Signaling Technology, Danvers, MA, USA), phosphorylated STAT3 (Cell Signaling Technology), phosphorylated STAT6 (Cell Signaling Technology) and α-Tubulin (Cell Signaling Technology).
After washing, the membranes were incubated with horseradisch peroxidase (HRP) –conjugated anti-rabbit IgG secondary antibody (Cell Signaling) for 1.5 hour at room temperature. Antibody detection was performed using SuperSignal West Pico Luminol Enhancer Solution and SuperSignal West Pico Stable Peroxide Solution (Thermo scientific). Western blot analyses were quantified using Image J (U.S. National Institutes of Health, Bethesda, Maryland, USA).

Immunohistological analysis

Synovial samples from 3 different donors (n=3, male: 1, mean age: 70 years) were collected and 6 μm thick cryosections were cut. Sections were fixed in acetone for 10 minutes and subsequently washed with PBS. Following blocking with 10% goat serum (Southern Biotech #0060-01) for 30 minutes, sections were incubated for 1 hour at room temperature

with an antibody against CD68 (Abcam, clone KP-1 ready-to-use) as pan-macrophage marker, CD86 (Genetex, clone EP1158Y; 0.45 µg/ml) as M1 marker, CD206 (Abcam, #64693; 2.5 µg/ml) as M2a marker, and CD163 (Abcam, #182422; 1.6 µg/ml) as M2c marker. Sections were incubated for 30 minutes with either second antibody biotinylated goat-anti-mouse Ig link (BioGenex, HK-325-UM) or a biotinylated goat-anti-rabbit Ig link (Biogenex, HK-326-UR) diluted with PBS/1%BSA. This was followed by incubation with a third antibody: alkaline phosphatase-conjugated streptavidin label diluted 1:50 in PBS/1%BSA (BioGenex, HK-321-UK). After a final wash step in Tris-HCl, sections were incubated in freshly prepared substrate mixture of Neu Fuchsin (Chroma Gesellschaft, 1g/25ml 2M HCl), Sodiumnitrate (Sigma, #S2252) and Naphtol AS-MX phosphate (Sigma, #N5000) that was dissolved in di-methylformamid (Sigma, D4551). Subsequently tissue sections were counterstained with Haematoxylin Gill's (Sigma, #GHS232) to evaluate the overall staining pattern better. An isotype-matched control antibody; either monoclonal mouse IgG1 (Dako Cytomation #X0931) or rabbit IgG1 antibody (Dako Cytomation #X0903) was used as negative control for each staining. Sections were dried overnight and mounted with VectaMount (Vector Laboratories, #H5000).

Human monocyte isolation and culture

Monocytes were isolated from human buffy coats of healthy male donors (Sanquin Blood bank, Amsterdam, the Netherlands) by Ficoll density gradient separation and $CD14^+$ selection as described before[14]. Isolated monocytes plated in 24-well plates (Corning Incorporated, NY, USA) at a density of 500,000 cells/cm^2 in X-VIVO 15 medium (Lonza, Verviers, Belgium) supplemented with 20% heat-inactivated fetal calf serum (FCS, Lonza), 50 µg/mL gentamycin and 1.5 µg/mL fungizone. Monocytes were differentiated towards different macrophage phenotypes using 10 ng/mL IFNγ (Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL TNFα (Peprotech) to obtain M(IFNγ + TNFα), 10 ng/mL IL4 (Peprotech) to obtain M(IL4) or 10 ng/mL IL10 (Peprotech) to obtain M(IL10).

Activated macrophages were harvested 30, 60, and 90 minutes after plating. These cells were suspended in 180 µL M-PER (Thermo Scientific, Rockford, USA) with 0.1% HALT™ – Protease Inhibitor Single-Use Cocktail (100x) (Thermo Scientific) and 0.1% Phosphatase Inhibitor (Thermo Scientific) and stored at -80°C until Western Blot analysis was performed. After 24 hours, the medium of macrophages was collected, centrifuged at 200x g and the supernatants were stored at -80°C for cytokine measurement. Harvested cells were resuspended in PBS/0.1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) for DNA quantification or in TRIzol Reagent (Ambion, Carisbad, CA, USA) for mRNA isolation.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics for Windows (version 21.0, IBM Corp., Armonk, NY). A mixed linear model with a Bonferroni post-hoc test

was used after log transformation to statistically analyze the data and to take into account donor variability between different donors. In all experiments, an individual experiment was considered as a random factor. For macrophages cultured in monolayers, polarization states were considered as a random factor. In the experiments with synovial explants, treatment with an inhibitor was considered as a random factor.

Differences were considered to be statistically significant if $p < 0.05$.

RESULTS

STAT phosphorylation in end-stage OA synovium

Phosphorylated STAT1 was detectable in synovial explants obtained immediately after surgery in 3 of the 5 OA donors evaluated. pSTAT3 could be detected in synovial tissue of all donors, although the levels of expression seem variable. No pSTAT6 could be detected in the collected synovial explants, only when the explants were stimulated with IL4 (as a control) (Figure 1).
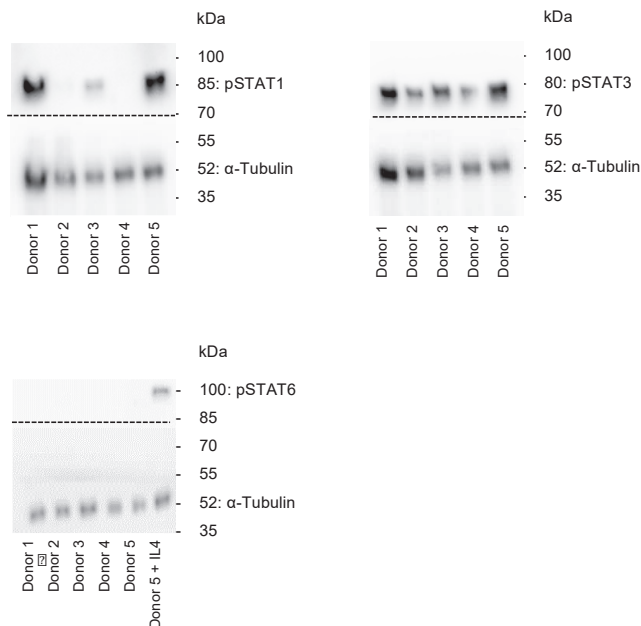
Figure 1. Presence of phosphorylated STATs in OA synovial tissue.
Western Blots analysis of OA synovial tissue in 5 donors. The dotted line indicates grouping of images from different parts of the same gel.

Therefore, presence of phosphorylated STATs was examined in explants that were cultured in synovial fluid (SF). Indeed, in the presence of synovial fluid, pSTAT6 was detectable in all explants in variable degrees (Figures 2A, D and G), next to pSTAT1 and pSTAT3.
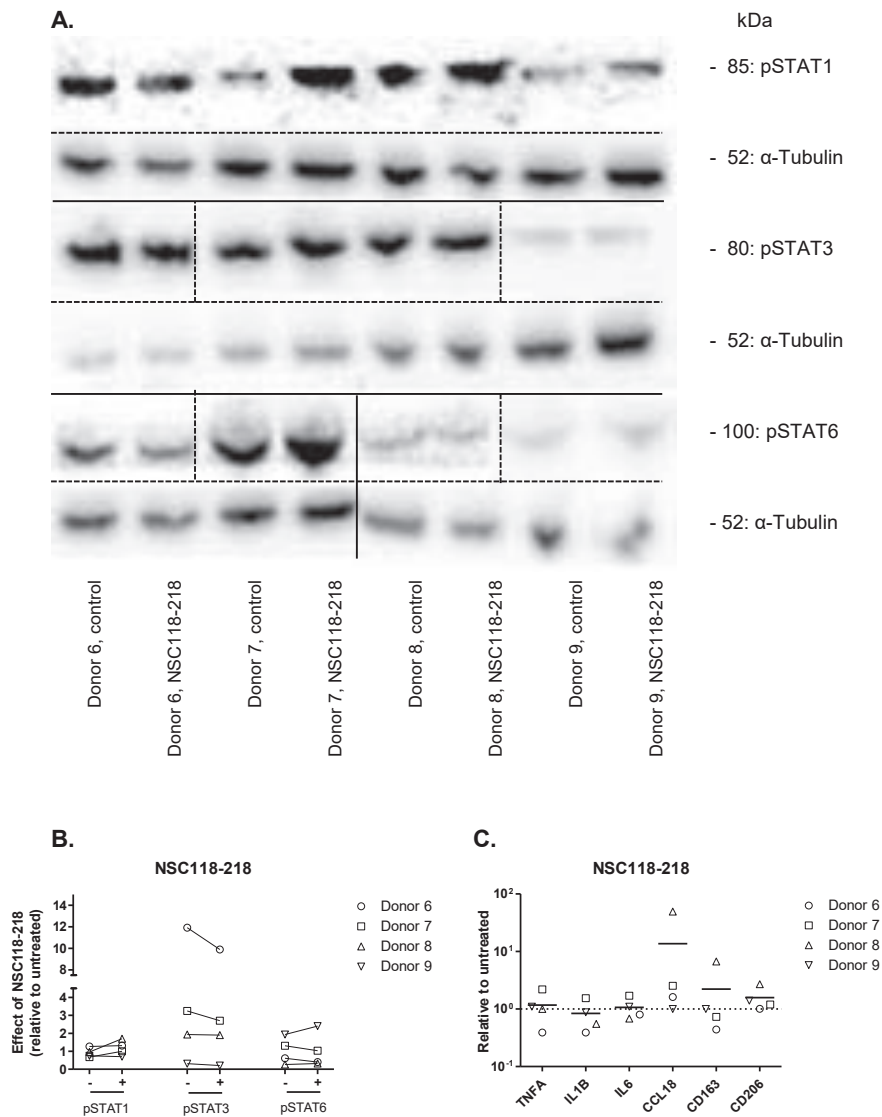
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Figure 2. Modulation of STAT phosphorylation in OA synovial tissue cultured in synovial fluid.

D.

E.

F.

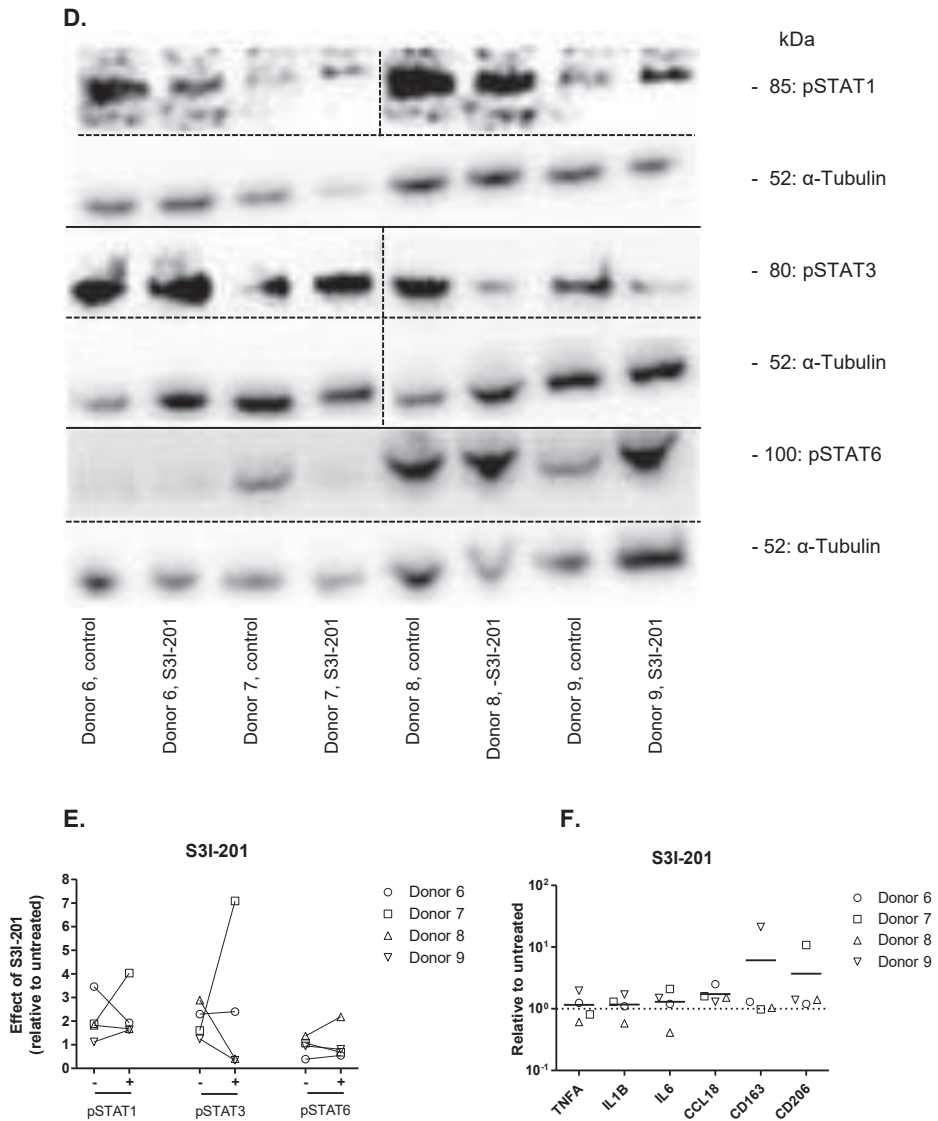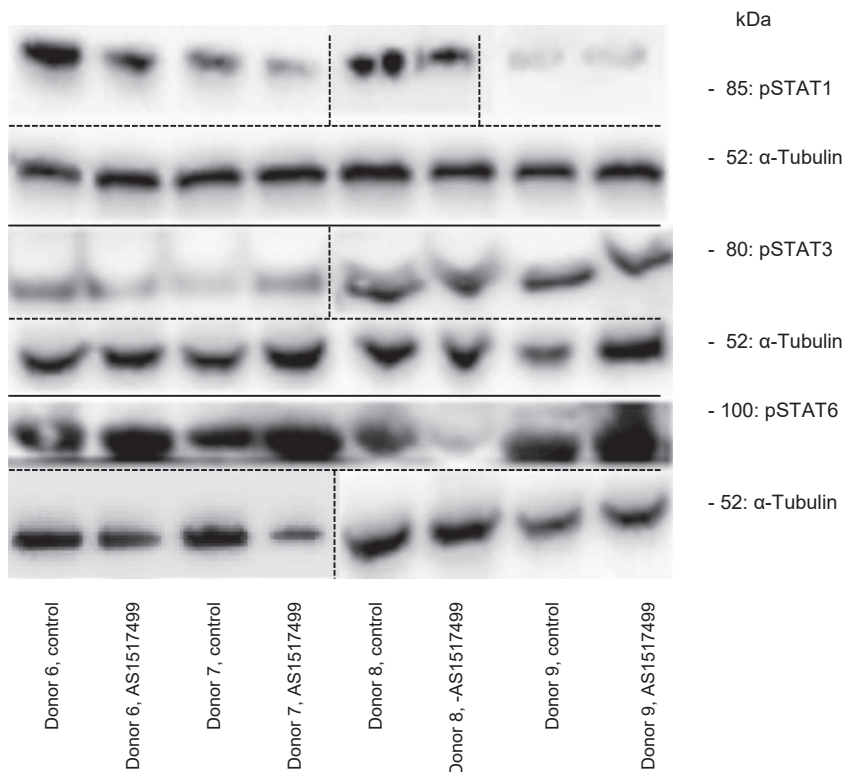**Figure 2. Modulation of STAT phosphorylation in OA synovial tissue cultured in synovial fluid.**

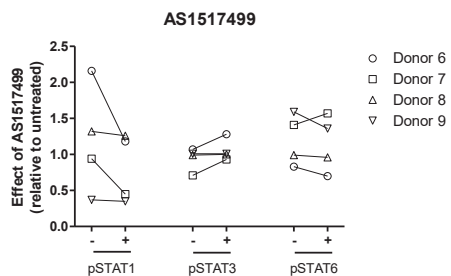**G.**

H.

I.

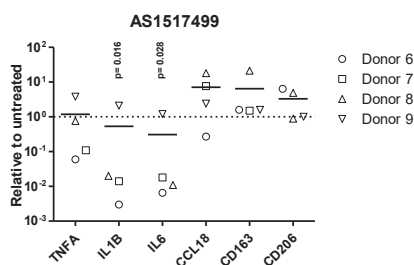**Figure 2. Modulation of STAT phosphorylation in OA synovial tissue cultured in synovial fluid.**
A, D, G) Western Blot analysis of synovial tissue after treatment with the STAT inhibitors. The dotted line indicates grouping of images from different parts of the same gel, whereas the continuous line indicates grouping of different parts from different gels. -; control, +; treated with STAT inhibitor. B, E, H) Quantification of the Western Blots relative to α Tubulin. C, F, I) Gene expression corrected for GAPDH and relative to the untreated control, which is represented by the dotted line. Data is shown as mean (as indicated by line) for n=4 analysed in 2-fold.

Modulation of OA synovial tissue with STAT-inhibitors

After determining STAT phosphorylation in human OA synovium, the effect of STAT1, 3, and 6 inhibition on the inflammatory profile was assessed in the tissue. Since especially STAT6 phosphorylation seems only detectable in OA synovium when certain stimuli are present, we decided to culture all the explants in the donor's own SF. Culturing synovial explants with 50 μM NCS118-218, known as STAT1 inhibitor, did not affect STAT1 phosphorylation, nor did it affect pSTAT3 or pSTAT6 levels (Figure 2A and B). 100 μM S3I-201, known as STAT3 inhibitor, did not influence STAT3 phosphorylation in explants cultured in SF, nor did it affect STAT1 or STAT6 phosphorylation (Figure 2D and E). 100 nM AS1517499, chosen as STAT6 inhibitor, decreased STAT1 phosphorylation levels, without affecting pSTAT6 levels (Figure 2G and H). This decrease in STAT1 phosphorylation levels was accompanied by a significant decrease of *IL1B* and *IL6* levels (Figure 2I).

The effect of the STAT1 and 3 inhibitors was also tested in cultures without synovial fluid. Here, NSC-118218 did result in a significant decrease of pSTAT1 levels (Figure 3A and B), without affecting gene expression levels (Figure 3C) or secreted protein production (Figure 3D). Treatment of synovial explants with S3I-201 decreased phosphorylated STAT3 levels in 4 of the 5 donors, and increased pSTAT3 in one of the donors (Figure 3E and F). This treatment led to a significant upregulation of *TNFA* and a significant downregulation of *IL6* and *CD163* (Figure 3G). S3I-201 treatment also did not alter protein secretion after 24 hours of culture (Figure 3H). As pSTAT6 is not present at detectable levels in OA synovial tissue in absence of the corresponding environmental cues, STAT6 phosphorylation levels were not determined in synovial explants in culture medium nor was AS1517499 tested under these conditions.

Characterization of human macrophage phenotypes and their presence in OA synovial tissue

Using immunohistochemistry, the presence of macrophages was analysed in OA synovium. CD68 used as pan-macrophage marker, was mainly found in the lining of OA synovium explants. The presence of CD86, indicating pro-inflammatory macrophages, and CD206, indicating tissue repair macrophages, was mainly seen in the lining of OA synovium explants. CD163, a marker indicating anti-inflammatory cells, was present in the synovial lining of donor 1 and in the sublining of donor 2 and 3. Isotype controls were negative for all stainings (Figure 4A).

To investigate the activity of the different STATs related to different macrophage subtypes and cytokine expression, we stimulated macrophages towards specific phenotypes *in vitro*. pSTAT1 was abundant in M(IFNγ+TNFα) at all three time points after activation and not detectable in M(IL10) and M(IL4). pSTAT3 was highly present in M(IL10). pSTAT6 was only detected in M(IL4), but not in M(IFNγ+TNFα) or M(IL10), and phosphorylation
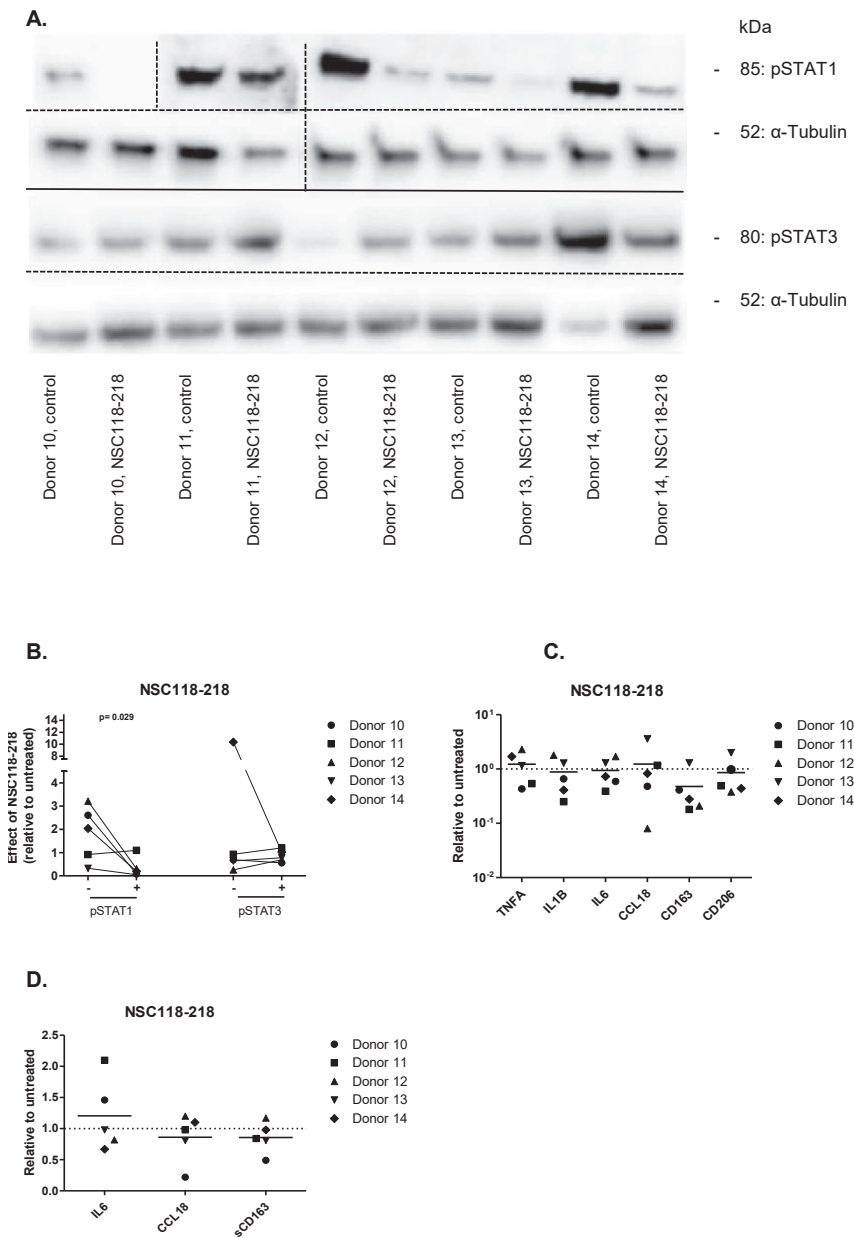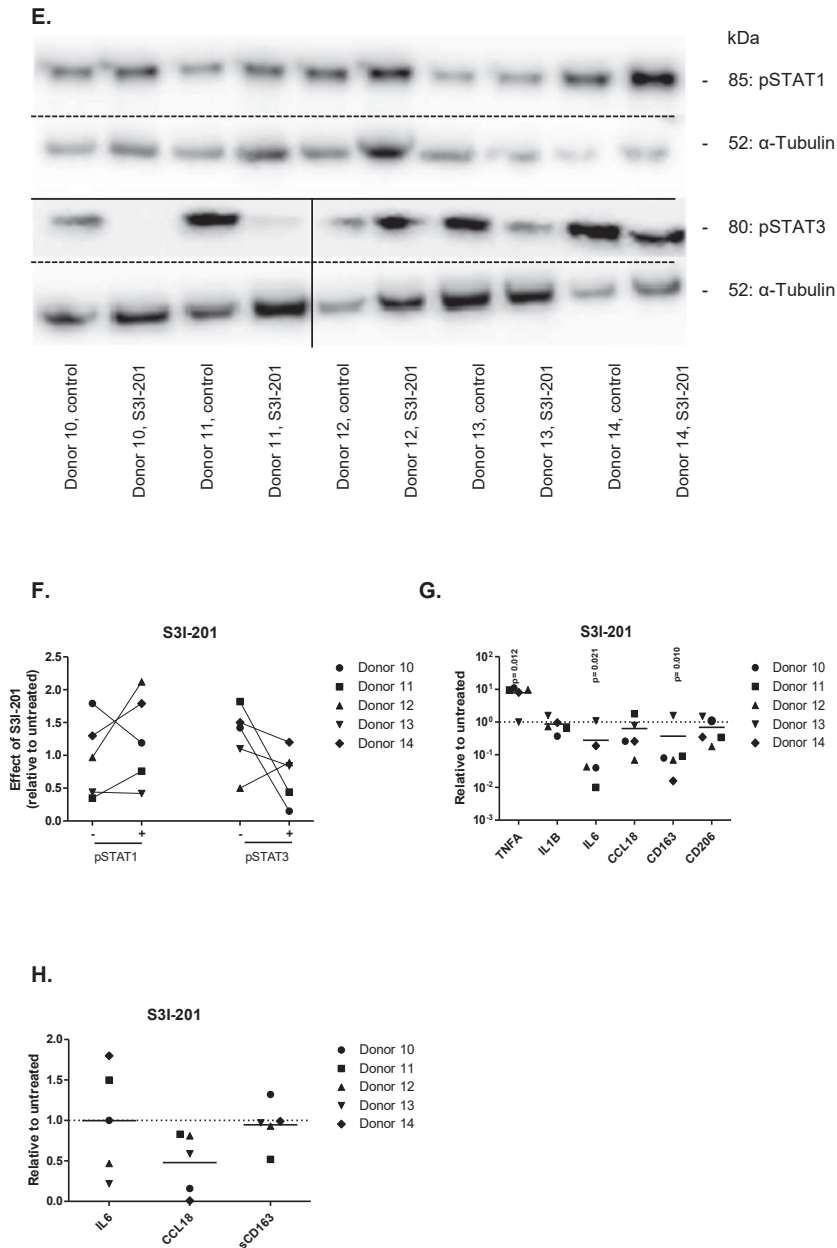
Figure 3. Modulation of STAT phosphorylation in OA synovial tissue cultured in medium.

Figure 3. Modulation of STAT phosphorylation in OA synovial tissue cultured in medium.

A, E) Western Blot analysis of synovial tissue after treatment with the STAT inhibitors. The dotted line indicates grouping of images from different parts of the same gel, whereas the continuous line indicates grouping of different parts from different gels. -; control, +; treated with STAT inhibitor. B, F) Quantification of the Western Blots relative to α Tubulin. C, G) Gene expression corrected for GAPDH and relative to the untreated control, which is represented by the dotted line. D, H) Protein production of IL6, CCL18 and sCD163. Data is shown as mean (as inidcated by the line) for n=5 donors analysed in 2-fold.

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rapidly decreased after stimulation (Figure 4B). M(IFNγ+TNFα) had significantly higher gene expression levels of *TNFA, IL6* and *IL1B*, than the other macrophage phenotypes. M(IL4) expressed the highest levels of *CCL18* and *CD206*. M(IL10) expressed significantly more *CD163* than M(IFNγ+TNFα) (Figure 4C). Although not significant, IL6 protein was the highest in culture medium of M(IFNγ+TNFα), CCL18 was the highest in M(IL4) and sCD163 in M(IL10) (Figure 4D).

To examine whether the analysed pSTATs in synovium are differentially activated in different macrophage phenotypes, we analysed pSTAT1, 3, and 6 in different *in vitro* obtained macrophage phenotypes. Samples were taken at different time points after stimulation since STAT activation can be very dynamic.

A.
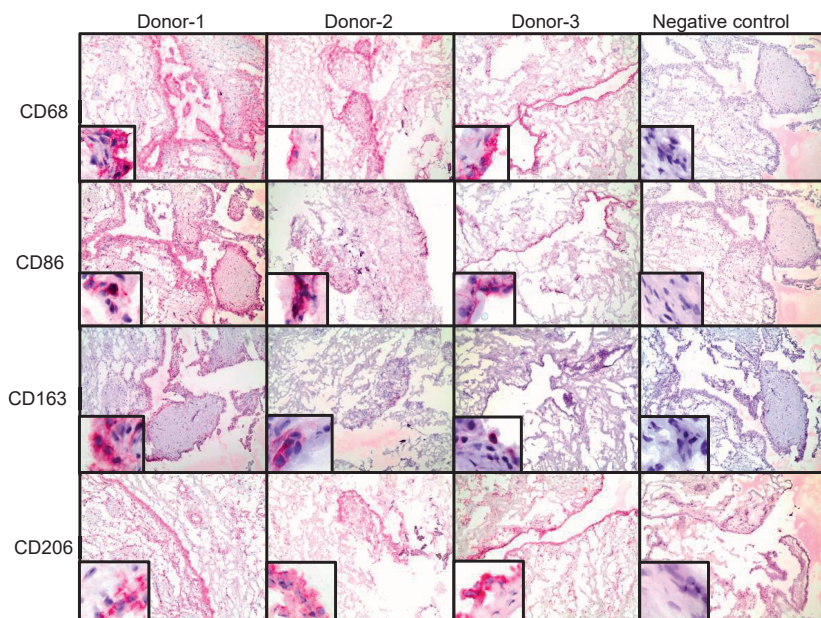
Figure 4. Differences in macrophage phenotypes in OA synovium and characterization of primary human macrophage phenotypes, stimulated with IFNγ and TNFα (M (IFNγ + TNFα)), IL4 (M(IL4)) or IL10 (M(IL10))

B.

Figure 4. Differences in macrophage phenotypes in OA synovium and characterization of primary human macrophage phenotypes, stimulated with IFNγ and TNFα (M (IFNγ + TNFα)), IL4 (M(IL4)) or IL10 (M(IL10))
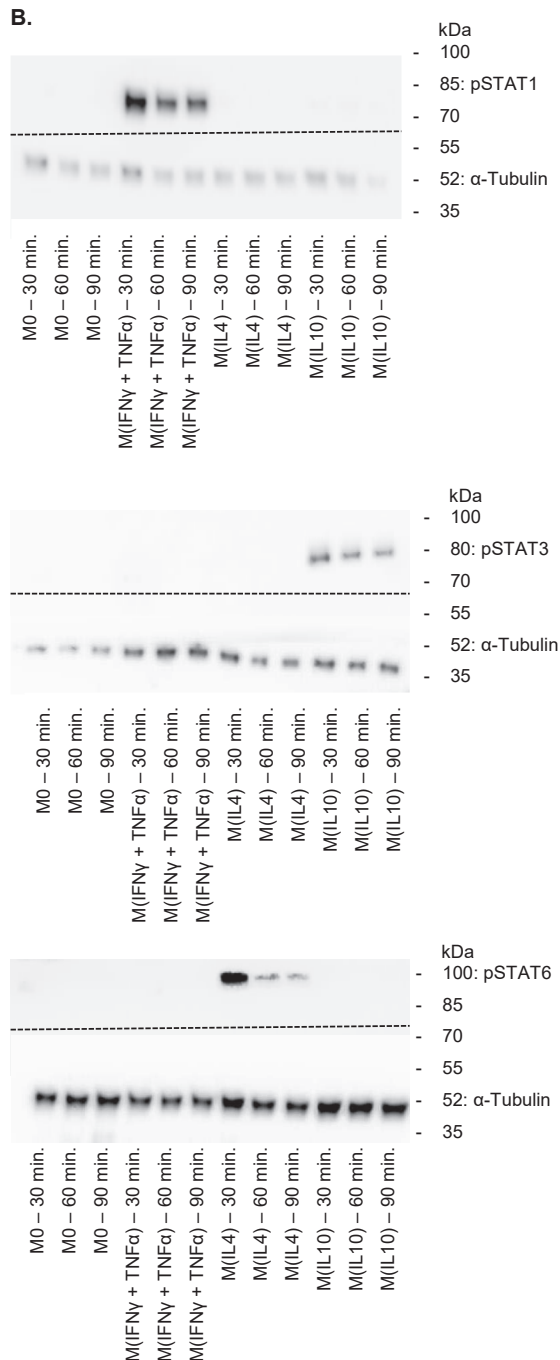
C.

Figure 4. Differences in macrophage phenotypes in OA synovium and characterization of primary human macrophage phenotypes, stimulated with IFNγ and TNFα (M (IFNγ + TNFα)), IL4 (M(IL4)) or IL10 (M(IL10))
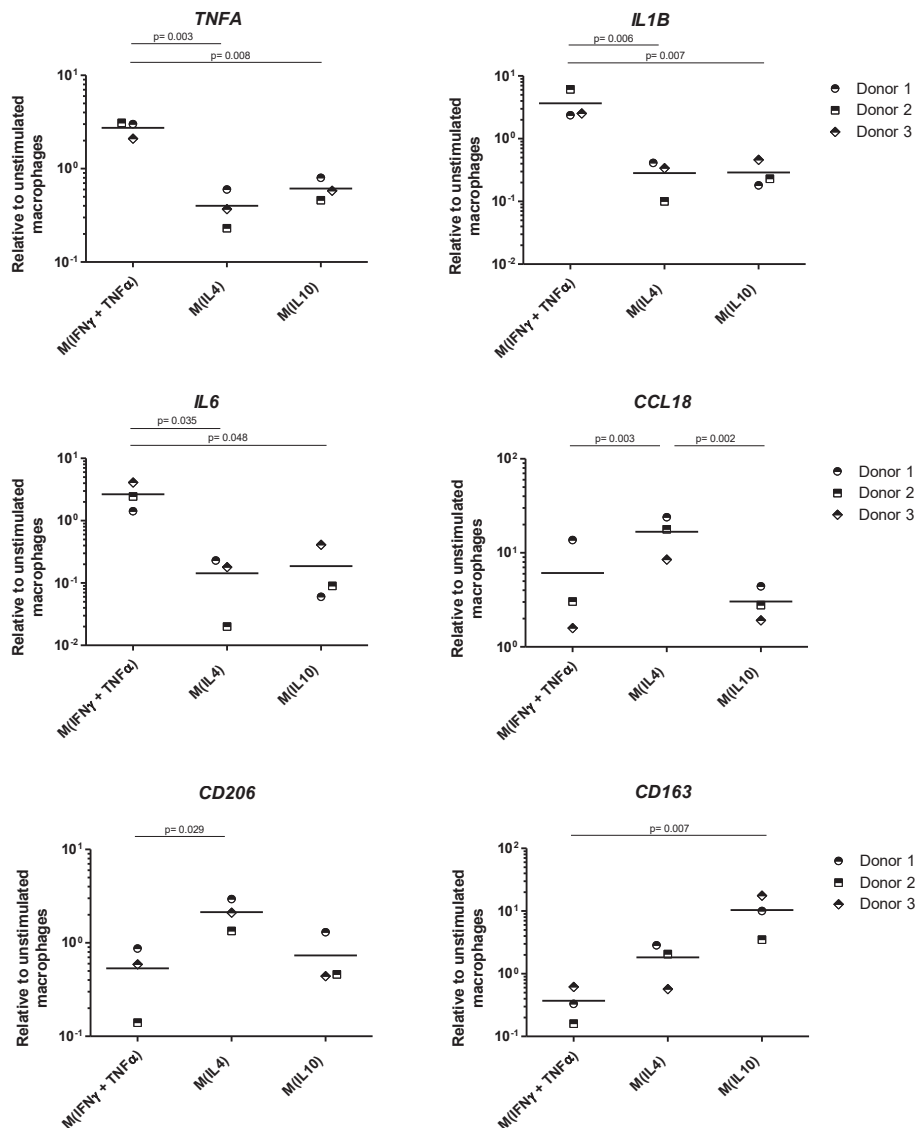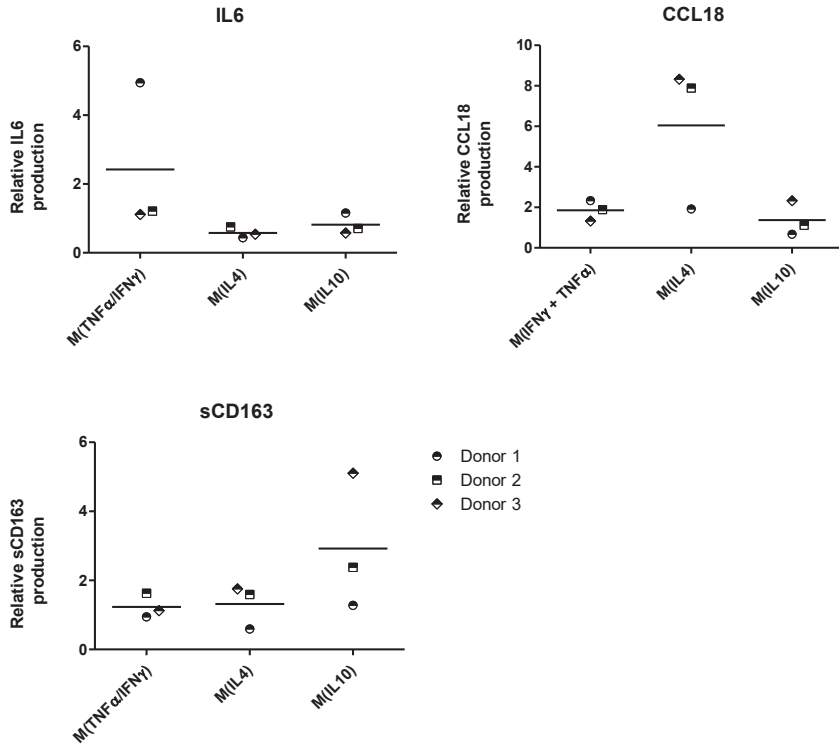
D.

Figure 4. Differences in macrophage phenotypes in OA synovium and characterization of primary human macrophage phenotypes, stimulated with IFNγ and TNFα (M (IFNγ + TNFα)), IL4 (M(IL4)) or IL10 (M(IL10))

A) Immunohistochemistry for markers indicating different macrophage phenotypes in OA synovium. B) Western Blot analysis of in vitro differentiated macrophages, at three time points after the start of differentiation. C) Gene expression corrected for GAPDH in the differentiated macrophages 24 hours after the start of stimulation, and D) Protein production by differentiated macrophages after 24 hours of IL6, CCL18 and sCD163 corrected for amount of DNA. Data is shown as mean (indicated by line) for n=3 donors analysed in 3-fold.

DISCUSSION

In the current paper, we show that STAT1 and STAT3 phosphorylation levels differ among the OA synovial donors and that pSTAT6 is only detectable in presence of corresponding stimuli. Although NSC118-218 is the known STAT1 inhibitor, STAT1 phosphorylation was only inhibited by AS1517499 (known as STAT6 inhibitor) in presence of synovial fluid and resulted in a significant decrease of *IL1B* and *IL6* gene expression levels. In absence of synovial fluid, the STAT3 inhibitor S3I-201 inhibited STAT3 phosphorylation

and resulted in more *TNFA*, and less *IL6* and *CD163*. Since macrophage phenotypes had specific STAT1, 3, and 6 phosphorylation levels and presence of the macrophage phenotypes was confirmed in OA synovium, the targeting of phosphorylated STAT proteins within macrophage phenotypes might be a potential new approach to modulate synovial inflammation.

Nowadays, many anti-inflammatory compounds are being tested as potential new strategies for OA, focusing on complete suppression of inflammation, either via macrophages or via directly inhibiting cytokines. However, this approach may be too aspecific as sometimes a certain level of inflammation is required for a proper healing[20] and the composition of macrophage phenotypes in the synovium can differ at different stages of OA[3] and even between patients[12]. Many cytokines use JAK-STAT signaling pathways to transduce intracellular signals. As phosphorylated STAT proteins are found in the synovial membrane of patient with rheumatoid arthritis[9, 34, 36], modulation of intracellular signaling pathways have shown to be a promising intervention[2]. Compounds used in the current study share a comparable mechanism of action, as NSC-118218 and AS1517499 are known to inhibit tyrosine phosphorylation and S3I-201 binds to SH2 binding sites[6, 13, 21, 26]. This prevents STAT proteins to get phosphorylated and detached from its receptor. Ultimately resulting in an inability to form STAT dimers and therefore an inability to bind DNA recognition sequences. To our knowledge, this is the first study showing modulation of the inflammatory phenotype of osteoarthritic synovial tissue using STAT phosphorylation inhibitors.

Different culture set-ups were used when testing the effect of the inhibitors. Since pSTAT6 was only detectable when stimuli were present, we cultured synovial explants with synovial fluid while adding the three STAT-inhibitors. Here surprisingly enough only AS1517499, chosen as STAT6 inhibitor, decreased the phosphorylation of STAT1 but not STAT6. When the synovial explants were cultured in medium without synovial fluid, no STAT6 was detectable and thus no STAT6 inhibitor was tested. Without the presence of synovial fluid, NSC-118218 decreased STAT1 phosphorylation, but without changing expression of the analysed genes and S3I-201 decreased STAT3 phosphorylation in 4 of the 5 synovial explants donors in the presence of S3I-201. Upregulation of *TNFA* in response to inhibition of STAT3 phosphorylation may be explained by the action of different STATs: selective blockade of one STAT molecule might be compensated by more activation of another STAT molecule[7], although we did not see this for the analysed STATs. Another explanation for the increase of *TNFA* when STAT3 phosphorylation is inhibited might be that inhibition of anti-inflammatory markers abolishes a more pro-inflammatory response, as has been shown in previous work[30]. The loss of inhibitory function of NSC-118218 and S3I-201 in the presence of synovial fluid might be explained by a continuous presence of JAK-STAT pathway-activating stimuli in the synovial fluid[24], such as the STAT1 activators IL-1β and IL-6 or the STAT3 activator IL-10.

Our study showed that different macrophage phenotypes had specific STAT phosphorylation patterns *in vitro*. Presence of these corresponding macrophage phenotypes in OA synovium was confirmed and in line with previous reports[12, 28]. The levels of phosphorylated STAT proteins that could be detected in OA synovium appeared to be strongly donor dependent. This suggests that the stage of inflammation of the synovial tissue and possibly also the different macrophage phenotypes residing in the synovium varies among patients. Differences in the response to treatment with STAT-inhibitors might be explained by these findings.

A potential approach to modulate synovial inflammation might be via modulation of macrophages with high STAT1 phosphorylation levels, as these phosphorylated proteins are predominantly found in pro-inflammatory macrophages. On the other hand, one should avoid the modulation of macrophages with high pSTAT3 or pSTAT6 levels, since these phosphorylated STATs are associated with macrophages that have an anti-inflammatory phenotype. Therefore, modulating synovial inflammation by targeting phosphorylated STAT proteins in macrophages might be a suitable approach to delay the progression of OA.

To quantify the effect of the compounds on STAT phosphorylation we semi-quantitated the Western blot data. Besides, using total protein measurements to load an equal amount of protein per sample, we used α-tubulin as an extra control for normalization. We specifically chose to use α-tubulin for this purpose because we were interested in the total amount of pSTAT1, 3, and 6 irrespective of how much unphosphorylated STAT was present in the cell. Moreover, since phosphorylation of STATs might result in an altered ratio between STAT and pSTAT, unphosphorylated STATs cannot be used as control protein for equal loading.

In conclusion, different macrophage phenotypes have specific STAT phosphorylation levels. OA synovium contains these macrophage phenotypes but has varying STAT phosphorylation levels among the donors. This suggests that the composition of synovial macrophages and herewith the degree of synovial inflammation is strongly donor dependent. In addition, this study shows that inhibition of STAT phosphorylation in OA synovium modulates its inflammatory phenotype. Considering the varying STAT phosphorylation levels in OA synovium among patients, inhibition of STAT phosphorylation is a potential personalized therapeutic approach to direct the synovial inflammation seen in OA.

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