Adrenergic receptors in osteoarthritic and rheumatoid synovial fibroblasts: Identification of β3 as novel player to modulate IL-6 and p38 activation

Ding Liang
Universitätsklinikum Düsseldorf: Universitätsklinikum Dusseldorf

Torsten Lowin (torsten.lowin@med.uni-duesseldorf.de)
University Hospital Duesseldorf  https://orcid.org/0000-0001-7368-2776

Xinkun Cheng
Universitätsklinikum Düsseldorf: Universitätsklinikum Dusseldorf

Tim Classen
St. Elisabeth-Hospital Meerbusch-Lank

Georg Pongratz
Universitätsklinikum Düsseldorf: Universitätsklinikum Dusseldorf

Research article

Keywords: synovial fibroblast, osteoarthritis, rheumatoid arthritis, adrenergic receptors, IL-6, p38

DOI: https://doi.org/10.21203/rs.3.rs-299542/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

**Background:** Rheumatoid arthritis (RA) is influenced by the activity of the sympathetic nervous system (SNS). In animal models of RA, the SNS promotes severity of the disease and its manipulation modulates experimental arthritis depending on timing of the intervention. Synovial fibroblasts (SF) are major contributors to RA pathology but their modulation by the SNS has been rarely investigated. In this study we assessed the expression and function of adrenergic receptors in RA and osteoarthritis (OA) synovial fibroblasts and investigated their downstream signaling.

**Methods:** We used western blot and quantitative PCR (qPCR) to determine protein and mRNA of adrenergic receptors in OASF/RASF. Furthermore we determined α<sub>1a</sub> and β<sub>2</sub> protein in synovial tissue by immunofluorescence. ELISA was employed to determine IL-6 production. p38 kinase activation and translocation was analyzed by cell-based ELISA and immunofluorescence.

**Results:** We detected α<sub>1a</sub>, α<sub>2b</sub>, β<sub>1</sub>, β<sub>2</sub> and β<sub>3</sub> protein in OASF/RASF and α<sub>1a</sub> and β<sub>2</sub> protein in synovial tissue of OA and RA patients. The pro-inflammatory cytokines IFN-γ and TNF downregulated β<sub>3</sub> adrenergic receptor. Activation of α<sub>1a</sub>, α<sub>2b</sub>, β<sub>2</sub> and β<sub>3</sub> increased production of TNF-induced IL-6 which was inhibited by specific antagonists. Furthermore, β<sub>3</sub> agonism enhanced p38 phosphorylation and translocation to the nucleus.

**Conclusion:** Among a comprehensive characterization of the adrenergic system of OASF/ RASF, we report for the first time β<sub>3</sub> expression and demonstrated that this adrenergic receptor participates in the inflammatory response of synovial fibroblasts. Therefore, modulation of β<sub>3</sub> might pose a new therapeutic opportunity to modulate synovial fibroblast function in patients with RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease which is characterized by inflammation of the joints, autoantibody and proinflammatory cytokine production. The immune reaction in RA is supported by the sympathetic nervous system (SNS), since sympathectomy before the induction of experimental arthritis in mice results in less severe symptoms [1]. The SNS supports migration of immune cells to lymph nodes and enhances IFN-γ production in the spleen [2, 3]. However, in the late phase of the disease the influence of the SNS changes from pro- to anti-inflammatory, which might be explained by the appearance of IL-10 producing, regulatory B cells which are generated under the influence of norepinephrine [4]. In addition, tyrosine hydroxylase (TH) positive immune cells, capable of producing own catecholamines, appear in inflamed tissue [5, 6], which is considered as a compensatory mechanism, since SNS fibre density decreases in inflamed tissue [7, 8]. As a consequence, late sympathectomy further aggravates experimental arthritis, most likely due to deletion of these TH<sup>+</sup> cells [9]. While the effects of norepinephrine on T cells, B cells and monocytes/macrophages are well described, the situation is less clear in rheumatoid arthritis synovial fibroblasts (RASF) [10]. RASF are a major contributor of joint destruction and they produce copious amounts of proinflammatory cytokines such as
IL-6 and matrix degrading enzymes [11]. RASF migration and cytokine production are influenced by dopamine [12, 13], which is synthesized by blood lymphocytes [14]. Norepinephrine is released by sympathetic nerve terminals but also by TH+ cells [15] and it influences cytokine production of RASF [16, 17]. Although the effects of norepinephrine on RASF have been described it is still unclear which adrenergic receptors (AR) in particular are involved. Wu et al. showed the surface expression of β2 adrenergic receptors [17], but it is not known whether β1 and β3-AR are also expressed. Similarly, α-adrenergic stimulation by low-dose norepinephrine influences cytokine levels in RASF, but the respective receptor subtypes have not been identified [16].

In this study, we determined the AR subtypes expressed by RASF and osteoarthritis synovial fibroblasts (OASF) and determined the effects of AR receptor stimulation on the production of the pro-inflammatory cytokine IL-6 and on activation of the MAP kinase p38, which has been shown to be a downstream target of β2 AR [18].

Results

RASF express α1a, α2b, β1, β2 and β3 AR and protein levels of AR are regulated by cortisol and proinflammatory cytokines

First, we detected α1a, α2b, β2 and β3 in unstimulated OASF and RASF on protein level (Fig. 1A). Next, we were interested in the regulation of AR by pro-and anti-inflammatory factors. For this purpose, we incubated OASF and RASF with either cortisol, TNF, IFN-γ or left untreated (Fig. 1B, C). Western blot densitometric analyses revealed that cortisol upregulated the protein levels of α2b (p=0.062), β2 (p=0.066) and β3 AR (p=0.026) in OASF (Fig. 1B) but not in RASF (Fig. 1C), whereas TNF reduced α2b (p=0.052), β2 (p=0.015) and β3 (p=0.002) in OASF (Fig. 1B) and β3 (p=0.004) in RASF (Fig. 1C). In addition, IFN-γ downregulated β3 in OASF (p=0.062) and RASF (p=0.036) (Fig. 1B, C). Furthermore, we detected several bands when using the anti-β1 antibody (Fig. 1D) although not at the predicted molecular weight. Whole blots are shown in supplementary Fig. 1.

Since antibodies against AR can be unspecific [19], we also employed quantitative PCR to detect α1a, α2b, β1 and β3 mRNA expression in RASF (Fig. 1E). Here, we confirmed expression and the downregulation of AR by cytokines as demonstrated in western blot analyses. In addition, we detected β1 AR mRNA which verifies the presence of this AR as shown on the protein level (Fig. 1E). β2 was not investigated by PCR since we used several different antibodies and techniques (flow cytometry, western blotting, immunofluorescence) to detect and verify this AR (data not shown).

α and β AR in synovial tissue

Besides isolated OASF and RASF we were also interested in the expression of AR in OA and RA synovial tissue since it might be possible that in vitro culture conditions alter the levels of AR on synovial fibroblasts. We detected α1a, β1 (RA only, Fig.2B) and β2 AR in OA and RA synovial tissue and these AR co-stained with CD55, a marker for synovial fibroblasts (Fig. 2A). The antibodies against α2b and β3 used in western blot were not suitable for immunofluorescence. In addition, we also co-stained the AR with CD68.
(marker for macrophages, suppl. Fig. 2A) and CD3 (marker for T cells, suppl. Fig. 2B) and found that macrophages and some T cells also express $\alpha_{1a}$ and $\beta_2$ AR. Furthermore, we detected $\alpha_{1b}$ AR to be expressed by a few CD3$^{low}$ cells in synovial tissue (Fig. suppl. 2C), suggesting that a T cell subpopulation expresses this AR.

**Modulation of IL-6 release from OASF and RASF stimulated with TNF by AR agonists**

TNF, a proinflammatory cytokine, is one important factor in RA and anti TNF treatment ameliorates disease activity in around 30% - 50% of patients [20]. TNF is a potent inducer of IL-6 expression [21], and, therefore we assessed the influence of AR agonists on IL-6 production. We found that 1h preincubation with the $\alpha_1$ agonist phenylephrine, the $\alpha_2$ agonist dexmedetomidine hydrochloride and the unselective $\beta$ agonist isoproterenol increased TNF-induced IL-6 production by OASF (Fig. 3A, B, C), whereas dexmedetomidine and BRL increased IL-6 production by RASF (Fig. 3G, I). These effects were specific since the effects by AR agonists were inhibited by their respective antagonists in OASF (Fig. 3A, B) and RASF (Fig. 3F, G, I). Interestingly, when we combined the selective $\beta_3$ agonist BRL37344 ($10^{-7}$M) with Isoproterenol ($10^{-7}$M and $10^{-6}$M), the stimulatory effect of both agonists was lost in RASF (Fig. 3J) but not OASF (Fig. 3E).

**AR agonists increase IL-6 without further stimulation with proinflammatory cytokines**

We demonstrated that AR ligands can further increase TNF-induced IL-6 production by OASF and RASF. However, we were also interested whether AR stimulation increases IL-6 levels without concomitant cytokine stimulation. For this purpose, we incubated OASF and RASF with the $\beta_2/3$ agonist BRL37344 (Fig. 3K) and found that BRL alone was able to increase IL-6 in RASF (~50% increase at $10^{-10}$M) (Fig. 3K).

**Activation of $\beta_3$ AR regulates p38 phosphorylation and translocation into the nucleus**

Our results show that the $\beta_3$ AR is expressed and functional in OASF and RASF and the importance of this receptor is underscored by the potential to upregulate IL-6 in RASFs without further cytokine stimulus. Therefore, we focused on this receptor for further characterization of downstream signaling. Since it is known that $\beta_3$ agonists foster p38 activation in cardiomyocytes and adipocytes [22, 23], we investigated p38 phosphorylation and translocation to the nucleus in OASF and RASF by cell-based ELISA and immunocytofluorescence. Cell-based ELISA demonstrated a time-dependent increase in p38 phosphorylation in OASF and RASF (Fig. 4A). The activation and translocation of p38 induced by BRL37344 ($10^{-7}$M) was visualized in Fig. 4B and C. In OASF, the signal for phosphorylated p38 appeared already after 1min and remained for at least one hour (Fig. 4B). In RASF, phosphorylated p38 was detectable after 3 minutes and nuclear translocation was still evident after 1h following stimulation with BRL (Fig. 4C).

**Discussion**

In this study, we assessed for the first time the protein expression of ARs in OASF and RASF. Besides the already described $\beta_2$ AR, we detected $\alpha_{1a}$, $\alpha_2b$, $\beta_1$ and $\beta_3$ AR protein and mRNA in OASF and RASF lysates.
The discovery of $\beta_3$ expression in fibroblasts was unexpected since this receptor is only expressed by very few cell types such as adipocytes [23]. Overall, stimulation of AR resulted in increased IL-6 production, which is usually considered as proinflammatory since IL-6 neutralization is one important therapeutic intervention in RA [24] and is required for the successful induction of collagen-induced arthritis in mice [25]. However, proinflammatory properties of IL-6 are mediated by trans signaling (stimulation of cells devoid of own IL-6 receptor by acquiring soluble IL-6 receptor and signaling transducer gp130) whereas classical signaling is mostly anti-inflammatory [26]. IL-6 is important for metabolic control as IL-6$^{-/-}$ mice develop metabolic disturbances [27]. Therefore, $\beta$ AR stimulation might increase IL-6 to control metabolism and energy expenditure since one major function of $\beta$ ARs is to mobilize energy rich substrates [28]. This is beneficial in short term inflammatory episodes but detrimental in chronic inflammation [28]. On the other hand, IL-6 production induced by $\alpha$ ARs might directly support proinflammatory immune cells. Of note, we did not investigate the effect of $\alpha$ AR stimulation without the addition of TNF. It might be that activation of $\alpha$ ARs in the absence of inflammation does not influence IL-6 production. We found that $\alpha$ and $\beta$ ARs both increase IL-6 production and this might not depend on G protein but $\beta$-arrestin signaling which can be employed by both $\alpha$ and $\beta$ ARs [29, 30]. In line with this, $\beta$-arrestin signaling can be pro- or anti-inflammatory, but under TNF-stimulated conditions, proinflammatory effects predominate [31].

Since $\beta_3$ ligation alone entailed upregulation of IL-6 in RASF without further cytokine stimulus, we investigated downstream signaling of this AR and found that BRL37344 was able to induce p38 phosphorylation and nuclear translocation in OASF and RASF.

Western blotting, quantitative PCR and immunofluorescence in OA and RA synovial tissue showed the expression of $\alpha_{1a}$, $\alpha_{2b}$, $\beta_1$, $\beta_2$ and $\beta_3$ in OASF, RASF but also in other immune cell populations present in synovial tissue emphasizing the important role of adrenergic mechanisms for regulation of joint inflammation [10]. Western blotting further revealed that Cortisol increased the expression of $\alpha_{1a}$ (in RASF), $\alpha_{2b}$ (in OASF), $\beta_2$ (in OASF) and $\beta_3$ (in OASF) protein. This is in line with early results from Lefkowitz who showed a stimulatory effect of cortisol on $\beta$ AR [32]. In addition, upregulation of the $\alpha_{1b}$ AR by the glucocorticoid dexamethasone was demonstrated due to enhanced transcription of the respective mRNA [33]. Since only OASF showed an upregulation of $\alpha_{2b}$, $\beta_2$, and $\beta_3$ in response to cortisol, RASF might have lost their ability to adequately respond to this glucocorticoid. In fact, it has been demonstrated that some loss-of function polymorphisms in the glucocorticoid receptor $\alpha$ gene are present in RA patients [34]. In addition, some RA patients have a relative preponderance of glucocorticoid $\beta$ over glucocorticoid $\alpha$ receptors, which is considered a proinflammatory signal, since glucocorticoid $\beta$ receptors antagonize the DNA binding of the $\alpha$ receptor subtype [35]. We do not consider drug therapy with glucocorticoids as contributing factor for this difference between OASF and RASF, since SFs were used after several passages and therefore any acute effect of GC therapy would not be relevant anymore. Nevertheless, there might be epigenetic alterations by GC therapy [36]. We also observed decreased $\beta_3$
(and β2 in OASF) protein levels after stimulation with TNF or IFN-γ. Similar effects are known for b2 AR since it was shown that TNF blunts the ability of the unselective β agonist Isoproterenol to relax smooth muscle cells by desensitization and therefore possible downregulation of the receptor [37]. Although western blot results for β1 AR were inconclusive due to incorrect molecular weight of detected bands, immunofluorescence showed expression of this receptor in RASF. However, as discussed below, antibodies against β AR in general are not utterly specific and the molecular weight of β AR can vary widely [19].

The functional impact of AR stimulation was assessed by analyzing IL-6 production by OASF and RASF. We found that α1a, α2b, β1/2 and β3 activation increased TNF-induced IL-6 production by OASF and RASF (albeit weaker) which was inhibited by respective antagonists, except for the unselective β agonist isoproterenol. Similarly, β3 activation without additional TNF also increased IL-6 levels in RASF. This is in line with data from Burger et al. and Tanner et al. who demonstrated enhanced IL-6 production in cardiac fibroblasts solely in response to norepinephrine in an α and β AR dependent manner [38, 39]. Also, Raap et al. showed a stimulatory or inhibitory effect of norepinephrine on IL-6 and IL-8 production by OASF and RASF, respectively, without further cytokine stimulation and depending on used norepinephrine concentration [16].

Interestingly, when we combined the β3-AR agonist BRL37344 with the unselective β-AR agonist Isoproterenol, we detected a decrease rather than an increase of IL-6 production by RASF. This suggests some degree of antagonism between the three types of β-AR, since activation of β3-AR alone increased IL-6. This might be related to differential signaling induced by individual β-AR. While e.g. the β2-AR couples to the PKA activating G protein Gαs with a switch to inhibitory Gαi after prolonged incubation, β3-AR can bind both Gαs and Gαi simultaneously leading to distinct signaling events [40].

One important kinase involved in IL-6 production by synovial fibroblasts is p38 [41], and therefore we investigated the activation of this map kinase in response to β3-AR activation. We found that BRL37344 induced p38 phosphorylation and translocation to the nucleus in OASF and RASF. Similar results have been obtained in adipocytes, where p38 was identified as downstream target of β3-AR [42]. In addition, p38 was also involved in β3-AR signaling in cardiomyocytes [22]. The activation of p38 is not restricted to the β3-AR, since earlier studies by our group also demonstrated p38 phosphorylation by ligation of β2-AR [18].

**Limitations of our study**

One major challenge detecting specific isoforms of α and β AR is the lack of specificity with a lot of the commercially available antibodies. In a study by Hamdani it was shown that antibodies raised against β1 or β2 recognized all three β isoforms without distinction [19]. The same was true for antibodies raised against β3, which also labelled β1 and β2 [43]. Similar problems have been demonstrated by using an
antibody against $\alpha_1$ AR, which detected all $\alpha_1$ subtypes including additional non-specific bands [44]. Although we did not use any of the $\alpha/\beta$ AR antibodies investigated in the above mentioned studies, there is still the possibility that the antibodies used in our experiments are not specific. Therefore, we also confirmed the expression of respective ARs by quantitative PCR. Similar problems might arise with the ligands used in our study. Although e.g. BRL37344 is sold as a specific $\beta_3$ AR agonist, it also binds to $\beta_2$ with similar affinity [45]. However, we also used the $\beta_3$ antagonist L-748,337, which is 20 fold (vs $\beta_2$) and 45 fold (vs $\beta_1$) more selective at $\beta_3$ AR [46].

**Conclusion**

In this study, we investigated for the first time in a comprehensive manner, which ARs are present on OASF and RASF, respectively. A completely novel finding is the presence of $\beta_3$ AR on OASF and RASF which might turn out to be a major responder to sympathetic stimuli in the joint, as this receptor was able to modulate IL-6 without further cytokine stimulus. In addition, we found that $\alpha$ and $\beta$ AR stimulation modulates IL-6 production and also revealed that $\beta_3$ activation induces p38 phosphorylation and translocation into the nucleus. These data suggest that intervention with the AR system especially the $\beta_3$ AR poses a therapeutic possibility to dampen proinflammatory activity of synovial fibroblasts in RA.

**Materials And Methods**

**Patients**

In this study, 18 patients with long-standing RA fulfilling the American College of Rheumatology revised criteria for RA [47] and 22 patients with OA, who underwent elective knee joint replacement surgery, were included. Mean age was 70.2 ± 9.1 years for OA and 67 ± 11.3 years for RA. Mean CRP was 3.3 ± 4.1 for OA and 8.7 ± 10.8 for RA. Rheumatoid factor was 11.8 ± 7.8 in OA and 182 ± 319 in RA. In the RA patient group 6/18 received MTX, 7/18 glucocorticoids and 4/18 received biologicals or JAK inhibitors. All patients in this study were informed about the purpose and gave written consent before surgery. This study was approved by the Ethics Committees of the University of Düsseldorf (approval number 2018-87-KFogU).

**Compounds and chemicals**

Phenylephrine hydrochloride (selective $\alpha_1$ AR agonist), dexmedetomidine hydrochloride (selective $\alpha_2$ AR agonist), L-748, 337 ($\beta_3$ AR antagonist), doxazosin mesylate ($\alpha_1/\alpha_2$ AR antagonist) and RS79948 hydrochloride (selective $\alpha_2$ AR antagonist) were obtained from Tocris/Bio-Techne (Wiesbaden, Germany). Isoproterenol (unselective $\beta$-AR agonist), norepinephrine (unselective AR agonist), BRL 37344 (selective $\beta_3$-AR agonist) and nadolol (non-selective $\beta$ AR agonist) were obtained from Sigma Aldrich (St. Louis, USA).

**Synovial fibroblast and tissue preparation**
Samples from RA and OA synovial tissue were isolated and prepared as described previously [48]. After opening of the knee joint capsule, synovial tissue samples were obtained immediately. Synovial tissue of 9 cm² was excised, part of which was cut off and stored in a protective freezing medium at −80°C until further use (Tissue Tek, Sakura Finetek, Zoeterwoude, The Netherlands). The other part was chopped and treated overnight at 37 °C with liberase (Roche Diagnostics, Mannheim, Germany). The resulting suspension was filtered (70 µm) and centrifuged at 300 g for 10 minutes. The pellet was then treated with erythrolysis buffer (20.7 g NH₄Cl, 1.97 g NH₄HCO₃, 0.09 g EDTA ad 1L H₂O) for 5 minutes, and centrifuged again for 10 minutes at 300 g. Cells were resuspended in RPMI-1640 (sigma Aldrich, St. Louis, USA) with 10% FCS. The number of cells was calculated by a Neubauer cell counting chamber. A total of 1,000,000 cells were transferred to a 75 square centimeter tissue culture flask. After overnight culture, cells were supplemented with fresh medium.

**Stimulation of OA and RASFs**

5000 cells were seeded onto 96 well microtiter plates, grown for three days and were then incubated with or without TNF (10 ng/ml) and AR agonists and antagonists for 24h in RPMI medium containing 2% FCS to minimize proliferation; for all assays. Cell-free supernatants were collected (18-24h after TNF-α stimulation).

**IL-6 ELISA**

Cell culture supernatants were used for ELISAs 24 h (IL-6) after addition of related AR ligands. The test was carried out according to the supplier’s description (BD, OptEIA, Heidelberg, Germany). The coefficient of variation between and within batches was less than 10%.

**Immunofluorescence I (staining of synovial tissue)**

For immunofluorescent visualization of α₁a (antibody ab137123, 1.049mg/ml, Abcam, Cambridge, UK, 1:5000), α₂b (antibody ab151727, 1.049mg/ml, Abcam, Cambridge, UK, 1:20000), β₁ (antibody ab3442, 1mg/ml, Abcam, Cambridge, UK, 1:1000) and β₂ (antibody ab182136, 0.182mg/ml, Abcam, Cambridge, UK, 1:5000) in frozen tissue sections, antibodies, #9661-01 (CD55, Southern Biotech, Birmingham, AL, USA, 0.1 mg/ml), #ab5690 (CD3, Abcam, Cambridge, UK, 0.2mg/ml) and #MO718 (CD68, Dako/Agilent, Santa Clara, USA, 237 µg/ml) were used. Frozen tissue samples were cut, fixed and dried. After that, samples were rehydrated with PBS and then blocked with 2% normal goat serum and 0.3% Triton X-100 in PBS for 1 h at room temperature. Then samples were incubated with primary antibodies overnight at 4°C. Slides were washed and incubated with secondary antibodies (A-11037, Thermo Fisher, Alexa Fluor 594, goat anti-rabbit, 1:2000; A-11001, Thermo Fisher, Alexa Fluor 488, goat anti-mouse, 1:2000) for 2h at room temperature. Samples were covered with ProLong Gold Antifade Mountant (Thermo Fisher) and visualized.

**Immunofluorescence II (staining of OASF and RASF)**
For immunofluorescent visualization of phosphorylated p38, Cell Signaling Technology, Inc, USA, NB4511, 1:1500 was used. Cells were fixed with 2% formaldehyde for 20min and permeabilized with PBS containing 0.1% Triton-X 100. Slides were blocked with 1% BSA in PBS/0.1% Triton-X and were incubated with primary antibodies overnight at 4°C. After washing, culture slides were incubated with secondary antibodies (A-11037, Thermo Fisher, AlexaFluor 594, goat anti-rabbit, 1:2000) for 2h at room temperature. Samples were covered with ProLong Gold Antifade Mountant (Thermo Fisher) and visualized. Isotype IgG was used as negative control.

Western blot
The following antibodies were used: α1a (antibody ab137123, 1.049mg/ml, Abcam, Cambridge, UK, 1:5000), α2b (antibody ab151727, 1.049mg/ml, Abcam, Cambridge, UK, 1:20000), β1 (antibody ab3442, 1mg/ml, Abcam, Cambridge, UK, 1:1000), β2 (antibody ab182136, 0.182mg/ml, Abcam, Cambridge, UK, 1:5000) and β3 (PA5-50914, ThermoFisher Scientific, Cambridge, UK, 1:1500) and Anti-cyclophilin B (abcam, USA, 1:5000). 1,000,000 cells were lysed subsequently with two buffers with increasing detergent strengths to obtain a cytosolic and a membrane-bound organelle/nuclear fraction. Buffer 1 (Cytosol, 150 mM NaCl, 50 mM HEPES (Sigma), 25 µg/ml digitonin (Sigma)); buffer 2 (membrane-bound organelle/nuclear proteins, RIPA buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl). All buffers were supplemented with complete protease inhibitor (Roche, Mannheim, Germany) and protein content was determined. The protein fractionation was performed as described by Baghirova et al.[49]. Gels (separation gel: 10% acrylamide) were loaded with 10 µg protein and run for 60 min at 20 mA (Biorad, Puchheim, Germany). Gels were blotted at 80 V for 90 min on nitrocellulose membranes (Biorad). Membranes were blocked in 5% milk in TBS for 1 h and incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with the detection antibody (goat anti-rabbit IgG HRP, DAKO P0448, 1:2000) for 2 h at room temperature. Proteins complexed with HRP-conjugated antibody were stained by addition of ECL Prime (GE Healthcare, Freiburg, Germany) and visualized in a V3 Western Workflow (Biorad). The membranes were then washed and dried at room temperature overnight. After that, membranes were incubated with anti-cyclophilin B antibody (housekeeper) (abcam, USA, 1:5000) overnight at 4°C. After washing, membranes were incubated with the secondary antibody (goat anti-rabbit IgG HRP, DAKO P0448, 1:2000) for 2 h at room temperature. Specific signals for ARs were normalized to the anti-cyclophilin B signal.

Quantitative Polymerase chain reaction (qPCR)
RASF were seeded in 6-well plates (10⁵ cells/well) and were grown into an adherent cell monolayer of 70%-80% confluency within 1 week. Stimulation of RASF with TNF-α and IFN-γ for 12 or 24 hours was performed afterwards.
Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.
cDNA was synthesized from equal amounts of RNA (1µg) of different samples using the iScript™ gDNA clear cDNA Synthesis Kit (BIO-RAD).
qPCR was performed by using qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems) and StepOnePlus™ Real-Time PCR System. The total reaction volume was 20µl in all cases. The sequences for the relevant primers are shown in table 1, and GAPDH was used as a quantitative control for mRNA levels. Calculation of results: Ct values were determined for the gene of interest (ARs) and the housekeeper (GAPDH). Then, delta ct was calculated (ct gene of interest – ct gene housekeeper) for each sample. For each condition, mean delta ct values from the control group were subtracted from individual ct values obtained for control, TNF and IFN treatment yielding delta delta ct. Fold expression was calculated by the formula $2^{(\text{delta delta ct})}$.

Following thermocycling conditions were used: 95°C for 2 min, 40 cycles of 95°C for 5 s and 66°C for 30 s, followed by the melt curve stage of 1 cycle of 95°C for 15 s, 60°C for 1 min and 95 °C for 15 s. Samples from 3 RA patients were determined in triplicates.

**Cell-based ELISA**

The following antibodies were used: phosphorylated p38 (Cell Signaling Technology, Inc, USA, NB4511, 1:1500) and isotype control (Abcam, Cambridge, UK, ab171870, 1:10,000). 5000 cells were seeded onto 96 well microtiter plates and were stimulated with BRL or TNF (10 ng/ml). Then, cells were fixed with 3.7% formaldehyde at room temperature for 20 min. After permeabilizing with 0.1% Triton-X in PBS, cells were blocked with Casein blocking buffer (Abcam, ab171532) in 0.1% Triton-X for 1 h at room temperature. Each well was incubated with primary antibody overnight at 4°C. Phosphorylated p38 was then visualized after addition of secondary antibody for 1 h (Goat anti-Rabbit IgG (H+L) Poly-HRP, Thermo Fisher, #32260, 1:1500) with 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher, #34029).

**Statistical analysis**

All the data are presented from at least of three independent experiments. Statistical analysis was performed with GraphPad Prism (GraphPad software Inc, California, USA) and SPSS 25 (IBM, Armonk, USA). The statistic tests used are given in the figure legends. The level of significance was p < 0.05. When data are presented as line plots, the line represents the mean. When data are presented as bar charts, the top of the bar represents the mean and error bars depict the standard error of the mean (sem).

**Declarations**

**Competing interests:** The authors declare that they have no competing interests.

**Ethics approval:** This study was approved by the local ethics committee of Düsseldorf (2018-87-KFogU)

**Funding:** This work was supported by an unlimited grant of the Hiller Foundation and the Deutsche Forschungsgemeinschaft (DFG, PO801/8-1).

**Disclosure:** The authors have nothing to disclose.

**Data availability statement:**
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions:**

D.L. and C.C. and T.C. performed the experiments and analysed the data. T.L. and G.P. designed the study and performed statistical analysis. T.L., D.L., T.C., and C.C. participated in data collection. D.L., C.C., T.L., and G.P. interpreted the data and prepared the draft manuscript. The final version of manuscript was read and approved by all authors.

**Authors’ information:**

Ding Liang and Torsten Lowin are co-first authors.

Poliklinik, Funktionsbereich & Hiller Forschungszentrum für Rheumatologie, University Hospital Duesseldorf, D-40225 Duesseldorf, Germany

Ding Liang, Torsten Lowin, Xinkun Cheng and Georg Pongratz

Klinik für Orthopädie / Orthopädische Rheumatologie, St. Elisabeth-Hospital Meerbusch-Lank, D-40668 Meerbusch, Germany

Tim Claasen

**Corresponding authors:**

Torsten Lowin and Georg Pongratz

**Acknowledgement:** We thank Birgit Opgenoorth for excellent technical assistance.

**References**

1. Härle P, Pongratz G, Albrecht J, Tarner IH, Straub RH: *An early sympathetic nervous system influence exacerbates collagen-induced arthritis via CD4+CD25+ cells*. *Arthritis Rheum* 2008, 58(8):2347-2355.

2. Straub RH, Rauch L, Fassold A, Lowin T, Pongratz G: *Neuronally released sympathetic neurotransmitters stimulate splenic interferon-gamma secretion from T cells in early type II collagen-induced arthritis*. *Arthritis Rheum* 2008, 58(11):3450-3460.

3. Klatt S, Stangl H, Kunath J, Lowin T, Pongratz G, Straub RH: *Peripheral elimination of the sympathetic nervous system stimulates immunocyte retention in lymph nodes and ameliorates collagen type II arthritis*. *Brain Behav Immun* 2016, 54:201-210.

4. Pongratz G, Melzer M, Straub RH: *The sympathetic nervous system stimulates anti-inflammatory B cells in collagen-type II-induced arthritis*. *Ann Rheum Dis* 2012, 71(3):432-439.

5. Jenei-Lanzl Z, Capellino S, Kees F, Fleck M, Lowin T, Straub RH: *Anti-inflammatory effects of cell-based therapy with tyrosine hydroxylase-positive catecholaminergic cells in experimental arthritis*. 
6. Capellino S, Cosentino M, Wolff C, Schmidt M, Grifka J, Straub RH: Catecholamine-producing cells in the synovial tissue during arthritis: modulation of sympathetic neurotransmitters as new therapeutic target. *Ann Rheum Dis* 2010, 69(10):1853-1860.

7. Fassold A, Falk W, Anders S, Hirsch T, Mirsky VM, Straub RH: Soluble neuropilin-2, a nerve repellent receptor, is increased in rheumatoid arthritis synovium and aggravates sympathetic fiber repulsion and arthritis. *Arthritis Rheum* 2009, 60(10):2892-2901.

8. Koeck FX, Bobrik V, Fassold A, Grifka J, Kessler S, Straub RH: Marked loss of sympathetic nerve fibers in chronic Charcot foot of diabetic origin compared to ankle joint osteoarthritis. *J Orthop Res* 2009, 27(6):736-741.

9. Capellino S, Weber K, Gelder M, Harle P, Straub RH: First appearance and location of catecholaminergic cells during experimental arthritis and elimination by chemical sympathectomy. *Arthritis Rheum* 2012, 64(4):1110-1118.

10. Pongratz G, Straub RH: The sympathetic nervous response in inflammation. *Arthritis Res Ther* 2014, 16(6):504.

11. Nygaard G, Firestein GS: Restoring synovial homeostasis in rheumatoid arthritis by targeting fibroblast-like synoviocytes. *Nat Rev Rheumatol* 2020, 16(6):316-333.

12. Capellino S, Cosentino M, Luini A, Bombelli R, Lowin T, Cutolo M, Marino F, Straub RH: Increased expression of dopamine receptors in synovial fibroblasts from patients with rheumatoid arthritis: inhibitory effects of dopamine on interleukin-8 and interleukin-6. *Arthritis Rheumatol* 2014, 66(10):2685-2693.

13. van Nie L, Salinas-Tejedor L, Dychus N, Fasbender F, Hulser ML, Cutolo M, Rehart S, Neumann E, Muller-Ladner U, Capellino S: Dopamine induces in vitro migration of synovial fibroblast from patients with rheumatoid arthritis. *Sci Rep* 2020, 10(1):11928.

14. Buttarelli FR, Fanciulli A, Pellicano C, Pontieri FE: The dopaminergic system in peripheral blood lymphocytes: from physiology to pharmacology and potential applications to neuropsychiatric disorders. *Curr Neuropharmacol* 2011, 9(2):278-288.

15. Schaible HG, Straub RH: Function of the sympathetic supply in acute and chronic experimental joint inflammation. *Auton Neurosci* 2014, 182:55-64.

16. Raap T, Justen HP, Miller LE, Cutolo M, Scholmerich J, Straub RH: Neurotransmitter modulation of interleukin 6 (IL-6) and IL-8 secretion of synovial fibroblasts in patients with rheumatoid arthritis compared to osteoarthritis. *J Rheumatol* 2000, 27(11):2558-2565.

17. Wu H, Chen J, Wang C, Liu L, Wu Y, Zhang Y, Zhou A, Zhang L, Wei W: beta2-adrenoceptor signaling reduction is involved in the inflammatory response of fibroblast-like synoviocytes from adjuvant-induced arthritic rats. *Inflammopharmacology* 2019, 27(2):271-279.

18. Pongratz G, McAlees JW, Conrad DH, Erbe RS, Haas KM, Sanders VM: The level of IgE produced by a B cell is regulated by norepinephrine in a p38 MAPK- and CD23-dependent manner. *J Immunol* 2006, 177(5):2926-2938.
19. Hamdani N, van der Velden J: **Lack of specificity of antibodies directed against human beta- adrenergic receptors.** *Naunyn Schmiedebergs Arch Pharmacol* 2009, **379**(4):403-407.

20. D'Souza A, Meissner BL, Tang B, McKenzie RS, Piech CT: **Effectiveness of anti-tumor necrosis factor agents in the treatment of rheumatoid arthritis: observational study.** *Am Health Drug Benefits* 2010, **3**(4):266-273.

21. Harigai M, Hara M, Kitani A, Norioka K, Hirose T, Hirose W, Suzuki K, Kawakami M, Masuda K, Shinmei M *et al*.: **Interleukin 1 and tumor necrosis factor-alpha synergistically increase the production of interleukin 6 in human synovial fibroblast.** *J Clin Lab Immunol* 1991, **34**(3):107-113.

22. Ma MM, Zhu XL, Wang L, Hu XF, Wang Z, Zhao J, Ma YT, Yang YN, Chen BD, Liu F: **beta3-adrenoceptor impacts apoptosis in cultured cardiomyocytes via activation of PI3K/Akt and p38MAPK.** *J Huazhong Univ Sci Technolog Med Sci* 2016, **36**(1):1-7.

23. Bordicchia M, Pocognoli A, D'Anzeo M, Siquini W, Minardi D, Muzzonigro G, Dessi-Fulgheri P, Sarzani R: **Nebivolol induces, via beta3 adrenergic receptor, lipolysis, uncoupling protein 1, and reduction of lipid droplet size in human adipocytes.** *J Hypertens* 2014, **32**(2):389-396.

24. Hammer HB, Hansen I, Jarvinen P, Leirisalo-Repo M, Ziegelasch M, Agular B, Terslev L: **Major reduction of ultrasound-detected synovitis during subcutaneous tocilizumab treatment: results from a multicentre 24 week study of patients with rheumatoid arthritis.** *Scand J Rheumatol* 2021:1-9.

25. Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G, De Benedetti F, Poli V, Ciliberto G: **Interleukin 6 is required for the development of collagen-induced arthritis.** *J Exp Med* 1998, **187**(4):461-468.

26. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S: **The pro- and anti-inflammatory properties of the cytokine interleukin-6.** *Biochim Biophys Acta* 2011, **1813**(5):878-888.

27. Matthews VB, Allen TL, Risis S, Chan MH, Henstridge DC, Watson N, Zaffino LA, Babb JR, Boon J, Meikle PJ *et al*.: **Interleukin-6-deficient mice develop hepatic inflammation and systemic insulin resistance.** *Diabetologia* 2010, **53**(11):2431-2441.

28. Straub RH, Schradin C: **Chronic inflammatory systemic diseases: An evolutionary trade-off between acutely beneficial but chronically harmful programs.** *Evol Med Public Health* 2016, **2016**(1):37-51.

29. DeGraff JL, Gagnon AW, Benovic JL, Orsini MJ: **Role of arrestins in endocytosis and signaling of alpha2-adrenergic receptor subtypes.** *J Biol Chem* 1999, **274**(16):11253-11259.

30. van Gastel J, Hendrickx JO, Leysen H, Santos-Otte P, Luttrel LM, Martin B, Maudsley S: **beta-Arrestin Based Receptor Signaling Paradigms: Potential Therapeutic Targets for Complex Age-Related Disorders.** *Front Pharmacol* 2018, **9**:1369.

31. Freedman NJ, Shenoy SK: **Regulation of inflammation by beta-arrestins: Not just receptor tales.** *Cell Signal* 2018, **41**:41-45.

32. Davies AO, Lefkowitz RJ: **Regulation of beta-adrenergic receptors by steroid hormones.** *Annu Rev Physiol* 1984, **46**:119-130.

33. Sakaua M, Hoffman BB: **Glucocorticoids induce transcription and expression of the alpha 1B adrenergic receptor gene in DTT1 MF-2 smooth muscle cells.** *J Clin Invest* 1991, **88**(2):385-389.
34. Quax RA, Koper JW, Huisman AM, Weel A, Hazes JM, Lamberts SW, Feelders RA: Polymorphisms in the glucocorticoid receptor gene and in the glucocorticoid-induced transcript 1 gene are associated with disease activity and response to glucocorticoid bridging therapy in rheumatoid arthritis. Rheumatol Int 2015, 35(8):1325-1333.

35. Kozaci DL, Chernakovske Y, Chikanza IC: The differential expression of corticosteroid receptor isoforms in corticosteroid-resistant and -sensitive patients with rheumatoid arthritis. Rheumatology (Oxford) 2007, 46(4):579-585.

36. Zannas AS, Chrousos GP: Epigenetic programming by stress and glucocorticoids along the human lifespan. Mol Psychiatry 2017, 22(5):640-646.

37. Shore SA: Cytokine regulation of beta-adrenergic responses in airway smooth muscle. J Allergy Clin Immunol 2002, 110(6 Suppl):S255-260.

38. Burger A, Benicke M, Deten A, Zimmer HG: Catecholamines stimulate interleukin-6 synthesis in rat cardiac fibroblasts. Am J Physiol Heart Circ Physiol 2001, 281(1):H14-21.

39. Tanner MA, Thomas TP, Maitz CA, Grisanti LA: beta2-Adrenergic Receptors Increase Cardiac Fibroblast Proliferation Through the Galphas/ERK1/2-Dependent Secretion of Interleukin-6. Int J Mol Sci 2020, 21(22).

40. Collins S: beta-Adrenoceptor Signaling Networks in Adipocytes for Recruiting Stored Fat and Energy Expenditure. Front Endocrinol (Lausanne) 2011, 2:102.

41. Suzuki M, Tetsuka T, Yoshida S, Watanabe N, Kobayashi M, Matsui N, Okamoto T: The role of p38 mitogen-activated protein kinase in IL-6 and IL-8 production from the TNF-alpha- or IL-1beta-stimulated rheumatoid synovial fibroblasts. FEBS Lett 2000, 465(1):23-27.

42. Mottillo EP, Shen XJ, Granneman JG: beta3-adrenergic receptor induction of adipocyte inflammation requires lipolytic activation of stress kinases p38 and JNK. Biochim Biophys Acta 2010, 1801(9):1048-1055.

43. Cernecka H, Ochodnicky P, Lamers WH, Michel MC: Specificity evaluation of antibodies against human beta3-adrenoceptors. Naunyn Schmiedebergs Arch Pharmacol 2012, 385(9):875-882.

44. Jensen BC, Swigart PM, Simpson PC: Ten commercial antibodies for alpha-1-adrenergic receptor subtypes are nonspecific. Naunyn Schmiedebergs Arch Pharmacol 2009, 379(4):409-412.

45. Baker JG: The selectivity of beta-adrenoceptor agonists at human beta1-, beta2- and beta3-adrenoceptors. Br J Pharmacol 2010, 160(5):1048-1061.

46. Candelore MR, Deng L, Tota L, Guan XM, Amend A, Liu Y, Newbold R, Cascieri MA, Weber AE: Potent and selective human beta(3)-adrenergic receptor antagonists. J Pharmacol Exp Ther 1999, 290(2):649-655.

47. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD et al: 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010, 62(9):2569-2581.
48. Lowin T, Bleck J, Schneider M, Pongratz G: Selective killing of proinflammatory synovial fibroblasts via activation of transient receptor potential ankyrin (TRPA1). *Biochem Pharmacol* 2018, 154:293-302.

49. Baghirova S, Hughes BG, Hendzel MJ, Schulz R: Sequential fractionation and isolation of subcellular proteins from tissue or cultured cells. *MethodsX* 2015, 2:440-445.

**Table**

Table 1 was not provided with this version.