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

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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_{1a} and β_2 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_{1a}, α_{2b}, β_1, β_2 and β_3 protein in OASF/RASF and α_{1a} and β_2 protein in synovial tissue of OA and RA patients. The pro-inflammatory cytokines IFN-γ and TNF downregulated β_3 adrenergic receptor. Activation of α_{1a}, α_{2b}, β_2 and β_3 increased production of TNF-induced IL-6 which was inhibited by specific antagonists. Furthermore, β_3 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_3 expression and demonstrated that this adrenergic receptor participates in the inflammatory response of synovial fibroblasts. Therefore, modulation of β_3 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^+ 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 β_2 adrenergic receptors [17], but it is not known whether β_1 and β_3-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 β_2 AR [18].

Results

RASF express α_{1a}, α_{2b}, β_1, β_2 and β_3 AR and protein levels of AR are regulated by cortisol and proinflammatory cytokines

First, we detected α_{1a}, α_{2b}, β_2 and β_3 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 α_{2b} (p=0.062), β_2 (p=0.066) and β_3 AR (p=0.026) in OASF (Fig. 1B) but not in RASF (Fig. 1C), whereas TNF reduced α_{2b} (p=0.052), β_2 (p=0.015) and β_3 (p=0.002) in OASF (Fig. 1B) and β_3 (p=0.004) in RASF (Fig. 1C). In addition, IFN-γ downregulated β_3 in OASF (p=0.062) and RASF (p=0.036) (Fig. 1B, C). Furthermore, we detected several bands when using the anti-β_1 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 α_{1a}, α_{2b}, β_1 and β_3 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 β_1 AR mRNA which verifies the presence of this AR as shown on the protein level (Fig. 1E). β_2 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 α_{1a}, β_1 (RA only, Fig.2B) and β_2 AR in OA and RA synovial tissue and these AR co-stained with CD55, a marker for synovial fibroblasts (Fig. 2A). The antibodies against α_{2b} 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 α_{1a} and β_2 AR. Furthermore, we detected α_{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 preincuabtion with the α_1 agonist phenylephrine, the α_2 agonist dexmedetomidine hydrochloride and the unselective β 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 β_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 β_3 AR regulates p38 phosphorylation and translocation into the nucleus

Our results show that the β_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 β_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 β_2 AR, we detected α_{1a}, α_{2b}, β_1 and β_3 AR protein and mRNA in OASF and RASF lysates.

The discovery of β_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, β AR stimulation might increase IL-6 to control metabolism and energy expenditure since one major function of β 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 α ARs might directly support pro-inflammatory immune cells. Of note, we did not investigate the effect of α AR stimulation without the addition of TNF. It might be that activation of α ARs in the absence of inflammation does not influence IL-6 production. We found that α and β ARs both increase IL-6 production and this might not depend on G protein but β-arrestin signaling which can be employed by both α and β ARs [29, 30]. In line with this, β-arrestin signaling can be pro- or anti-inflammatory, but under TNF-stimulated conditions, pro-inflammatory effects predominate [31].

Since β_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 α_{1a}, α_{2b}, β_1, β_2 and β_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 α_{1a} (in RASF), α_{2b} (in OASF), β_2 (in OASF) and β_3 (in OASF) protein. This is in line with early results from Lefkowitz who showed a stimulatory effect of cortisol on β AR [32]. In addition, upregulation of the α_{1b} AR by the glucocorticoid dexamethasone was demonstrated due to enhanced transcription of the respective mRNA [33]. Since only OASF showed an upregulation of α_{2b}, β_2, and β_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 α gene are present in RA patients [34]. In addition, some RA patients have a relative preponderance of glucocorticoid β over glucocorticoid α receptors, which is considered a proinflammatory signal, since glucocorticoid β receptors antagonize the DNA binding of the α 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 β_3

(and β_2 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 β_1 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 α_{1a}, α_{2b}, $\beta_{1/2}$ and β_3 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, β_3 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 β_3-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 β_3-AR alone increased IL-6. This might be related to differential signaling induced by individual β-AR. While e.g. the β_2-AR couples to the PKA activating G protein Gαs with a switch to inhibitory Gαi after prolonged incubation, β_3-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 β_3-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 β_3-AR [42]. In addition, p38 was also involved in β_3-AR signaling in cardiomyocytes [22]. The activation of p38 is not restricted to the β_3-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 β_1 or β_2 recognized all three β isoforms without distinction [19]. The same was true for antibodies raised against β_3, which also labelled β_1 and β_2 [43]. Similar problems have been demonstrated by using an

antibody against α_1 AR, which detected all α_1 subtypes including additional non-specific bands [44]. Although we did not use any of the α/β 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 β_3 AR agonist, it also binds to β_2 with similar affinity [45]. However, we also used the β_3 antagonist L-748,337, which is 20 fold (vs β_2) and 45 fold (vs β_1) more selective at β_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 β_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 α and β AR stimulation modulates IL-6 production and also revealed that β_3 activation induces p38 phosphorylation and translocation into the nucleus. These data suggest that intervention with the AR system especially the β_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 α_1 AR agonist), dexmedetomidine hydrochloride (selective α_2 AR agonist), L-748, 337 (β_3 AR antagonist), doxazosin mesylate (α_1/α_2 AR antagonist) and RS79948 hydrochloride (selective α_2 AR antagonist) were obtained from Tocris/Bio-Techne (Wiesbaden, Germany). Isoproterenol (unselective β-AR agonist), norepinephrine (unselective AR agonist), BRL 37344 (selective β_3-AR agonist) and nadolol (non-selective β 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 cm^2 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 NH_4Cl, 1.97 g NH_4HCO_3, 0.09 g EDTA ad 1L H_2O) 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 α_{1a} (antibody ab137123, 1.049mg/ml, Abcam, Cambridge, UK, 1:5000), α_{2b} (antibody ab151727, 1.049mg/ml, Abcam, Cambridge, UK, 1:20000), β_1 (antibody ab3442, 1mg/ml, Abcam, Cambridge, UK, 1:1000) and β_2 (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 containg 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: α_{1a} (antibody ab137123, 1.049mg/ml, Abcam, Cambridge, UK, 1:5000), α_{2b} (antibody ab151727, 1.049mg/ml, Abcam, Cambridge, UK, 1:20000), β_1 (antibody ab3442, 1mg/ml, Abcam, Cambridge, UK, 1:1000), β_2 (antibody ab182136, 0.182mg/ml, Abcam, Cambridge, UK, 1:5000) and β_3 (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 (10^5 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 preformed 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 substracted 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, 00000). 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)

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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.

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Table

Table 1 was not provided with this version.

Figures

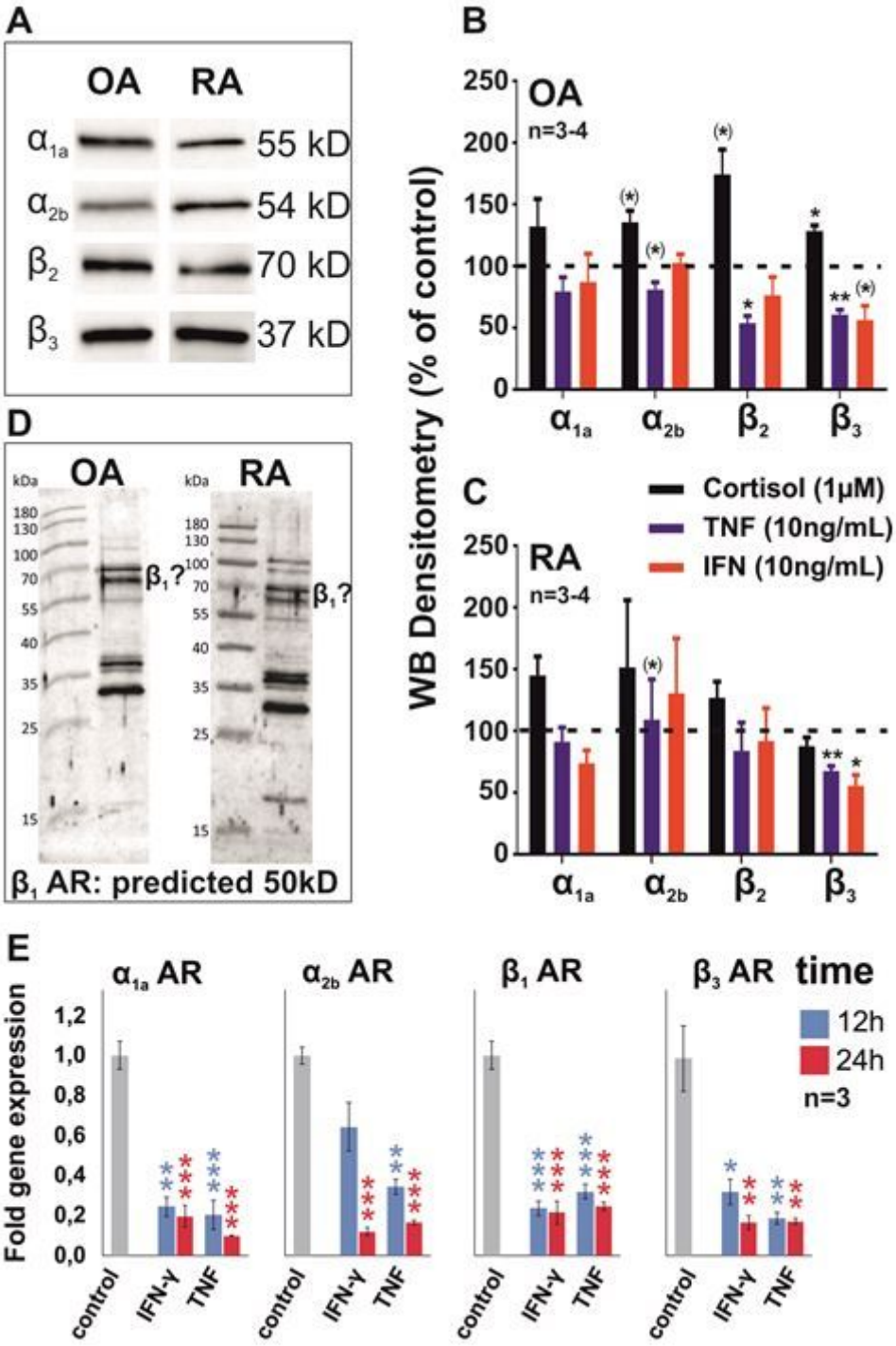

Figure 1

Protein and mRNA levels of α1a, a2b, β1, β2 and β3 adrenergic receptors (AR) detected by western blot (chemiluminescence) and quantitative PCR. A) Visualization of α1a, a2b, β2 and β3 protein levels in OASF and RASF under basal conditions. B, C) Densitometric analyses of of α1a, a2b, β2 and β3 AR protein levels after 72h stimulation with cortisol, TNF or IFN-γ in OASF (B) and RASF (C). D) Visualization of β1 AR in OASF and RASF cell lysates after western blotting. The putative β1 AR bands are marked in the graph. E) Quantitative PCR for the detection and regulation of α1a, a2b, β1 and β3 by IFN-γ and TNF in RASF. Unstimulated RASF served as control for both time points. Fold expression (control vs treatment)

was quantified by the delta-delta Ct method and GAPDH served as housekeeper. The number of patients included are given in the graph. P<0.05 was the level of significance. *p<0.05, **p<0.01, (*)p>0.05 and <0.1. Paired t-test was used for comparisons in B, C and ANOVA with Bonferroni post-hoc test was used for comparisons in E. ***p<0.001, **p<0.01, *p<0.05. Data are given as bar charts ±standard error of the mean.

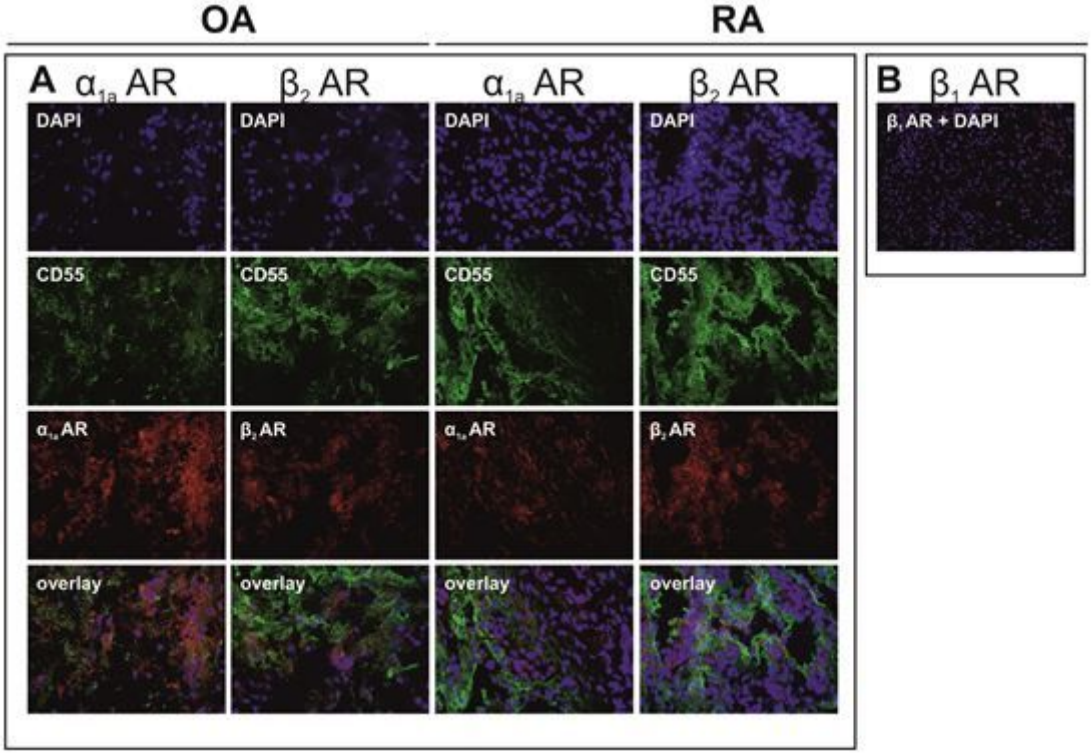

Figure 2

Detection of α1a, β1, β2 adrenergic receptors (AR) by immunofluorescence of OA and RA synovial tissue. A) Visualization of α1a and β2 AR in OA (left) and RA (right) synovial tissue. The first row depicts the staining of nucleii by DAPI, the second row visualizes CD55, a marker for synovial fibroblasts. The third row shows the specific staining for the AR investigated and the forth row shows an overlay which allows to pinpoint the cell population expressing the AR of interest. B) Visualization of β1 AR in RASF. Only the overlay is shown. Magnification is 200x.

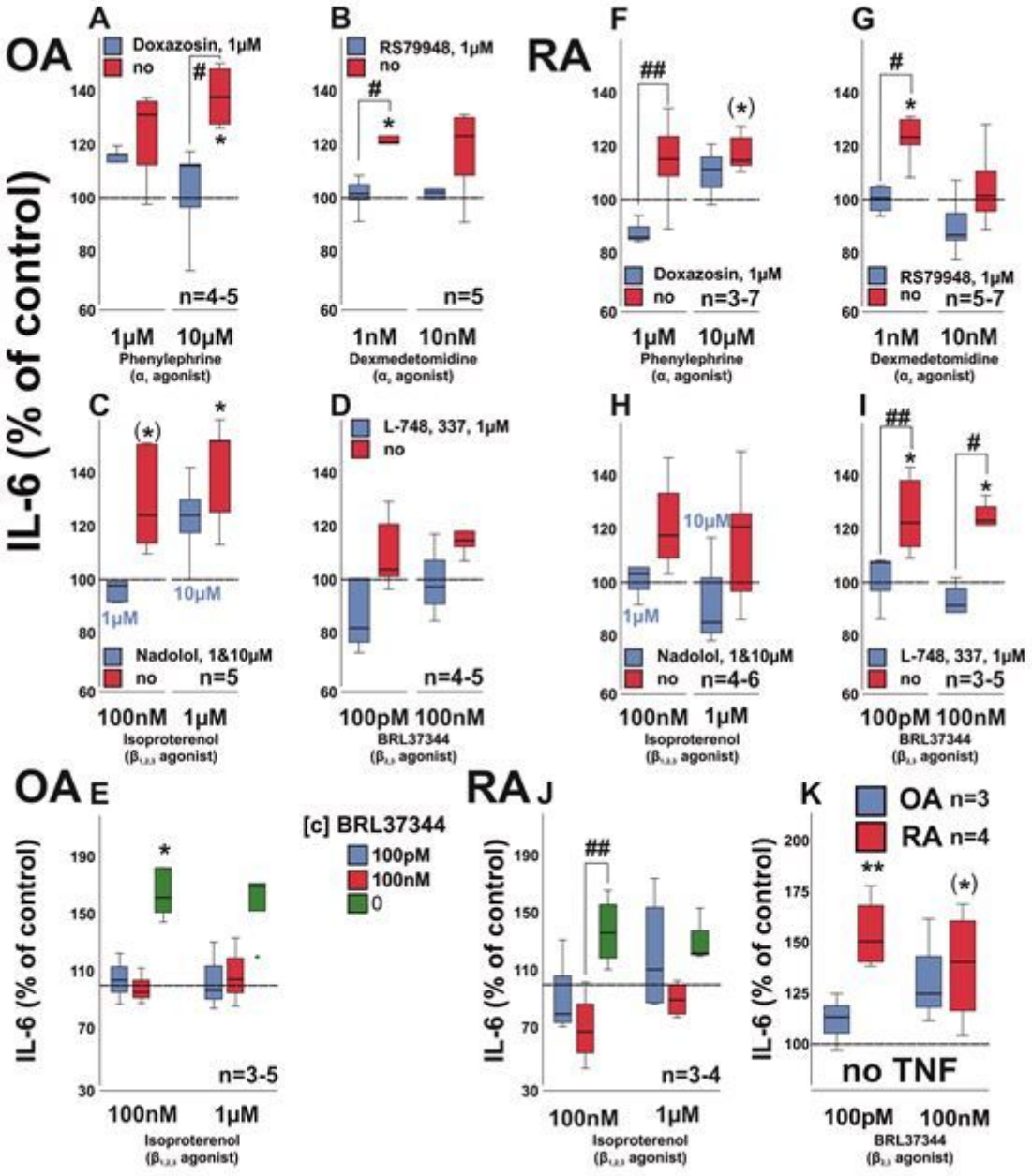

Figure 3

Modulation of IL-6 production by adrenergic receptor (AR) ligands. A, F) Effects of the α1 agonist Phenylephrine (1 and 10μM) alone and together with the antagonist Doxazosin (1μM) on TNF-induced IL-6 production by OASF (A) and RASF (F). B, G) Effects of the α2 agonist Dexmedetomidine (1 and 10nM) alone and together with the antagonist RS79948 (1μM) on TNF-induced IL-6 production by OASF (B) and RASF (G). C, H) Effects of the unselective β agonist Isoproterenol (0.1 and 1μM) alone and together with the antagonist Nadolol (1 and 10μM) on TNF-induced IL-6 production by OASF (C) and RASF (H). D, I) Effects of the β2/3 agonist BRL37344 (0.1 and 100nM) alone and together with the β3 antagonist L-748, 337 (1μM) on TNF-induced IL-6 production by OASF (D) and RASF (I). E, J) Combined effects of the β2/3 agonist BRL37344 (0.1 and 100nM) with Isoproterenol (0.1 and 1μM) on TNF-induced IL-6 production by OASF (E) and RASF (J). A-J) TNF was used at a concentration of 10ng/mL. K) Effects of the β2/3

agonist BRL37344 (0.1 and 100nM) on basal IL-6 production (no added TNF) by OASF and RASF. *p<0.05, (*)p>0.05 and <0.1, **p<0.01 vs control and #p<0.05, ##p<0.01 agonist vs agonist+antagonist treatment. ANOVA with Bonferroni post-hoc test was used for comparisons. Data are shown as box plots where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles.

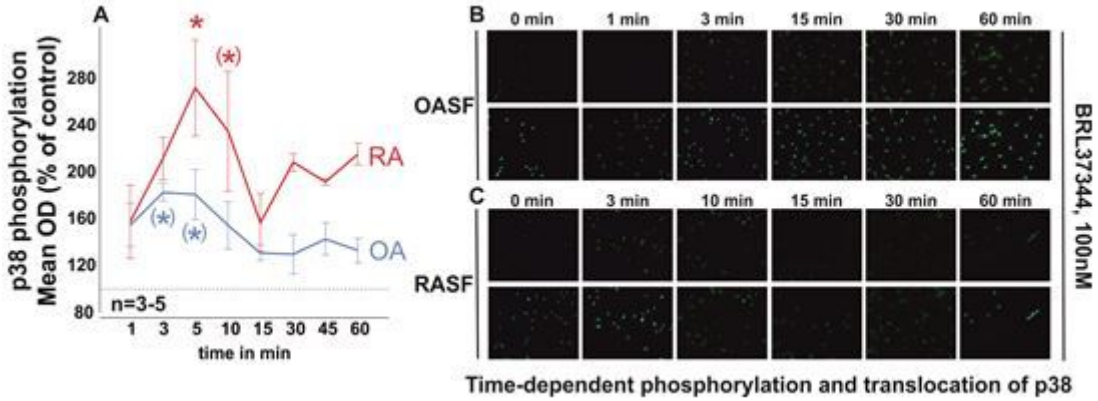

Figure 4

p38 phosphorylation and nuclear translocation in repsonse to the β2/3 agonist BRL37344. A) Time course of p38 phosphorylation in response to 100nM BRL in OASF (blue curve) and RASF (red curve). The number of patients included are given in the graph. P<0.05 was the level of significance. *p<0.05, (*)p>0.05 and <0.1. ANOVA with Bonferroni post-hoc test was used for comparisons. B, C) Time-dependent activation and nuclear translocation of p38 in response to BRL37344 (100nM) in OASF (B) and RASF (C) visualized by immunofluorescence. Maginfication is 200x. Data are given as line chart ±standard error of the mean.