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An elementary description of the functional catecholaminergic pathway in rheumatoid arthritis synovial fibroblasts

Dissertation

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Zusammenfassung

Katecholamine, als wichtige Neurotransmitter des sympathischen Nervensystems (SNS), sind von zentraler Bedeutung für die Regulierung von Entzündungen und zahlreichen physiologischen und pathologischen Prozessen. Tatsächlich wurde bereits ein Einfluss des SNS und seiner Neurotransmitter Noradrenalin (NE) und Dopamin (DA) in der Pathologie der rheumatoiden Arthritis (RA) beschrieben. Obwohl die Expression von Tyrosin-Hydroxylase (TH) im Verlauf der RA berichtet wurde, gibt es nur begrenzte Hinweise für die Expression und Modulation der gesamten Katecholamin-Maschinerie in Synovialzellen. Ziel dieser Arbeit ist es, eine detaillierte Beschreibung des Katecholaminweges und seiner entzündlichen Modulation in humanen RA synovialen Fibroblasten (RASFs) zu präsentieren.

RASFs exprimieren alle katecholaminergen Zielmoleküle einschließlich der synthetisierenden Enzyme (TH, DOPA Decarboxylase, Dopamin Beta-Hydroxylase und Phenylethanolamin N-Methyltransferase), vesikulären Monoamintransporter 1/2 (VMAT1/2), Dopamintransporter (DAT), Noradrenalintransporter (NET) und Abbaurenzyme (Monoaminoxidase (MAO)-A, MAO-B und Catechol-O-Methyltransferase (COMT)). DA, NE und Epinephrin (EPI) wurden durch RASFs synthetisiert. Die Funktion all dieser Katecholamin-bezogenen Komponenten wurde in RASFs durch Versuche mit entsprechenden Inhibitoren validiert. Hierbei fand sich eine Erhöhung der extrazellulären Spiegel durch MAO-, COMT-, VMAT- oder DAT/NET-Hemmung. Tumornekrosefaktor (TNF) erhöhte die Expression von VMAT2, MAO-B und NET in RASFs sowie die extrazellulären Katecholaminspiegel. Während eine hohe Konzentration [100µM] von exogenem DA die interleukin-6 (IL-6) Produktion und das Zellüberleben reduzierte, erhöhte NE in Konzentrationen über 1µM die IL-6 Produktion bei gleichzeitiger Reduktion des Zellüberlebens. Die Wirkung der MAO-Hemmung in RASF unterschied sich abhängig von der inhibierten Isoform und TNF Vorbehandlung. Die IL-6-Produktion wurde durch MAO-A-Hemmung mit Clorgyline (CLG) [10nM -1µM] in unstimulierten, aber nicht TNF-stimulierten RASFs erhöht, während sie bei hohen Konzentrationen [100µM] des Inhibitors unabhängig von der TNF Stimulation reduziert war. Die höchste Konzentration von CLG führte zudem zu einem reduzierten Überleben der RASF. Im Gegensatz dazu erniedrigte der MAO-B-Inhibitor Lazabemidhydrochlorid (LB) das Zellüberleben nur geringfügig bei 100µM. Außerdem erhöhte LB die IL-6-Produktion in unstimulierten RASFs, während sie in TNF vorbehandelten Zellen herunterreguliert wurde.

RASFs besitzen eine komplette und funktionelle katecholaminerge Systemmaschinerie, dessen Funktion durch TNF reguliert wird. Unsere Ergebnisse zeigen nicht nur die mögliche Beteiligung sympathischer Neurotransmitter an der RA-Pathologie, sondern eröffnen auch therapeutische Möglichkeiten generell Entzündungen entgegenzuwirken, wobei die MAO-Enzyme potenzielle Ziele für die Wirkstoffe sind.

Summary

Catecholamines, as major neurotransmitters of the sympathetic nervous system (SNS), are of pivotal importance in regulating inflammation and numerous physiological and pathological processes. In fact, some evidence has been described about the influence of the SNS and its neurotransmitters norepinephrine (NE) and dopamine (DA) in rheumatoid arthritis (RA) pathology. Although the expression of tyrosine hydroxylase (TH) has been reported in the course of RA, there is only limited evidence demonstrating the expression and modulation of the whole catecholamine machinery in synovial cells. The involved enzymes, receptors, transporters, ligands and the function of each in human RA synovial fibroblasts (SFs) were investigated in this project. This study aims to present a detailed description of the catecholaminergic pathway and its modulation under inflammatory conditions in human RASFs.

RASFs express all components of the catecholaminergic machinery including the synthesizing enzymes (TH, DOPA decarboxylase, dopamine beta-hydroxylase, and phenylethanolamine N-methyltransferase), vesicular monoamine transporters 1/2 (VMAT1/2), dopamine transporter (DAT), norepinephrine transporter (NET) and degradation enzymes (monoamine oxidase (MAO)-A, MAO-B and catechol-O-methyltransferase (COMT)). DA, NE and epinephrine (EPI) were synthesized by RASFs. The function of all catecholamine-related components was validated in RASFs, since extracellular catecholamine levels were elevated by either MAO, COMT, VMAT or DAT/NET inhibition. Tumor necrosis factor (TNF) upregulated the expression of VMAT2, MAO-B and NET levels and increased extracellular catecholamine levels in RASF supernatants. While high concentrations [100μM] of exogenous DA reduced interleukin-6 (IL-6) production and cell viability, NE above 1μM enhanced IL-6 levels with a concomitant suppression in cell viability. The effect of MAO inhibition on RASF was dependent on isoform (A or B). IL-6 production was augmented by MAO-A inhibition by Clorgyline in unstimulated but not TNF stimulated RASFs [10nM-1μM], while it was diminished at 100μM of inhibitor. Cell viability was decreased dose-dependently in TNF stimulated and unstimulated RASFs. In contrast, the MAO-B inhibitor Lazabemide hydrochloride (LB) only modestly reduced cell viability at 100μM. Besides, LB enhanced IL-6 production in unstimulated RASFs, but downregulated it in TNF pre-treated cells.

RASFs possess a complete and functional catecholamine machinery whose function is regulated by TNF. Our findings not only shed further light on the involvement of sympathetic neurotransmitters in RA pathology, but also open therapeutic avenues to counteract inflammation with the MAO enzymes as potential drug candidates.

List of Abbreviations

Abbreviation	Full Name
ACPAs	Anti-citrullinated protein antibodies
ARs	Adrenergic receptors
APRIL	Proliferation-inducing ligand
BAFF	B-cell-activating factor
BZD	Benserazide hydrochloride
CLG	Clorgyline
COMT	Catechol-O-methyltransferase
CRP	C-reactive protein
DA	Dopamine
DAT	Dopamine transporter
DBH	Dopamine-beta-hydroxylase
DCs	Dendritic cells
DDC	DOPA decarboxylase
DRs	Dopamine receptors
ELISA	Enzyme-linked immunosorbent assay
EPI	Epinephrine
FLS	Fibroblast-like synoviocyte
GABA	γ-aminobutyric acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
HLA-DR	Human leukocyte antigen D-related
IDA	Indatraline hydrochloride
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
IRF-5	Interferon regulatory factor 5
JNK	Jun activated kinase
LB	Lazabemide hydrochloride
LPS	Lipopolysaccharide
M30	M30 dihydrochloride
MAO	Monoamine oxidase
M-CSF	Macrophage colony-stimulating factor
MLS	Macrophage-like synoviocyte
MMPs	Matrix metalloproteinases
NE	Norepinephrine
NET	Norepinephrine transporter
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NPY	Neuropeptide Y
NSS	Normal swine serum
OA	Osteoarthritis
PTPN22	Protein tyrosine phosphatase non-receptor type 22
PNMT	Phenylethanolamine N-methyltransferase
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROS	Reactive oxygen species
RSP	Reserpine
SEM	Standard error of the mean
SF	Synovial fibroblast
SNS	Sympathetic nervous system
TGF-β	Transforming growth factor-β

TH	Tyrosine hydroxylase
Th17	IL-17 producing helper T cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF1/C5	Tumors necrosis factor-receptor associated factor 1 and complement component 5
VMAT	Vesicular monoamine transporter
WB	Western blotting

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1. INTRODUCTION

1.1 RA and RASFs

1.1.1 Clinical features

Rheumatoid arthritis (RA) has been widely described as a systemic autoimmune disease, which is characterized by chronic joint inflammation, bone erosion and cartilage destruction leading to complete loss of function. [1] The diagnosis of RA followed the classification criteria of the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) in 2010, taking joint involvement, serology test, acute phase reactants and symptom duration into consideration. [2]

Normally, RA primarily targets the small joints and then the large ones, implicating multiple human organs leading to widespread pain, stiffness and severe disability. [3] The most common targeted sites are proximal interphalangeal joints, metacarpophalangeal joints and wrists, sometimes with visible synovitis-caused swelling and synovial thickening. Meanwhile, large joints (shoulders, elbows, hips, knees, and ankles) can also be affected during the progression of RA. [4] In addition, RA also involves extra-articular manifestations, including the skin, eye, heart, lung, kidney, digestive system, and nervous system, causing an increase in immortality. [5-8] In the acute phase of the disease, patients may also present with systemic symptoms, such as loss of weight, fatigue, and low-grade fever. [4]

1.1.2 Epidemiology

In recent decades, the prevalence and risk factors for RA have been extensively studied by scientists from different areas. RA is a global disease regardless of age, nationality, sex and race. However, prevalence differs according to population characteristics and fluctuates over time. [9] Some studies described low prevalence ratios in Serbia (0.18%) [10], France (0.31%) [11] and Italy (0.33%) [12], while higher ratios were reported in Japan and Argentina (1.7% and 1.97%, respectively). [13, 14] Besides, gender differences are also apparent in the prevalence of RA. Studies have demonstrated a two- to three-fold higher prevalence of RA in females than in males. [15] The personal lifetime risk of developing RA has been roughly elucidated at 3.6% for women and 1.7% for men in the United States. [16]

Genetic, environmental and stochastic factors play a crucial role in RA development. [17] According to the existence of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), RA can be classified into two subtypes. In the ACPA-positive subtype, human leukocyte antigen D-related (HLA-DR) alleles [18], tumor necrosis factor-receptor associated factor 1 and complement component 5 (TRAF1/C5) [19] and protein tyrosine phosphatase non-receptor type 22 (PTPN22) risk alleles [20] are important genetic components. Meanwhile, interferon regulatory factor 5 (IRF-5) has a great impact on the ACPA-negative subtype. [21]

RA heritability is approximately 60% proportion in twin studies, while identical twins have only 12–15% disease concordance. [22] The obvious difference in concordance implies the contribution of environmental factors to susceptibility.

Smoking and silica exposure are important environmental risk factors for developing RA. The increased risk of contracting RA was validated, having a linear relationship with the amount and duration of smoking. [23, 24] This impact is related to the genetic subtype of RA, because it has been revealed that RF- or ACPA-positive RA can be triggered by smoking, but there is no or little influence on the ACPA-negative subtype. [25] Silica exposure was identified as a crucial occupational hazard principally activating ACPA-positive RA, while chronic exposure can lead to rheumatoid pneumoconiosis. [26, 27] Moreover, periodontal disease can induce the development of RA due to its effect on mediating protein citrullination and promoting osteoclastogenesis. [28]

1.1.3 Pathogenesis

Synovitis is the major manifestation of RA, and it involves the joint capsule composed of the synovial membrane, synovial fluid, and the respective bones. Tissue inflammation and cartilage destruction are triggered and maintained by the interaction of T cells, B cells, macrophages, neutrophils, dendritic cells (DCs) and fibroblasts. [29] Sustained immune cell activation promotes an inflammatory milieu, leading to the subsequently synovial membrane pannus formation, bone erosion and cartilage degradation. [3, 29]

1.1.3.1 T cell-mediated immune response in RA

Numerous researches have reported that RA is driven by $CD4^+$ T lymphocytes, especially interleukin (IL) -17 producing helper T cells (Th17). [30] Th17 cells can be generated by many cytokines in synovial joints, including IL-6, IL-1β, IL-21, transforming growth factor-β (TGF-β), and IL-23, and they have vital contributions to B-cell activation, neutrophil recruitment and osteoclastogenesis. [31-34] The migration of Th17 cells to inflammatory areas can be promoted by joint-specific mesenchymal cells, resulting in homeostatic proliferation and an increase in IL-17 production, thereby accelerating the inflammatory phase of RA. [35] Pro-inflammatory cytokines, such as IL-17A, IL-17F, and IL-22, produced by Th17 cells have an important contribution to RA pathogenesis. [36] However, Th17 cells were also found to secrete IL-10, which can dampen the immune response, implying an immunosuppressive capacity of Th17 cells in RA development. [37, 38] Since therapeutic interventions targeting either IL-17A or IL-17R are ineffective, the function of IL-17A in RA is still controversial. [39, 40]

1.1.3.2 B cells and autoantibodies in RA development

Autoantibodies, especially RF and ACPA, are produced by B cells after differentiation into plasma cells under Toll-like receptor (TLR) stimulation. [41] RF and ACPA have been widely discussed as principal prognostic markers regarding RA onset in symptomatic patients. [42]

Sixty-nine percent of RA patients are positive for RF, whose specificity is estimated at approximately 60%–85%. [43, 44] RF is an accepted diagnostic immunoglobulin (Ig) M antibody of RA pathology that recognizes the Fc portion of IgG. It was the first-discovered autoantibody in RA and served as a marker in early classification criteria for RA (1987 ACR). [45, 46] Although the activity of RF has been confirmed in numerous immunoglobulins (IgA, IgG, and IgM), the correlation between RF levels and clinical disease activity is still controversial. [47] RF levels have the potential to revert and convert during the early stage of RA, and have limited correlation with clinical prognosis. [48] Moreover, RF is also detectable in other diseases, including infections, cancers, other rheumatic diseases and even in healthy people. [43]

ACPAs also have pivotal contributions to early RA diagnosis, as 60-80% of patients with RA are ACPA-positive with a specificity of up to 85-99%. [49, 50] There is an increase of ACPA levels in preclinical RA, which is highly specific for the prediction of RA development. [51-53] Thus, its role in diagnosis was highlighted in the ACR/EULAR 2010 classification criteria. [54] Over the course of RA, the concentration of pro-inflammatory cytokines is enhanced alongside the concentrations and epitope diversity of ACPAs. Besides, ACPAs promote the progression of RA by triggering the activation of macrophages and osteoclasts via immune complex formation. [55] The citrulline residues on proteins or peptides are the main targets of ACPAs. By binding to citrullinated vimentin on the surface of osteoclast precursor cells, ACPAs can initiate the process of bone loss by causing efficient osteoclast generation. Nevertheless, ACPAs can also accelerate bone resorption via the activation of macrophages by immune complexes and subsequent generation of pro-inflammatory cytokines (tumor necrosis factor (TNF) and RANK-L), resulting in osteoclast differentiation. [46, 56]

1.1.3.3 The synovium and synovial fibroblasts (SFs)

Normal healthy synovium is described as a quiescent relatively acellular structure, while it becomes hyperplastic and invasive abound with immunocompetent cells in RA. [57] The pathogenetic synovium features by hyperplasia of the intimal lining, occasional aggregation of lymphocytes, and sublining infiltration of mononuclear cells. [58]

Moreover, non-immune cells play critical roles in autoimmune and chronic inflammatory diseases. [59] Fibroblast-like synoviocytes are the major non-immune cell type in the pathogenesis of RA and can be classified into type A and type B synoviocytes. Type A synoviocytes originate from the monocyte/macrophage lineage and are classified as

macrophage-like synoviocytes (MLS), while type B synoviocytes are recognized as fibroblast-like synoviocytes (FLS) or SF, derived from mesenchymal lineage cells. [60]

Certain aggressive phenotypes of SFs are considered to contribute to the development of RA by regulating the local production of cytokines, chemokines and matrix-degraded proteolytic enzymes. [57] RASFs interact with immune and non-immune cells, prompting the activation of effector immune cells. [61]

By interacting with T cells, SFs are thought to be responsible for local chronic inflammation and the destruction of bone and cartilage. Chemokines (such as CCL2, CCL20, and CXCL9/10/11), cytokines (such as TGF-β), adhesion molecules, and membrane proteins are involved in the communication between SFs and T cells, along with T cell recruitment and activation. [57, 62, 63]

Furthermore, RASFs are able to produce two B cell-related factors (B cell-activating factor (BAFF) and proliferation-inducing ligand (APRIL)) through TLR3 stimulation, accounting for B cell maturation and differentiation. [64] Anti-BAFF antibody treatment in RA patients suppresses RF and disease activity, with alleviating clinical features. [65] In addition, IL-6 is a crucial cytokine produced by RASFs which exerts a strong effect on B cell activation, development, maturation, expansion and survival. [66]

Peripheral monocytes/macrophages can be recruited into the inflamed synovial tissues by inflammatory mediators, such as specific chemokines such as monocyte chemoattractant protein-1 and angiogenic factors produced by RASFs. [57] A positive inflammatory loop was illustrated in the interaction between RASFs and macrophages, causing the release of cytokines and enzymes, recruitment of immune cells, and synovial hyperplasia. Granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and IL-6 are assumed to be secreted mainly by RASFs, while TNF and IL-1β are considered to originate from macrophages. The secretion of GM-CSF from RASFs resulting in further macrophage expansion can be provoked by IL-1β and TNF released by activated macrophages. [67, 68] Beyond that, anti-IL-6, anti-TNF and anti-GM-CSF receptor therapies alleviated clinical presentations in RA patients. [69-72] As a non-immune cell, MLS also interacts with RASF, leading to the activation and development of inflammation and subsequent joint destruction. [73] In an *in vivo* co-culture of MLS and RASF, obvious cartilage damage was emphasized in arthritis. [74] Furthermore, MLS is able to release cytokines such as IL-1β and TNF, inducing the activation of RASFs. [75]

RASF also exerts a strong effect on RA joint and cartilage destruction through the synthesis of matrix metalloproteinases (MMPs). [57, 76] Moreover, the expression of MMPs

can be enhanced by inflammatory cytokines such as IL-17, TNF, and IL-1β, which may contribute to further joint degradation. [57]

1.2 The sympathetic nervous system (SNS) and its influence on the immune system

1.2.1 The SNS and inflammation

The SNS is associated with the fight and flight reaction under stressful situations and it plays a major role in the interaction between the central nervous system and the immune system. [77-79] Early in 1936, the aggregation of endotoxin in sympathetic tissue and further activation of sympathetic nerve fibres were demonstrated along with a subsequent systemic typhoid fever. [80] This finding preliminarily revealed the crosstalk and interaction between the SNS and inflammation. Extensive innervation with sympathetic nerve fibres was found in secondary lymphoid tissue in the mid-1980s, while the sympathetic nerve terminals in lymphoid tissue were recorded closely around immune cells. [81]

The expression of sympathetic neurotransmitter receptors, such as adrenergic receptors (ARs), was described in immune cells in the 1990s, and they contribute to the dynamic interaction between the immune system and the neuroendocrine system. [82] Additionally, the secretion of neurotransmitters can be elicited by SNS activation under inflammatory stimulation. Catecholamines are the major neurotransmitters secreted by sympathetic nerves, however, ATP, neuropeptide Y (NPY), and nitric oxide also play crucial roles in immune regulation. [83] Several approaches can unravel the sympathetic regulation of immune cells, including interfering with lymphocyte distribution and generation [84, 85], regulating the release of proinflammatory peptides [86], regulating blood or lymph flow [87] and directly combining with ARs on immune cells [88]. TNF is the first-reported cytokine whose level can be regulated by catecholamines and ARs. [89, 90] Meanwhile, many other cytokines and immune cells have been described to be modulated by AR stimulation and the levels of sympathetic neurotransmitters. [89] Actually, the role of catecholamines in inflammatory regulation can hardly be categorized as activation or repression. Rather, there is a concentration-dependent modulation of catecholamine levels and effects during an immune response. [90, 91]

The pro-inflammatory effects of the SNS on clinical outcomes have also been illustrated in several disease models like allergic asthma [92, 93], chronic eye inflammation [94], arthritis [95, 96], endometriosis [97], sepsis [98], colitis [99, 100] and others. In an acute model of septic shock, cell proliferation, apoptosis, and immune cell migration were all regulated by the adrenergic system. [101]

1.2.2 The SNS and arthritis

To exemplify the impact of the SNS on the immune response, arthritis is particularly well suited as it was reported already that pain and joint swelling were alleviated after sympathectomy in RA patients. [102] Similar results were observed in carrageenan-induced arthritis [103] and adjuvant arthritis [104] in rats, which revealed mild clinical outcomes and less pro-inflammatory cytokine levels after sympathectomy. In addition, enhanced activity of the SNS was reported in a study of spontaneously hypertensive rats that developed aggravated arthritis. [105] The proinflammatory influence of the SNS was also observed in collagen type II arthritis (CIA) in mice [106], although another study from 1986 only demonstrated mitigation of overall pain but not attenuation of morning stiffness or joint tenderness. [107] The time point of sympathetic intervention is a likely explanation for these contradictory results, since late sympathectomy in the chronic phase of CIA aggravates arthritis, while early sympathectomy attenuates CIA. [95]

There is a loss of innervation in synovial tissue during chronic inflammation. [108] and the concomitant loss of sympathetic neurotransmitters during experimental arthritis can be compensated by catecholamine-producing cells in the joint. [109] Although these cells alleviate experimental arthritis, their role in other conditions might be different. Catecholamine-producing phagocytes have potential proinflammatory effects in a lung injury model via α2-AR. [110] Catecholamines and ARs mediate anti-inflammatory functions in an autocrine or paracrine manner. Moreover, the production of IL-10 from B cells in arthritic animals can be upregulated by norepinephrine (NE) via β2-AR, which has an anti-inflammatory effect in CIA. [111]

1.3 Catecholamines and RA

Catecholamines, as a major neurotransmitter of the SNS, are of pivotal importance in regulating inflammation and numerous physiological and pathological processes. In the central nervous system, abundant evidence has illustrated the constitution and function of the catecholaminergic pathway. Catecholamine synthesis, including the synthesis of dopamine (DA), NE and epinephrine (EPI), requires the participation of tyrosine hydroxylase (TH), DOPA decarboxylase (DDC), dopamine-beta-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT). Monoamine oxidase (MAO)-A/B and catechol-O-methyltransferase (COMT) contribute to the degradation of catecholamine. Moreover, catecholamines can be released by exocytosis and then invoke downstream reactions in target cells. Excess catecholamines are taken up through dopamine transporter (DAT) or norepinephrine transporter (NET) and are stored in vesicles through transport by the vesicular monoamine transporters (VMAT) 1 and 2. [112, 113] Catecholamines have been extensively demonstrated to have immune modulating properties in diseases such as Parkinson’s disease [114, 115], multiple sclerosis [116, 117] and especially RA [118, 119].

1.3.1 Catecholamines and immune cells in RA

In previous studies, neurotransmitter receptors were found on RA immune cells, implying a crosstalk between neuronal signals and immune signals. [82] In a mouse model of arthritis, secreted DA from DCs induced the production of IL-17 by T cells, causing Th17 lymphocyte differentiation and subsequently exacerbating cartilage destruction. Additionally, this impact was prevented by a DRD1 antagonist. [118, 120] D2-like DR is expressed by B cells in RA synovium and in mast cells from the synovial fluid and there are more B cells expressing D2DR in RA than in OA synovial tissue. [121]

It was suggested that G protein-coupled receptors (for example, DR and AR) can switch from Gαs to Gαi signaling under chronic inflammation. [122] Besides, cytokine production (like TNF) is regulated by stimulating ARs by catecholamines in macrophages and THP-1 cells. [89, 123] However, this crosstalk between ARs and immune cells can be influenced by many factors, such as age [124], the activation state of respective cells stimulated by neurotransmitters [111, 125], or the regulation of receptor expression on different cell types [126].

1.3.2 Catecholamines and synovial cells in RA

In previous studies, a high concentration of DA was measured in the synovial fluid of RA patients. [118] Later, newly appearing TH positive, catecholamine-producing cells were detected in the synovial tissue of RA and osteoarthritis (OA) patients. [109] Mixed synovial cells produce and release DA, representing a noncanonical mechanism in the modulation of local joint inflammation. TH-, DBH-, DDC-, PNMT-, VMAT2- and COMT-positive cells were visualized by immunofluorescence staining in mixed synovial cells. The treatment of mixed synovial cells with reserpine, which induces a rapid release of stored DA (together with noradrenaline) from cells, led to a strong inhibition of TNF release in isolated cells from RA patients. [119]

Moreover, it was reported that RASFs possess a dopaminergic system, including dopamine receptors (DRs), DAT, TH and DA. The number of SFs positive for DRs was significantly higher in RA patients than in OA patients, and the activation of DRs resulted in a reduction in the inflammatory cytokine release of RASFs. [127] These results imply that the catecholaminergic pathway is a potential target to modulate RASF function. Therefore, a detailed investigation of the catecholaminergic pathway in RASFs is needed.

1.4 Aims of this study

The objectives of our project are to investigate the expression and function of the catecholaminergic pathway and its potential modulation by inflammatory mediators (TNF) in human RASFs.

2. MATERIAL AND METHODS

2.1 Patients

In total, 17 RA patients who underwent elective knee joint replacement surgery were recruited. The diagnosis of RA fulfilled the RA revised criteria of the American College of Rheumatology [2] and laboratory data were collected before surgical intervention. The mean age of the RA patients was 70.12 ± 7.89 years. The mean C-reactive protein (CRP) and RF levels were 8.41 ± 11.94mg/L and 61.24 ± 69.57IU/mL, respectively. Previous specific RA-related treatment of each patient was also recorded. A total of 4/17 patients received methotrexate treatment, while 5/17 underwent glucocorticoid therapy and 5/17 received biologicals or Janus kinase inhibitors. All patients were informed about the aim and gave written consent to participate in our study before surgery. Our project was approved by the Ethics Committees of the University of Düsseldorf (approval number 2018-87-KFogU).

2.2 Compounds and chemicals

Compounds and chemicals with abbreviations, order numbers, companies and concentrations used are presented in Table 1.

Compound	Abbreviation	Order number	Company	Concentration used in this study
Tumor necrosis factor	TNF	300-01A	PeroTech	10ng/mL
Benserazide hydrochloride	BZD	B7283	Sigma-Aldrich	50µM
Clorgyline	CLG	M3778	Sigma-Aldrich	1µM
Reserpine	RSP	2742	Tocris / Bio-Techne	10µM
M30 dihydrochloride	M30	6067	Tocris / Bio-Techne	10µM
Lazabemide hydrochloride	LB	2460	Tocris / Bio-Techne	10µM
OR-486	OR-486	0483	Tocris / Bio-Techne	10µM
Indatraline hydrochloride	IDA	1588	R&D / Bio-Techne	10µM

Table 1: List of compounds and proteins used in this study

2.3 Synovial tissue preparation and SF culture

The preparation of synovial tissue and isolation of RASFs was performed as described previously for in-vitro experiments [128, 129]. First, synovial samples were immediately obtained (up to 9cm^2) and excised upon exposing the knee joint capsule. The tissue pieces were carefully minced into tiny fragments and digested with liberase (628825, Roche Diagnostics, Mannheim, Germany). After overnight digestion at 37℃, the suspension was filtered (70µm, MACS SmartStrainer) and centrifuged (300g, 10min). After centrifugation, the

supernatant was discarded and the pellet was treated with erythrolysis buffer (20.7g NH_4Cl, 1.97g NH_4HCO_3, 0.09g EDTA ad 1L H_2O) (Red blood cell lysis solution, 130-094-183, Miltenyi Biotec, Bergisch Gladbach, Germany) for 5min. Then, the suspension was centrifuged again for 10min at 300g and the pellet contained synovial cells including RASFs. The pellet was resuspended in RPMI-1640 (Sigma Aldrich, St. Louis, USA, 10% FCS). Finally, dead cells and debris were removed after overnight culture and cells were supplied with fresh medium. Pure RASF cultures were obtained after passaging, as lymphocytes, granulocytes and macrophages are either washed off or die during cell culture over prolonged periods.

2.4 Western Blotting (WB)

Cells were detached and harvested with Accutase solution (A6964, Sigma-Aldrich) for 10min. Cell lysis and isolation of protein were performed using RIPA lysis buffer (R0278; Sigma) with complete protease inhibitor (Roche, Mannheim, Germany). Protein was quantified by a Bradford assay and then equal amounts of protein per sample were subjected to electrophoresis in 12.5% SDS-polyacrylamide gels (60min, 20mA, Biorad, Puchheim, Germany). Protein was then transferred from gels to 0.2µm nitrocellulose membranes (300mA, 90min, Biorad, Germany). Membranes were washed 3 times (10min each) with TBS-T and then blocked with 5% nonfat milk in TBS-T (Tris-glycine-SDS buffer from Sigma containing 0.1% Tween 20) at room temperature for 1 hour. After three washing steps, membranes were treated with the primary antibodies shown in Table 2 overnight at 4℃. Afterwards, the membranes were washed with TBS-T and incubated with secondary antibody (goat anti-rabbit IgG HRP, DAKO P0448, 1:2000 in 5% nonfat milk) for 2 hours. After washing, immunoreactive protein bands were visualized by treating membranes with ECL Prime (GE Healthcare, Freiburg, Germany). Proteins of interest were quantified in a V3 Western Workflow (Biorad) and the signals were normalized against that of GAPDH (antibody 2118, Cell Signalling, 1:2000), which was chosen as a reference protein.

2.5 Immunofluorescence (IF)

RASFs were seeded in 96-well plates and cultured until confluence reached approximately 80-90%. Cells were washed with pre-cooled PBS 5 times and fixed with cold methanol for 20min at -20℃. Cells were rinsed with PBS for 4 times and then permeabilized with 0.3% (v/v) Triton X-100 in PBS for 5min at room temperature. Subsequently, the cells were blocked with blocking buffer (PBS with 5% normal swine serum (NSS, Dako, X0901)) for one hour at room temperature. Primary antibodies (Table 2) and the same amount of rabbit IgG polyclonal isotype (ab37415, Abcam) were diluted in blocking buffer separately. After overnight culture with primary antibodies or IgG isotype at 4℃, cells were washed with 0.3% (v/v) Triton X-100 diluted in PBS 4 times followed by 2 washing steps with PBS only. Afterwards, secondary antibody (Goat anti-rabbit IgG (Alexa Fluor 488), ab150077, Abcam) was diluted in blocking

buffer, and the cells were incubated for 1 hour at room temperature. Finally, the cells were rinsed 6 times, overlaid with ProLong™ Gold Antifade Mountant with DAPI (P36931, Invitrogen), covered with coverslips and kept in the dark overnight at 4℃. Images of each target protein were captured using an Axio Observer microscope (Zeiss-Germany) with a digital camera AxioCam (Zeiss-Germany). Zen 2.6 software (Blue edition-Zeiss-Germany) was applied for image analysis.

	Catalog	Company	Dilution in WB	Dilution in IF
DAT	22524-1-AP	Proteintech	1:1000	1:250
TH	25859-1-AP	Proteintech	1:10000	1:500
VMAT1	AMT-007	Alomone	1:200	1:100
VMAT2	AMT-006	Alomone	1:400	1:1000
MAOA	10539-1-AP	Proteintech	1:3000	1:300
MAOB	12602-1-AP	Proteintech	1:4000	1:50
GAPDH	2118	CellSignalling	1:2000	—

Table 2: Primary antibodies used in this study.

2.6 Catecholamine enzyme-linked immunosorbent assay (ELISA)

To determine of extracellular catecholamine levels, RASFs were seeded in 96-well plates. When confluence reached 90%, the supernatant was discarded, and replaced with 50μL/well fresh complete medium (10% FCS) with specific inhibitors. After incubation(varied depending on treatment), cell culture supernatants were collected followed by 20min of centrifugation (1000×g at 2-8℃). The resulting supernatants were used to carry out the assay, according to the given protocols (E-EL-0045, E-EL-0046, E-EL-0047, Elabscience). Control samples without inhibitor were collected after 2, 6, 12 and 24 hours of incubation to establish the optimal incubation time.

To quantify intracellular catecholamines, cell lysates were prepared according to the manufacturer's instructions. First, cells seeded in 6-well plates were incubated until 90% confluence. After stimulation with inhibitors, cells were gently washed with pre-cooled PBS and detached by trypsin (Trypsin-EDTA Solution, Sigma, 59418C). Cell pellets were prepared by centrifugation at 1000×g for 5min. The pellets were washed 3 times with pre-cooled PBS and resuspended in distilled water. The suspension was treated with an ultrasonic cell disrupter for 10min and repeated freeze-thaw cycles until the cells were fully lysed. Supernatants from those lysates were generated by centrifugation (10min, 1500×g at 2-8℃) and were used for ELISA.

All reagents and samples were equilibrated to room temperature (18-25℃) before use. After mixing of the samples, 50μL standard, blank and sample was added to the wells of the ELISA plate. The plate was covered by a sealer and incubated at 37℃ for 45min. After that, the solution was decanted from each well, followed by 3 washes (1min soaking, decanting, and patting it dry). Then, 100μL of HRP conjugate working solution was added to each well, along with a new cover sealer, for 30min at 37℃. Then, the conjugate solution was discarded and the plate was washed 5 times as mentioned above. 90μL of substrate reagent was added to each well of the ELISA plate for 15min at 37℃ in a light-protected place. After completion of the reaction, 50μL of stop solution was applied to each well in the same order as the substrate solution. A micro-plate reader (450nm, Tecan, Infinite® 200 PRO) was used to determine the optical density of each well as within 30min after stopping the reaction.

2.7 Quantitative polymerase chain reaction (qPCR)

Cultured cells treated with or without TNF [10ng/mL] or not were harvested after 6, 12 and 24 hours. Total RNA was extracted with the RNA Mini Kit (GR8RNA, Qiagen, Hilden, Germany) according to the manufacturer's instructions. After washing twice with PBS, each cell pellet (no more than 5×10^6 cells) was disrupted in 350μL RLT buffer containing β-mercaptoethanol (β-ME, v/v=1/100) and homogenized by vortexing. The solution was then moved to a QIA shredder and centrifuged at maximum speed for 2min. Afterwards, the bottom solution was kept and treated with 350μL 70% ethanol and mixed well by pipetting. The whole 700μL solution was transferred to a RNeasy Mini spin column placed in the supplied 2mL collection tube. The tube was centrifuged for 15sec at ≥ 8000×g. Then, the flow-through was discarded and the column was used for DNase digestion as described below. Then, 350μL of RW1 buffer was added to the RNeasy column, followed by 15sec of centrifugation at ≥ 8000×g. After discarding the flow-through, 10μL DNase I stock solution was gently mixed with 70μL RDD buffer and was moved to the RNeasy column membrane for a 15-min incubation at 20-30℃. Subsequently, the RNeasy column was rinsed with 350μL of RW1 buffer and centrifuged for 15sec at ≥ 8000×g. After on-column DNase digestion, 500μL of RPE buffer was added (this step was conducted twice) to the spin column and centrifuged at ≥ 8000×g for 15sec and 2min. A new 2mL collection tube was applied for a 1min centrifugation to dry the membrane. The RNeasy spin column was placed in a new 1.5mL collection tube and eluted with 50μL RNase-free water after 1min centrifugation at ≥ 8000×g.

By spectrophotometry (260nm), the total RNA content was determined. Afterwards, the synthesis of cDNA was performed with an iScript™ gDNA clear cDNA Synthesis Kit (172-5035, BIO-RAD) with the same amount of RNA (1μg) from the different samples. qPCR was performed using qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems) in a total volume of 20μL and the StepOnePlus real-time PCR system with the primers shown in Table 3. GAPDH was used as a quantitative control for normalization. The relative expression fold-change was

expressed by the values of $2^{-\Delta\Delta CT}$ [130]. Each qPCR analysis was conducted at least in duplicate.

Genes	Primer sequences	
	Forward	Reverse
TH	5′-TGTCCACGCTGTACTGGTTC-3′	5′-AGCTCCTGAGCTTGTCCTTG-3′
DDC	5′-GAACAGACTTAACGGGAGCCTTT-3′	5′-AATGCCGGTAGTCAGTGATAAGC-3′
DBH	5’-GACGCCTGGAGTGACCAGAA-3’	5’-CAGTGACCGGAACGGCTC-3’
PNMT	5’-ATGATGTCAAGGGCGTCTTC-3’	5’-CCACTTCAAAGAACAGGGAATC-3’
VMAT1	5’-TGACATGGAGTTCAAAGAAGTCAAC-3’	5’-GAGAGGCGAGGGCATGTG-3’
VMAT2	5’-CGGATGTGGCATTTTGTATGG-3’	5’-TTCTTCTTTGGCAGGTGGACTTC-3’
MAOA	5′-TAAATGGTCTCGGGAAGGTG-3′	5′-CCCAGGGCAGTTACTGATGT-3′
MAOB	5′-GCTCTCTGGTTCCTGTGGTATGTG-3′	5′-TCCGCTCACTCACTTGACCAGATC-3′
COMT	5′-GAAGGGGACAGTGCTACTGG-3′	5′-CAGGAACGATTGGTAGTGTGTG-3′
DAT	5’-TCACCAACGGTGGCATCTAC-3’	5’-CACTCCGATGGCTTCGATGA-3’
NET	5’-ACTGTTCCTTATCATCGCGG-3’	5’-CGATCAGGATGACAGCATAGC-3’
DRD1	5’-TGTTTCCTGTCGCTGCTCATCC-3’	5’-TCTGACACAGCCAAGGAGATGAC-3’
DRD2	5’-TGTACAATACGCGCTACAGCTCCA-3’	5’-ATGCACTCGTTCTGGTCTGCGTTA-3’
DRD3	5’-ACATGCCTACTATGCCCTCTCCTAC-3’	5’-ATTCCAGACTCCACCTGTCACCTC-3’
DRD4	5’-TTCGTCTACTCCGAGGTCCA-3’	5’-CGCACAGGTTGAAGATGGAG-3’
DRD5	5’-CCATCCTCATCTCCTTCATTCC-3’	5’-AGTCACAGTTCTCTGCATTCACG-3’
GAPDH	5′-ACAACTTTGGTATCGTGGAAGGAC-3′	5′-CAGGGATGATGTTCTGGAGAGC-3′

Table 3: Primer sequences used in this study.

2.8 IL-6 ELISA

RASFs (10,000cells/well) were seeded in 96-well plates at least 24 hours before stimulation. Supernatants were collected after indicated time for the quantification of IL-6. Experiments were performed as described in the manufacturer's protocol (human IL-6, 555220, from BD, OptEIA, Heidelberg, Germany).

ELISA plates were coated with 100µL/well of capture antibody diluted in coating buffer and incubated overnight at 4℃. Wells were aspirated and washed 3 times with wash buffer. Plates were blocked with assay diluent and placed at room temperature for 1 hour. After

discarding the blocking solution, standard, control and samples(in assay diluent) were added into appropriate wells. Plates were sealed and incubated at room temperature for 2 hours. After 5 washes, detection antibody with streptavidin-HRP reagent was added to each well for 1 hour at room temperature. Wells were washed 7 times (1min soak every time) and substrate solution was added for 30 minutes at room temperature in the dark. The reaction was terminated by the addition of 100µL of stop solution (1M H_2SO_4) and the absorbance of each well at 450nm was determined in a microplate reader (450nm, Tecan, Infinite® 200 PRO).

2.9 CellTiter-Blue cell viability assay

After collecting the supernatants of treated RASFs, the cells were incubated with CellTiter-Blue reagent following the instruction of manufacturer (G8081, Promega). After 2 hours of incubation, the fluorescence in both, the samples and negative control wells at 560/590nm was recorded by a microplate reader (450nm, Tecan, Infinite® 200 PRO). By determining the reduction from resazurin to resorufin, the cell viability was estimated and quantified to reflect the toxic effects of individual treatments.

2.10 Statistics

All data were summarized from at least three independent experiments. The statistical tests used are given in the figure legends. GraphPad Prism (GraphPad Software Inc., California, USA) was applied for data analysis. For line plot data, the line represents the mean. For bar plots, the top of the bar represents the mean and error bars depict the standard error of the mean (SEM). For box plot data, the boxes represent the 25th to 75th percentiles. The median is presented by lines within the boxes, and the lines outside the boxes represent the 10th and 90th percentiles. The level of significance was $p<0.05$.

3. RESULTS

3.1 Key proteins for synthesis, transport, storage, and degradation of catecholamines are expressed in RASFs

In the first step, the expression of key proteins involved in the catecholamine synthesis (TH), uptake (DAT), storage (VMAT 1/2) and degradation (MAO-A/B) was analyzed by IF and WB in RASFs (Fig.1 a-f). Original and uncropped WB images of each key protein are shown in supplementary figure (Supplement Fig.1). It was found that RASFs express TH, DAT,VMAT1/2 and both MAO isoforms under unstimulated conditions.

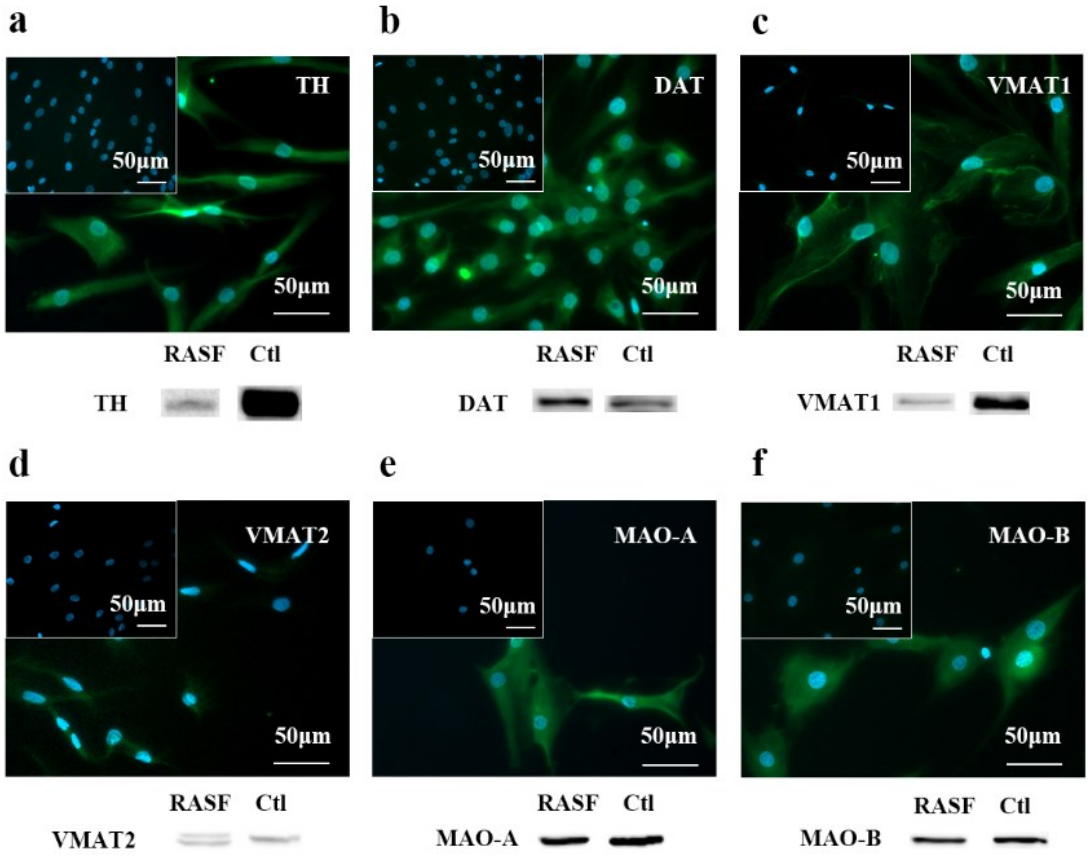
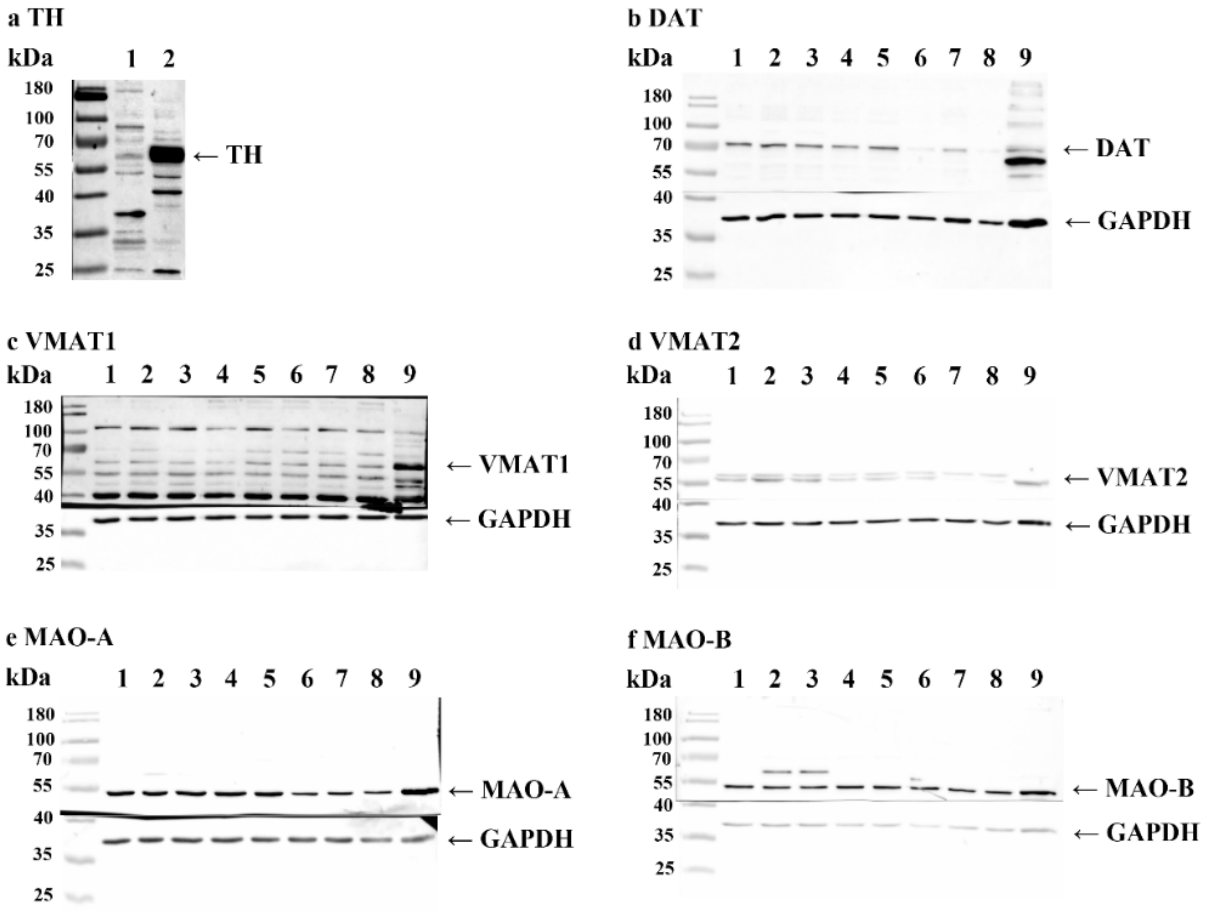


Fig.1 Expression of the major components of the catecholaminergic pathway in RASFs.

IF and WB images of TH **(a)**, DAT **(b)**, VMAT1 **(c)**, VMAT2 **(d)**, MAO-A **(e)** and MAO-B **(f)**. Staining with respective antibody isotypes (images in upper left corners) served as the negative control, target proteins are shown in green, and cell nuclei (blue) were stained with DAPI (Bar: 50µm). In WB experiments, mouse brain homogenate was used as a positive control (Ctl).



Supplement Fig. 1 Original and uncropped images of WB.

(a-f) Original and uncropped WB images of TH **(a)** (Column 1, RASFs; Column 2, mouse brain protein as a positive control), DAT **(b)** (Column 1-8, RASFs under different stimulations; Column 9, mouse brain protein as a positive control), VMAT1 **(c)** (Column 1-8, RASFs under different stimulations; Column 9, mouse brain protein as a positive control), VMAT2 **(d)** (Column 1-8, RASFs under different stimulations; Column 9, mouse brain protein as a positive control), MAO-A **(e)** (Column 1-8, RASFs under different stimulations; Column 9, mouse brain protein as a positive control), and MAO-B **(f)** (Column 1-8, RASFs under different stimulations; Column 9, mouse brain protein as a positive control).

3.2 RASFs express all components of the catecholaminergic pathway which are selectively regulated by TNF

Since WB for TH and VMAT1 showed multiple bands, making interpretation difficult, expression of these target proteins along with DDC, DBH, PNMT, NET and COMT was confirmed by qPCR. (Fig. 2). In addition, since TNF is widely considered as a major pro-inflammatory cytokine with a vital impact on RASFs in the joint, its effect on the regulation of the above-mentioned mRNAs at different stimulation time points was assessed. It was found that DAT was slightly reduced at 6 hours (down 20.68±7.825%, p=0.0166) under TNF stimulation (Fig. 2 e). There was an increase in NET (up 179.4±68.20%, p=0.0170) mRNA expression after 24 hours of TNF stimulation (Fig. 2 f). The magnitude of regulation in

catecholamine transporters (DAT and NET) was small, whereas a much stronger upregulation of VMAT2 and MAO-B by TNF was found. The mRNA expression of VMAT2 (Fig. 2 h) after TNF stimulation increased significantly at all time points, with a maximum at 12 hours (up 796.3±217.3%, p=0.0018). Additionally, MAO-B (Fig. 2 j) mRNA expression was significantly enhanced at 24 hours under TNF treatment (up 650.4±229.2%, p=0.0109).

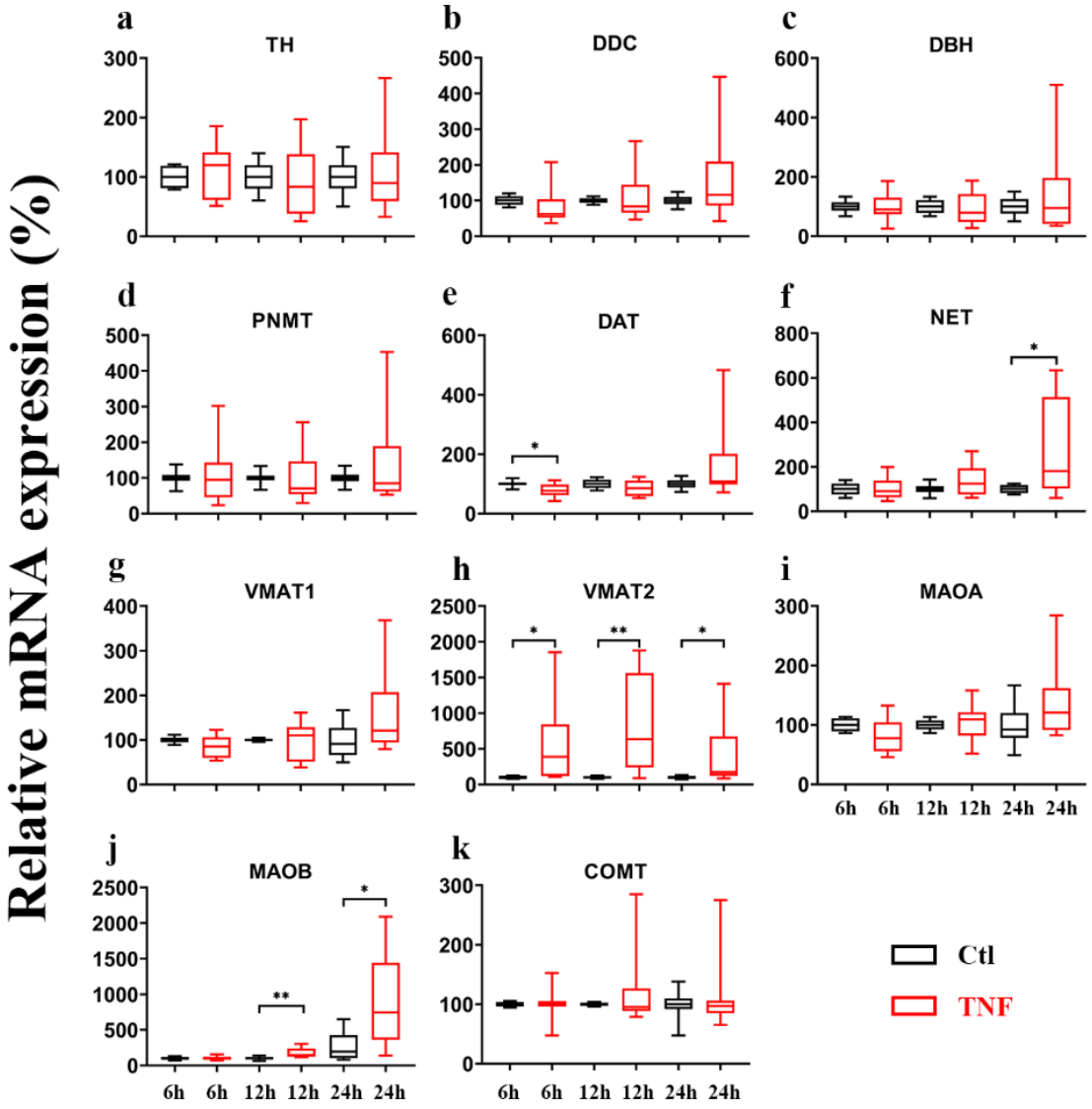


Fig.2 mRNA expression of all components of the catecholaminergic pathway in RASFs under basal conditions and after stimulation with TNF.

Relative mRNA expression of components of the catecholaminergic pathway in RASFs with and without TNF stimulation for 6, 12 and 24 hours including TH **(a)**, DDC **(b)**, DBH **(c)**, PNMT **(d)**, NET **(e)**, DAT **(f)**, VMAT1 **(g)**, VMAT2 **(h)**, MAO-A **(i)**, MAO-B **(j)** and COMT **(k)**. (n=5) * p<0.05, ** p<0.01, for comparisons of TNF-stimulated with control groups (no TNF) for each stimulation at a given time point by paired Student's t test. Black, control conditions; Red: stimulated with TNF (10ng/mL); 6h, 12h, 24h =stimulation time in hours.

Since VMAT and MAO were strongly upregulated by TNF at the mRNA level, we also confirmed these findings at the protein level by WB (Fig. 3). However, a significant increase was found only for MAO-B and VMAT2 (Fig. 3 b, d) but not for MAO-A and VMAT1 expression (Fig. 3 a, c), confirming the results obtained from the mRNA expression analysis. Remarkably, WB of MAO-B revealed the appearance of a second band with a higher molecular weight, which might be a TNF-induced specific isoform of MAO-B or a posttranslational modification (Fig. 3 b).

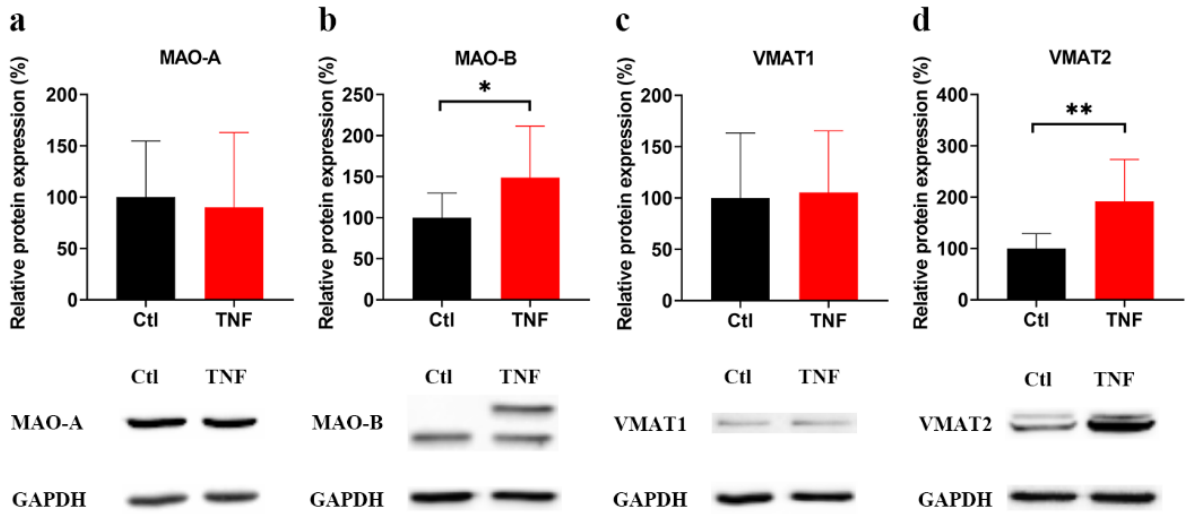


Fig.3 Relative protein expression of MAO-A, MAO-B, VMAT1 and VMAT2 after TNF stimulation.

Synovial fibroblasts were treated with or without TNF [10ng/mL] for 72h. Protein levels of MAO-A **(a)**, MAO-B **(b)**, VMAT1 **(c)** and VMAT2 **(d)** were determined by WB. (n=5) * $p<0.05$, for comparisons between TNF-treated and untreated cells by paired t test. Ctl (black), control group without TNF stimulation; TNF (red), TNF stimulated.

3.3 The catecholamine machinery in RASFs is functional

Since all necessary enzymes for catecholamine synthesis were found in RASFs, we confirmed whether RASFs could actually produce catecholamines. First, the supernatant was collected (50µL/well) after 2, 6, 12 and 24 hours of culture with medium and catecholamine levels were determined by ELISA. As shown in Fig. 4, extracellular DA, NE and EPI were increased time-dependently. The DA level at baseline (2 hours) was approximately 49pg/mL [0.33nM], with an increase to 110pg/mL [0.72nM] after 24 hours (+60.54±13.17pg/mL, p=0.0002, Fig. 4 a). Similar results were obtained for NE, whose extracellular concentration also increased from 246pg/mL [1.46nM] at 2 hours to 417pg/mL [2.47nM] after 24 hours (+171.2±61.88pg/mL, p=0.0077, Fig. 4 b). EPI levels (Fig. 4 c) remained relatively stable when compared to DA and NE levels. The concentration of extracellular EPI was moderately increased from 32pg/mL [0.17nM] at 2 hours to 44pg/mL [0.24nM] after 24 hours (+11.93±6.53pg/mL; p=0.0002).

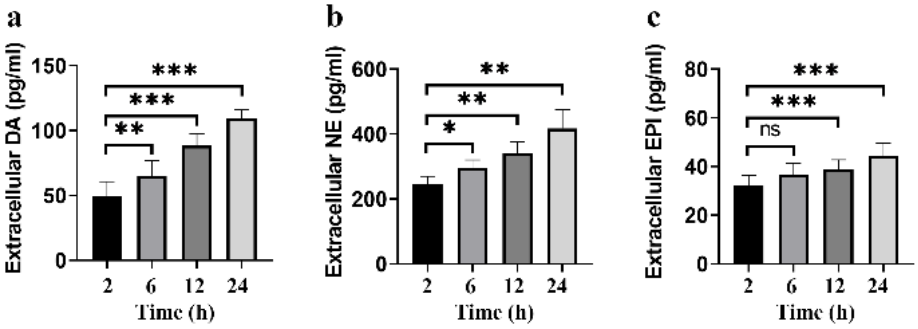


Fig.4 Catecholamines in RASF supernatants

Time-dependent synthesis of DA **(a)**, NE **(b)** and EPI **(c)** by RASF. Extracellular levels of catecholamines after 2-, 6-, 12- and 24-hour incubation with complete medium are shown. (n=4-5) * p<0.05, ** p<0.01, *** p<0.001, for comparisons between the 2-hour incubation group and the other three incubation time groups by one-way ANOVA with Tukey's post-hoc test.

In addition, BZD (50μM), a DDC inhibitor that blocks the conversion from L-DOPA to DA, was applied to confirm the synthesis of catecholamines. (Fig. 5) There was a significant decline in extracellular DA (p=0.007) and NE (p=0.038) levels (Fig. 5 a, b) but not EPI (Fig. 5c) when BZD was applied. (Fig. 5 c). The intracellular catecholamine content was not altered after BZD treatment (Fig. 5 d-f).

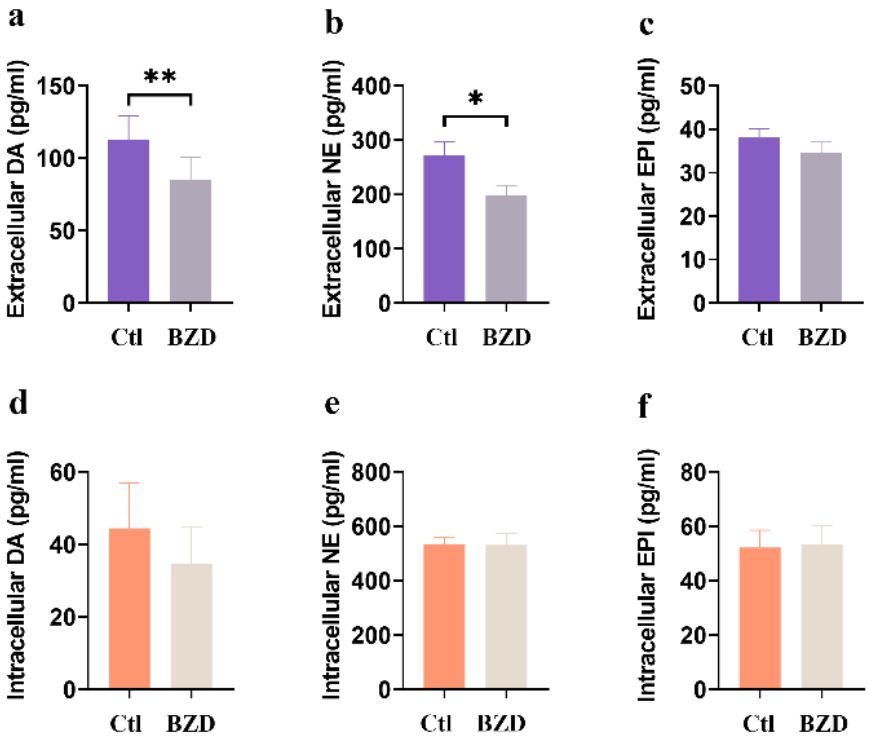


Fig.5 Effects of DDC inhibition on catecholamine production by RASFs.

Extracellular **(a-c)** and intracellular **(d-f)** catecholamine levels after 72 hours of inhibition of DDC with BZD (50μM). (n=4-5) * p<0.05, ** p<0.01, for comparisons between inhibitor treated and untreated groups by the Mann-Whitney U test.

To confirm the function of other proteins in the catecholaminergic pathway in RASFs, we investigated alterations in catecholamine levels after inhibition of degrading enzymes (MAO and COMT), catecholamine transport (DAT and NET) and storage (VMAT1 and VMAT2).

When MAO and COMT were inhibited by the combination of M30 (MAO inhibitor, 10μM) and OR486 (COMT inhibitor, 10μM), extracellular NE (p=0.028) and EPI (p=0.028) levels were augmented (Fig. 6 b, c), while neither intracellular DA, NE and EPI nor extracellular DA levels were affected (Fig. 6 a, d, e, f).

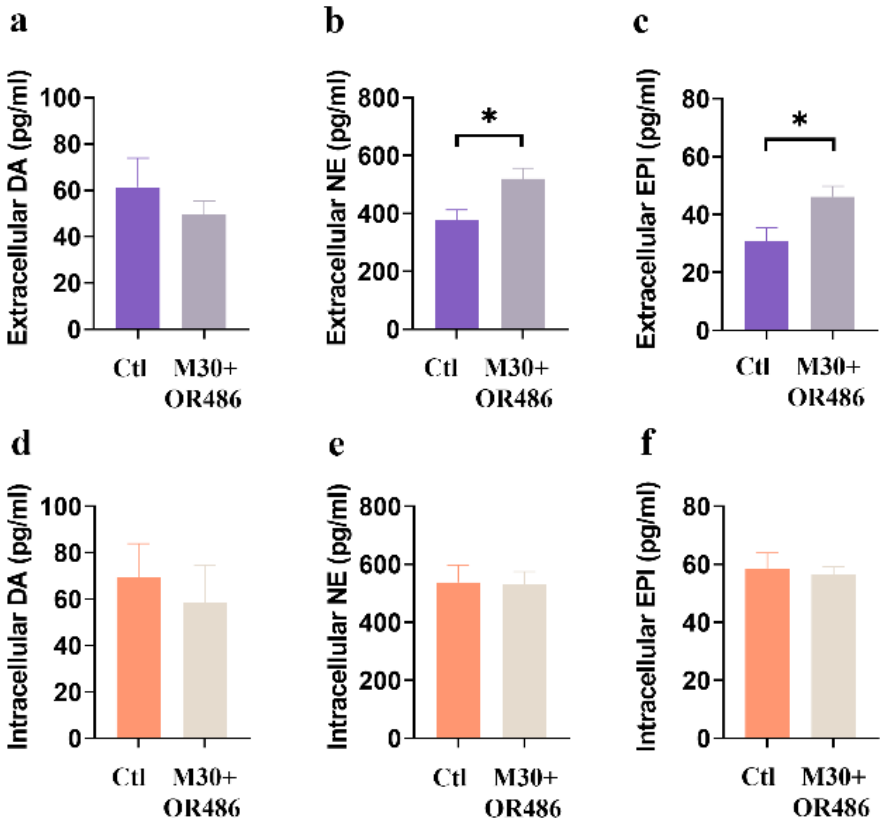


Fig.6 Effects of MAO and COMT inhibition on catecholamine production by RASFs.

Extracellular **(a-c)** and intracellular **(d-f)** catecholamine levels after 24 hours of inhibition of MAO and COMT with M30 (MAO inhibitor, 10μM) and OR486 (COMT inhibitor, 10μM). (n=4-5) * p<0.05, for comparisons between inhibitor treated and untreated groups by the Mann-Whitney U test.

In addition, when the VMAT inhibitor and releaser RSP (10μM) was applied to block catecholamine storage, extracellular DA (p=0.010) and intracellular DA (p=0.032) concentrations were elevated (Fig. 7 a, d). However, no obvious difference in extracellular or intracellular NE and EPI was observed after RSP treatment (Fig. 7 b, c, e, f).

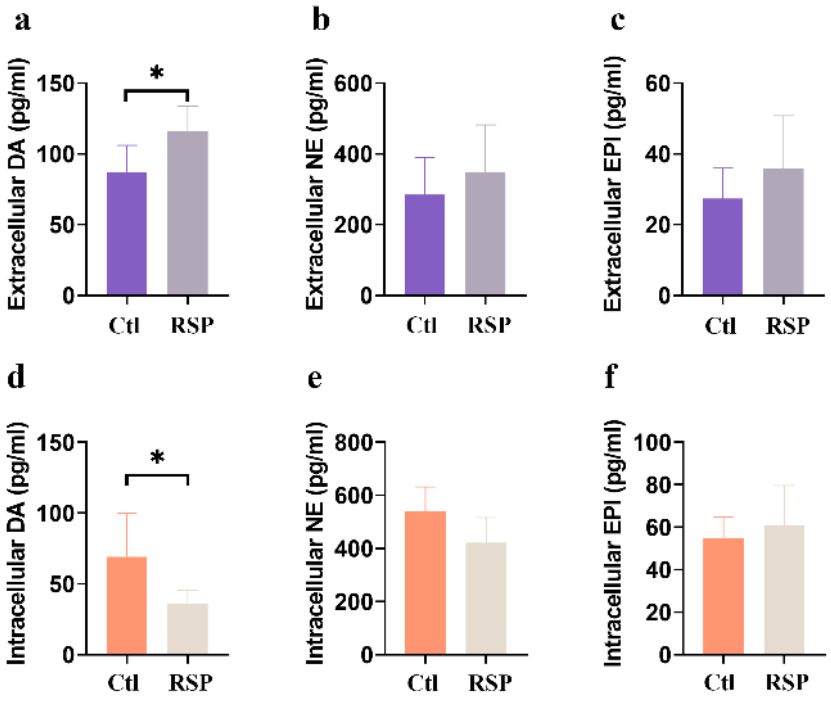


Fig.7 Effects of VMAT inhibition on catecholamine production by RASFs.

Extracellular **(a-c)** and intracellular **(d-f)** catecholamine levels after 24 hours of inhibition of VMAT with RSP (10μM). (n=4-5) * p<0.05, for comparisons between inhibitor treated and untreated groups by the Mann-Whitney U test.

When targeting uptake/reuptake of catecholamines with an inhibitor of DAT and NET (IDA, 10μM), a significant elevation of extracellular levels of NE (p=0.0317, Fig. 8 b) was found, while extracellular levels of DA and EPI only tended to increase. No difference was found in the intracellular levels of DA, NE and EPI (Fig. 8 d-f).

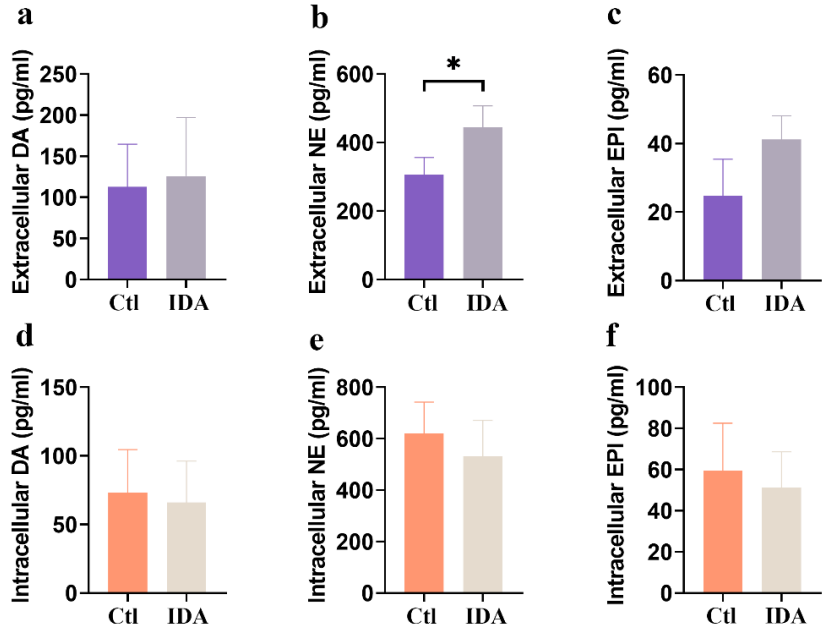


Fig.8 Effects of DAT and NET inhibition on catecholamine production by RASFs.

Extracellular **(a-c)** and intracellular **(d-f)** catecholamine levels after 24 hours of inhibition of DAT and NET with IDA (inhibitor of both DAT and NET, 10μM). (n=4-5) * p<0.05, for comparisons between inhibitor treated and untreated groups by the Mann-Whitney U test.

3.4 TNF modulates catecholamine synthesis and IL-6 production

Since RA is characterized by an inflammatory environment, we also investigated whether TNF has an impact on the synthesis of catecholamines and the production of IL-6, a major cytokine produced by RASFs. Intracellular and extracellular catecholamine production was evaluated after 24 hours of stimulation with TNF [10ng/mL]. As demonstrated in Fig. 9, no obvious difference was found in intracellular catecholamine levels (Fig. 9 a-c). However, extracellular DA (+18%), NE (+55%) and EPI (+208%) were significantly elevated by TNF treatment (p<0.05, Fig. 9 d-f). This increase in catecholamines was accompanied by elevated production of IL-6 (+890%, p=0.0022, Fig. 9 g).

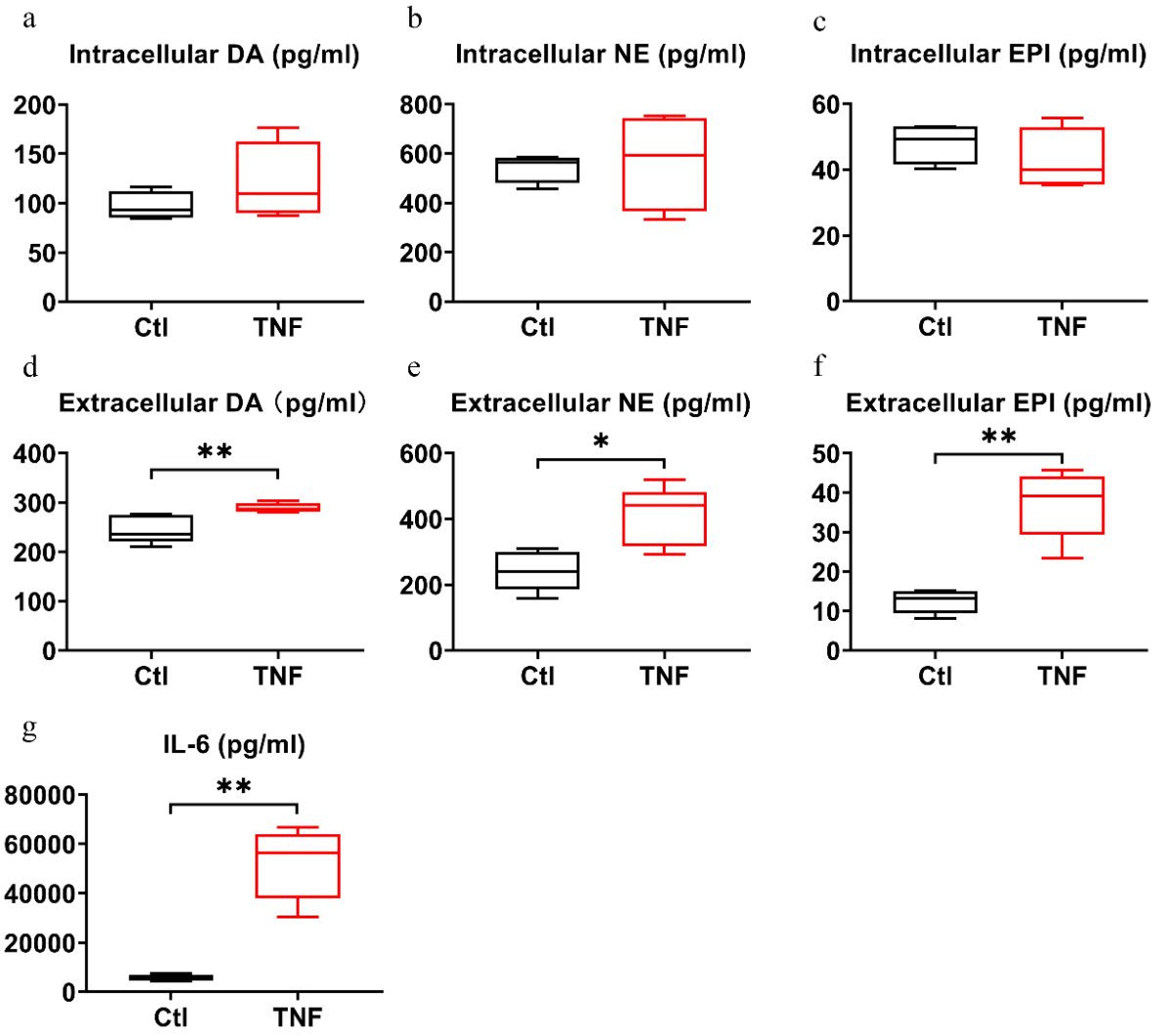


Fig.9 Modulation of intra- and extracellular catecholamine levels and IL-6 production by TNF.

Intracellular **(a-c)** and extracellular **(d-f)** levels of dopamine (DA) **(a, d)**, norepinephrine (NE) **(b, e)** and epinephrine (EPI) **(c, f)** levels under basal conditions and after stimulation with TNF [10ng/mL] for

24h. (n=4-5) IL-6 production in 24h after stimulation with TNF vs unstimulated controls (n=3). * p<0.05, ** p<0.01, for comparisons of catecholamine levels versus control by unpaired t-test in a-f, for the comparison of IL-6 production in TNF-treated versus control RASF, the Mann-Whitney U test was employed. Ctl (black), control group; TNF (red).

3.5 Modulation of IL-6 production and cell viability by exogenous DA and NE in RASFs

Since the presence of a complete and functional catecholaminergic pathway in RASFs along with catecholamine synthesis was confirmed, the impact of catecholamines in regulating cell viability and production of IL-6 was investigated. When RASF was stimulated with DA for 24 hours, there was a slight decrease in IL-6 production at 100µM DA (Fig. 10 a, upper panel). However, cell viability was reduced already at concentrations above 10^{-7}M (100nM) (Fig. 10 a, lower panel). In contrast, NE promoted IL-6 production at concentrations above 10^{-6}M (1µM) reaching a maximum at 10^{-4}M (100µM) (+40%, p<0.0001, Fig. 10 b, upper panel). Moreover, this pro-inflammatory influence was accompanied by a dose-dependent reduction of cell viability. The reduction in cell viability started at 10^{-6}M (1µM) with a maximal decrease of 24% at 10^{-4}M (100µM) (p<0.0001, Fig. 10 b, lower panel).

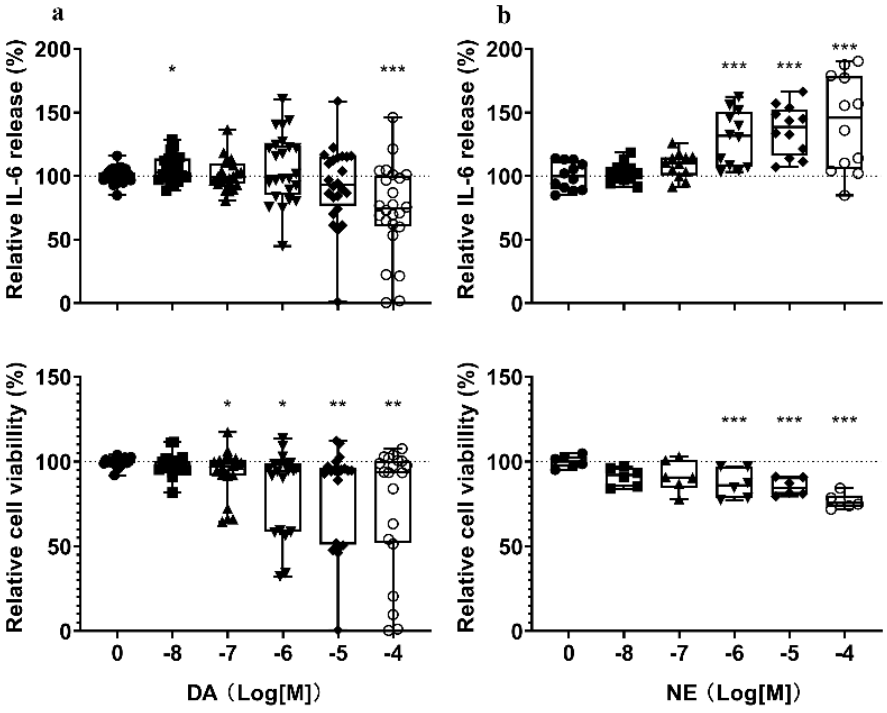


Fig.10 Dopamine and norepinephrine modulate IL-6 production and cell viability of RASFs.

IL-6 production **(a, upper panel)** and cell viability **(a, lower panel)** after stimulation with dopamine (DA, 10nM-100µM) for 24 hours (n=6). IL-6 production **(b, upper panel)** and cell viability **(b, lower panel)** after stimulation with norepinephrine (NE, 10nM-100µM) for 24 hours. (n=3). * p<0.05, ** p<0.01, *** p<0.001, for comparisons with the control group (without DA or NE) by one-way ANOVA with Tukey's post-hoc test.

3.6 MAO-A, but not MAO-B inhibition enhances extracellular catecholamine levels

Both, MAO-A and MAO-B are recognized as major enzymes that degrade catecholamines, and therefore, selective inhibitors were used to pinpoint the isoform involved in catecholamine degradation by RASFs. After 24 hours of stimulation, CLG (selective MAO-A inhibitor) significantly increased extracellular DA (p=0.0449) and NE (p=0.0335), but not EPI (Fig. 11 a-c), while no obvious modulation was found in intracellular catecholamine levels (Fig. 11 d-f, p>0.05). When using the selective MAO-B inhibitor LB, the intra- and extracellular catecholamine levels remained unchanged (Fig. 12, p>0.05).

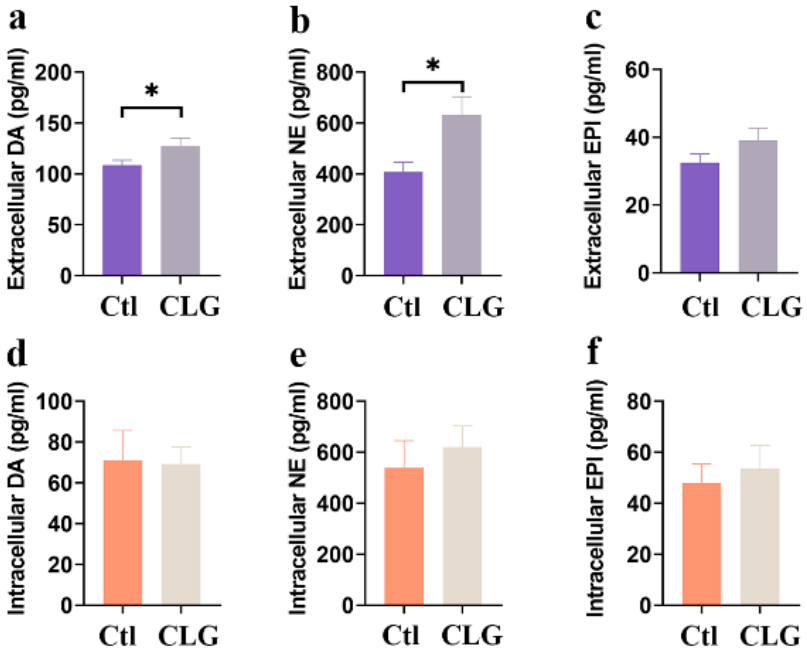


Fig.11 Extracellular and intracellular catecholamine levels after selective MAO-A inhibition.

Extracellular **(a-c)** and intracellular **(d-f)** catecholamine levels after 24-hour inhibition of MAO-A with CLG (1μM). (n=4-6) * p<0.05, for comparisons between inhibitor treated and untreated groups by the Mann-Whitney U test.

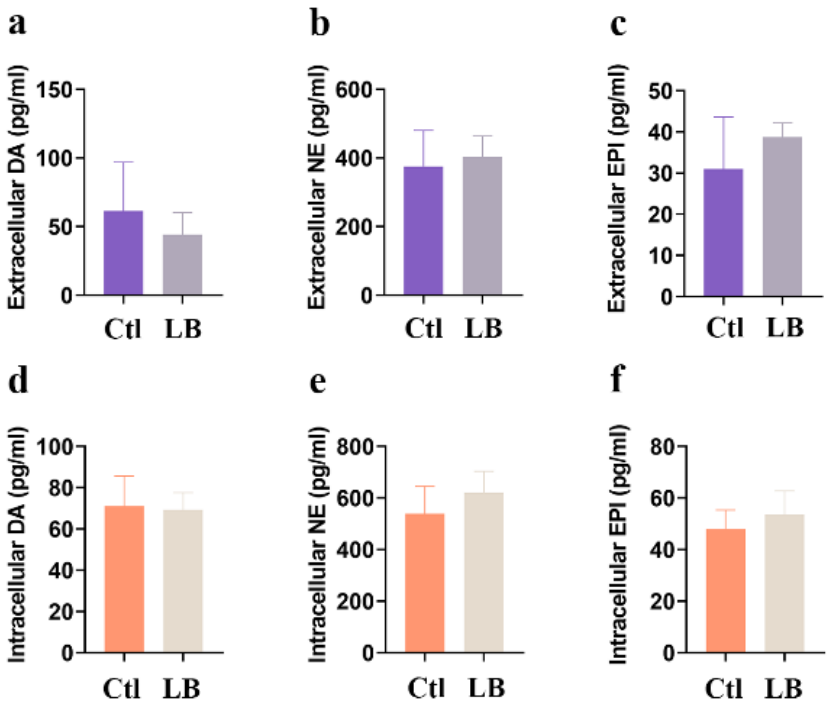


Fig.12 Extracellular and intracellular catecholamine levels after selective MAO-B inhibition.

Extracellular **(a-c)** and intracellular **(d-f)** catecholamine levels after 24 hours of inhibition of MAO-B with LB (10µM). (n=4-6) * $p<0.05$, for comparisons between inhibitor treated and untreated groups by the Mann-Whitney U test.

3.7 Selective MAO-A and MAO-B inhibition have opposite effects on IL-6 production and cell viability under basal and TNF-induced conditions in RASFs

Since MAO-B was highly upregulated by TNF at the mRNA and protein levels, we investigated whether inhibition of this enzyme with a selective inhibitor (LB) has any effect on cell viability and IL-6 production in RASFs. As a control, we applied a selective MAO-A inhibitor (CLG) for comparison. In cells without TNF pre-treatment, IL-6 levels were elevated by low concentrations of CLG (from 10^{-8} to 10^{-6}M; $p<0.05$) with a maximum at 1µM (+19%), while production was diminished at 10^{-4}M CLG (-38%; $p<0.0001$, Fig. 13 a). In contrast, no significant impact on IL-6 was detectable after CLG treatment in RASFs stimulated with TNF (Fig. 13 a). Meanwhile, the cell viability of RASFs was dose-dependently reduced by MAO-A inhibition regardless of TNF pre-treatment ($p<0.05$, Fig. 13 b).

Interestingly, MAO-B inhibition by LB resulted in the opposite: IL-6 production was significantly suppressed by MAO-B inhibition in TNF-pre-treated RASFs at concentrations above 10^{-6}M (1µM). The maximum suppression of IL-6 production was observed at 10^{-4}M (100µM) (-19%, $p<0.0001$, Fig. 13 c). When cells were not pre-treated with TNF, LB elevated IL-6 production at 10^{-4}M (100µM) (+31%, $p=0.0038$, Fig. 13 c). Moreover, cell viability was slightly dampened by 10^{-4}M (100µM) LB in the control group ($p<0.01$, Fig. 13 d).

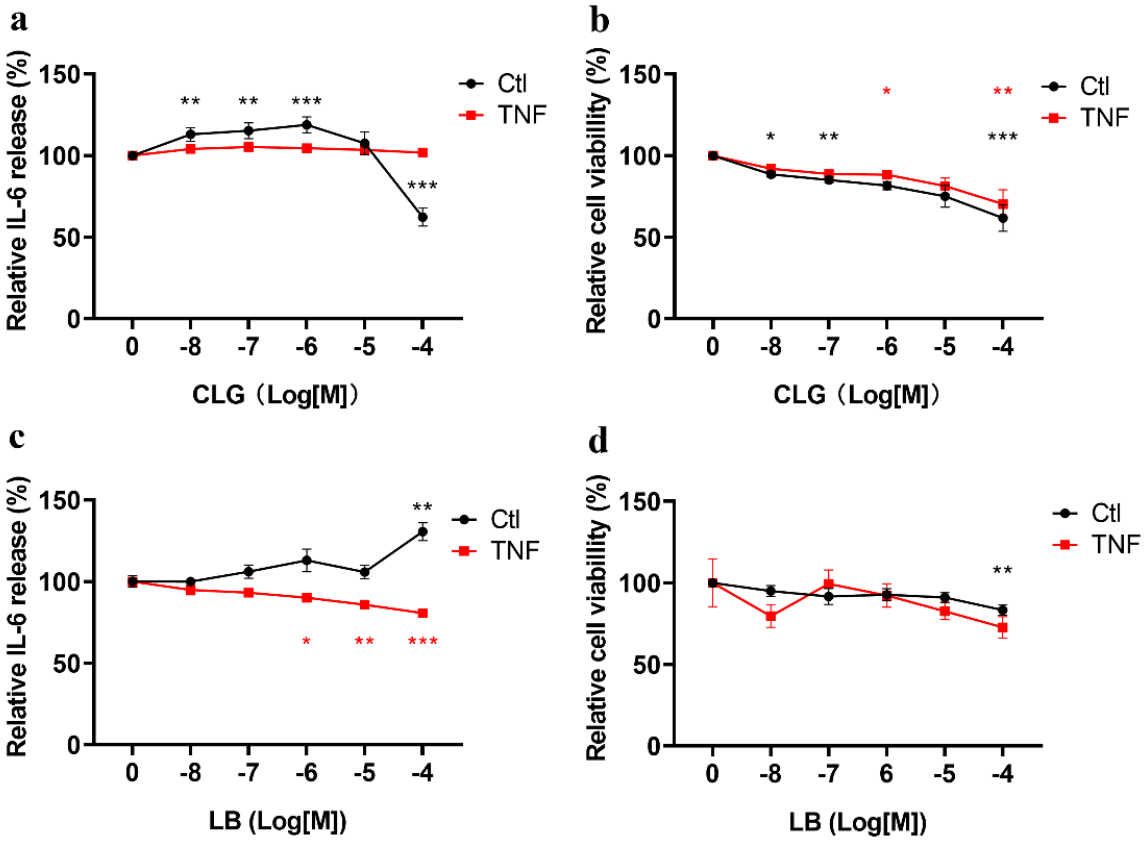


Fig.13 Modulation of IL-6 production and cell viability by selective MAO inhibition.

IL-6 production and cell viability of RASFs treated with different concentrations of the MAO-A inhibitor CLG (n=6) **(a, b)** or the MAO-B inhibitor LB (n=5) **(c, d)**. RASFs were pre-treated with TNF for 72 hours (red) or left untreated (black). MAO-A and MAO-B inhibitors were applied thereafter for 24 hours. * p<0.05, ** p<0.01, *** p<0.001, compared with the control group without MAO inhibitors by one-way ANOVA with Tukey's multiple comparison test.

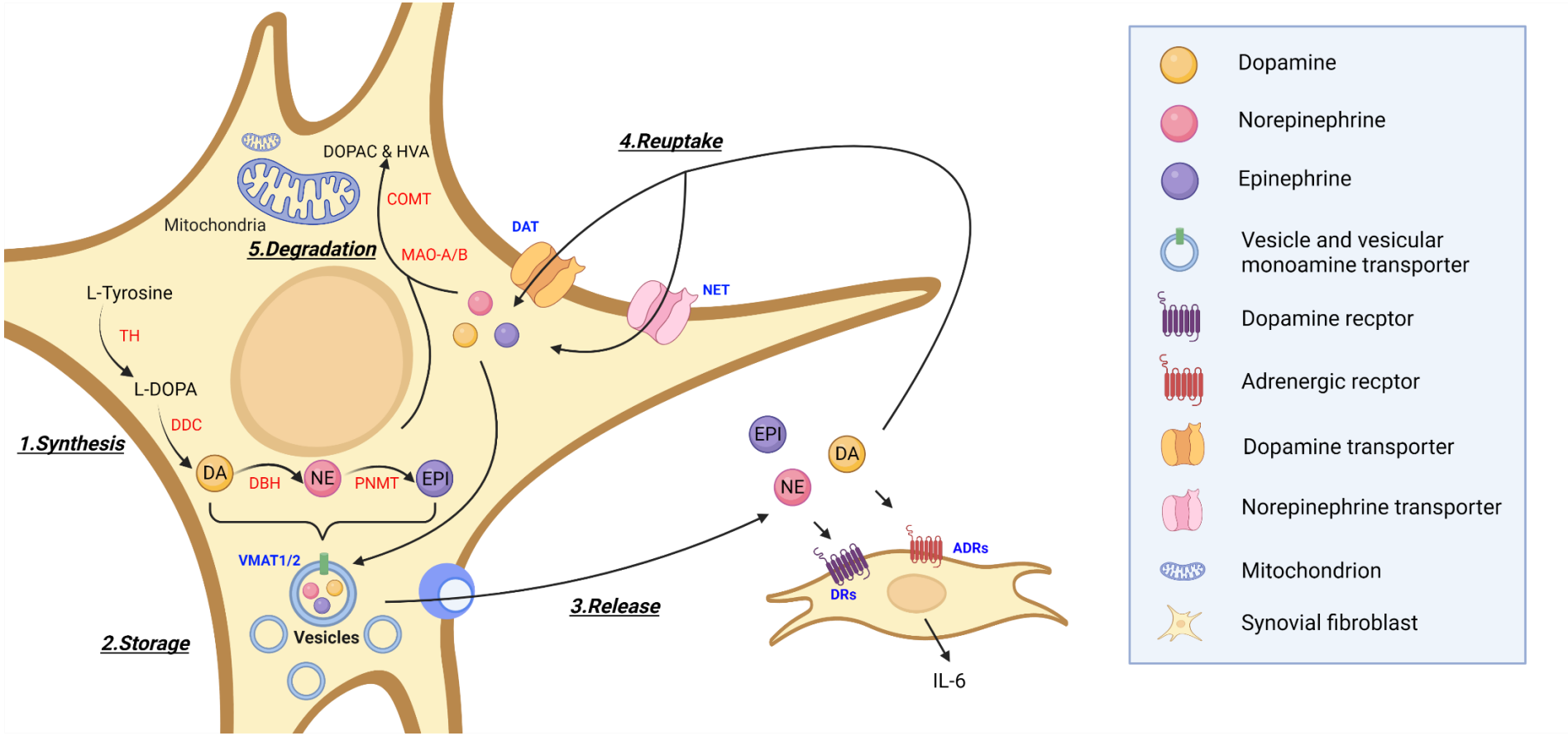

DOPAC & HVA
COMT
Mitochondria
5.Degradation
MAO-A/B
DAT
4.Reuptake
NET
L-Tyrosine
TH
L-DOPA
DDC
1.Synthesis
DA
DBH
NE
PNMT
EPI
VMAT1/2
Vesicles
2.Storage
3.Release
EPI
DA
NE
ADRs
DRs
IL-6
Dopamine
Norepinephrine
Epinephrine
Vesicle and vesicular monoamine transporter
Dopamine recptor
Adrenergic recptor
Dopamine transporter
Norepinephrine transporter
Mitochondrion
Synovial fibroblast

Fig. 14 Schematic representation of the catecholaminergic pathway in RASFs.

1. Synthesis: RASFs are able to synthesize DA, NE and EPI. This requires the participation of the enzymes TH, DDC, DBH and PNMT. 2. Storage: Vesicles are able to store catecholamines by transport through VMAT1 and VMAT2. 3. Release: Catecholamines can be released into the extracellular space by exocytosis and invoke downstream reactions via DRs and ADRs in an autocrine and paracrine fashion. Released catecholamines are able to ligate receptors (ADRs and DRs) and foster or inhibit the secretion of IL-6. 4. Reuptake: Exogenous catecholamines are taken up through DATs or NETs and repacked into vesicles or are degraded. 5. Degradation: MAO-A, MAO-B and COMT catalyze the degradation of excess catecholamines and thereby produce the metabolites DOPAC and HVA. (DA, dopamine; NE, norepinephrine; EPI, epinephrine; TH, tyrosine hydroxylase; DDC, DOPA decarboxylase; DBH, dopamine-beta-hydroxylase; PNMT, phenylethanolamine N-methyltransferase; VMAT1/2, vesicular monoamine transporter 1/2; DRs, dopamine receptors; ADRs, adrenergic receptors; DAT, dopamine transporter; NET, norepinephrine transporter; MAO-A/B, monoamine oxidase-A/B; COMT, catechol-O-methyltransferase; DOPAC, dihydroxy-phenyl acetic acid; HVA, homovanillic acid; IL-6, interleukin-6.) Illustrations were made using Biorender© (Biorender.com).

4. DISCUSSION

To the best of our knowledge, our study presented the first comprehensive description of the catecholaminergic machinery in RASF, although there are several previous studies highlighting some aspects of the catecholaminergic system in RASF [119, 127]. In this study, the whole pathway from the synthesis, reuptake and degradation of catecholamines was investigated. Furthermore, the regulation of key proteins of the catecholaminergic system by TNF, mimicking the inflammatory situation in the joint, was validated. Figure 14 depicts a graphic summary of the catecholaminergic pathway in RASF. In addition, evidence is provided that MAO-B, which is regulated by TNF, influences IL-6 production and might therefore be employed as a potential therapeutic target.

It has been noted over the past decades that there is extensive crosstalk between the SNS and the immune system. [91] Early studies already speculated that sympathectomy limits RA pathology. [131] which was substantiated in experimental arthritis models, where β2-AR signaling ameliorated the disease. [111, 132] Additionally, DRs are also involved in the regulation of RA, as inhibition of D1-like or activation of D2-like DRs mitigates collagen-induced arthritis in mice. [120, 133] Furthermore, the loss of SNS fibres in synovial tissue during the course of RA is accompanied by the appearance of anti-inflammatory TH positive cells which are anti-inflammatory due to catecholamine synthesis. [109, 119] Similarly, when treating mixed synovial cells with the VMAT inhibitor reserpine, causing a rapid release of stored catecholamines, a strong inhibition of TNF production was observed. [119] However, although some target proteins of the catecholaminergic pathway able to modulate inflammation have been identified, it was unclear until now whether RASFs actively synthesize, store, take up or degrade catecholamines.

4.1 Catecholamine synthesis and its modulation under inflammatory conditions in RASF

All necessary enzymes (TH, DDC, DBH, PNMT) to synthesize catecholamines were detected in RASF. Accordingly, the contents of DA, NE and EPI both intra- and extracellularly were quantified by ELISA. To elucidate the function of the catecholaminergic pathway, several enzyme inhibitors were used in this study. These inhibitors have already been described to influence catecholamine levels. For instance, the production of extracellular DA level was reduced when a DDC inhibitor was applied in the striatum of rats. [134] This was confirmed in this study, as cells treated with the DDC inhibitor BZD showed a decrease in intracellular and extracellular catecholamine levels. Remarkably, the increase in extracellular catecholamines over time in culture was also recorded, revealing an on-going synthesis of catecholamines in RASFs. Although no regulation of TH, DDC, DBH, PNMT, VMAT1, MAO-A and COMT was detected under TNF stimulation, we recorded augmented extracellular catecholamine levels in

response to TNF. Previous studies indicated that TH expression and catecholamine synthesis were dampened by TNF in neuronal tissue. [135] However, our observed outcomes are in line with the findings from Miller et. al. who assumed that higher TH immunoreactivity and NE production in synovial tissue were ascribed to increased inflammation. [136] Thus, enhanced catecholamine synthesis may result from the activation or production of cofactors associated with TH activity, such as tetrahydrobiopterin as reported in glioma cells. [137, 138]

Catecholamines play a crucial role in the negative feedback modulation of inflammation in postganglionic sympathetic neurons. [139] Catecholamines can activate β-ARs which leads to anti-inflammatory effects in macrophages by enhancing IL-10 production and suppressing TNF secretion. [140-143] However, catecholamines, dependent on concentration, can also have α-AR-mediated pro-inflammatory effects in certain immune cells. [89, 144, 145] Inflammatory regulation by catecholamines in RA patients remains to be thoroughly clarified. Up to now, there is only limited evidence on the modulation of the catecholaminergic pathway in RA synovial cells by inflammatory mediators. One study showed that RSP treatment of mixed synovial cells decreased TNF secretion. [119] and activation of DRs in RASFs entailed a suppression of IL-6 and IL-8 secretion [127] In addition, it was demonstrated that there is a switch in coupling from Gαs to Gαi at G protein-coupled receptors (GPCRs), in the RA synovium. [122] Hence, inflammation modulates GPCR function in RA, dependent on disease duration and NE modulates immune function in a context-dependent manner. [91] Thus, receptor activity, affinity and G-protein coupling should be taken into account when assessing the effects of catecholamines in arthritis. [146] Although we found an increase in catecholamines after TNF stimulation, it is still unclear whether the increased levels of catecholamines exert any effect on inflammatory cytokine release. However, the impact of exogenous DA and NE on RASF cell viability and IL-6 production was assessed in this study. The results presented here imply that low-dose DA reduces IL-6 secretion and suppresses cell viability, whereas high concentrations of DA showed the opposite effects. Besides, we found that NE upregulated IL-6 levels while concomitantly dampening cell viability. In a previous study, intermediate levels of DA reduced IL-6 and IL-8 production by RASF, however, under different culture conditions and incubation times. [127] Our results are in line with the upregulation of IL-6 by NE in a β-AR dependent fashion. [147] The important role of β-ARs in RA pathology has already been described, and the results showed that beta-adrenergic agonists but not NE decreased cell viability in RASFs derived from rats with adjuvant-induced arthritis. [148, 149] Catecholamines reduce cell viability and it has been demonstrated previously that free intracellular DA promotes the generation of reactive oxygen species (ROS) and subsequent cell death in neuronal cells. [150] The spontaneous oxidation of DA accounts for DA neuron damage via the production of o-quinones and the polymerized neuromelanin. [151]

4.2 Catecholamine storage and its modulation under inflammatory conditions in RASF

Besides synthesizing enzymes, RASFs also express VMAT1 and VMAT2 which are responsible for packaging free catecholamine into vesicles. This sequestration is important to prevent their oxidation which would disrupt cellular function. [152] A significant increase in extracellular catecholamines was found after 24 hours of inhibition of VMAT with reserpine, but this might be in part dependent on negative regulation of DAT and NET function. [153, 154] Under TNF stimulation, overexpression of VMAT2 but not in VMAT1 was observed, which might indicate that the vesicle composition is altered when RASFs are stimulated with TNF. VMAT2 was reported to have a 3-fold higher affinity towards monoamines than VMAT1. [155] Thus, the overexpression of VMAT2 might be caused by increased extracellular catecholamines induced by TNF, as it was demonstrated that high catecholamine levels during stress or drug intake can elevate VMAT2 expression. [156, 157] Interestingly, two bands of VMAT2 were identified, which have slightly different molecular weights in WB images. This indicates that TNF might not only regulate overall levels but also VMAT2 activity, since glycosylation, phosphorylation and nitrosylation play crucial roles in VMAT2 localization and function. [158]

4.3 Catecholamine uptake and its modulation under inflammatory conditions in RASF

DAT and NET account for the reuptake of released catecholamines, and thereby prevent excessive DR or AR stimulation in postsynaptic neurons. Therefore, blocking DAT results in large amounts of extracellular free DA that are associated with increased susceptibility to addictive behavior. [159] As a membrane-spanning protein, DAT through internalization, is responsible for the modulation of DA levels. [160] In this study, DAT and NET expression of RASF was confirmed and inhibiting both transporters elevated extracellular catecholamine levels while leading to intracellular catecholamine depletion. This is in line with results obtained with nomifensine, another inhibitor of DAT and NET, which has similar effects in healthy volunteers. [161]

4.4 Catecholamine degradation and its modulation under inflammatory conditions in RASF

Besides synthesis, storage and uptake, catecholamine degradation is also a pivotal process in the catecholaminergic pathway. MAOs and COMT are responsible for the degradation of released neurotransmitters by catalyzing the oxidative deamination. MAO-A and MAO-B are two subtypes of MAOs, which have over 70% homogeneity. MAO-A is regarded as having higher affinity for hydroxylated amines (NE and serotonin), while MAO-B presents greater affinity for non-hydroxylated amines. The affinity of DA and NE for MAO-A

and MAO-B seems similar. [162] The distribution of these two degradation enzymes differs between cell types. [163, 164] It is shown here that RASFs express both MAO isoforms along with COMT and the combined inhibition of MAOs and COMT enhances extracellular catecholamine levels. This confirms the *in vivo* and *in vitro* effects of the COMT inhibitor OR-486 or non-selective MAO inhibitors from previous studies. [165, 166] Analogous to VMAT2, we found a strong upregulation of MAO-B by TNF at both the mRNA and protein levels. As a second band specific for MAO-B appeared in WB after TNF stimulation, it is likely that MAO-B is post-translationally modified which, similar to VMAT2, might enhance its function. There is no evidence in previous studies regarding these modifications in RASF, but results in yeast-produced MAO-B showed acetylation [167] and demonstrated that ubiquitination elicits a strong effect on MAO-B insertion into mitochondrial membranes [168]. However, the specific function and identification of this modification in RASF was not clarified in our study. Further experiments are necessary for a more detailed assessment of MAO-B function on a molecular level.

In previous studies, the regulation effect of MAO inhibitors on inflammatory cytokine expression in several disease models was investigated. Tomaz [169] found that nonselective MAO inhibition suppresses the expression of IL-1β, IL-6, TNF and IFN-γ in the brains of rats in a lipopolysaccharide (LPS) -induced depression model. In a rat model of Parkinson's disease, mRNA expression of TNF in brain homogenates was downregulated by MAO inhibition. [170] Moreover, in the epithelium of periodontal tissues, TNF and hydrogen peroxide levels were enhanced while the treatment with phenelzine, a nonselective and irreversible MAO inhibitor, can prevent the elevation of TNF and hydrogen peroxide and dampen cell proliferation and migration. [171] Therefore, MAO inhibition was corroborated to diminish pro-inflammatory cytokine and chemokine expression in numerous diseases. [172] Recently, a study in rats with collagen type II-induced arthritis assessed the effect of the MAO inhibitor selegiline on the regulation of inflammation. The results showed that MAO inhibition protects against the progression of experimental arthritis via suppressing pro-inflammatory cytokines (IL-6 and TNF) with concomitant reductions in clinical features (paw thickness, arthritic scores and radiographic joint damage) of arthritis. [173]

4.5 MAO inhibition and its modulation under inflammatory conditions in RASF

The potential therapeutic value and translational implications of MAO inhibitors in a variety of diseases have been extensively discussed over the past decades. [174-179] Therefore, we further delineated the impact of MAO-A and MAO-B inhibition on IL-6 production and cell viability of RASFs under basal or TNF-stimulated conditions. It was shown in our study that IL-6 production by RASF was not influenced by MAO-A when RASF was stimulated with TNF,

but was inhibited without TNF pre-treatment. In addition, MAO-A inhibition reduced the cell viability of RASFs regardless of TNF stimulation. Although no studies regarding the effect of MAO-A inhibition in RASFs have been published, studies in macrophages have been reported that MAO-A degrades monoamines and by doing so, upregulates the production of ROS which facilitates anti-inflammatory M2 polarization and this effect was reversed by MAO-A inhibition. [180] Consistent with this, the increase in IL-6 induced by MAO-A inhibition and suppression of cell viability presented in our study might result from the decrease in ROS at high concentrations of MAO-A inhibitor. ROS limit the cell viability of RASFs at high concentrations, but low to intermediate concentrations of ROS actually promote cell survival and proliferation. [181, 182]

MAO-B inhibition reduced IL-6 production after TNF pre-treatment, but increased it in TNF-naïve RASFs. We observed a slight decline in cell viability only at high concentrations of the MAO-B inhibitor in the TNF-naïve group. *In vivo*, MAO-B was considered to account for γ-aminobutyric acid (GABA) synthesis and ROS generation [183-185], while MAO-A might be predominantly responsible for catecholamine degradation [183, 186]. Moreover, we observed an elevation of catecholamines only with MAO-A, not MAO-B inhibition, which is in line with the anti-inflammatory influence of MAO-B inhibition on the levels of inflammatory cytokines and experimental arthritis in mice. [185] However, GABA mainly has an anti-inflammatory impact in the periphery [187], thus the reduction of ROS seems to be the major cause for the effects seen with MAO-B inhibition. Besides, the synthesis of ROS can be provoked by TNF, which leads to pro-inflammatory results through the activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB). [188, 189] Therefore, suppression of ROS fostered by MAO-B inhibition might inhibit the NFκB signaling activity and disrupt the functions of other pro-inflammatory ROS-dependent pathways such as Jun activated kinase (JNK) [190] or mitogen-activated kinase [191].

5. CONCLUSIONS

All components necessary for catecholamine production, storage, reuptake and degradation were identified in our study, which is the first comprehensive description of the catecholaminergic system in human RASF. As cells from a mesenchymal origin, RASFs have a functional catecholaminergic pathway which resembles pre-synaptic sympathetic neuron-like cells. We speculate that RASF might play a similar role as sympathetic neuron-associated macrophages which take up excess catecholamines released from sympathetic nerves and store them for further secretion or participate in their degradation [192]. Nevertheless, when there is a local deficit of catecholamines, for example during sympathetic nerve fibre repulsion caused by arthritis, RASFs might be involved in *de novo* catecholamine synthesis similar to TH-positive lymphocytes and macrophages in the joint.

The promising therapeutic value of MAO inhibition in RA treatment is elucidated in our study. As previous reports have already shown, MAO inhibition targets excess inflammation and provides an improvement in RA pathology. [193] Besides, MAO-B inhibition alone strongly suppresses inflammation in experimental arthritis. [185] Furthermore, MAO-A also participates in catecholamine degradation and oxidative stress, implying future studies of both isoforms (peripherally) as adjunct therapy for RA.

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