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Interference between adrenergic pathways and P38 MAPK signaling pathway in synovial fibroblasts from OA and RA patients.

Dissertation

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Zusammenfassung

Das Synovium, als eine Art semipermeable Membran, reguliert den molekularen Verkehr zum Gelenkknorpel, um die physiologische Homöostase aufrecht zu erhalten. Synoviale Fibroblasten (SF) sind die wichtigsten stromalen Zellen des Gelenksynoviums, die die extrazellulären Matrix (ECM) Komponenten der Synovialflüssigkeit produzieren und somit für die Integrität des Knorpels und die Schmierung des Gelenks verantwortlich sind. Außerdem haben SF eine Vielzahl von Mustererkennungsrezeptoren (pattern recognition receptors) und sind in der Lage, proentzündliche Zytokine sowie Knochen- und Knorpel destruirende Enzyme zu produzieren. Es ist bekannt, dass Synovitis und die daraus resultierenden proentzündlichen Mediatoren eine zentrale Rolle bei der Pathogenese von Osteoarthritis (OA) und rheumatoider Arthritis (RA) spielen. Frühere Studien haben gezeigt, dass adrenerge Rezeptoren (AR) bei chronischen Entzündungs- und Immunprozessen ebenfalls eine bedeutende Rolle spielen. Ziel dieser Studie war es deshalb, das Vorhandensein von ARs auf SF nachzuweisen und ihre Rolle im proinflammatorischen Kontext zu untersuchen, indem die Wirkung adrenerg aktiver Substanzen auf den p38 MAPK-Signalweg und die Zytokinproduktion durch SF in vitro untersucht wurde.

SF von OA- und RA-Patienten wurden kultiviert und mit TNF (10 ng/ml), IFN (10 ng/ml) oder Cortisol für 72h stimuliert. Anschließend wurden α1a-, α2b-, β1-, β2- und β3-AR mittels Western Blot detektiert. Zusätzlich wurden mittels Immunfluoreszenzfärbung α1a-, α2b- und β2-AR in synovialem Gewebe dargestellt und nach Stimulation von β3-AR phosphoryliertes p38-Protein in SF dargestellt. ELISA wurde verwendet, um zu untersuchen, ob AR-agonisten die IL-6- und BAFF-Freisetzung nach TNF- bzw. IFN-Stimulation in SF modulieren können. Die Wirkung des β3-Agonisten BRL 37344 auf den p38 MAPK Signalweg wurde mittels Zell-ELISA untersucht.

Die Ergebnisse zeigen, dass SF α1a-, α2b- und β1-3-AR exprimieren, wohingegen keine α1b-AR nachgewiesen werden konnten. α - und β -AR wurden durch Cortisol reguliert, β3-AR wurde von TNF und IFN-γ herunterreguliert. Über β-adrenerge Mechanismen konnte die Produktion von IL-6 in SF, die mit TNF bereits behandelt wurden, erhöht werden, die Stimulation hatte jedoch keine Auswirkung auf die Proliferation der SF. BRL 37344 erhöhte die IL-6-Produktion durch SF auch ohne TNF-Stimulation, eine Synergie zwischen BRL37344 und Isoproterenol bzgl. der IL-6 Produktion wurde aber nicht beobachtet. Isoproterenol beeinflusste die BAFF-Produktion in SF, die mit IFN-γ in unterschiedlichen Konzentrationen zuvor stimuliert wurde. Zudem aktivierte die β3-Stimulation den p38 MAPK Signalweg in SF.

Zusammenfassend ergab unsere Studie, dass SF α1a-, α2b-, β1-, β2- und β3-AR exprimieren, welche unter pro- bzw. anti-entzündlichen Bedingungen moduliert wurden. Darüber hinaus ist in SFs die Produktion von IL-6 durch β3 AR reguliert. Während frühere Studien nur β3-Expression im Fettgewebe und im Herzen identifizierten, haben wir gezeigt, dass SF ebenfalls β3-AR exprimieren und dass β3-Agonismus über eine Aktivierung des p38

MAPK Signalwegs zu einer Modulation der IL-6-Produktion führt. Diese Befunde geben neue Einblicke in die, durch das sympathische Nervensystem beeinflussbaren pathophysiologischen Prozesse bei RA.

Summary

The synovium, as a kind of semipermeable membrane, controls the molecular traffic into and maintain the normal physiological state of articular cartilage. Synovial fibroblasts (SFs) are the main stromal cells of the joint synovium, which produce the extracellular matrix (ECM) components of the synovial fluid and thus are important for cartilage integrity and lubrication of the joint. In addition, SFs carry a variety of innate immune receptors and are able to produce pro-inflammatory cytokines and bone and cartilage destructive enzymes. More and more evidence show that synovitis and the resulting pro-inflammatory mediators play important roles in the pathogenesis of osteoarthritis (OA) and rheumatoid arthritis (RA).

Previous studies have demonstrated that adrenergic receptors (AR) play significant roles in chronic inflammatory and immune response processes. The aim of this study is to demonstrate the existence of ARs on SFs and elucidate their pathological role under proinflammatory condition by assessing the effects of adrenergic drugs on P38 MAPK signaling and cytokine production in SFs in vitro.

SFs from OA and RA patients were cultured, stimulated with TNF (10 ng/ml), IFN (10 ng/ml) or cortisol for 72h. Then, α1a, α2b, β1, β2 and β3 protein were detected by Western Blot. Immunocytofluorescent staining was conducted to visualize α1a, α1b, α2b and β2 protein in synovial tissue and phosphorylated p38 protein in SFs after β3 ligation. ELISA was used to determine whether AR agonists modulate IL-6 and BAFF release in SFs after TNF and IFN stimulation respectively. The effect of the β3 AR agonist BRL 37344 on p38 MAPK signaling pathway was investigated by cell ELISA.

Results showed that SFs express α1a, α2b and β1-3 receptors, but no α1b protein. Alpha and beta ARs were up-regulated by cortisol, β3 AR was down-regulated by TNF and IFN-γ. Beta AR agonism increased IL-6 production in SFs pretreated with TNF, but had no significant effects on proliferation of SFs. BRL 37344 increased IL-6 production in SFs even without TNF stimulation, yet no synergy was found for IL-6 production when BRL 37344 and isoproterenol were added together. Isoproterenol affected BAFF production in SFs pretreated with IFN-γ at different concentrations. Beta3 agonism activated P38 MAPK signaling pathway in SFs.

In conclusion, our study found that SFs expressed α1a, α2b, β1, β2 and β3 ARs, and the levels of ARs changed under pro-inflammatory or anti-inflammatory conditions. In addition, β3 AR regulated IL-6 expression in SFs. While previous studies only identified β3 expression in adipose tissue and the heart, we demonstrated that SF also express β3 AR, and that β3 agonism activates P38 MAPK signaling pathway to modulate IL-6 production. These findings provide a new view on pathophysiological processes with respect to the involvement of the sympathetic nervous system in the pathogenesis of RA.

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1. Introduction

1.1 Characteristics of RA and OA

The most well-known arthritides are osteoarthritis (OA) and rheumatoid arthritis (RA) [1, 2], which morbidity rates are respectively reported to be about 10-12% [3] and 0.5-1% of the adult population [4, 5], respectively. The socio-economic burden of arthritis, as well as the cost of pharmacology and surgical treatment (biological agents, joint replacement, etc.), is enormous [6, 7]. In fact, in the United States, Canada, the United Kingdom, France, and Australia, the cost of OA is estimated at 1-2.5% of the gross national product [8, 9].

RA is an autoimmune disease of the musculoskeletal system, which causes progressive articular destruction and associated comorbidities in vascular, metabolic, bone, and psychological domains [4, 10]. The most common joints affected are the hands, feet, wrists, elbows, knees and ankles, and disease usually manifests symmetrical [11]. RA is characterized by synovitis and is accompanied by inflammatory and abnormal immune responses [10, 12]. The cause of RA is not clear despite of some identified genetic and environmental factors [13, 14, 15, 16]. The underlying mechanism involves the body's immune system attacking the joints [17], which results in thickening and inflammation of the joint capsule.

OA has been widely accepted as a whole joint disease[18, 19]. Synovitis and meniscal damage are common, and changes in inflammatory mediator levels are detected in synovial fluid [20, 21]. The pathogenesis of OA is unclear at present, yet studies have shown that it is due to an imbalance between the synthesis and decomposition of chondrocytes, cell matrices and subchondral bone, thus leading to the degeneration of articular cartilage [22]. The hallmark changes of OA include narrowing of joint space, formation of subchondral cyst, subchondral sclerosis and osteophyte formation [22, 23]. These pathological changes of subchondral bone were observed in both human patients and animal models of OA [24, 25].

1.2 Functions of synovial fibroblasts (SFs)

Articular cartilage has no internal blood vessels or lymph supply, so it relies on adjacent tissues (subchondral bone and synovium) to provide the nutrients that are necessary for the health of chondrocytes and articular cartilage [26]. The synovium, as a kind of semipermeable membrane, controls the molecular traffic into and out of the joint space and maintains the composition of synovial fluid, which is very important to maintain the normal physiological state of articular cartilage. SFs are the main stromal cells of the joint synovium [27], which produce extracellular matrix (ECM) components of the synovial fluid and thus are important for cartilage integrity and lubrication of the joint. In addition, SFs carry a variety of innate immune receptors and are able to produce pro-inflammatory cytokines and bone and cartilage destructive enzymes [27, 28]. Membrane-bound TNF on lymphocytes promote Interleukin-6 (IL-6) and IL-8 production by SF [29], and activated B cells can induce

inflammatory fibroblasts with cartilage-damaging properties [29, 30, 31]. Under inflammatory conditions, SFs produce a large number of cytokines, such as IL-6 and, B cell activating factor (BAFF), as well as matrix degrading enzymes such as matrix metalloproteinases (MMPs) and cathepsins [32]. IL-6 is a pro-inflammatory cytokine with pleiotropic biological activities, which plays an important role in immunity, inflammation, tissue regeneration, and metabolism [33]. Levels of IL-6 are increased in synovial fluid and sera from patients with RA or from those with OA [34] and were positively correlated with the clinical severity and radiological joint damage of OA and RA [35, 36]. Moreover, several studies have shown that IL-6 upregulates the expression of MMP-1 and MMP-13 in human cartilage [37]. Additionally, IL-6 also plays a role in angiogenesis by inducing intracellular adhesion molecules [38]. These functions of IL-6 in the pathogenesis of RA makes it as a remarkable target for the RA therapy. BAFF, known as a B-cell proliferation and survival factor, is produced by immune cells (monocytes, dendritic cells and macrophages) [39], and also by non-immune cells, such as salivary gland epithelial cells [40], prostate epithelium [41], and SFs [42]. Excessive BAFF levels can be detected in patients with autoimmune diseases, and particularly in the synovium of patients with RA [43]. It is harmful for patients with autoimmune diseases (such as systemic lupus erythematosus, Sjogren's syndrome and RA) to increase the survival rate of B cells in response to BAFF, because, excessive B-cell responses increase circulating autoantibody level[42, 44].

It is now widely accepted that low-level inflammation is a key driver for progressive joint damage [18, 20, 26]. Synovial cells react by producing proinflammatory mediators, which attract immune cells, increase angiogenesis and induce phenotypic changes of chondrocytes [45]. This vicious cycle is perpetuated by chondrocytes which produce additional cytokines and proteolytic enzymes, which eventually increase cartilage degradation and induce further synovial inflammation [46]. Therefore, the altered cellular composition might reduce the concentration of cartilage protective factors and increase the production of factors that lead to the degradation of articular matrix.

RA is characterized by macroscopic changes in the synovial lining, including inflammation, hyperplasia, and invasion into local cartilage and bone [30, 31]. SF proliferate, are activated and invade and destroy the adjacent cartilage. SF promote synovial inflammation by producing mediators which recruit and activate immune cells.

Similarly, more and more evidence [18, 20, 26] show that synovitis and the resulting pro-inflammatory mediators play an important role in the pathogenesis of OA with effects on articular cartilage. Modern imaging methods such as magnetic resonance imaging (MRI) have confirmed a high incidence of "macroscopic" inflammation, and have supported the role of synovitis as an active component of the OA process, which is related to pain and structural progress [47]. The histological features of the synovium in OA patients are hyperplasia of the synovium lining, subsynovial fibrosis and interstitial vascularization [18, 20, 26].

1.3 The relationship between ARs and fibroblast function

G-protein-coupled receptors (GPCRs) are the largest single protein family, which compose around 4% of all proteins encoded by the human genome [48]. ARs , members of GPCRs, are coupled to G protein（β ARs are coupled to Gs, α1-AR is coupled to Gq, α2-AR is coupled to Gi） to activate adenylate cyclase (AC) and produce cyclic AMP (cAMP), which activates signaling pathways that regulate proliferation, differentiation, maturation and effector functions in cells[49, 50]. The α-ARs are divided into two categories: α1-and α2-ARs [51, 52], and are located on both neural and non-neural tissue (lymphoid tissues, lymphocytes, monocytes, macrophages, microglia and astrocytes [53]). The β-ARs are divided into three categories: β1, β2 and β3-ARs [54, 55]. β1 and β2 play an important role in regulating the excitation contraction coupling of the myocardium. However, the β1 AR accounts for 75-80% of β ARs found in the heart [56]. β2 AR is expressed in kidney, lung, blood vessel and heart, accounting for 20-25% of cardiac β ARs, while β3 AR is expressed in adipose tissue and bladder, with very little expression in the heart [57, 58].

Several studies have emphasized that α-ARs are promising therapeutic targets because they are involved in inflammation [59, 60, 61], stress response [62] and regulation of movement [63], pain [64] and spasm [65] in the central nervous system. These functions can be activated by directly regulating ARs on neural networks of the brain and spinal cord [66]. Many studies have reported that β2 and β3 AR regulate the proinflammatory cytokines (such as TNFα, IL-1β and IL-6) to influence the development of functional pain [67, 68, 69 ,70]. Therefore, β2 AR can also regulate the expression of IL-6 in choroidal endothelial cells (ChECs), retinal pigment epithelial (RPE) cells and pericytes [71]. The trend of β3-AR-dependent IL-6 expression was observed in retinal pericytes [71]. Stimulation of β1 or β2 ARs releases the G-protein subunit Gs, which activates adenylate cyclase to produce cAMP [72,73], a process generally considered as anti-inflammatory. In contrast, β3 AR couples Gs/Gi protein simultaneously leading to distinct intracellular signaling events [74]. However, there is an unusual biphasic effect on cAMP production in response to β3 AR agonist, in which the agonist could either stimulate or inhibit adenylate cyclase activity in some cells [75]. The β3 AR coupling to Gi could serve to restrain Gs mediated activation of adenylate cyclase and to initiate additional signal transduction pathways [74]. Some neuroendocrine factors, like dopamine, glutamate, and endocannabinoids, can alter SF function, which may be particularly important because RA is characterized by an increase in sensory nerve fibers and a decrease in sympathetic nerve fibers in joints and spleen [75]. This alters the composition of neurotransmitters / neuropeptides in the joint which impacts not only SF directly but also B- and T-lymphocytes which in turn might also modulate SF function [32]. Norepinephrine stimulates IFN-γ production in T-cells during early experimental arthritis and this cytokine contributes to the activation of SF [76] and stimulates the production of B cell modulating cytokines, like B-lymphocyte activating factor [32]. On the other hand, β-adrenergic stimulation leads to increased production of IL-10 by

regulatory B cells, which inhibits RASF-induced cartilage destruction [77] and suppresses many pro-inflammatory activities of neutrophils and monocytes. The β ARs, which might directly influence SFs function, are expressed in OA and RA synovial tissue [78, 79]. However, there is little information on the expression of ARs under pro-inflammatory conditions in SFs.

Mitogen-activated protein kinases (MAPKs) are involved in many important cellular processes, including cell differentiation, proliferation, inflammation, cell growth and cell death [80]. There are three major subsets, including extracellular signal regulated kinase (ERK1/2), c-jun-NH2-terminal kinase (JNK) and p38 MAPK [81, 82]. Many kinds of extracellular stimuli, such as growth factors, cytokines, mechanical stress, ultraviolet light, osmotic stress and heat shock, can activate the MAPK signal cascade [83]. There is increasing evidence that β-ARs also regulate p38 MAPKs, especially ERK1/2 MAPKs [84]. P38 MAPK is activated by β-AR through a Gi-dependent mechanism in cultured adult rat cardiomyocytes, protecting cardiomyocytes from β-AR/Gs-mediated apoptosis [85]. P38 is activated by β3-AR in brown fat [86], but the mechanism is unclear [87].

1.4 AIM

The aim of this study is to elucidate whether SFs express ARs and determine their role in modulating SF function under normal and proinflammatory conditions. In addition, the effect of adrenergic drugs on P38 MAPK signaling in SF will be determined in vitro. This study will help to elucidate the role of ARs in chronic inflammation and establish ARs as a potential therapeutic target to silence activated SFs from OA and RA.

2. Experimental Procedures

2.1. Patients

In this study, 18 patients with long-standing RA fulfilling the American College of Rheumatology revised criteria for RA [88] 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).

2.2. Compounds and chemicals

Phenylephrine hydrochloride (selective α1 AR agonist), dexmedetomidine hydrochloride (selective α2 AR agonist), L-748, 337 (β3 AR antagonist), doxazosin mesylate (α1/α2 AR antagonist) and RS79948 hydrochloride (selective α2 AR antagonist) were obtained from

Tocris/Bio-Techne (Wiesbaden, Germany). Isoproterenol (unselective β-AR agonist), norepinephrine (unselective AR agonist), BRL 37344 (selective β3-AR agonist) and nadolol (non-selective β AR agonist) were obtained from Sigma Aldrich (St. Louis, USA).

2.3. Synovial fibroblast and tissue preparation

Samples from RA and OA synovial tissue were isolated and prepared as described previously [89]. 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 particles were 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.

2.4. Stimulation of OA and RA SFs

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 contents were determined by ELISA.

2.5. 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%.

2.6. Cytotoxicity Assays

SFs were seeded onto 96-well plates (200 µl; 3.0×10^3 cells/well) in 10% FBS and cultured 2-3 days at 37°C prior to treatment. Cells were cultured for 96h with BRL, phenylephrine, dexmedetomidine hydrochloride or norepinephrine or isoproterenol in decreasing concentrations (10^{-5} -10^{-10} M) at 37°C, and the number of viable cells was determined using the Cell Titer-blue reagent (Promega, Madison, WI) and a microplate reader (TECAN, Infinite 200 Pro) capable of detecting the corresponding wavelengths.

2.7. Immunofluorescence I (staining of synovial tissue)

For immunofluorescent visualization of α1a (antibody ab137123, 1.049mg/ml, Abcam, Cambridge, UK, 1:5000), α1b (antibody ab169523, 0.607mg/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.

2.8. Immunofluorescence II (staining of SFs)

For immunofluorescent visualization of phosphorylated p38 (Cell Signaling Technology, Inc, USA, NB4511, 1:1500) were 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.

2.9. Western blot

The following antibodies were used: α1a (antibody ab137123, 1.049mg/ml, Abcam, Cambridge, UK, 1:5000), α1b (antibody ab169523, 0.607mg/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. [90].

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 being washed, 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.

2.10. 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).

2.11. 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 16 (IBM, Armonk, USA). The statistic tests used are given in the figure legends. The level of significance was $p < 0.05$.

2.8 Personal Contribution

Ding Liang performed most of the experiments and was supervised for his thesis by Georg Pongratz and Torsten Lowin. Ren Tingting participated in part of the work of Western blot and ELISA.

3. Results

3.1. SFs express α1a, α2b and β receptors, but no α1b

In a first step, we assessed whether RASF and OASF express α1a, α1b, α2b, β1, β2 and β3 ARs by western blot and immunofluorescence (Fig. 1 and 2A). Western blot analysis showed that α1a, α2b, β2 and β3 (Fig. 1A) but not α1b (Fig. 1B) ARs were expressed by SFs. In OA patients, the expression of β2 and β3 AR in SFs was higher than that in RA patients, but the difference was not significant ($P > 0.05$) (Fig. 1D). Immunofluorescence showed that α1a and β2 ARs were expressed in synovial tissue from OA (Fig. 2A) and RA (Fig. 2B) patients, and it also showed α1b was expressed by other cells in synovial tissue but not SFs (Fig. 2B). Although the western blot for β1 AR detected multiple bands without a band at the predicted size, data from Hamdani suggest that it might be a post-translationally modified variation of β1 [91] (Fig. 1C). In addition, we confirmed β1 protein expression in synovial tissue by immunofluorescence (Fig. 2C). Previous data have already shown that, α1 and β AR antibodies lack specificity in WB analysis [91, 92].

The inflammatory response in OA and RA is mainly localized to the synovial joint where immune cells composed of T cells, B cells, macrophages, and dendritic cells infiltrate the synovium [93, 94]. Immunofluorescence showed that CD3 + T cells (Fig 2A, Fig 2B) and CD68 + macrophages (Fig 2A, Fig 2B) expressing α1a and β2 AR infiltrated into the synovium. These results also showed α1b AR was expressed in some types of CD3 + T cells (Fig 2C).

Fig1.

A B

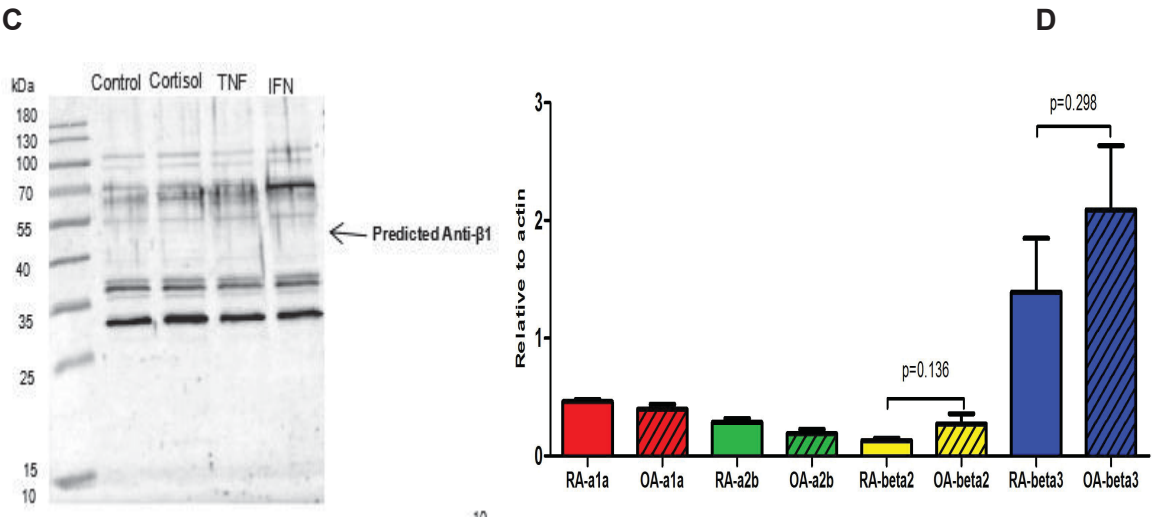
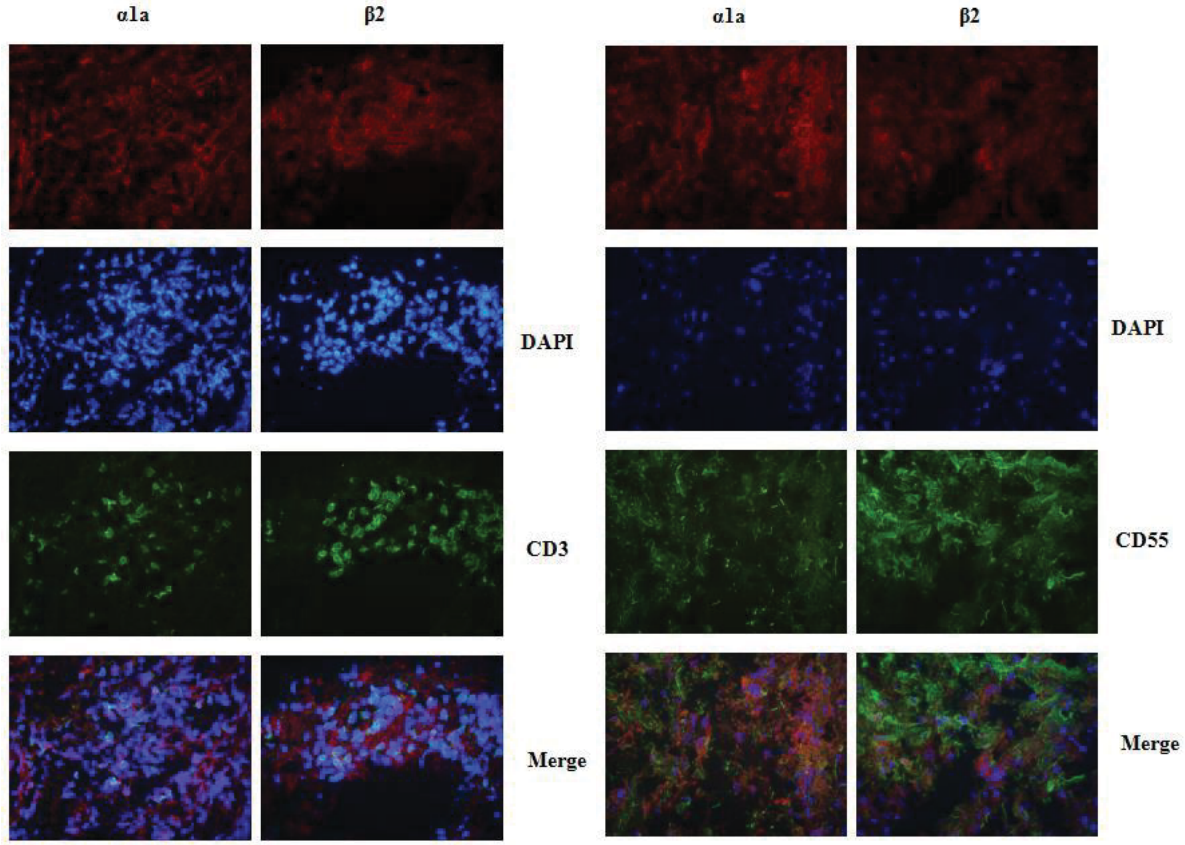


Fig. 1. **Visualization of AR protein in isolated OA and RA SFs (n=4-5).** (A) Western Blot; staining of α1a, α1b, α2b, β1, β2 and β3 protein. Values are expressed as the means ± SEM. Paired t-test was used for comparisons in Western Blot (B). $p < 0.05$ was the level of significance. (TNF, Tumor Necrosis Factor-α; IFN, interferon-γ)

Fig. 2A (OA X400)



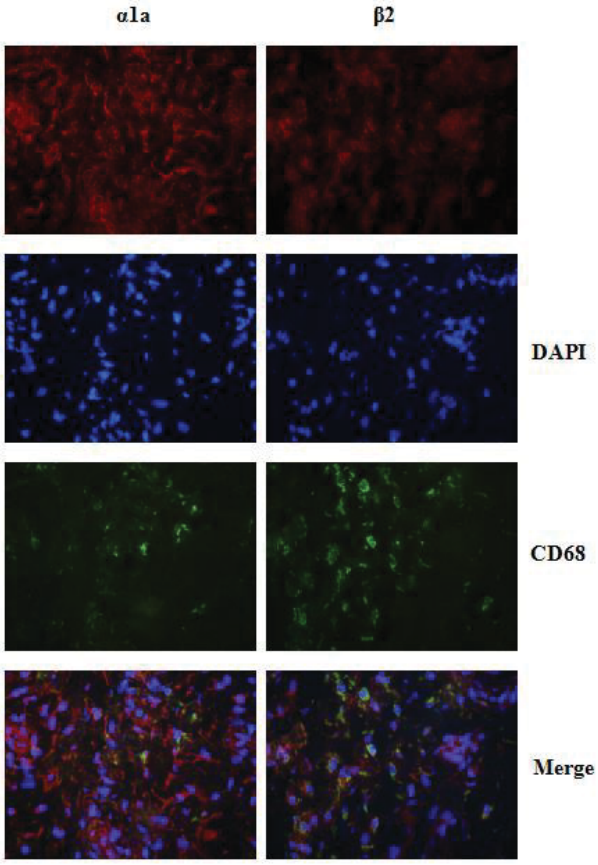
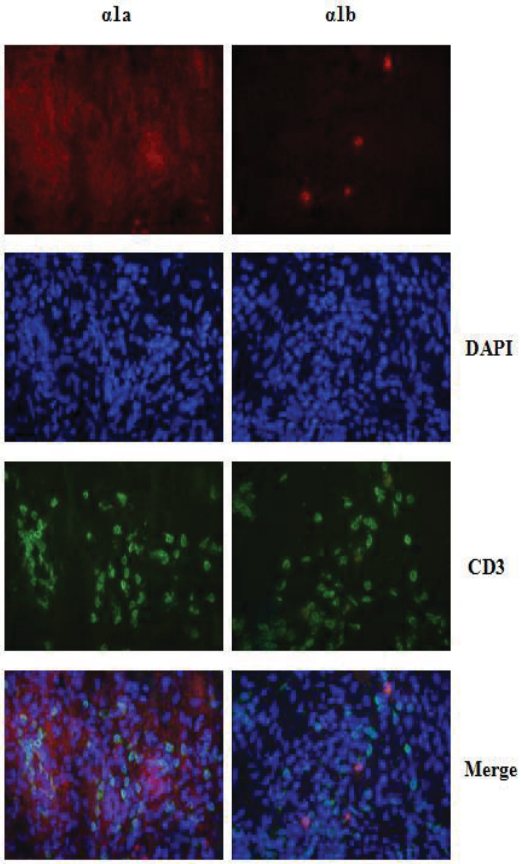


Fig. 2B (RA X400)



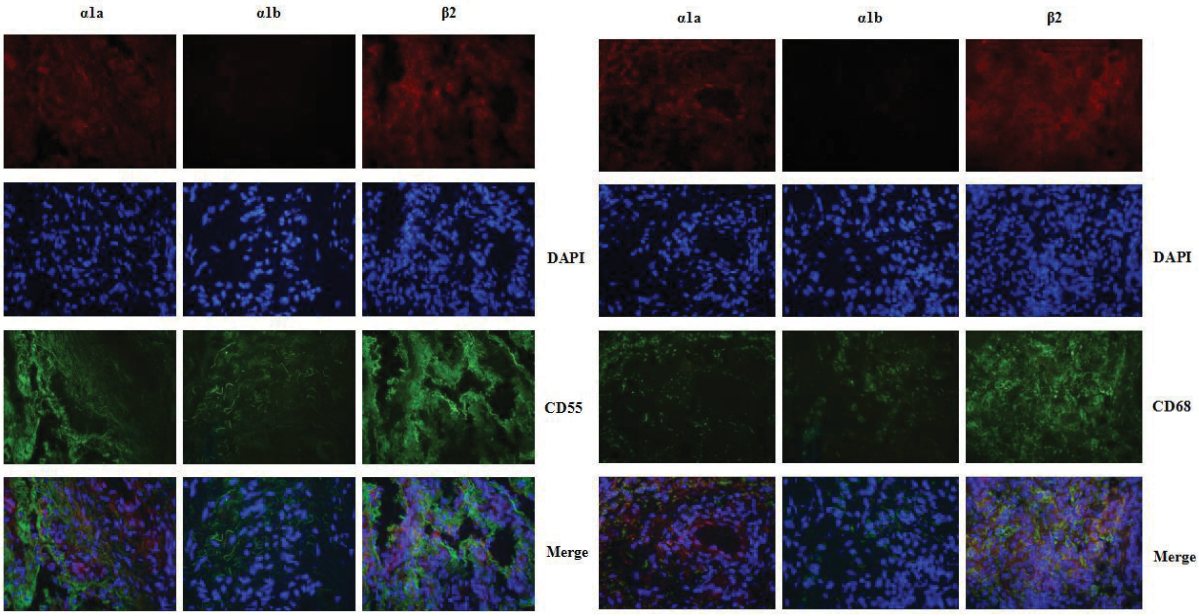


Fig. 2C (merge X200)

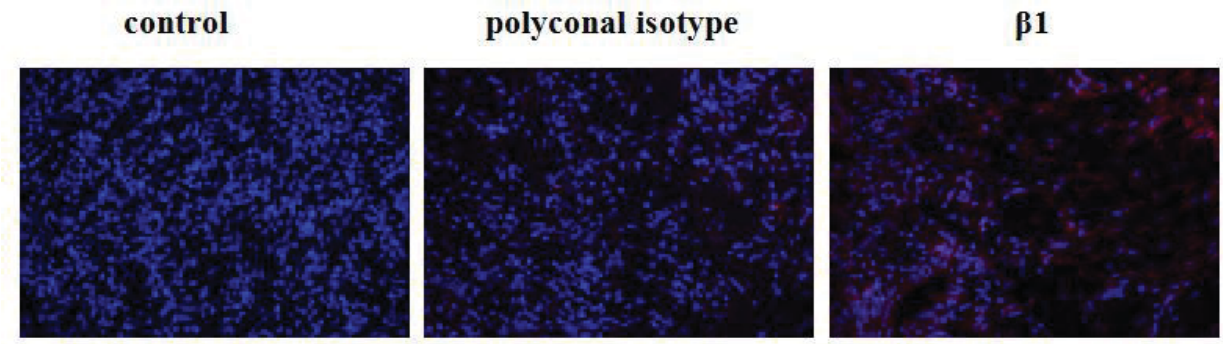
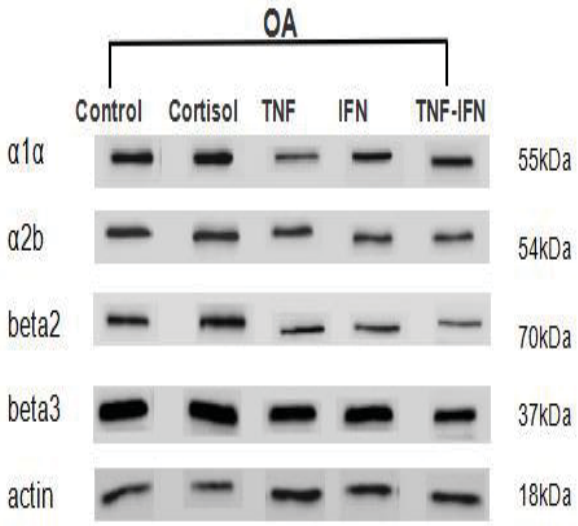


Fig. 2. **Visualization of AR protein in synovial tissue.** A) Immunofluorescent staining of α1a and β2 protein in OASF. B) Immunofluorescent staining of α1a, α1b and β2 protein in RASF. CD3 (in green) is a marker for T cells. C)Immunofluorescent staining of β1 protein in RASF. CD55 (in green) is a marker for SF. CD68 (in green) is a marker for macrophages.

3.2 alpha and beta AR were up-regulated by cortisol, β3 AR was down-regulated by TNF or IFN-γ

SFs are usually exposed to many proinflammatory mediators in the rheumatoid and osteoarthritic joint [24], and, therefore, we aimed to investigate the effect of prolonged exposure to the pathomechanistically central cytokines in RA, TNF and IFN-γ on the expression of ARs. In addition, we also used cortisol as the anti-inflammatory counterpart. Western blot showed that the expression of α2b, β2 and β3 AR was up-regulated in SFs from OA patients (OASF) ($P< 0.05$) (Fig. 3A), and the expression of a1a was up-regulated in SFs from RA patients (RASF) $P< 0.05$) (Fig. 3B). Conversely, the expression of β3 AR was down-regulated significantly in both OASF and RASF after TNF or IFN stimulation ($P< 0.05$) (Fig. 3A-B).

Fig.3A


OA
Control
Cortisol
TNF
IFN
TNF-IFN
α1α
α2b
beta2
beta3
actin
55kDa
54kDa
70kDa
37kDa
18kDa

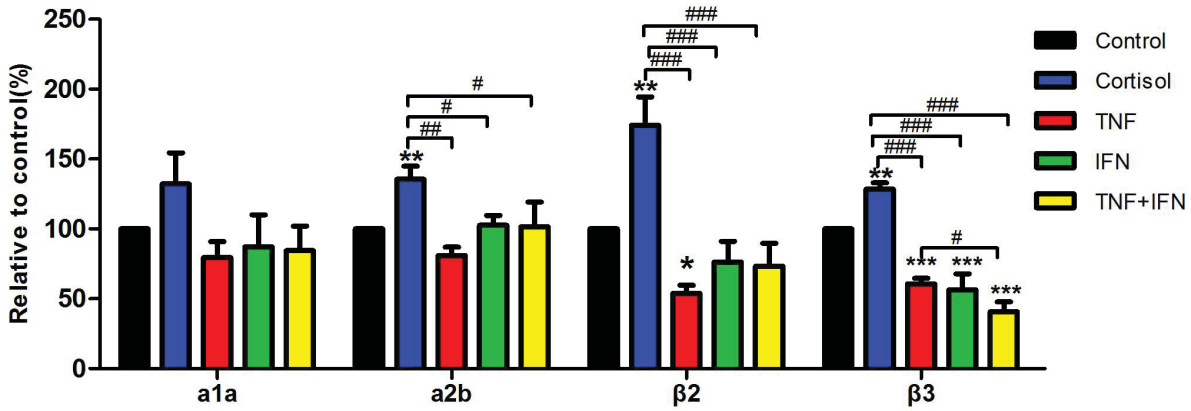
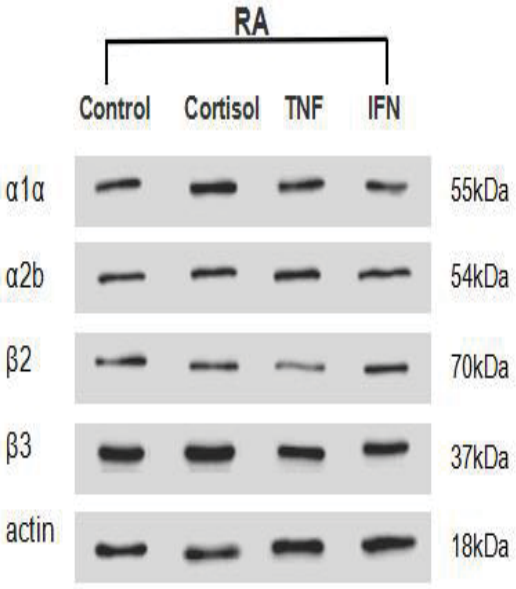

Relative to control(%)
250
200
150
100
50
0
a1a
a2b
β2
β3
Control
Cortisol
TNF
IFN
TNF+IFN

Fig.3B


RA
Control
Cortisol
TNF
IFN
α1α
α2b
β2
β3
actin
55kDa
54kDa
70kDa
37kDa
18kDa

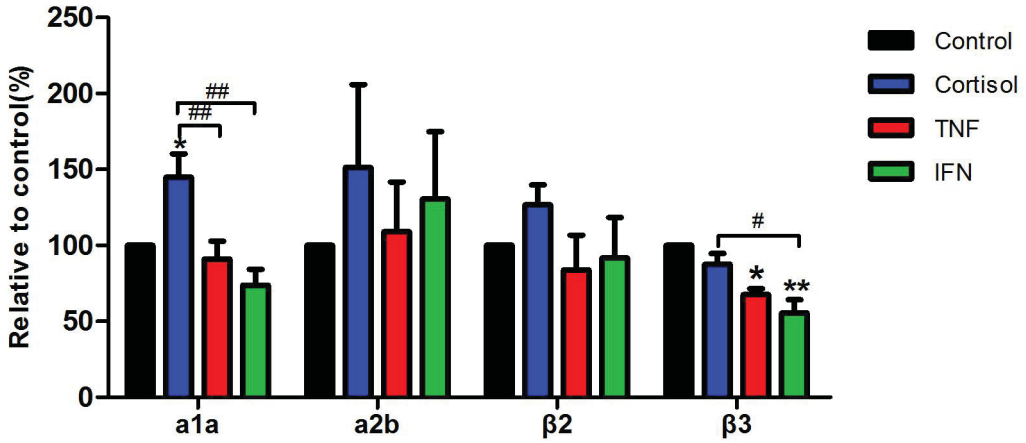


Fig.3C

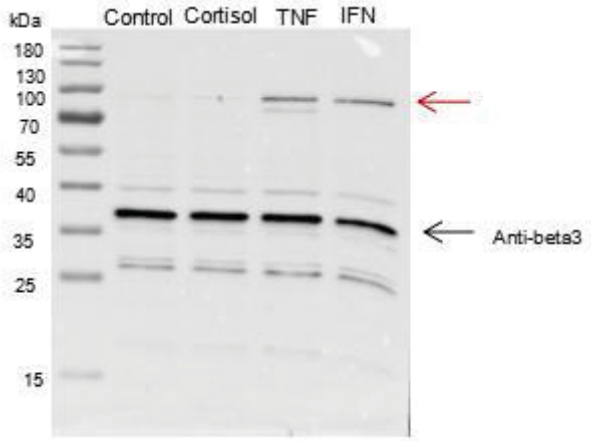


Fig. 3. **Visualization of AR protein in isolated OA and RA SFs under basal and stimulated (72 h) conditions.** Western Blot; staining of α1a, α2b, β2 and β3 protein under basal and cortisol, TNF, IFN, TNF+IFN stimulated (72 h) conditions in OASF (A) and RASF (B). An expression at 85KD with β3 antibody was shown (C, red arrow) after TNF and IFN stimulation (72 h). Values are representative of at least three different experiments and are expressed as the means ± SEM. Paired t-test was used for comparisons in Western Blot (A, B). $p < 0.05$ was the level of significance. (* means compared with the control group; # means compared with each other; TNF, Tumor Necrosis Factor-α; IFN, interferon-γ)

3.3. β AR agonism increased IL-6 production in SFs pretreated with TNF.

IL-6 is an important proinflammatory mediator in the pathology of RA and OA, and SF is one prominent producer of IL-6 in the joint. Tocilizumab, a humanized monoclonal antibody that acts as an IL-6 receptor antagonist, is effective in the treatment of adult patients with RA [95]. So, investigation of potential regulation of this mediator by ARs is of interest. We investigated the effects of the ARs agonists phenylephrine (α1), dexmedetomidine hydrochloride (α2), isoproterenol (β1, 2, 3), norepinephrine (α1/2,> β1/2/3) and BRL 37344 (β3> β2/ β1) on IL-6 production by RASF and OASF. When the concentration of phenylephrine was greater than 10^{-8} M in RA and 10^{-7} M in OA patients, the production of IL-6

increased significantly ($p< 0.05$). However, at the highest dose of 10^{-4} M, there was no significant effect on IL-6 in both OASF and RASF (Fig. 4A). The α2 agonist dexmedetomidine hydrochloride, increased, IL-6 production at 10^{-9} M significantly in both OASF and RASF (Fig. 4B). The non-selective β agonist isoproterenol induced a dose-dependent increase in IL-6 production at low concentrations (10^{-5} – 10^{-8} M), which reached its maximum at a concentration of 10^{-7} M (Fig. 4C). Norepinephrine increased IL-6 production only at the highest concentration used (10^{-5} M) (Fig. 4D). When using the β3 selective agonist BRL 37344, RASF showed a dose-dependent increase in IL-6 production at the concentrations 10^{-7} M, 10^{-8} M and 10^{-10} M, whereas this was not evident in OASF (Fig. 4E). When antagonists (Nadolol 10^{-5} M for beta ARS, Doxazosin 10^{-6} M for α1, RS79948 10^{-6} M for α2 AR, and L-748, 337 10^{-6} M for β3-AR) were incubated 30min prior to respective agonists, the effects of AR agonists on IL-6 production were blocked in OASF and RASF, indicating that ARs play an important role in IL-6 regulation (Fig. 5A, B).

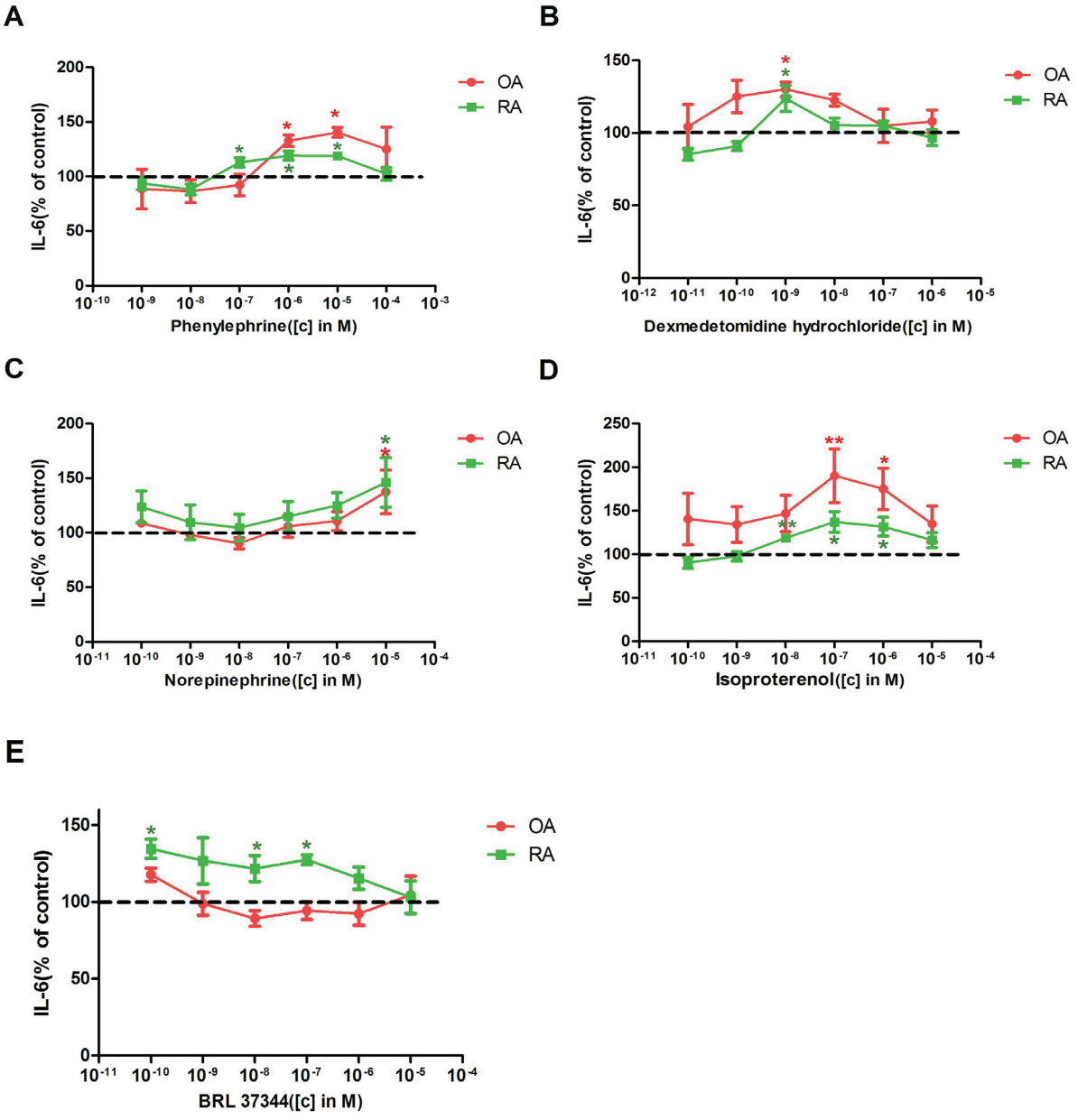


Fig 4. **Modulation of IL-6 release by AR agonists in SFs stimulated with TNF**. SFs were preincubated for 1h with different concentrations of AR agonists. Then, TNF was added to the fibroblast monolayers yielding a final concentration of 10ng/ml. Cell-free supernatants were collected 18-24h after TNF-α stimulation. IL-6 levels were determined by ELISA. The data of five OA patients and four RA patients are given. The unstimulated control (OA:3.93 ±0.87 ng/ml, RA: 7.50±1.25 ng/ml) was set to 100%. Values are shown as percent of control and expressed as mean±SEM. One-way ANOVA was used for comparisons between different doses of agonist/antagonist vs control. $p < 0.05$ was the level of significance. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

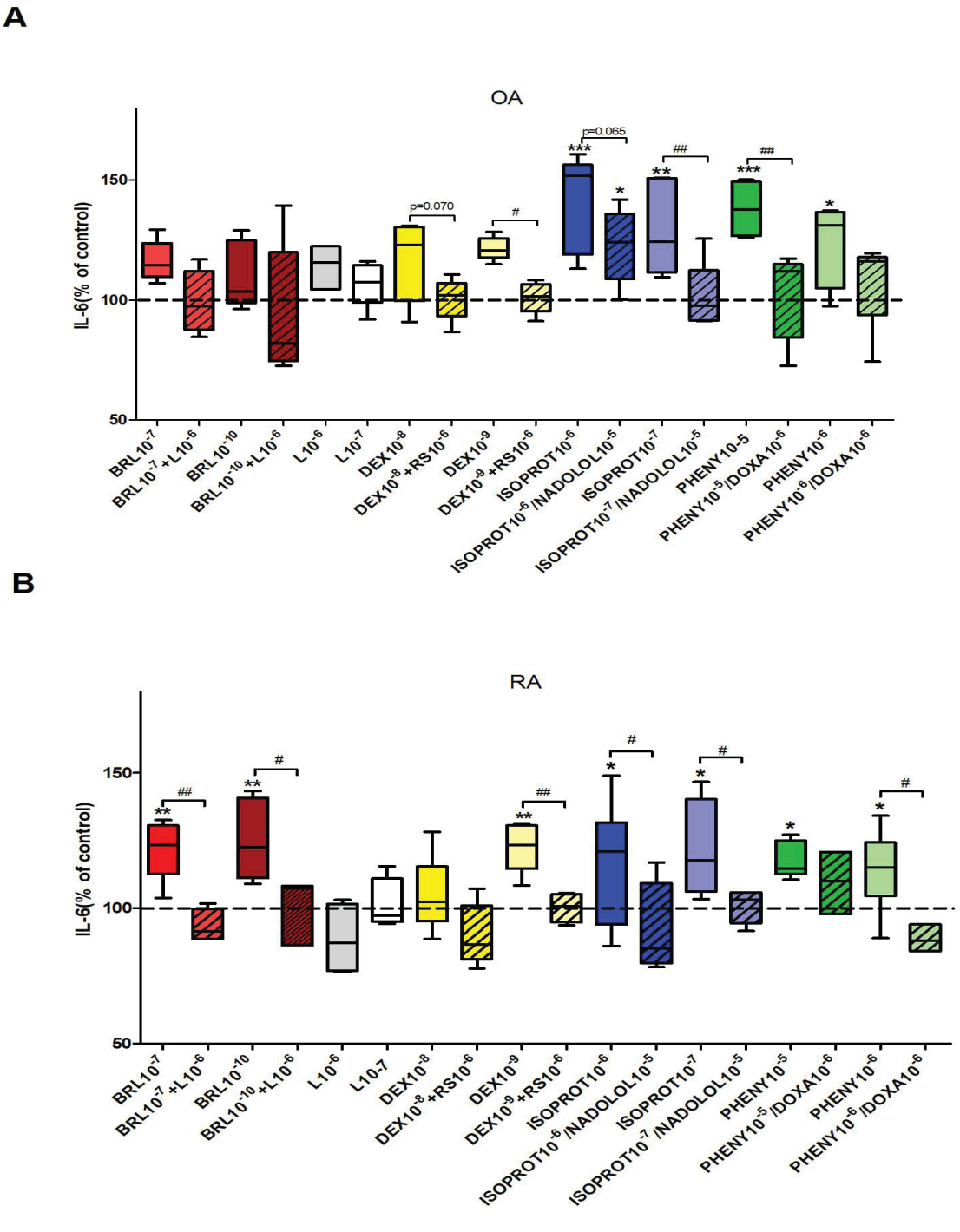


Fig 5. **Modulation of IL-6 release by AR agonists and antagonists in SFs stimulated with**

TNF. SFs were preincubated for 1h with different concentrations of AR agonists and antagonists (respective antagonists were added 30 min prior to agonists). The data of OA (n=3-5) and RA patients(n=3-8) are given. The control (OA: from 3.45 to 4.71 ng/ml, RA: from 3.46 to 14.38 ng/ml) was set to 100%. Values are shown as percent of control and expressed as mean±SEM. Paried t test was used for comparisons between different doses (agonist, agonist/antagonist vs control (*); agonist vs agonist/antagonist (#)). $p < 0.05$ was the level of significance. ***p or ###p< 0.001; **p or ##p < 0.01; *p or #p< 0.05.(BRL, BRL37344; L, L-748, 337; ISOPROT, isoproterenol; DEX, dexmedetomidine hydrochloride; RS, RS79948; PHENY, phenylephrine; Doxa, doxazosin)

3.4. BRL 37344 increased IL-6 production in SFs without TNF stimulation.

While the experiments described in section n3.3. were conducted with TNF as inflammatory stimulus, we also investigated the impact of AR stimulation without the addition of TNF. Here we found that in RA patients, low concentrations of BRL 37344 (Fig. 6A) but not norepinephrine (Fig. 6B) increased IL-6 production in SFs. Interestingly, when BRL 37344 (at 10^{-7}M or 10^{-10}M BRL 37344 30min prior to isoproterenol) and isoproterenol were added together, and cells were stimulated with TNF 1 hour later, no synergy in IL-6 production was observed in OASF and RASF (Fig. 6C -D).

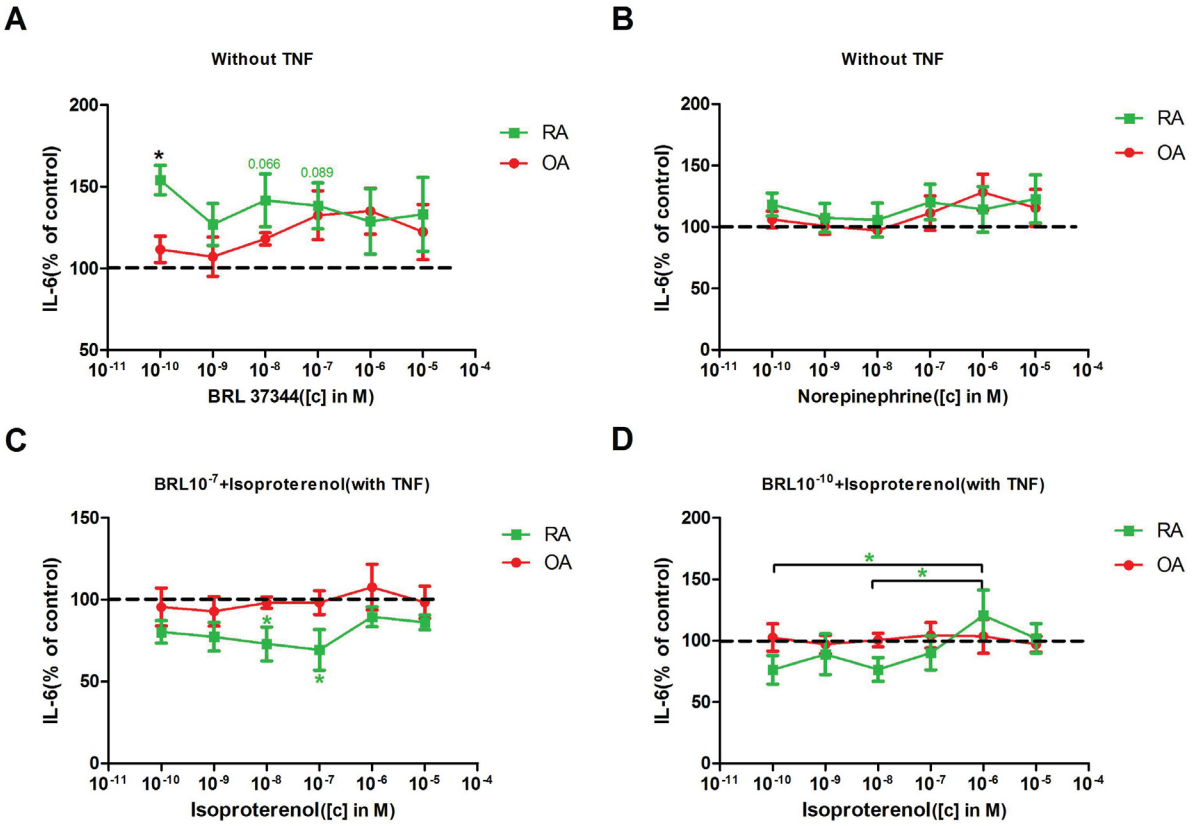
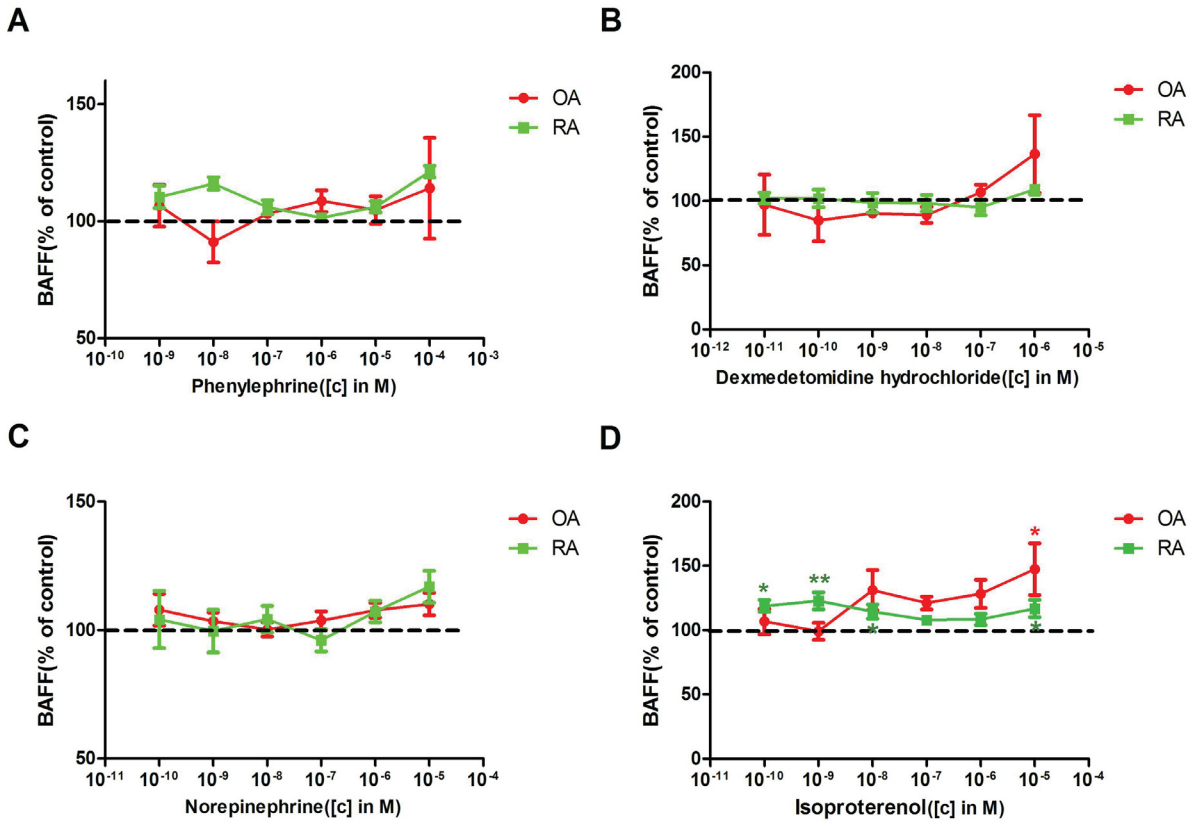


Fig 6. **Modulation of IL-6 release by AR agonists in SFs in the absence of TNF or not.** SFs were preincubated for 1h with different concentrations of BRL37344 (BRL) and norepinephrine (NE) alone or in combination with isoproterenol (ISO) (A, B, C, D). ISO was

added 30min prior to BRL, then cells were stimulated with TNF (10ng/ml final concentration) 1 hour later (C, D). The data of three OA patients and three RA patients are given. Values are shown as percent of control and expressed as mean±SEM. One-way ANOVA was used for comparisons between different doses of agonist/antagonist vs control. $p < 0.05$ was the level of significance. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

3.5. Isoproterenol affects BAFF production when SFs were concomitantly stimulated with IFN-γ.

B cell activating factor (BAFF) is a critical cytokine for the maturation of immature B cells. Elevated levels of BAFF have been identified in patients with rheumatoid arthritis [96], and the levels of BAFF are known to correlate with disease activity [97]. Here, we sought to investigate whether AR agonists modulate IFN-induced BAFF production by OASF and RASF .The results showed that phenylephrine, dexmedetomidine hydrochloride, norepinephrine and BRL 37344 have no significant effects on the production of BAFF after IFN-γ stimulation (Fig. 7A, B, C, E). Only the highest concentrations of isoproterenol increased BAFF production in OASF, while only low concentrations of isoproterenol increased BAFF production in RASF (Fig. 7D).



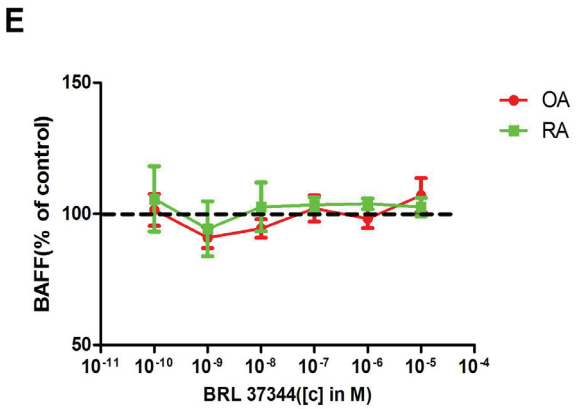
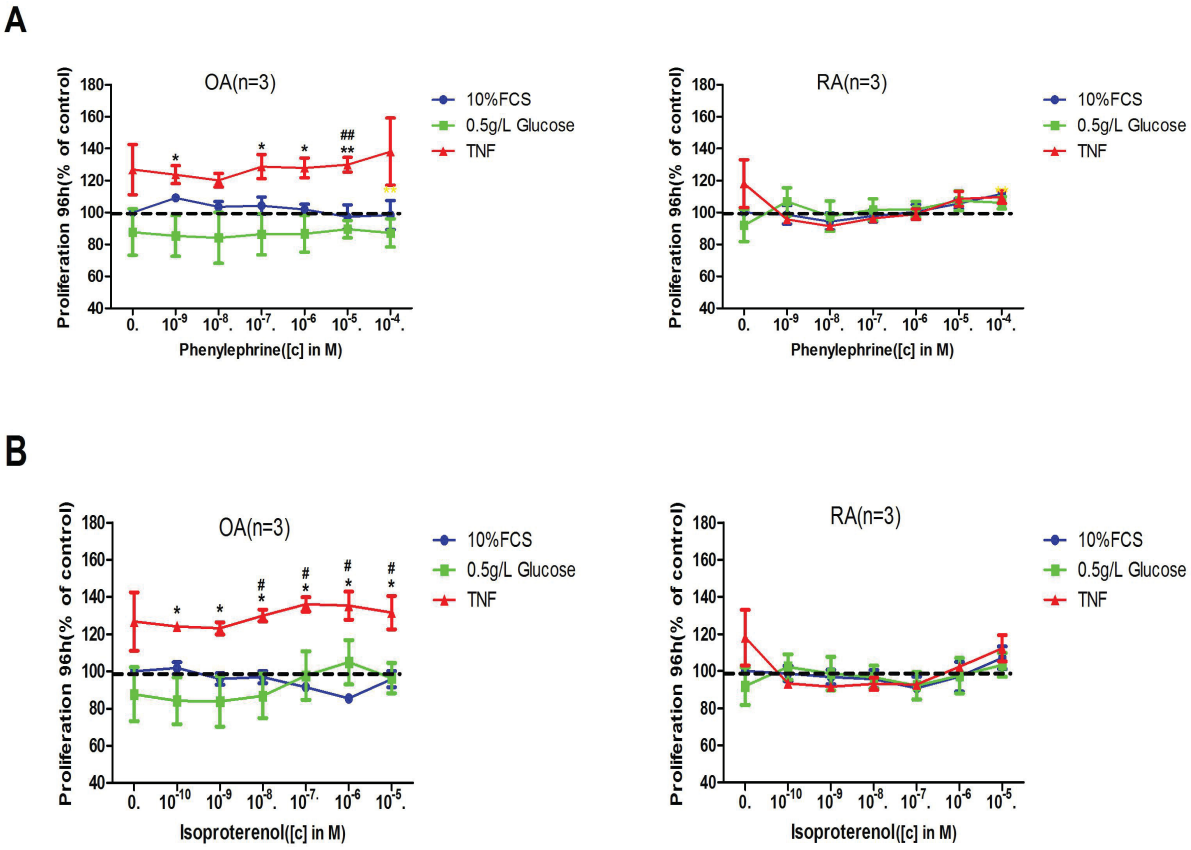


Fig 7. **Modulation of BAFF release by AR agonists in SFs stimulated with IFN-γ.** The data of five OA patients and three RA patients are given. The control (OA:42.77 ±14.64 pg/ml, RA: 48.46±7.72 pg/ml) was set to 100%. Values are shown as percent of control and expressed as mean±SEM. One-way ANOVA was used for comparisons between different doses of agonist/antagonist vs control. $p < 0.05$ was the level of significance. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.(BAFF, B-cell activating factor)

3.6 α and β AR have no significant effects on the proliferation of SFs

RASF are considered as hyperproliferative [98] and therefore, we assessed the ability of AR ligands to modulate SF proliferation. As shown in Figure 8A-B, TNF (10ng/ml final concentration) promoted the proliferation of SFs. However, α and β AR agonists showed no significant effect on proliferation of SFs (P<0.05).



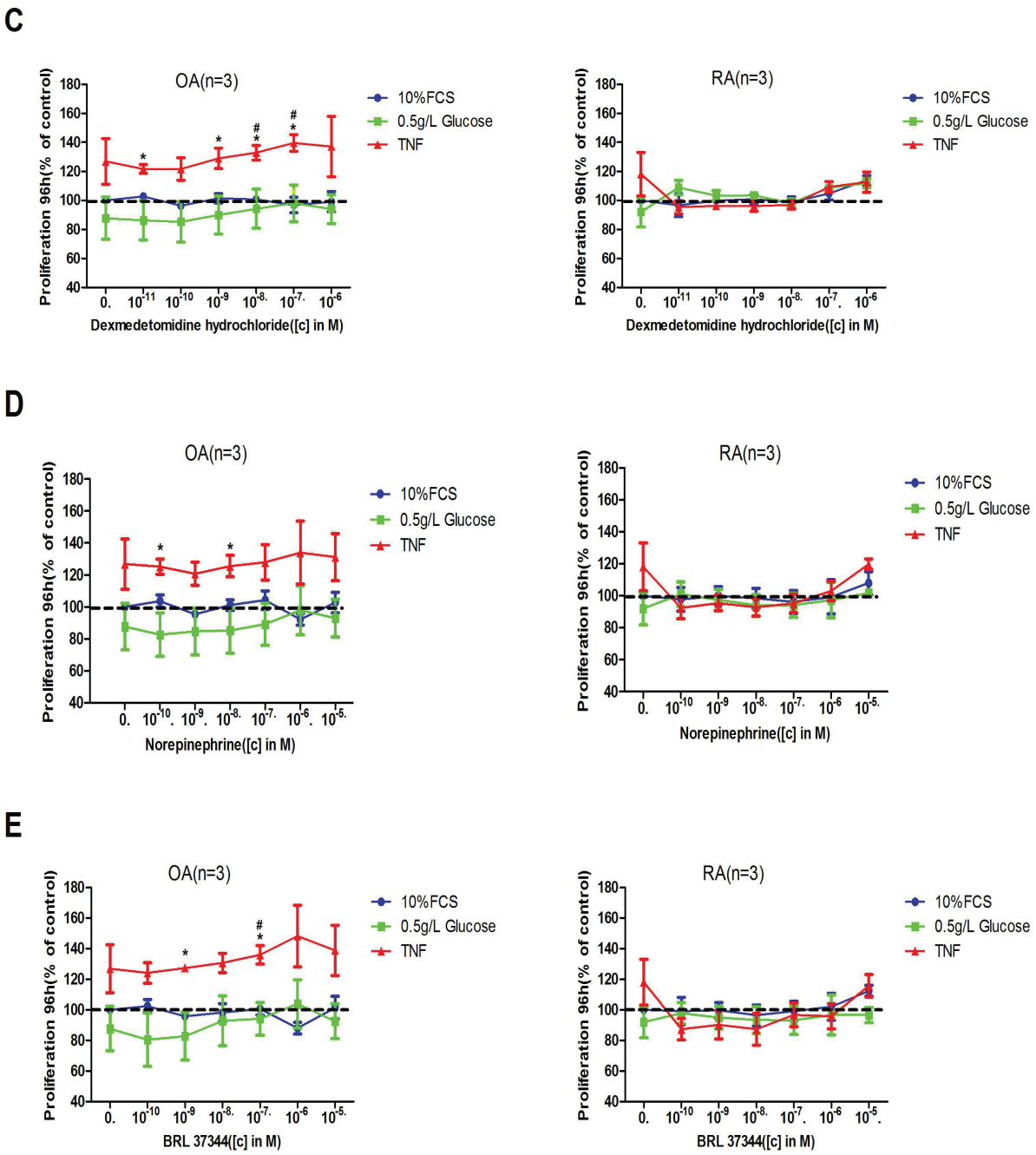
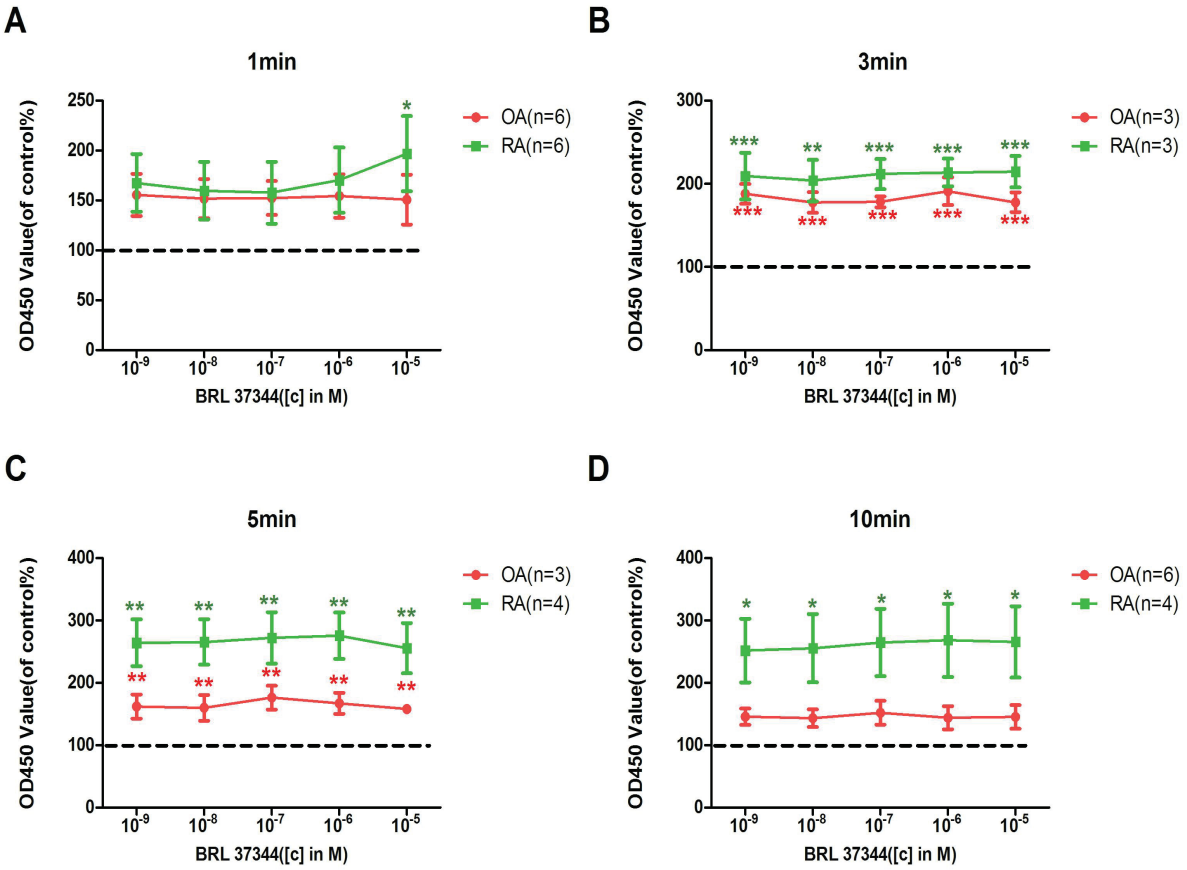


Fig 8. **Proliferation of SFs under the influence of AR agonists（phenylephrine, dexmedetomidine hydrochloride, isoproterenol, norepinephrine and BRL 37344）.** SFs were preincubate for 96h in 10% FCS or 10% FCS+TNF (10ng/ml) or low glucose (0.5g/L) medium with different doses of AR agonists. Then cell proliferation was assessed. The data of three OA patients and three RA patients are given. Values are shown as percent of control and expressed as mean±SEM. One-way ANOVA test was used for comparisons between each group (TNF vs 0.5g/L Glucose (*) or vs 10% FCS (#)). $p < 0.05$ was the level of significance. ***p or ###p< 0.001; **p or ##p < 0.01; *p or #p< 0.05.

3.7. β3 agonism active P38 MAPK signaling pathway in SFs

In recent studies we already demonstrated p38 activation by β2 agonists in B cells and

therefore we also investigated whether β-AR agonists also induce p38 phosphorylation in SF [99]. Aactivated (phosphorylated) p38 might increase the transcription of IL6 and therefore, we sought to investigate whether β3 agonism activates the P38 MAPK signaling pathway in SFs. Cell ELISA results showed that β3 agonism (BRL 37344) increased phosphorylated P38 in SFs (Fig. 9B-H). Furthermore, it showed that phosphorylated p38 changes dynamically over time (Fig. 9I). When the β3 antagonist L-748, 337 was included (10^{-6} M 30min prior to BRL37344) phosphorylated P38 decreased in OASF and RASF (Fig. 10A-E). In immunocytofluorescent analysis, we showed that phosphorylated p38 resided in the nucleus under basal conditions (Fig. 11, Fig. 12), while after activation through β3 agonism p38 relocalized to the cytoplasm (Fig. 11, Fig. 12), and then returned into the nucleus again (Fig. 12).



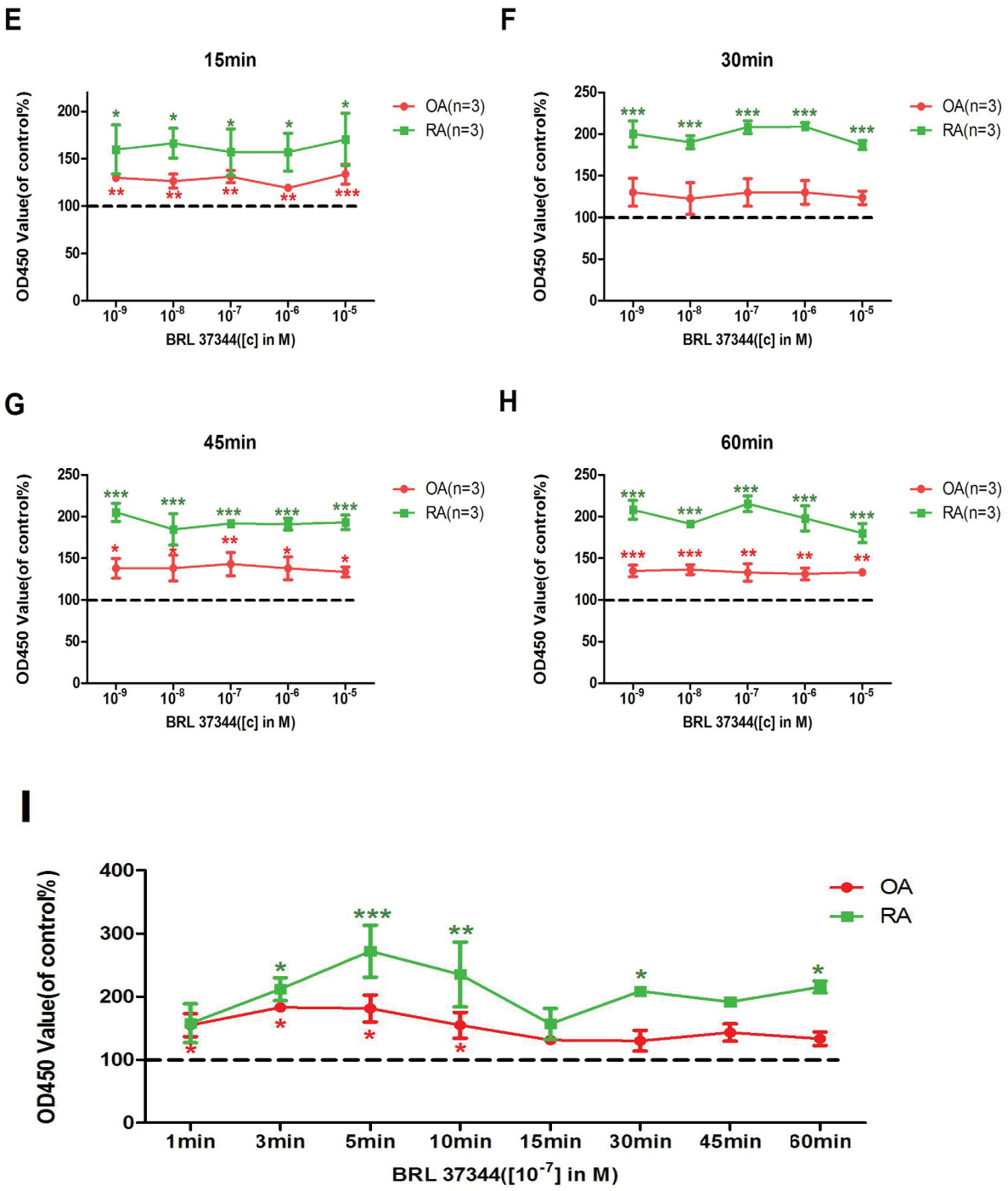


Fig 9. **The effect of β3 AR agonist (BRL 37344) on p38 MAPK signaling in SFs.** SFs were incubated for different times (1min, 3min, 5min, 10min, 15min, 30min, 45min and 1h) and different concentrations of BRL 37344. Values are shown as percent of control and expressed as mean±SEM. One-way ANOVA was used for comparisons vs control. $p < 0.05$ was the level of significance. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

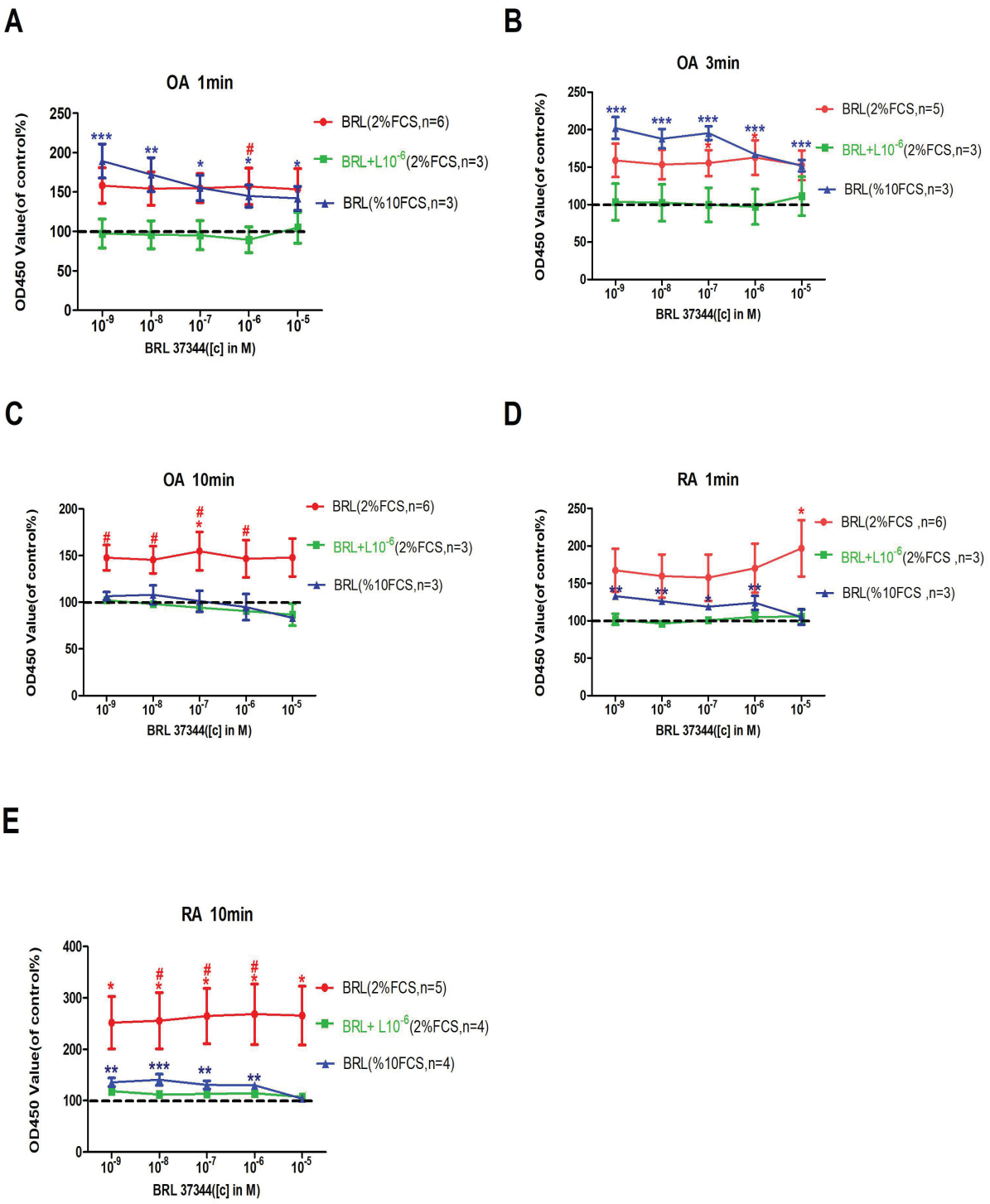


Fig 10. **The effect of the β3 AR agonist BRL 37344 and the antagonist L-748, 337 on p38 MAPK signaling pathway in SF**. SF were incubated for different times (OASF:1min, 3min, 10min; RASF:1min, 10min) with different concentrations of BRL 37344 (L-748, 337 was added 30min prior to BRL 37344) and different concentrations of FCS (2% or 10%). Values are shown as percent of control and expressed as mean±SEM. One-way ANOVA was used for comparisons vs control (*). One-way ANOVA was used for comparisons between different doses of agonist vs agonist/antagonist (#). $p < 0.05$ was the level of significance. ***p or ###p< 0.001; **p or ##p < 0.01; *p or #p< 0.05.(L, L-748, 337)

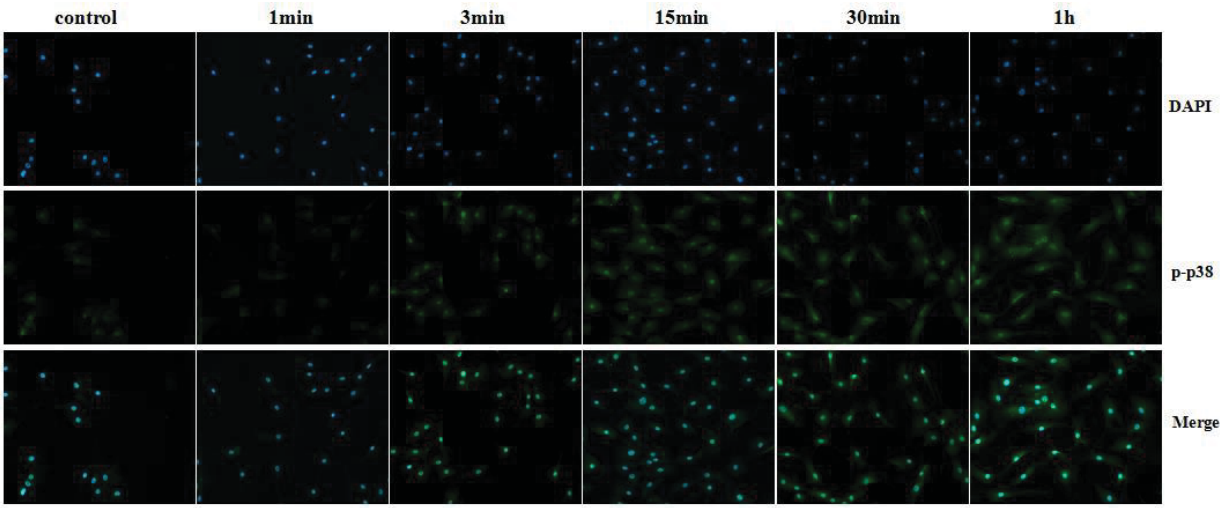


Fig 11. **Visualization of phosphorylated p38 protein in OASF (X200).** A). Immunocytofluorescent staining of phosphorylated p38 protein in OASF stimulated by β3 AR agonist (BRL 37344) after 1min, 3min, 15min, 30min and 1h.

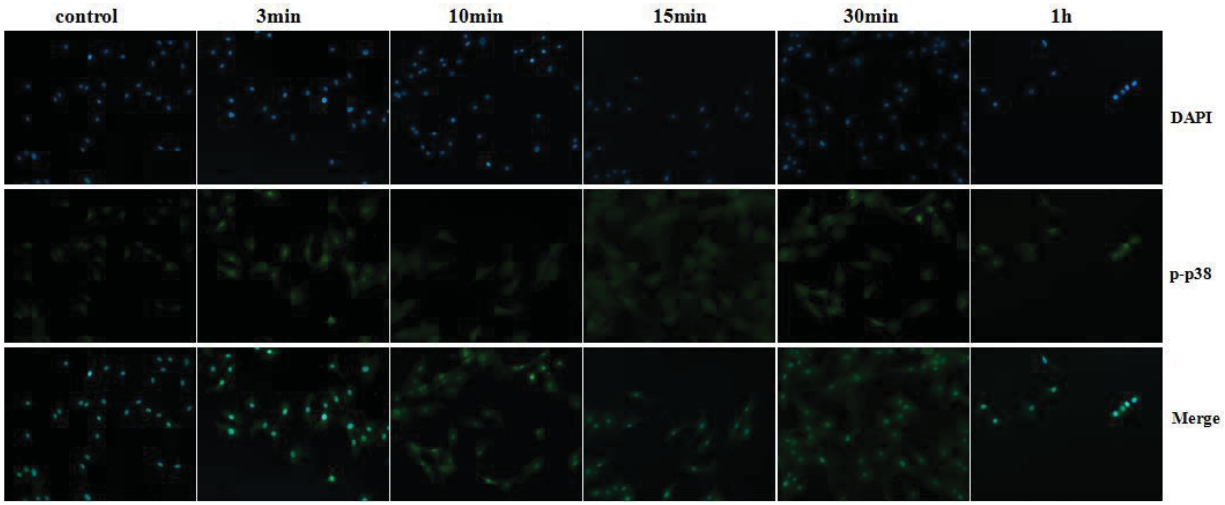


Fig 12. **Visualization of phosphorylated p38 protein in RASF (X200).** A) Immunocytofluorescent staining of phosphorylated p38 protein in RASF stimulated by β3 AR agonist (BRL 37344) after 3min, 10min, 15min, 30min and 1h.

4. Discussion

1.Expression of ARs in SF

In this study, we demonstrated that SFs express α1a, α2b, β1, β2 and β3. The expression of the latter has never been shown in SF or synovial tissue. It has been previously reported that ARs are expressed in human neural networks, heart, kidney, lung, blood vessels, immune cells, microglia and astrocytes [53-58,66]. Moreover, there are few studies about the function of ARs in SFs. In a study by Mishima et al., [100], exposure of synoviocytes to epinephrine or norepinephrine apparently inhibited the spontaneous increase in total cell number in a dose- and time-dependent manner. These responses were blocked by phentolamine (α-AR antagonist) or yohimbine (α2- AR antagonist), and were mimicked with

selective α2-AR agonists indicating the involvement of α2-AR stimulation in this response. The expression of β2-AR on fibroblast-like synoviocytes from rats was identified, and β2-AR signaling is decreased in the adjuvant arthritis model, which might be related to excessive desensitization of β2-AR under inflammatory conditions [101]. Similarly, in western blot, we have shown that the expression of β2 and β3 ARs decreased under pro-inflammatory conditions in SF. A loss of β-ARs on peripheral and synovial immune cells has been described in patients with RA and in arthritis animal models [102]. A significant decrease in β2-AR density on peripheral blood mononuclear cells was found in RA patients [103, 104]. This down-regulation has also been correlated with higher RA disease activity [102,103]. The modulation of β2 AR is possibly due to the influence of pro-inflammatory cytokines [104].

Cortisol as an anti-inflammatory drug, increased the expression of α2b, β2 and β3 in our study. In physiological situations, cortisol released from the adrenal cortex [105], impacts many different physiological systems (e.g., immunity, metabolism) and plays a role in augmenting the activity of the sympathetic nervous system (SNS), such as enhancing the sympathetically mediated cardiovascular response to stress (e.g., increased heart rate) [106]. The anti-inflammatory effect of cortisol is underpinned by our findings that β-ARs were upregulated by cortisol, which might enhance the effects of drugs acting on ARs. In this study, we detected for the first time β3 protein in SFs. While western blot revealed a band at the predicted size, we also observed a band at 85KD with the β3 antibody used. A possible explanation is that β3 either couples with other protein or the receptor dimerizes under inflammatory conditions. Studies have demonstrated the existence of AR dimers/digomers both in vitro and in living cells [107-110]. The different biochemical nature of the dimers and oligomers may be indicative of distinct biological roles in cells [110]. Whether β3 AR really dimerizes is not clear and additional studies may be required to clarify this. For the β1 AR, we found weak expression in immunofluorescent staining and multiple bands in western blot, but not a band at the predicted site. This is due to a lack of specificity for β1 AR, which was already demonstrated in studies by Hamdani N [91].

2. The effects of adrenergic ligands on pro-inflammatory function of SF

2.1 β AR agonism increases IL-6 production by SFs stimulated with TNF

In this study, we demonstrated that β3-AR regulates IL-6 expression in SFs. Enhanced IL-6 production by OASF and RASF was detected when using NE at high concentrations. This effect is due to β-AR agonism since NE binds to stimulatory β-ARs only at high NE concentrations [111]. Under proinflammatory conditions when SFs were pre-treated with TNF, the non-selective β agonist isoproterenol increased IL-6 expression and this was blocked by the β2 antagonist nadolol. Similarly, the specific β3 agonist BRL 37344 increased IL-6 expression and this was blocked by the β3 antagonist L-748,337, which suggests pro-inflammatory effects of β2 and β3 agonism in a proinflammatory environment. This was

also true under basal conditions since β3 agonism elevated IL-6 without TNF pretreatment. Interestingly, IL-6 was decreased when both, isoproterenol and BRL 37344 were used together, which suggests an antagonistic effect of β-1 /β-2 on β-3 activation.

The expression of IL-6 was elevated when using α1 and α2 agonists and this effect was blocked by their respective antagonists. Since the influence of alpha agonists on TNF-induced IL-6 production is rather small, the biological significance has yet to be determined. Moreover, the expression of IL-6 was not dose-dependently modulated by α1 and α2 agonists. This result may be explained by the cross-talk between α- and β-ARs [112, 113]. In α2a-AR/β1-AR co-transfected cells, α2a-AR stimulation resulted in internalization of both α2a-AR and β1-AR, whereas stimulation of β1 AR resulted in internalization of only β1 AR [112]. When α1D-AR and β2-AR are co-expressed in HEK-293 cells, heterodimerization of β2-AR with α1D-AR also conferred the ability of α1D-AR to co-internalize upon β2-AR agonist stimulation [113]. The cross talk between α1- and β2-ARs is mediated through heterodimerization and cross-internalization [114]. Similarly, co-expression of β2-AR with α2C-AR enhanced α2C-AR-mediated activation of extracellular signal-regulated kinase 1/2 [115]. Furthermore, enhanced α2C-AR internalization occurs in response to α2 AR agonists was observed when α2C-AR and β2-AR were co-expressed [115].

However, Western Blot results showed that the expression of β2 and β3 ARs decreased in response to TNF, the decreased expression of these receptors might also be involved in the regulation of IL6 expression.

2.2 The effect of AR agonisms on BAFF production of SFs stimulated with IFN-γ

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway has been associated with cellular proliferation [116-118]. Few studies on direct adrenergic coupling to JAK / STAT signaling is reported. β-Adrenergic stimulation acutely induces STAT1 and STAT3 phosphorylation [119]. In vascular smooth muscle cells, direct α-adrenergic stimulation of JAK / STAT signaling has been described [120]. The α1 agonist induced tyrosine phosphorylation of JAK2 and STAT1 in vascular smooth muscle cells, while α1 AR antagonist prevented JAK2 phosphorylation, which suggested that α1 AR is linked to the JAK/STAT pathway [121]. IFN-γ triggers the prolonged activation of the transcription factor STAT1 via the IFN-γ receptor (IFNR) and JAK [122]. In addition, changes between STAT homodimers and STAT1/3 heterodimers may represent a biologically relevant approach to determining the crosstalk between IFN-γ and other pathways (such as IL6) [123]. However, the interactions between IFN-γ and AR signals are not fully understood. We observed BAFF production by RASF and OASF when treated with IFN-γ [124]. BAFF production was only modulated by high and low concentrations of isoproterenol in RASF and OASF respectively. Our result suggests that β-ARs may play a role in regulating the expression of BAFF related to IFN-γ signaling, but further studies are needed to support this view.

2.3 ARs have no significant effects on the proliferation of SFs

The previous studies demonstrated that glucose deficiency is related to inhibition of proliferation [125-127]. So, we assessed the ability of AR ligands to modulate SF proliferation under glucose deficiency or pro-inflammatory conditions (TNF stimulation). The results showed that low glucose levels had a tendency to inhibit OASFs proliferation, and pro-inflammatory conditions promoted OASFs proliferation, and neither such effects happened in RASFs. TNF is considered to be the central mediator of a series of biological activities from cell proliferation, cell death and differentiation to inflammation induction and immune regulation [128-129]. These effects are mainly mediated by TNF receptor 1 (TNFR1, p55), which is widely expressed in almost all cell types and is considered to be the main receptor of soluble TNF [130]. Furthermore, TNF contributes to proliferation based on different cell types, various activation states, and a variety of microenvironment factors [128].

The β-AR activation has been reported to modulate proliferation in retinal endothelial cell [131] and tumor cell [132]. However, our results showed α and β AR agonists had no significant effect on the proliferation of SFs.

3 β3-ARs and p38 MAPK

It is established p38 as the major regulator of inflammatory genes during β-AR activation [84,86,133,134]. The β-adrenergic activation of p38 MAPK has been shown to occur through cAMP-dependent mechanisms involving PKA [134]. The activated p38 translocates to the nucleus, and phosphorylates transcription factors, such as nuclear factor kappa-B(NF-κB) [135] and signal transducer and STAT3 [136], which stimulates the transcription of pro-inflammatory cytokines such as IL6 [135]. Several studies have identified β3-AR affects apoptosis [137] and inflammation through p38 Mak pathway [138]. However, how β3-AR activation produces inflammation is not fully understood.

We demonstrated here that, β3 agonism increases IL6, and p38 MAPK is usually upstream in the regulation of IL6 [139]. We speculated that β3 influences IL-6 production through the p38 MAPK signaling pathway. Cell ELISA experiments, showed that phosphorylated p38 is activated rapidly after β3 activation. In addition, IF experiments found that phosphorylated p38 resides in the nucleus at the basal state, and relocates to the cytoplasm after activation of β3, and then shuttles back to the nucleus again. We didn't look into the relationship between p38 MAPK and nuclear hormone receptor in this study. There is some precedent for nuclear hormone receptors to be modulated by p38 MAPK [140,141]. For example, PPAR γ coactivator-1 is phosphorylated by p38 MAPK under cytokine stimulation and this event regulates the control of genes involved in energy expenditure [140]. Phosphorylation of the glucocorticoid receptor by p38 MAPK appears to reduce receptor activity and this may be involved in feedback regulation of the receptor in the setting of inflammation [141]. The nature of how p38 MAPK interacts with nuclear hormone receptor is not clear from this work, but our

experiments do suggest a time-dependent phosphorylation of p38 in the nucleus in response to β3 AR stimulation. Further investigation will be required to determine the molecular determinants of this event.

In summary, our data indicate that P38 MAPK pathway is a mechanism for the activation of β3-AR in SFs. However, our data does not demonstrate directly that p38 activation is responsible for the increase of IL6 in SF.

5. Conclusions

1. SFs express α1a, α2b, β1, β2 and β3, and the expression of ARs is changed under pro-inflammatory and anti-inflammatory conditions.
2. The α- and β-ARs regulate IL-6 expression in SFs, but not so much BAFF.
3. β3 agonism activates P38 MAPK signaling pathway in SFs. At present, we have only carried out the cell experiment, and the role of β3-AR in vivo needs to be further confirmed. We hope that β3-AR will become a new target for the treatment of RA, which may have great value for the RA patients.

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Abbreviations

SI units and symbols were used in this thesis.

OA	osteoarthritis
RA	rheumatoid arthritis
SFs	synovial fibroblasts
ECM	extracellular matrix
MMPs	matrix metalloproteinases
HA	hyaluronic acid
MGCs	multinucleated giant cells
MRI	magnetic resonance imaging
MW	molecular weight
AR	adrenergic receptors
cAMP	Cyclic Adenosine monophosphate
MAPK	Mitogen-activated protein kinase
AITC	Allyl isothiocyanate
FCS	Fetal Calf Serum
Doxa	Doxazosin
BRL	BRL37344
L	L-748, 337
ISOPROT	Isoproterenol
DEX	Dexmedetomidine hydrochloride
RS	RS79948
PHENY	Phenylephrine
GPCRs	G-protein-coupled receptors
AC	adenylate cyclase
IL-6	Interleukin-6
BAFF	B-cell proliferation and survival factor
ERK	extracellular signal regulated kinase
JNK	c-jun-NH2-terminal kinase
PKA	protein kinase

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