The dopaminergic pathway: A potential approach to target specific leukocyte subpopulations in chronic inflammatory joint diseases

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Karolin Stephanie Wieber

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1. Gutachterin: Prof. Dr. Silvia Capellino
2. Gutachterin: Prof. Dr. Wiebke Hansen

Vorsitzender des Prüfungsausschusses: Prof. Dr. Sven Brandau

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Table of contents

Abbreviations

µ:	micro
ACPA:	anti-citrullinated protein antibody
ACR:	American College of Rheumatology
AF:	Alexa Fluor®
ALDH:	aldehyde dehydrogenase
ANOVA:	analysis of variance
ASAS:	Assessment of Spondylo Arthritis International Society
ASDAS:	Ankylosing Spondylitis Disease Activity Score
ATF-1:	activating transcription factor 1, synonym for CREB
BASDAI:	Bath Ankylosing Spondylitis Disease Activity Index
BASFI:	Bath Ankylosing Spondylitis Functional Index
BSA:	bovine serum albumin
BV:	Brilliant Violet™
C:	Celsius
cAMP:	3'-5'-cyclic adenosine monophosphate
CCL:	C-C motif chemokine ligand
CD:	cluster of differentiation
CFDA-SE:	carboxyfluorescein diacetate succinimidyl ester
CIA:	collagen-induced arthritis
CO_2:	carbon dioxide
COMT:	catechol-o-methyltransferase
CREB:	cAMP response element-binding protein
CTLA-4:	cytotoxic T lymphocyte antigen-4
CXCL:	CXC ligand
CXCR:	chemokine receptor
Cy:	cyanine
DA:	dopamine
DAG:	diacylglycerol
DARPP-32:	32-kDa dopamine and cAMP-regulated phosphoprotein
DAS28:	Disease Activity Score 28
DAT:	dopamine transporter
DBH:	dopamine β-hydroxylase
DMARD:	disease-modifying anti-rheumatic drug
DMSO:	dimethyl sulfoxide
DOPAC:	3,4-dihydroxyphenylacetic acid
DR:	dopamine receptor
ELISA:	enzyme-linked immunosorbent assay
Epi:	epinephrine
ER:	endoplasmic reticulum
ERK:	extracellular-signal regulated kinase
EULAR:	European League Against Rheumatism
FACS:	fluorescence activated cell sorting
FBS:	fetal bovine serum
FFbH:	Funktionsfragebogen Hannover, measuring functional capacity of RA patients
FITC:	fluorescein isothiocyanate
FMO:	fluorescence minus one
FSC:	forward scatter
g:	gram or gravity

G protein:	guanine nucleotide binding regulatory protein
GC:	germinal center
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GPCR:	G protein coupled receptor
GRK:	G protein coupled receptor kinase
GTP:	guanosine triphosphate
h:	hour
HC:	healthy control(s)
HLA:	human leukocyte antigen
HVA:	homovanillic acid
ICOS(L):	inducible T-cell costimulator (ligand)
IfADo:	Leibniz Research Centre for Working Environment and Human Factors
IFN:	interferon
Ig:	immunoglobulin
IL:	interleukin
ILC:	innate lymphoid cells
Iono:	ionomycin
IP_3:	inositol triphosphate
L-DOPA:	levodopa
m:	milli or meter
M:	molar
MAO-B:	monoamine oxidase B
MFI:	mean fluorescence intensity
MHC:	major histocompatibility complex
min:	minute(s)
n:	nano-
NE:	norepinephrine
NK:	natural killer
ODN:	oligodeoxynucleotide
p:	pico-
PBMCs:	peripheral blood mononuclear cells
PE:	phycoerythrin
Pen/Strep:	penicillin-streptomycin
PerCP:	peridinin-chlorophyll-protein
PFA:	paraformaldehyde
PKA:	protein kinase A
PLC:	phospholipase C
PNMT:	phenyl ethanolamine N-methyltransferase
pNPP:	p-nitrophenyl phosphate
PMA:	phorbol-12-myristate-13-acetate
PsA:	psoriasis arthritis
RA:	rheumatoid arthritis
RANKL:	receptor activator of nuclear factor kappa B ligand
RGS:	regulators of G protein coupled receptors
RF:	rheumatoid factor
SD:	standard deviation
SEB:	staphylococcal enterotoxin B
SF:	synovial fibroblast
SpA:	ankylosing spondylitis

SSC:	side scatter
T_{FH}:	T follicular helper
T_{PH}:	T peripheral helper
TCR:	T cell receptor
TGF:	transforming growth factor
Th:	T helper
TH:	tyrosine hydroxylase
TLR:	Toll-like receptor
TMB:	tetramethyl benzidine
TNF:	tumor necrosis factor
Treg:	regulatory T cell
VMAT2:	vesicular monoamine transporter 2

List of figures

List of tables

Abstract

Chronic inflammatory diseases of the joints are complex diseases leading to pain, disability, and loss of life quality. Although being effective, available disease-modifying anti-rheumatic drugs also implicate severe side effects as systemic immunosuppression. Thus, specific treatments targeting only disease pathomechanisms involving fewer side effects would be advantageous. Several studies indicate a role of the catecholamine dopamine (DA) in immunity and limited evidence also points to DA involvement in rheumatoid arthritis (RA). However, data on DA in RA is mostly restricted to local joint inflammation in late phase of disease. Moreover, a possible influence of DA in psoriasis arthritis (PsA) and ankylosing spondylitis (SpA) has not been investigated so far. Aims of this thesis were the investigation of possible disease-related changes and immune-modulatory functions of the dopaminergic system in peripheral immune cells of patients with chronic inflammatory joint diseases that could ultimately serve as new targets for directed modulation of immune pathomechanisms.

Peripheral blood mononuclear cells (PBMCs) from healthy controls (HC), RA, PsA, and SpA patients were investigated for expression of D_1-D_5 dopamine receptors (DR) and tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, by comprehensive flow cytometric approaches. Additionally, specific DR were stimulated with selective agonists *in vitro* to assess effects on immune cell activation, apoptosis, proliferation, and differentiation as well as intracellular signaling. Cytometric bead arrays and ELISAs were conducted to measure effects of DR stimulation on cytokine secretion.

Among peripheral T cells, B cells, NK cells and monocytes of HC and rheumatic patients all five DR and TH were expressed. An elevated expression of D_1 DR was identified in peripheral B cells of RA patients compared to HC. Expression of D_1 DR in B cells differed between sexes in HC and particularly female RA patients revealed increased D_1 DR levels that additionally correlated with clinical parameters. Deeper phenotyping revealed increasing D_1 DR expression during B cell maturation and especially naive B cells of RA patients displayed increased levels of D_1 DR. Importantly, elevation of D_1 DR expression during maturation was also found in peripheral NK cells and $CD4^+$ T cells. Circulating T follicular helper cells of RA patients exhibited increased expression of D_1 DR compared to HC as well.

Functionally, acute DR stimulation showed no effects on activation and apoptosis of immune cells. In T cell-dependent B cell stimulation additional D_1-like receptor stimulation did not elicit effects on B cell proliferation or plasmablast differentiation. In T cell-independent B cell stimulation, differentiation towards antibody-secreting cells was unaltered by D_1-like receptor stimulation. Notably, D_1-like receptor agonist increased proliferation of D_1 DR expressing B cells independent of T cells, both in HC and RA. Moreover, D_1-like receptor stimulation up- or downregulated cytokine secretion donor-dependently. Significantly, only in cultures of RA PBMCs D_1-like receptor stimulation increased IL-8 secretion which is relevant for bone

destruction in RA. Ki-67 expression and phosphorylation of ERK and CREB as classical signaling molecules within the dopaminergic pathway were unaltered after D_1-like receptor stimulation. Nevertheless, increased expression and phosphorylation was detected in RA.

In conclusion, peripheral immune cells contain components of the dopaminergic pathway thus being able to communicate via DA. Increased levels of D_1 DR are present in peripheral naive B and T_{FH} cells of RA patients and may play pivotal roles in RA by enhancing development and function of pathogenic B cells. These findings are mostly in line with literature and highlight the blockade of D_1 DR on peripheral B cells as potential new therapeutic target in therapy of RA. Further studies are planned to clarify exact mechanisms.

Zusammenfassung

Chronisch entzündliche Gelenkerkrankungen sind komplex und führen bei Patienten zu Schmerzen, Einschränkungen und geringerer Lebensqualität. Die Behandlungsmöglichkeiten mit Antirheumatika sind zwar effektiv, jedoch einhergehend mit systemischer Immunsuppression. Gerichtete Therapien, die einzig die Pathomechanismen anzielen und dadurch weniger Nebenwirkungen hervorrufen, sind daher wünschenswert. Viele Studien zeigen, dass das Katecholamin Dopamin (DA) eine wesentliche Rolle im Immunsystem spielt und einige Befunde deuten auch auf eine Beteiligung von DA bei der rheumatoiden Arthritis (RA). Jedoch sind diese Daten zur Beteiligung von DA in RA auf die lokale Gelenkentzündung und späte Krankheitsstadien beschränkt. Ein möglicher Einfluss von DA in Psoriasis Arthritis (PsA) und Spondylitis Ankylosans (SpA) wurde darüber hinaus noch nicht untersucht. Ziele dieser Arbeit waren die Untersuchung von möglichen Krankheitsspezifischen Veränderungen und immunmodulatorischen Funktionen des dopaminergen Systems in peripheren Immunzellen von Patienten mit chronisch entzündlichen Gelenkerkrankungen, welche schließlich als neues Target zur gerichteten Modulation der Immunpathomechanismen dienen könnten.

Mononukleäre Zellen aus dem peripheren Blut von gesunden Spendern (HC), RA, PsA und SpA Patienten wurden in umfassenden, durchflusszytometrischen Ansätzen auf die Expression der D_1-D_5 Dopamin Rezeptoren (DR) und Tyrosin Hydroxylase, dem geschwindigkeitsbestimmenden Enzym in der Katecholamin-Synthese, untersucht. Außerdem wurden selektive Agonisten für verschiedene DR in Zellkulturexperimenten verwendet, um Effekte auf Aktivierung, Apoptose, Proliferation und Differenzierung von Immunzellen sowie intrazelluläre Signalwege zu untersuchen. Mittels zytometrischer Bead Arrays und ELISA wurden zudem Effekte der DR Stimulation auf die Zytokin Sekretion ermittelt.

In peripheren T Zellen, B Zellen, NK Zellen und Monozyten von HC sowie RA, PsA und SpA Patienten konnte die Expression aller fünf DR und TH nachgewiesen werden. Hierbei wurde eine erhöhte Expression von D_1 DR in peripheren B Zellen von RA Patienten im Vergleich zu HC festgestellt. Die Expression von D_1 DR in B Zellen unterschied sich zwischen den Geschlechtern in HC und insbesondere weibliche RA Patienten offenbarten erhöhte D_1 DR Level, die zudem mit Krankheitsparametern korrelierten. Eine tiefergehende Phänotypisierung zeigte, dass die Expression von D_1 DR mit der Reifung der B Zellen anstieg und vor allem naive B Zellen von RA Patienten eine erhöhte D_1 DR Expression aufwiesen. Auch in peripheren NK und $CD4^+$ T Zellen stieg die Expression von D_1 DR mit dem Reifestadium der Zellen an. Zirkulierende follikuläre T Helferzellen von RA Patienten wiesen ebenfalls eine erhöhte Expression von D_1 DR auf.

Funktionell zeigte die reine Stimulation der DR keinen akuten Effekt auf die Aktivierung oder Apoptose von Immunzellen. Die Stimulation von D_1-like Rezeptoren hatte keinen Einfluss auf die Proliferation und Plasmablasten Differenzierung in T Zell-abhängiger Stimulation. In T Zell-

unabhängiger B Zell Stimulation induzierte die D_1-like Rezeptor Stimulation keine Differenzierung zu Antikörper-sekretierenden Zellen. Jedoch steigerte der D_1-like Rezeptor Agonist die Proliferation der D_1 DR exprimierenden B Zellen unabhängig von T Zellen, sowohl in HC als auch RA. Außerdem beeinflusste die D_1-like Rezeptor Stimulation die Zytokin-Sekretion spenderabhängig. Bedeutsam war hier, dass ausschließlich in Zellkulturen von RA Patienten die D_1-like Rezeptor Stimulation zum Anstieg der IL-8 Sekretion führte, welche maßgeblich in der Knochendestruktion der RA involviert ist. Die Expression von Ki-67, sowie die Phosphorylierung von ERK und CREB als klassische Signalmoleküle des dopaminergen Signalwegs, waren nach reiner D_1-like Rezeptor Stimulation unverändert. Nichtsdestotrotz konnten erhöhte Expressions- und Phosphorylierungslevel in RA nachgewiesen werden.

Abschließend kann zusammengefasst werden, dass Komponenten des dopaminergen Signalwegs in peripheren Immunzellen präsent sind und daher eine Kommunikation via DA ermöglichen. Periphere naive B und T_{FH} Zellen von RA Patienten weisen erhöhte Level von D_1 DR auf und könnten durch den positiven Einfluss auf die Proliferation und Funktion von pathogenen B Zellen eine zentrale Rolle in der RA spielen. Diese Ergebnisse sind mehrheitlich übereinstimmend mit beschriebener Literatur und unterstreichen damit die Blockade von D_1 DR auf peripheren B Zellen als potenziellen neuen Angriffspunkt bei der Therapie der RA. Weitere Studien sind geplant, um die exakten Mechanismen vollends zu entschlüsseln.

1. Introduction

1.1. Rheumatic diseases

Rheumatic diseases comprise a variety of musculoskeletal disorders. Foci of this thesis are specifically chronic inflammatory diseases of the joints including rheumatoid arthritis (RA), psoriasis arthritis (PsA) and ankylosing spondylitis (SpA). All three diseases are different entities that have some common features. Particularly, PsA and SpA belong to the group of spondylarthritis that summarizes different inflammatory diseases sharing clinical, genetic, and radiological characteristics. Sites of inflammation in spondylarthritis involve mainly the axial skeleton but also peripheral joints. Diseases in the group of spondylarthritis are moreover associated with other inflammatory disorders like uveitis, psoriasis or inflammatory bowel disease and presence of human leukocyte antigen (HLA)-B27 antigen (Raychaudhuri and Deodhar 2014).

1.1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoinflammatory disease primarily affecting synovial joints. Though, extra-articular manifestations can develop as well. In course of disease RA patients suffer from pain, joint destruction, functional impairment and premature mortality (Scott et al. 1987; Mitchell et al. 1986; Pincus et al. 1984). Generally, RA affects around 0.8% of the adult population with a three-fold higher incidence in women (Deutsche Gesellschaft für Rheumatologie e.V.). According to the 2010 Rheumatoid arthritis classification criteria by the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR), RA is classified based on joint swelling, serology, acute-phase reactants and duration of symptoms (Aletaha et al. 2010).

Etiology of RA is still not fully understood but is thought to rely on genetically predispositions, epigenetic modifications and environmental factors (Scherer et al. 2020). In the beginning of clinical disease both innate and adaptive immune cells are activated and migrate in the synovial compartment consisting of synovial tissue surrounding the cartilage and synovial fluid. Within the synovial tissue of RA patients autoimmune-associated B cells, peripheral helper T (T_{PH}) cells, follicular helper T (T_{FH}) cells, pro-inflammatory monocytes as well as immune effector synovial fibroblasts (SF) are enriched (Zhang et al. 2019; Croft et al. 2019). Pro-inflammatory cytokines like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 are secreted by local immune cells as well as SF and exacerbate joint inflammation represented in characteristic swelling of joints (McInnes and Schett 2007). SF develop towards an aggressively proliferating phenotype forming a hypertrophic synovial lining. Further on, they induce matrix degradation for instance through matrix metalloproteinases and upregulate adhesion molecules necessary for invasion of cartilage tissue (Croft et al. 2019; Huber et al. 2006). Beside SF, synovial tissue macrophages infiltrate the hypertrophied synovium called

pannus (Croft et al. 2019). While osteoclastogenesis is promoted by upregulation of receptor activator of nuclear factor kappa B ligand (RANKL) in SF and immune cells, function of osteoblasts is suppressed by TNF-α (Meednu et al. 2016; Söderström et al. 2010; Shim et al. 2018) consequently leading to bone erosions (Pettit et al. 2001). Levels of IL-8 are elevated in inflamed joints (Kraan et al. 2001) further contributing to osteoclastogenesis (Krishnamurthy et al. 2016) and attracting neutrophils (Gorlino et al. 2018). Neutrophils are also drawn by granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted from natural killer (NK) cells which infiltrate the inflamed synovium (Louis et al. 2020). In synovial fluid of inflamed joints, the amount of cytokine producing cluster of differentiation (CD)56^{bright} NK cells is elevated (Dalbeth and Callan 2002; Caligiuri 2008). The frequency of $CD56^{bright}$ NK cells in synovial fluid is even higher in patients with erosive and deformative RA resulting in increased interferon (IFN)-γ and TNF-α levels (Yamin et al. 2019). Moreover, NK cells induce monocyte differentiation towards dendritic cells (Zhang et al. 2007).

Role of B cells in RA

RA is a heterogeneous disease considering the presence or absence of autoantibodies. Rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) are the most significant autoantibodies studied in RA also serving as classification criteria. Their presence is no requirement for disease development but is connected to worse disease progression (Aletaha et al. 2015; van Gaalen et al. 2004). Both RF and ACPA are detectable years before RA diagnosis (Nielen et al. 2004) and decrease during therapy (Böhler et al. 2013).

For seropositive RA (meaning presence of either RF or ACPA) the most important susceptibility gene is HLA-DRB1 containing the shared epitope (Gregersen et al. 1987). A positively-charged amino acid motif within the shared epitope is able to present negatively-charged or even citrullinated self-peptides (Hill et al. 2003). Indeed, presence of the shared epitope is clearly enhancing the production of autoantibodies (Bellatin et al. 2012). RF is an antibody of either immunoglobulin class (Ig) M, IgG, or IgA directed against the Fc region of human IgG. Binding of RF to IgG induces formation of immune complexes able to activate the complement system (Volkov et al. 2020). The complement system in turn improves activation of autoimmune B cells (Nikitin et al. 2019). Moreover, by signaling via Fc receptor common γ subunit, IgG-RF is promoting osteoclastogenesis (Grötsch et al. 2019). IgM-RF together with ACPA boost TNF-α production by macrophages further supporting inflammation (Sokolove et al. 2014). ACPA is, as its name suggests, one type of autoantibody against the posttranslational protein modification citrulline. ACPAs are present as either IgG, IgM, or IgA and can target different citrullinated proteins like vimentin or type II collagen (Goules et al. 2013). B cells secreting ACPA are found in synovial fluid and peripheral blood (Rombouts et al. 2016; Kerkman et al. 2016a; Kerkman et al. 2016b; Kerkman et al. 2013). Through ACPA secretion, B cells are directly involved in initiation of synovial inflammation and bone destruction (Harre et al. 2012). Indirectly, ACPAs induce IL-8 secretion leading to local osteoclastogenesis

(Krishnamurthy et al. 2016). Also, transdifferentiation of dendritic cells into osteoclasts is initiated by ACPA further promoting bone erosions (Krishnamurthy et al. 2019).

The presence of different Ig classes of ACPA and RF points towards interaction with T cells. Locally in inflamed synovial tissue of RA patients B cells can undergo germinal center (GC)-like reactions which include proliferation, somatic hypermutation and differentiation into antibody secreting plasma cells (Schröder et al. 1996). So called ectopic lymphoid structures consisting of interacting B and T_{FH} cells are present in synovial joints of 6 - 35% of RA patients (Hutloff 2018). Lymphoid neogenesis occurs in degree of synovial inflammation rather than depending on seropositivity (Cantaert et al. 2008). B cells, central memory $CD4^+$ T cells as well as T_{FH} cells are attracted by CXC ligand (CXCL)-13 via CXC chemokine receptor (CXCR)-5 to these structures in RA (Shi et al. 2001; Chevalier et al. 2011). Additionally, T_{PH} cells lacking CXCR5 but expressing other chemokine receptors are markedly expanded in inflamed non-lymphoid but synovial tissue of seropositive RA patients and capable to trigger B cell responses too (Rao et al. 2017). In seropositive RA T_{PH} cells are even elevated in peripheral blood (Fortea-Gordo et al. 2019).

Apart from autoantibody production, B cells modulate local joint inflammation and bone destruction by cytokine secretion. B cells secrete C-C motif chemokine ligand (CCL)-3 and TNF-α, which inhibit osteoblast differentiation in RA (Sun et al. 2018). IL-6 production is enhanced in RA B cells and contributes to persistent inflammation (Dam et al. 2018). On the contrary, B regulatory cells produce IL-10 and exert an anti-inflammatory function in RA by suppressing $CD4^+$ T cells (Bankó et al. 2017).

1.1.2. Psoriasis arthritis

Psoriasis arthritis (PsA) is a chronic inflammatory disease of peripheral joints that can be distinguished from RA by additional enthesitis or dactylitis meaning inflammation of connective tissue or one complete finger/toe, respectively (Narváez et al. 2012; Kane et al. 1999). Furthermore, disease may also involve inflammation of skin, nails, or axial skeleton. PsA patients report pain, functional impairment, and health-related loss of quality of life (Gladman et al. 2007). Prevalence of PsA is generally 0.05 - 0.3%, but in established psoriasis drastically increased towards 10% (Deutsche Gesellschaft für Rheumatologie e.V.). It is diagnosed based on clinical phenotype according to Assessment of Spondylo Arthritis International Society (ASAS) classification criteria (Raychaudhuri and Deodhar 2014).

Genetically predisposition for PsA includes single-nucleotide polymorphisms in *HLA-B*, *IL12B* and *IL23R* genes among others indicating the importance of T cells in disease development (Veale and Fearon 2018). In contrast to RA, PsA is not characterized by thickening of synovial membrane but by neoangiogenesis within synovium facilitating the migration of inflammatory cells from peripheral blood (Espinoza et al. 1982). In synovial tissue of PsA, levels of pro-

inflammatory cytokines TNF-α, IL-6 and IL-1β are as high as in RA (van Kuijk et al. 2006). Furthermore, the IL-23/IL-17 axis contributes specifically to PsA pathogenesis. IL-17-expressing $CD4^+$ and $CD8^+$ T cells as well as innate lymphoid cells (ILC) 3 are enriched in synovial fluid of PsA patients (Menon et al. 2014; Benham et al. 2013; Leijten et al. 2015). Similar to RA, SF proliferate within the inflammatory environment in PsA and invade cartilage (Veale and Fearon 2018). Increased levels of RANKL again lead to osteoclastogenesis and bone erosions (Dalbeth et al. 2010). However, differences between RA and PsA are present regarding periarticular bone changes. Bone microenvironment of PsA presents more osteophytes indicating also bone repair (Finzel et al. 2011).

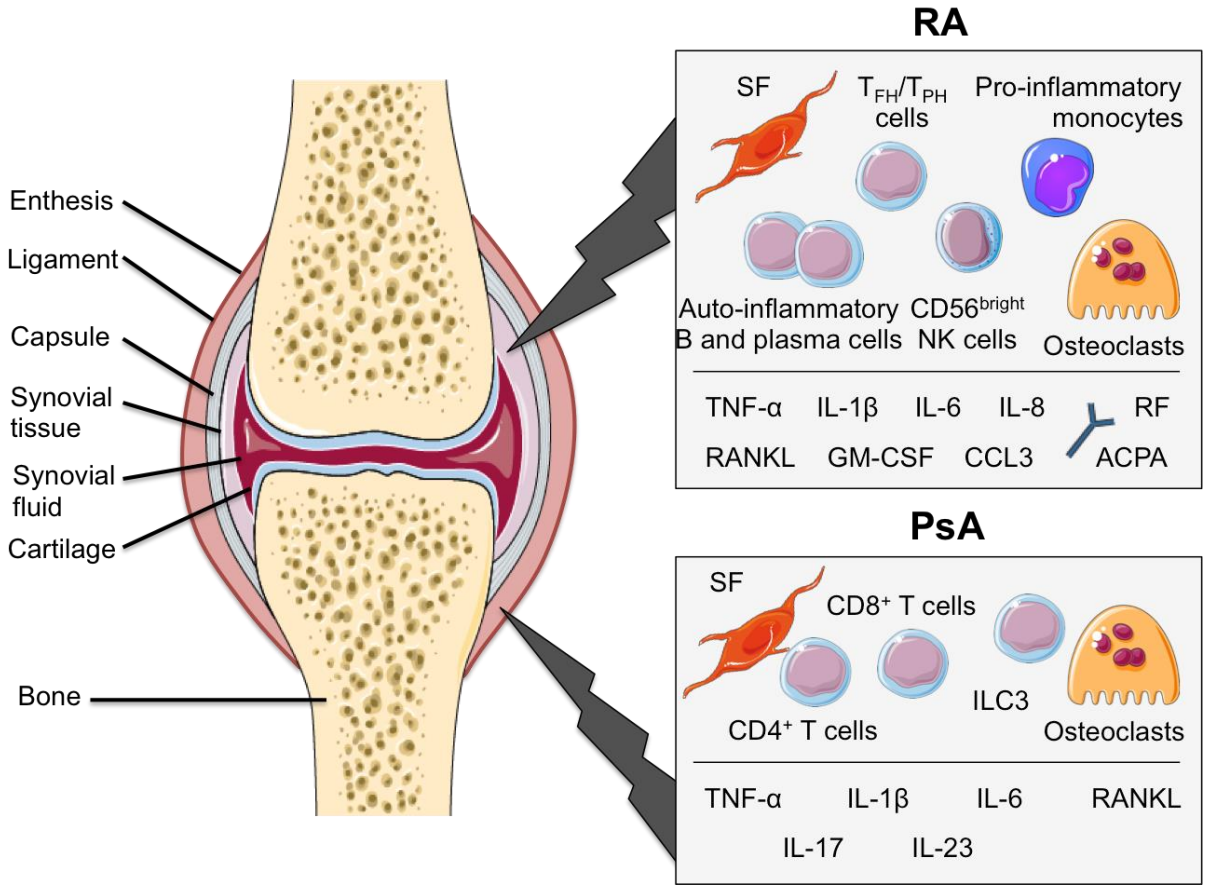

Figure 1: Involved sites of inflammation in RA and PsA.
Shown is the anatomical profile of a healthy peripheral joint. Arrows indicate sites of primary inflammation in RA (synovial tissue) and PsA (enthesis). Boxes indicate main cellular and soluble drivers of inflammation and bone destruction. Pictures were modified from Servier Medical Art. ACPA: anti-citrullinated protein antibody, CCL: C-C motif chemokine ligand, GM-CSF: granulocyte-macrophage colony-stimulating factor, IL: interleukin, ILC: innate lymphoid cell, RA: rheumatoid arthritis. RANKL: receptor activator of nuclear factor kappa B ligand, RF: rheumatoid factor, PsA: psoriasis arthritis, SF: synovial fibroblast, TNF: tumor necrosis factor

1.1.3. Ankylosing spondylitis

Ankylosing spondylitis (SpA) or former Morbus Bechterew is a chronic inflammatory disease affecting primarily the axial skeleton. Patients suffer from inflammatory back pain including stiffness in advanced stages. Its prevalence in the adult population is around 0.1 - 1.4% depending on globally varying HLA-B27 presence. Additionally, SpA usually develops between the second to fourth decade of life with a higher incidence in men (Deutsche Gesellschaft für

Rheumatologie e.V.). Based on the 1984 modified New York Classification Criteria and new classification criteria by ASAS, SpA can be detected in radiographs by defined structural changes in sacroiliac joints. Subsequently, inflammation is followed by either bone erosions or more prominently ossification of spinal ligaments connecting discs. Such bony protuberances called syndesmophytes grow over time forming “bamboo”-like structures consequently impeding patients mobility (Raychaudhuri and Deodhar 2014).

The pathogenesis of SpA is still unclear. Several genetic predispositions were identified, among them most importantly a polymorphism in *HLA-B27* gene but also genes involved in IL-17/23 axis, in CD8 T cell differentiation or latest for endoplasmic reticulum aminopeptidase 1/2. Environmental factors may add up to disease development in predisposed individuals (Zhu et al. 2019). IL-17 and IL-23 are elevated in serum and synovial tissue of SpA patients and strongly associated with chronic inflammation (Mei et al. 2011; Jethwa and Bowness 2016). In facet joints of SpA patients the frequency of IL-17 producing cells is increased (Appel et al. 2011). Mainly innate immune cells including neutrophils, mast cells, ILC3, γ/δ T cells, mucosal-associated invariant T cells but also $CD8^+$ T cells and Th17 cells contribute to high IL-17 levels in SpA (Pedersen and Maksymowych 2019). IL-17A can directly induce osteoblast generation from mesenchymal stem cells (Huang et al. 2009). Apart from that, IL-23 stimulates osteoproliferation indirectly via IL-22 secretion from lymphocytes (Babaie et al. 2018).

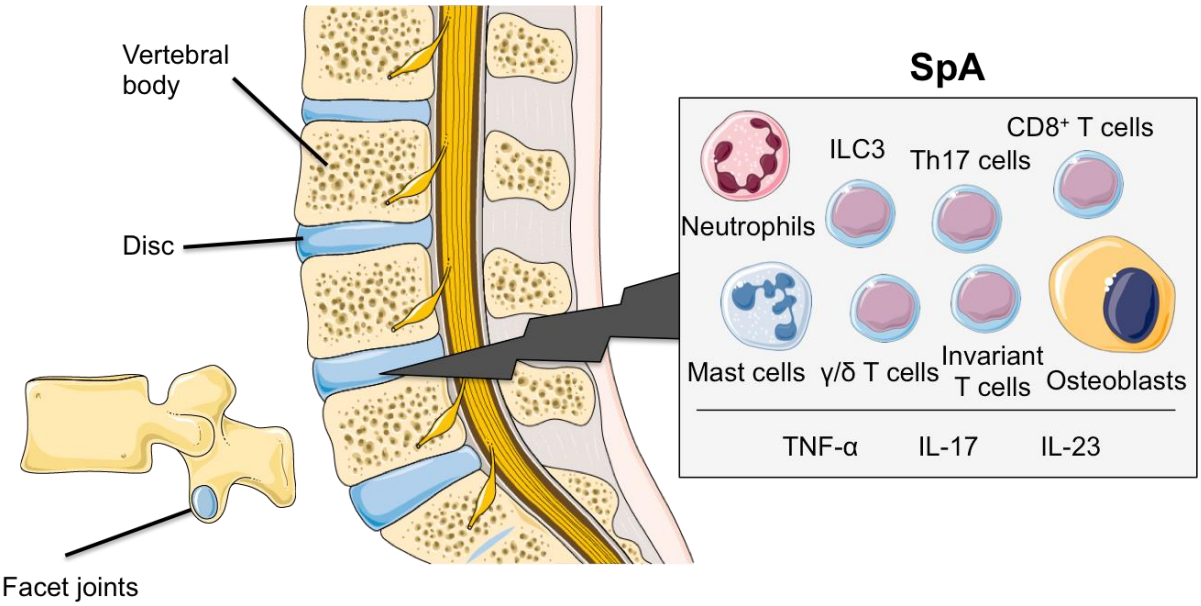

Figure 2: Involved sites of inflammation in SpA.
Shown is the anatomical profile of a healthy vertebra column and a single vertebra (sagittal view). Arrow indicates sites of primary inflammation in SpA. Box summarizes main cellular and soluble drivers of inflammation and bone formation. Pictures were modified from Servier Medical Art. IL: interleukin, ILC: innate lymphoid cell, SpA: ankylosing spondylitis, TNF: tumor necrosis factor

Table 1 summarizes the main features of the three chronic inflammatory joint diseases. All information is based on the above cited references.

Table 1: Main differences of chronic inflammatory joint diseases investigated.

	Rheumatoid arthritis	**Psoriasis arthritis**	**Ankylosing spondylitis**
Prevalence in adult population	0.8%	0.05 – 0.3%, increased to 10% in psoriasis	0.1 – 1.4%
Male:female ratio	1:3	1:1	Increased in males
Age at onset	50-70	Not specified	20-40
Clinical features	Synovitis	Enthesitis, dactylitis, inflammation of nails	Enthesitis
Involved sites of inflammation	Peripheral joints	Peripheral joints (and axial skeleton)	Axial skeleton (and peripheral joints)
Main genetic predispositions	*HLA-DRB1*	*HLA-B27, IL12B, IL23R*	*HLA-B27*, IL-17/23 axis, CD8 T cell differentiation
Typical comorbidities	-	Uveitis, psoriasis, inflammatory bowel disease	

1.1.4. Treatment options for RA, PsA and SpA

Generally, the overarching goal in treatment of RA, PsA and SpA is clinical remission and low disease activity meaning alleviation of symptoms, functional improvement and prevention or inhibition of structural damage to bone and cartilage. In this way patients should benefit in restored quality of life.

Disease-modifying anti-rheumatic drugs (DMARD) are particularly used for treatment of rheumatic diseases. For long time conventional DMARD comprising e.g. methotrexate, leflunomide, sulfasalazine or hydroxychloroquine were the only available therapies for management of rheumatic disorders and still serve as basic therapy. However, not every patient shows a good response or even reveals contraindications (Smolen et al. 2020). The need for alternative therapies led to the development of biologic or synthetic DMARD beginning in the early 90s. Biologic DMARD comprise different classes selectively targeting either cytokines or components in immune cell signaling which are critically involved in disease pathogenesis. Table 2 gives an overview about the available and already approved biologic DMARD. Beside these biological originator DMARD, biosimilars are available as well.

Table 2: Approved biologic DMARD for treatment of RA, PsA and SpA.

Group	Name	Structure and mechanism of action	Reference
TNF-α inhibitors	infliximab	chimeric monoclonal anti-TNF-α antibody containing human IgG1	(Elliott et al. 1994)
	etanercept	fusion protein of recombinant human p75 TNF-α receptor bound to Fc region of human IgG1	(Moreland et al. 1997)
	adalimumab	fully human monoclonal anti-TNF-α antibody blocking interaction of TNF-α with p55 and p75 subunits of TNF-α receptors	(den Broeder et al. 2002)
	certolizumab	human anti-TNF-α Fab' fragment linked to polyethylene glycol for increased half-life	(Choy et al. 2002)
	golimumab	fully human monoclonal anti-TNF-α antibody neutralizing TNF-α	(Kay et al. 2008)
IL-1 inhibitor	anakinra	recombinant human form of IL-1 receptor antagonist	(Cohen et al. 2002)

IL-6 inhibitors	tocilizumab	humanized monoclonal antibody inhibiting soluble and membrane bound IL-6 receptor	(Mihara et al. 2005)
	sarilumab	fully human monoclonal antibody inhibiting IL-6 receptor-α	(Huizinga et al. 2014)
IL-12/23 inhibitor	ustekinumab	fully human monoclonal antibody binding to shared p40 subunit of IL-12 and IL-23 thus inhibiting receptor binding	(Gottlieb et al. 2009)
IL-17A inhibitors	ixekizumab	humanized monoclonal anti-IL-17 antibody	(Genovese et al. 2010)
	secukinumab	fully human monoclonal anti-IL-17A antibody for neutralization	(McInnes et al. 2014)
CTLA-4 inhibitor	abatacept	fusion protein of recombinant cytotoxic T lymphocyte antigen-4 (CTLA-4) bound to Fc region of human IgG inhibiting co-stimulatory signal of antigen presenting cells and thus inhibiting T cell activation	(Linsley et al. 1992)
B cell depletion	rituximab	monoclonal antibody against CD20	(Anderson et al. 1997)
RANKL inhibitor	desonumab	fully human monoclonal anti-RANKL antibody inhibiting osteoclastogenesis and further bone damage	(Cohen et al. 2008)

As every disease relies on distinct inflammatory pathomechanisms, indications for biologic DMARD therapy of RA, PsA and SpA differ.

Latest recommended treatment options for management of RA from EULAR in 2019 include conventional DMARD as well as glucocorticoids and biologic or targeted synthetic DMARD (Smolen et al. 2020). After first diagnosis RA patients most frequently receive methotrexate in combination with glucocorticoids. However, in case of insufficient response and poor prognostic factors, therapy is supplemented with biologics or synthetic DMARD targeting janus kinases. Depending on clinical phenotype and preceding failure of therapy, RA patients receive either an TNF-α inhibitor, IL-6 inhibitor, anakinra or abatacept. In severe RA, B cell depletion with rituximab is considered. RA patients with erosive disease are additionally treated with denosumab. Disease activity score 28 (DAS28) and functional capacity (FFbH) of RA patients are assessed on regularly basis by medical examination and questionnaires to prove therapeutic success or to change therapy if necessary.

Choice of treatment for PsA is based on disease manifestation. Generally, therapeutic recommendations from the last EULAR 2015 update on management of PsA include non-steroidal anti-inflammatory drugs and methotrexate as first DMARD of choice. If treatment is ineffective or even toxic, therapy is combined with or changed to one biologic DMARD targeting either TNF-α, IL-12/23 or IL-17. Additionally, apremilast, a synthetic DMARD inhibiting phosphodiesterase-4, is used for PsA therapy (Gossec et al. 2016). Disease activity is measured by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) or Ankylosing Spondylitis Disease Activity Score (ASDAS). Functional impairment in PsA is assessed by the Bath Ankylosing Spondylitis Functional Index (BASFI) (Machado and Raychaudhuri 2014).

SpA treatment depends on individual disease manifestation as well. According to the 2016 update of the ASAS-EULAR management recommendations for axial spondylarthritis, non-steroidal anti-inflammatory drugs or even analgesics are used to relief pain. Further on, local glucocorticoid injections and sulfasalazine may be used to limit inflammation whereas TNF-α inhibitors are more common. In case of insufficient disease remission another TNF-α or IL-17A inhibitor should be used (van der Heijde et al. 2017). Disease activity and functional impairment in SpA are assessed with the same instruments as already introduced for PsA.

Unfortunately, despite the wide range of available treatment options, clinical remission is not always achieved even after repeated therapeutic adjustments. Moreover, DMARD treatment is accompanied with systemic immunosuppression and patients are prone to serious infections (Singh et al. 2015). Treatment with abatacept even increases risk for melanoma (Germay et al. 2019). Several clinical trials are conducted and already test the efficacy of new drugs in treatment of chronic inflammatory diseases of the joints. These drugs commonly target identical cytokines or molecules as already approved DMARD do or are biosimilars of them. Moreover, new targets of monoclonal antibodies include GM-CSF and IL-23(A). Also, monoclonal antibodies with dual functions in neutralizing both IL-17A and IL-17F, TNF-α and IL-17A, or drug antibody conjugates of anti-TNF-α and steroids are being tested. On the contrary, IL-2 mutein is examined in promoting anti-inflammatory effects in RA patients. In addition to that, synthetic drugs inhibiting Bruton's tyrosine kinase, interleukin-1 receptor-associated kinase 4, receptor interacting serine/threonine kinase 1, or tyrosine kinase 2 involved in cytokine signaling are currently under investigation (National Center for Biotechnology Information). However, these new approaches again focus on systemically expressed proteins which could as well induce side effects. Therapeutic options specifically targeting the disease pathomechanisms thereby involving fewer side effects are lacking.

1.2. Dopamine - basic neurotransmitter with underestimated features?

Dopamine (DA) is a neurotransmitter of the central nervous system known to operate in several important functions such as motor control, memory, or motivation. DA belongs among norepinephrine (NE) and epinephrine (Epi) to the group of catecholamines. Characteristic for such monoamines is their synthesis from free amino acids by short metabolic pathways including one rate-limiting enzymatic reaction. Additionally, monoamines signal via metabotropic receptors and activate second messengers (Klein et al. 2019).

1.2.1. Dopaminergic pathway

Synthesis of DA and further conversion to NE and Epi is depicted in Figure 3. Phenylalanine is converted to tyrosine by phenylalanine hydroxylase. However, this step is not essential since tyrosine exists in cytosol per se. The key reaction is rather the conversion of tyrosine to levodopa (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH) (NAGATSU et al. 1964). L-DOPA is further converted to DA by aromatic amino acid decarboxylase (Christenson et al. 1970). NE arises from DA by the enzyme DA β-hydroxylase (DBH) (Weinshilboum et al. 1971) and can be further converted to Epi by phenyl ethanolamine N-methyltransferase (PNMT) (Wurtman and Axelrod 1965).

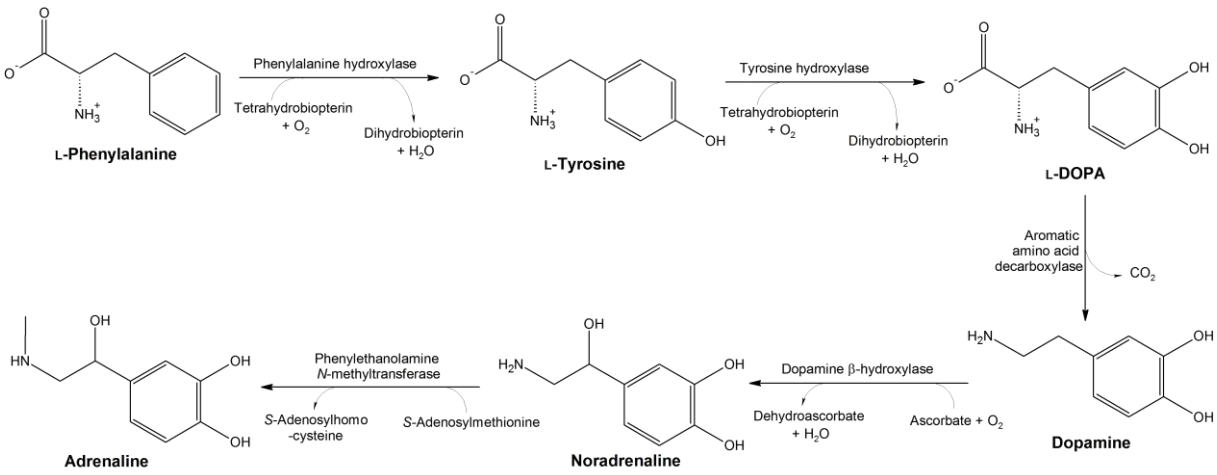

Figure 3: Synthesis of catecholamines.
Amino acids phenylalanine and tyrosine are hydroxylated by specific enzymes leading to L-DOPA, the precursor of DA. Particularly, TH is the rate-limiting enzyme in DA synthesis. L-DOPA decarboxylase then proceeds in conversion of L-DOPA to DA. DA can be converted to NE by DA β-hydroxylase. Further, PNMT converts NE to Epi. Image from http://www.brainprotips.com. DA: dopamine, Epi: epinephrine, L-DOPA: levodopa, NE: norepinephrine, PNMT: phenylethanolamine N-methyltransferase, TH: tyrosine hydroxylase.

After synthesis, DA is stored into synaptic vesicles via vesicular monoamine transporter 2 (VMAT2) (Eiden and Weihe 2011). The acidic lumen of vesicles is able to stabilize DA and prevents its oxidation (Guillot and Miller 2009). However, in non-acidic milieu such as cytosol DA is rapidly oxidized and metabolized by monoamine oxidase B (MAO-B) and aldehyde dehydrogenase (ALDH) to 3,4-dihydroxyphenylacetic acid (DOPAC). DOPAC is further

degraded to homovanillic acid (HVA) by catechol-o-methyltransferase (COMT) (Eisenhofer et al. 2004). Additionally, spontaneous oxidation of DA can result in neurotoxic quinones (Stokes et al. 1999). After release of the catecholamine by extrinsic triggers DA binds to its specific receptors or is taken back up by dopamine transporter (DAT) (Giros et al. 1992).

1.2.2. Dopamine signaling via dopamine receptors

Dopamine receptors (DR) belong to the class of neurotransmitter receptors and so far, knowledge regarding their structural and signaling properties is mainly based on findings in neuroscience. DR belong to G protein coupled receptors (GPCRs), which consist of seven transmembrane domains and transmit signals intracellularly via guanine nucleotide binding regulatory (G) proteins (Sibley et al. 1992). Five different DR were described in the early 1990s. Depending on their pharmacological, biochemical, and physiological profile they are assigned in two subclasses, namely D_1- and D_2-like receptors. D_1 and D_5 DR belong to the D_1-like and D_2, D_3 and D_4 DR to the D_2-like receptor family (Figure 4).

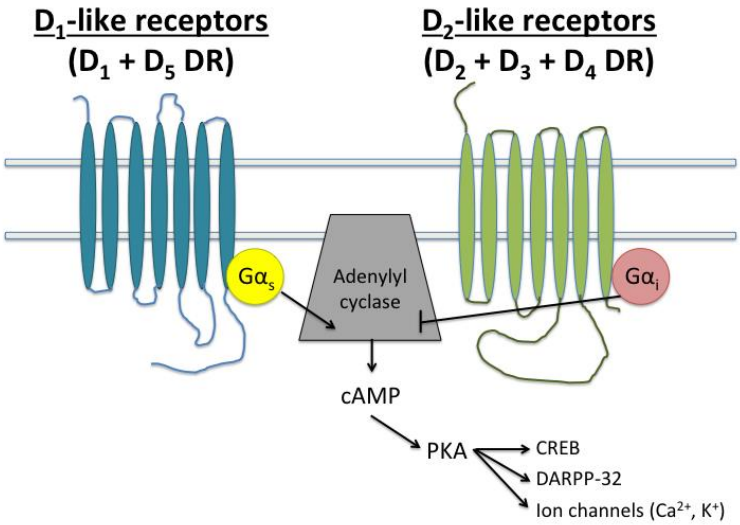

Figure 4: Canonical signaling of D_1- and D_2-like dopamine receptors.
DR are grouped into two classes based on their intracellular signaling pathways. D_1 -like receptors D_1 and D_5 DR are coupled to $G\alpha_s$ protein activating adenylyl cyclase and increasing cytosolic cAMP concentration. D_2-like receptors D_2, D_3, and D_4 DR are coupled to $G\alpha_i$ protein inhibiting adenylyl cyclase activity and decreasing cAMP concentration. cAMP is activating PKA further phosphorylating different target proteins such as CREB or DARPP-32. cAMP: 3'-5'-cyclic adenosine monophosphate, CREB: cAMP response element-binding protein, DARPP-32: 32-kDa dopamine and cAMP-regulated phosphoprotein, DR: dopamine receptor, PKA: protein kinase A.

D_1-like receptors typically contain a small third cytoplasmic loop and a long C-terminus for regulatory phosphorylation. Their extracellular N-terminus possesses sites for glycosylation. D_1-like receptors are coupled to $G\alpha_s$ thus enabling activation of adenylyl cyclase and increasing cytosolic 3'-5'-cyclic adenosine monophosphate (cAMP) concentration. In contrast, D_2-like receptors possess a large third cytoplasmic loop and a smaller C-terminus. They also possess sites for glycosylation at the N-terminus and for phosphorylation in the third cytoplasmic loop. Moreover, D_2-like receptors are coupled to $G\alpha_i$ and inhibit adenylyl cyclase activity (Sibley et

al. 1993). Typically, second messenger cAMP stimulates protein kinase A (PKA) further affecting several targets such as cAMP response element-binding protein (CREB), other neurotransmitter receptors or calcium and potassium channels (Greengard 2001). Also, many additional pathways have been described for DA in the brain indicating complex signal transduction. One additional target of PKA is 32-kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32) owing many sites for phosphorylation resulting in divergent effects, exemplarily extracellular-signal regulated kinase (ERK) activation (Santini et al. 2012). Apart from that, alternative receptor coupling of $G\alpha_q$ and activation of phospholipase C (PLC) is present in specific D_5 DR, heterodimers of D_1-D_2 DR and the $G_{\beta\gamma}$ subunit of some D_2-like receptors (Sahu et al. 2009; Rashid et al. 2007; Hernández-López et al. 2000). PLC then promotes production of inositol triphosphate (IP_3) and diacylglycerol (DAG) increasing intracellular calcium levels and activating protein kinase C further regulating various pathways. However, evidence for the formation of D_1-D_2 DR heterodimers resulting in altered signaling via $G\alpha_q$ and PLC is conflicting (Hasbi et al. 2014; Frederick et al. 2015). During chronic inflammation GPCRs are able to switch their intracellular signaling from $G\alpha_s$ to $G\alpha_i$ and notably, this includes DR in RA as well (Jenei-Lanzl et al. 2015).

Exact mechanisms in regulation of D_1-D_5 DR expression are yet unclear. Different factors as drug intake (Bordet et al. 1997; Schrader et al. 2019) or sex hormones (Lee and Mouradian 1999; Lammers et al. 1999) are discussed to impact DR expression but evidence is still lacking. As all GPCRs, DR are synthesized in the endoplasmic reticulum (ER). Here, they are successfully assembled and folded by ER resident chaperones as for instance calnexin that also mediate their export (Free et al. 2007). Some uniqueness is reported between different receptors. Especially, D_4 DR relies on oligomerization within the ER for subsequent export to the plasma membrane (van Craenenbroeck et al. 2011). In addition, further posttranslational modification is required for trafficking of D_2-like receptors (Zhang et al. 2016; Zhang and Kim 2016). Importantly, specific sequences within the proximal C-terminus of DR serve as binding sites for e.g. DR interacting protein 78 or the γ-subunit of COPI complex that represent key proteins in further DR trafficking via Golgi to the plasma membrane (Bermak et al. 2001; Bermak et al. 2002). Here, DR are expressed and sense external signals by agonist binding. Importantly, D_1-like and D_2-like receptors bind DA with varying affinity. D_2-like receptors are already activated by low concentrations of DA as they possess a 10- to 100-fold higher affinity than D_1-like receptors. D_1-like receptors sense only high concentrations of DA (Klein et al. 2019). Nonetheless, selective agonists and antagonists for D_1- or D_2-like receptors are available to target specific DR *in vivo* and enable the study of different DR *in vitro*.

Activity of expressed DR is modulated by regulators of G protein coupled receptors (RGS) and G protein coupled receptor kinases (GRK) (Klein et al. 2019). RGS catalyze guanosine triphosphate (GTP) hydrolysis from activated α-subunit of $G\alpha_i$ and $G\alpha_q$ proteins (Berman and

Gilman 1998). Upon sustained DR stimulation, specific GRKs and other kinases as PKA phosphorylate DR at the third intracellular loop inducing inactivation (Pitcher et al. 1998; Mason et al. 2002). Further, the phosphorylation provides basis for binding of the scaffolding protein β-arrestin (Marchese et al. 2003). After agonist-induced phosphorylation and β-arrestin binding, DR undergo endocytosis in clathrin-coated pits and either recycle to plasma membrane or degrade in the lysosomal compartment (Marchese et al. 2003). Particularly, D_1 DR is known to rapidly recycle to plasma membrane (Vargas and Zastrow 2004). Additionally, β-arrestins were shown to induce G protein-independent pathways such as mitogen-activated protein kinase, c-Src, Mdm2, Akt or glycogen synthase kinase-3 (Beaulieu and Gainetdinov 2011). Thus, expression and activation of DR is very dynamic.

1.2.3. Dopamine involvement in immunity

Beside its existence and classical function as neurotransmitter in the central nervous system, DA is present in the periphery. DA is secreted by nerve endings in different peripheral organs and critically involved in different body functions such as hormone secretion, vascular, or renal functions (Matt and Gaskill 2020). Generally, DA is detectable in human plasma at a concentration of 10 pg/ml and increases up to 45 pg/ml in individuals dealing with stress (Saha et al. 2001). Several studies indicate the ability of human immune cells to synthesize and store catecholamines (Bergquist et al. 1994; Musso et al. 1996; Bergquist et al. 1997; Bergquist and Silberring 1998; Kokkinou et al. 2009). Metabolites of catecholamines are detectable in peripheral blood mononuclear cells (PBMCs) and medium after cell culture. Likewise, concentrations of metabolites are altered by modulators of catecholamine metabolism (Marino et al. 1999; Cosentino et al. 2000).

Early studies assessed the expression of DR in human PBMCs by radioligand binding assays or PCR confirming presence of D_1-D_5 DR (Basu and Dasgupta 2000). The first study illustrating the expression of D_1-D_5 DR by flow cytometry in distinct human peripheral immune cells was by McKenna et al. (2002). They reported the highest expression of DR in B cells and NK cells, lowest in T cells and monocytes with large variabilities between donors (McKenna et al. 2002). Limited studies further analyzed DR expression in specific human T cells (Kustrimovic et al. 2014; Cosentino et al. 2018; Nasi et al. 2019; Keren et al. 2019), B cells (Meredith et al. 2006) or NK cells (Mikulak et al. 2014). Regulatory peripheral immune cells also express DR (Arce-Sillas et al. 2019). However, considering different subgroups of each immune cell type, the expression profile of D_1-D_5 DR is much more complex. Moreover, published findings are partially conflicting. Equally complicated and yet incompletely resolved are the effects of DA on immune regulation such as proliferation, apoptosis, differentiation, or secretion of soluble mediators. While some studies report DA signaling following the canonical route, some fail to detect the involvement of expected signaling molecules.

In PBMCs, proliferative response and cytokine secretion in response to different stimuli decrease after supplementation of L-DOPA, DA and NE. Simultaneously, apoptosis of immune cells rises after DA treatment (Bergquist et al. 1997). However, apoptosis was shown to depend on DA concentration. While high levels of DA increase apoptosis via reactive oxygen species, low levels of DA appear protective (Colombo et al. 2003).

Specifically for T cells, effects of DR stimulation are reported to be dependent on T cell status. While resting T cells mostly get activated by dopaminergic stimulation, activated T cells are suppressed by DA (Keren et al. 2019). Accordingly, DA stimulation leads to increased cytokine production in $CD3^+$ T cells (Besser et al. 2005; Torres et al. 2005). Focusing on peripheral human $CD4^+$ T cells, D_1-like receptors are expressed at higher levels than D_2-like receptors. Moreover, expression of D_1-D_5 DR seems to be dynamic as it differs between maturational stages and also increases after anti-CD3/28 stimulation (Kustrimovic et al. 2014). Only regulatory T cells (Treg) were shown to express TH and produce catecholamines. DA released by Treg on the one hand acts autocrine to decrease further IL-10 and transforming growth factor (TGF)-β secretion while paracrine signaling suppresses effector T cells, both via D_1-like receptors (Cosentino et al. 2007). Thus neuro-immune communication via DA may also provide regulatory functions. In line, DA was shown to decrease proliferation of IL-2 stimulated $CD4^+$ T cells primarily via D_1-like receptors and increase of cAMP levels (Saha et al. 2001). Yet, in anti-CD3/CD28 stimulated human naive $CD4^+$ T cells DA stimulation resulted in pro-inflammatory cytokine secretion primarily via D_1-like receptors (Nakano et al. 2011).

Of great interest, Papa et al. demonstrated the presence of T_{FH} cells containing DA in GC within human secondary lymphoid organs. Moreover, these T_{FH} cells are able to synthesize DA and release it after interaction with B cells via antigen. DA upregulates inducible T-cell costimulator ligand (ICOSL) in B cells via D_1 DR further inducing a positive feedback loop involving ICOS and CD40 signaling and increasing synaptic area. In fact DA was shown to enhance B cell differentiation (Papa et al. 2017). On the contrary, early studies report stimulation with either tyrosine, L-DOPA, DA or NE *in vitro* decrease the number of IgG, IgA and IgM secreting B cells (Bergquist et al. 1994; Bergquist et al. 1997). Furthermore, L-DOPA, DA, and pan-DR agonist apomorphine diminish B cell proliferation. However, the described effects are independent of canonical DA signaling and rather occurring by reactive oxygen species inducing apoptosis (Meredith et al. 2006; Bergquist et al. 1997). Solely stimulation of D_2-like receptors with specific agonist bromocriptine also suppresses proliferation, Ig secretion and expression of activation markers in B cells (Morkawa et al. 1993).

Cytotoxic $CD8^+$ T cells express all DR, TH and VMAT2. Here, D_1-like receptor stimulation impedes $CD8^+$ Treg differentiation and regulatory function (Nasi et al. 2019). Such as in $CD4^+$ T cells proliferation of $CD8^+$ T cells in response to IL-2 and cytotoxic capacity is diminished predominantly via D_1-like receptors and increase of cAMP levels (Saha et al. 2001). Additionally, D_3 DR in naive $CD8^+$ T cells is coupled to $G\alpha_i$ and important for migration

synergistically with chemokines (Watanabe et al. 2006). Mikulak et al. describe expression of all DR except D_1 DR in human natural killer (NK) cells and generally $CD56^{dim}$ NK cells express higher levels than $CD56^{bright}$ NK cells. Similar to T cells, DR expression increases after activation also in NK cells. Proliferation of activated NK cells is inhibited and IFN-γ secretion is reduced in presence of DA, primarily via D_1-like receptors (Mikulak et al. 2014). Also, peripheral human monocytes express TH, L-DOPA decarboxylase, VMAT2 and DAT (Gopinath et al. 2020; Gaskill et al. 2012) thus able to produce and transport catecholamines. Likewise, monocyte-derived dendritic cells store DA and release it upon interaction with naive $CD4^+$ T cells supporting naive $CD4^+$ T cell differentiation towards Th2 cells (Nakano et al. 2009). *In vitro*, DA decreases proliferation and cytokine secretion of monocytes via NF-κB suppression in response to LPS (Bergquist et al. 2000).

In murine splenocytes DA has an anti-inflammatory effect by reducing proliferation and secretion of immunoglobulins in response to LPS (Kouassi et al. 1988). Moreover, in this context DA was shown to reduce pro-inflammatory and enhance anti-inflammatory cytokine secretion mainly via D_2-like receptors and intracellularly via PLC pathway (Kawano et al. 2018). Murine $CD3^+$ T cells possess all five DR as well, and both D_1- and D_2-like receptor stimulation reduce IFN-γ production in pre-stimulated T cells. Unexpectedly, only D_2-like receptor stimulation induces canonical DR signaling being reversed by D_2-like receptor antagonist haloperidol (Huang et al. 2010). This finding is in line with D_3 DR coupling to $G\alpha_i$ in murine naive $CD4^+$ T cells. Here however, signaling via D_3 supports $CD4^+$ T cell activation and Th1 cell differentiation (Franz et al. 2015). Signaling via D_5 DR fulfills dual roles in murine $CD4^+$ T cells. While D_5 DR signaling promotes differentiation of Th17 cells it also supports anti-inflammatory role of Tregs (Osorio-Barrios et al. 2018). Challenge with antigen in DAT knockout mice which represent a hyperdopaminergic phenotype leads to increased humoral immune responses reflected in high levels of IgG (Kavelaars et al. 2005). Conversely to data on human NK cells, murine NK cells were shown to express all DR. D_1-like receptor stimulation leads to increased cytotoxicity whereas D_2-like receptor stimulation diminishes NK cell activity. Moreover, canonical DR signaling with in- or decrease in cAMP and pCREB level is present in murine NK cells after stimulation of either D_1- or D_2-like receptors, respectively. Supporting this observation, respective agonists or further PKA and adenylyl cyclase modulating substances counteract these effects (Zhao et al. 2013). However, NK cells of DAT knockout mice show reduced cytotoxic activity (Kavelaars et al. 2005). Knockout of D_3 DR in mice or specifically in dendritic cells promotes tumor clearance by increasing cytotoxic capacity of $CD8^+$ T cells via antigen cross-presentation (Figueroa et al. 2017). However, neuroimmune mechanisms are not fully conserved between species as they were already shown to differ between mice and men (Papa et al. 2017).

As outlined above several studies indicate the involvement of the catecholamine DA in immunity. Yet, studies are often too focused on certain cell types/single DR and their results are moreover partly conflicting.

1.3. Role of dopamine in rheumatoid arthritis

Interestingly, the incidence of RA is altered in patients with neurological diseases affecting the dopaminergic system. Early reports suggest that RA and schizophrenia hardly coexist (Malek-Ahmadi 1985). Blockade of D_2-like receptors is standard treatment for schizophrenia and may account for the reduced risk of RA development in schizophrenia patients (Chen et al. 2012). Studies on the coexistence of RA and Parkinson's disease are conflicting (Rugbjerg et al. 2009; Sung et al. 2016; Bes et al. 2014). Prevalence of restless legs syndrome is increased in RA patients compared to general population (Gjevre and Taylor Gjevre 2013) and interestingly, these patients reveal also elevated plasma levels of DA (Mitchell et al. 2018).

Limited studies already point to a critical role of DA in the pathogenesis of human RA. Locally in inflamed joints of RA patients synovial tissue innervation by sympathetic nerve fibers is reduced while density of cells expressing TH and VMAT2 is high (Capellino et al. 2010). Accordingly, synovial cells secrete catecholamines and especially DA is elevated in synovial fluid of RA patients (Nakano et al. 2011). SF in the inflamed tissue of RA patients possess DR which modulate cytokine secretion upon stimulation (Capellino et al. 2014). Peripheral B cells of RA patients present an altered expression of D_2 DR correlating with several clinical parameters (Wei et al. 2015). Additionally, frequency of D_2 DR expressing B cells of RA patients is increased in synovial tissue correlating with plasma TNF-α level, pointing towards a role in bone metabolism (Wei et al. 2016). In mast cells from synovial fluid D_3 DR expression is negatively correlated with DAS28 score and connected to oxidative stress (Xue et al. 2018). Studies on treatment of RA patients with dopaminergic drugs are limited. RA patients treated with D_2-like receptor agonist bromocriptine, in order to suppress pituitary prolactin secretion via dopaminergic regulation, show improvement in several disease parameters after three months. Moreover, proliferative potential of PBMCs decreases after bromocriptine treatment (Figueroa et al. 1997). However, combination treatment with methotrexate prevents bromocriptines effect (Salesi et al. 2013). Similarly, treatment with D_2-agonist cabergoline leads to an improvement of tender and swollen joint count (Mobini et al. 2011).

More experimental data is available in rodent models for RA. In collagen-induced arthritis (CIA) mice VMAT2 expressing cells are present in the joints with start of disease and increase with its progression (Capellino et al. 2010). Beneficial effects were observed by selective inhibition of D_1-like receptors with antagonist SCH23390 in decreasing disease score and joint swelling. Importantly, prior D_1-like receptor stimulation with D_1-like receptor agonist A68930 reverses the therapeutic effect (Nakashioya et al. 2011). Likewise, in human RA/severe combined

immunodeficiency mouse chimera model D_1-like receptor antagonism prevents vascular proliferation and tissue enlargement (Nakano et al. 2011). On the contrary, D_2-like receptor stimulation with quinpirole was shown to limit pathology in CIA mice by reducing serum anti-type II collagen-IgG and restoring the balance between pro- and anti-inflammatory cytokines locally in ankle joints (Lu et al. 2015). Remarkably, the peripheral blockade of conversion from L-DOPA to DA by carbidopa reduces joint inflammation and cartilage destruction as well (Zhu et al. 2017). In addition, DA was shown to affect osteoclasts involved in bone erosion. Specifically, SCH23390 inhibited osteoclastogenesis of bone marrow-derived macrophages *in vitro* (Nakashioya et al. 2011). Blockade of peripheral DA synthesis also reduces the presence of osteoclasts in inflamed joints (Zhu et al. 2017). Moreover, DA uptake inhibitor mazindol has anti-nociceptive effects in Freund's complete adjuvant-induced arthritis mice (Robledo-González et al. 2017).

Taken together, components of the dopaminergic system appear as attractive new targets for future therapies of RA. Yet, knowledge of DA function in peripheral cells of RA patients is limited. Additionally, a potential influence of DA in PsA and SpA was not investigated so far.

2. Aim of the thesis

Aim of this thesis was to clarify the systemic role of the catecholamine DA in chronic inflammatory joint diseases. Therefore, disease-related alterations of the dopaminergic pathway were first investigated in PBMCs. By comprehensive flow cytometric approaches several immune cell subtypes of HC and patients with RA, PsA and SpA were analyzed for expression of D_1-D_5 DR and TH. Second, the examination of immune-modulatory and possibly disease-specific effects of DR stimulation was analyzed. Here, *in vitro* experiments with selective DR agonists were performed to assess effects of specific DR stimulation on activation, apoptosis, proliferation, differentiation, and cytokine secretion of immune cells. Ultimately, potential targets for directed modulation of the underlying immune pathomechanism in chronic inflammatory diseases specifically should be identified.

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals

β-mercaptoethanol, Carl Roth #4227.3

Albumin fraction V (BSA), biotin-free, Carl Roth #0163.4

Dimethyl sulfoxide (DMSO), Sigma Aldrich #472301

DPBS (without magnesium and calcium), Gibco #14190-094

Fetal bovine serum (FBS, heat inactivated), Gibco #10270-106 (LOT #41Q7415K)

IMDM (+ L-Glut, HEPES), Gibco #21980-032

MEM non-essential amino acids (100X), Gibco #11140-035

Methanol, Carl Roth #8388.5

Pancoll human, Density: 1.077 g/ml, PAN-Biotech #P04-60500

Paraformaldehyde (PFA), Sigma Aldrich #8.18715

Penicillin/Streptomycin (Pen/Strep), Gibco #15140-122

Sodium pyruvate (100 mM), Gibco #11360-070

Tween 20, Carl Roth #9127.1

3.1.2. Media and buffers

Table 3: Composition of media and buffers used.

Freezing medium	FBS 10% DMSO
PBMC culture medium	IMDM 10% FBS 1% Pen/Strep
B cell culture medium	IMDM 10% FBS 1% Pen/Strep 1 mM Sodium Pyruvate 1% MEM-non essential amino acids 0.055 mM β-mercaptoethanol
ELISA wash buffer	PBS 0.05% Tween 20
ELISA assay buffer (solely for IgG ELISA)	ELISA wash buffer 0.1% BSA
FACS staining buffer	DPBS 2% FBS
FACS blocking buffer	DPBS 2% BSA
FACS fixation buffer	FACS staining buffer 2% PFA

Annexin V Binding Buffer, Biolegend #422201

BD FACS Permeabilizing Solution 2 (10x), BD Biosciences #340973

3.1.3. Antibodies for flow cytometry

Table 4: Panel description and antibody specifications for flow cytometric analysis of PBMCs.

Panel	Antigen/ Product	Clone	Fluoro-chrome	Company	Product #	Dilution
D_1, D_3 and D_5 DR in PBMC subtypes	Zombie NIR	-	-	Biolegend	423106	1/1000
	CD3	UCHT1	PerCP	Biolegend	300428	1/200
	CD56	NCAM16.2	BV421	BD	562751	1/100
	CD19	HIB19	BV510	Biolegend	302242	1/200
	CD14	M5E2	BV650	Biolegend	301836	1/400
	D_1 DR	Polyclonal (rabbit)	FITC	Bioss	bs-10610R-FITC	1/50
	D_3 DR	Polyclonal (rabbit)	Cy5	Bioss	bs-1743R-Cy5	1/100
	D_5 DR	#889022	PE	R&D Systems	FAB82861P	1/100
D_2 DR in PBMC subtypes	Zombie NIR	-	-	Biolegend	423106	1/1000
	CD3	UCHT1	PerCP	Biolegend	300428	1/200
	CD56	NCAM16.2	BV421	BD	562751	1/100
	CD19	HIB19	BV510	Biolegend	302242	1/200
	CD14	M5E2	BV650	Biolegend	301836	1/400
	D_2 DR	Polyclonal (rabbit)	-	LSBio	LS-A1405	1/200
	Donkey anti-rabbit	Poly4064	PE	Biolegend	406421	1/400
D_4 DR in PBMC subtypes	Zombie NIR	-	-	Biolegend	423106	1/1000
	CD3	UCHT1	PerCP	Biolegend	300428	1/200
	CD56	NCAM16.2	BV421	BD	562751	1/100
	CD19	HIB19	BV510	Biolegend	302242	1/200
	CD14	M5E2	BV650	Biolegend	301836	1/400
	D_4 DR	Polyclonal (rabbit)	-	Biorbit	orb39453	1/200
	Donkey anti-rabbit	Poly4064	PE	Biolegend	406421	1/400
TH in PBMC subtypes	Zombie NIR	-	-	Biolegend	423106	1/1000
	CD3	UCHT1	PerCP	Biolegend	300428	1/200
	CD56	NCAM16.2	BV421	BD	562751	1/100
	CD19	HIB19	BV510	Biolegend	302242	1/200
	CD14	M5E2	BV650	Biolegend	301836	1/400
	TH	Polyclonal (rabbit)	AF647	Bioss	bs-0016R-A647	1/100
D_1-like receptors in B cell subsets	Zombie NIR	-	-	Biolegend	423106	1/1000
	CD19	HIB19	BV510	Biolegend	302242	1/200
	TCRα/β	IP26	PE/Cy5	Biolegend	306710	1/400

	IgD	IA6-2	PE	Biolegend	348204	1/800
	CD27	M-T271	PE/Cy7	Biolegend	356412	1/400
	CD38	HB-7	BV650	Biolegend	356620	1/400
	D_1 DR	Polyclonal (rabbit)	FITC	Bioss	bs-10610R-FITC	1/50
	D_5 DR	#889022	AF405	R&D Systems	RAB82861V	1/100
D_1-like receptors in T cell subsets	Zombie NIR	-	-	Biolegend	423106	1/1000
	CD4	OKT4	BV510	Biolegend	317444	1/100
	TCRα/β	IP26	PE/Cy5	Biolegend	306710	1/400
	CXCR5	J252D4	PE/Cy7	Biolegend	356924	1/400
	CD45RA	HI100	PE	Biolegend	304108	1/800
	D_1 DR	Polyclonal (rabbit)	FITC	Bioss	bs-10610R-FITC	1/50
	D_5 DR	#889022	AF405	R&D Systems	RAB82861V	1/100
Activation + apoptosis of PBMC subtypes	Zombie NIR	-	-	Biolegend	423106	1/1000
	CD3	UCHT1	PerCP	Biolegend	300428	1/200
	CD56	NCAM16.2	BV421	BD	562751	1/100
	CD19	HIB19	BV510	Biolegend	302242	1/200
	CD14	M5E2	BV650	Biolegend	301836	1/400
	CD69	FN50	PE	Biolegend	310906	1/200
	Annexin V	-	FITC	Biolegend	640906	1/400
Analysis of B cell proliferation + differentiation in co-cultures with T cells	Zombie NIR	-	-	Biolegend	423106	1/1000
	CFDA-SE	-	-	Thermo Fisher	V12883	-
	CD4	OKT4	BV421	Biolegend	317434	1/200
	CD27	M-T271	PE/Cy7	Biolegend	356412	1/400
	CD38	HB-7	BV650	Biolegend	356620	1/400
Analysis of B cell proliferation + differentiation in PBMC cultures	Zombie NIR	-	-	Biolegend	423106	1/1000
	CFDA-SE	-	-	Thermo Fisher	V12883	-
	CD19	HIB19	BV421	Biolegend	302234	1/200
	CD27	M-T271	PE/Cy7	Biolegend	356412	1/400
	CD38	HB-7	BV650	Biolegend	356620	1/400
Analysis of Ki-67 expression in B cells	CD3	UCHT1	PerCP	Biolegend	300428	1/200
	CD19	HIB19	BV510	Biolegend	302242	1/200
	Ki-67	Ki-67	PE	Biolegend	350503	1/50
Analysis of ERK and CREB phosphorylation in B cells	CD3	UCHT1	FITC	Biolegend	300528	1/200
	CD19	HIB19	BV510	Biolegend	302242	1/200
	ERK1/2 (pT202/pY204)	20A	AF647	BD Phosflow	612593	1/50
	CREB (pS133) / ATF-1 (pS63)	J151-21	PE	BD Phosflow	558436	1/50

Table 5: Panel description and antibody specifications for flow cytometric sorting of PBMCs.

Panel	Antigen	Clone	Fluorochrome	Company	Product #	Dilution
Sorting of naive and memory B cells	CD19	HIB19	BV510	Biolegend	302242	1/100
	TCRα/β	IP26	PE/Cy5	Biolegend	306710	1/200
	IgD	IA6-2	PE	Biolegend	348204	1/400
	CD27	M-T271	PE/Cy7	Biolegend	356412	1/200
Sorting of T_{FH} cells	CD4	OKT4	BV510	Biolegend	317444	1/200
	TCRα/β	IP26	PE/Cy5	Biolegend	306710	1/200
	CXCR5	J252D4	PE/Cy7	Biolegend	356924	1/200
	CD45RA	HI100	PE	Biolegend	304108	1/400

3.1.4. Stimuli

(-)-Apomorphine hydrochloride, Biomol #16094

A68930 hydrocloride, Tocris #1534

CpG ODN 2006 (CpG), InvivoGen #tlrl-2006-1

Ionomycin (Iono), Merck #407952

Phorbol-12-myristate-13-acetate (PMA), Merck #524400

Ropinirole, Tocris #3680

Staphylococcal enterotoxin B (SEB), Sigma-Aldrich #S4881-1MG

3.1.5. Kits

Human IgG ELISA development kit, Mabtech #3850-1AD-6

- pNPP ELISA Substrate, Mabtech #3652-P10

Human IL-6 ELISA MAX™ Standard Set, Biolegend #430501

Human IL-8 ELISA MAX™ Standard Set, Biolegend #431501

- Coating Buffer A (5x), Biolegend #79008
- Assay Diluent A (5x), Biolegend #78888
- Avidin HRP (1,000x), Biolegend #79004
- Tetramethyl benzidine (TMB) Substrate, Biolegend #421101

LEGENDplex™ Human Th Cytokine Mix and Match Subpanel, Biolegend

- Human Th Cytokine Panel Standard, Cat #740759, Lot #B298184
- Capture Beads A7/IL-6, Cat #740044, Lot #B293967
- Capture Beads A10/IL-10, Cat #740046, Lot #B304331
- Capture Beads B3/TNF-α, Cat #740053, Lot #B304332
- Human Th Cytokine Panel Detection Antibodies, Cat #740758

LEGENDplex™ Human Proinflammatory Chemokine Subpanel

- Human Proinflammatory Chemokine Panel Standard, Cat #740398, Lot #B299198
- Capture Beads B2/CCL3, Cat #740077, Lot #B296423
- Human Proinflammatory Chemokine Panel Detection Antibodies, Cat #740072
- LEGENDplex™ Buffer Set A, Cat #740368

TriCat TM ELISA, Tecan #RE59395

Vybrant™ CFDA-SE Cell Tracer Kit, Thermo Fisher Scientific #V12883

Zombie NIR Fixable Viability Kit, Biolegend #423106 (used in 1/1000 dilution)

3.1.6. Equipment

BD FACSJazz, BD Biosciences

BD LSR Fortessa, BD Biosciences

CASY TT cell counter, OLS OMNI Life Science

Tecan infinite M200 PRO, TECAN

3.1.7. Software

BD FACSDiva, BD Biosciences

LEGENDplex™ Software v8, Biolegend

FlowJo Version 10.3, FlowJo

FlowJo Version 887, FlowJo

GraphPad Prism Version 8, GraphPad

Tecan i-control 1.10 (for infinite reader), TECAN

3.2. Methods

3.2.1. Sample collection

Study was approved by the ethics committee of the Leibniz Research Centre for Working Environment and Human Factors (IfADo) at the TU Dortmund.

HC were recruited at the IfADo. Patients with defined diagnosis of RA, PsA or SpA were recruited in cooperation with Prof. Dr. Jürgen Braun and PD Dr. Xenofon Baraliakos at the Rheumazentrum Ruhrgebiet in Herne. All donors were informed about the study and signed informed consent. Peripheral venous blood was collected in heparinized tubes.

Table 6: Characteristics of HC and rheumatic patients recruited for our cross-sectional study.
*Summary of all subjects whose peripheral blood mononuclear cells were investigated in different analyses; disease scores were transferred on actual basis, non-actual scores were stringently excluded, *n indicates the number of missing actual information at date of blood sampling; ACPA: anti-citrullinated protein antibody, ASDAS: Ankylosing Spondylitis Disease Activity Score, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, DAS28: disease activity score 28, DMARD: disease-modifying anti-rheumatic drug, FFbH: Funktionsfragebogen Hannover, HLA: human leukocyte antigen, SD: standard deviation, RF: rheumatoid factor*

	Healthy controls	*Rheumatoid Arthritis*	*Psoriasis Arthritis*	*Ankylosing Spondylitis*
Total, #	35	30	14	21
Age (years), mean ± SD	39.2 ± 15.8	55.5 ± 15.4	44 ± 12.8	44.5 ± 12.4
Female, # (%)	25 (71)	25 (83)	7 (50)	8 (38)
Years since first diagnosis, mean ± SD	-	7.9 ± 7.1	6 ± 4.7	13 ± 10.8
Patients with bone erosion, # (%)	-	6 (20) *4	1 (7)	-
RF positive patients, # (%)	-	13 (50) *4	-	-
ACPA positive patients, # (%)	-	7 (64) *19	-	-
HLA-B27 positive patients, # (%)	-	-	-	9 (90) *11
DAS28, mean ± SD	-	2.9 ± 1.1 *22	2.1 ± 0.8 *7	-
FFbH, mean ± SD	-	68 ± 24 *19	83 ± 20 *6	-
ASDAS, mean ± SD	-	-	1.7 ± 0.5 *11	2.2 ± 0.8 *11
BASDAI, mean ± SD	-	-	2.8 ± 1.7 *11	4.8 ± 2.1 *11
BASFI, mean ± SD	-	-	2 ± 1 *11	4.3 ± 2.7 *11
Receiving corticosteroids, # (%)	-	13 (48) *3	2 (14)	1 (5) *1
Receiving non-biologic DMARD, # (%)	-	14 (52) *3	12 (86)	2 (10) *1
Receiving biologic DMARD, # (%)	-	16 (59) *3	8 (57)	19 (95) *1

All analyses were performed with regard to biologic treatment. RA patients treated with rituximab almost completely lacked $CD19^+$ B cells. Therefore, data on B cells was excluded in these cases.

3.2.2. Cell biology

Isolation of PBMCs

PBMCs were isolated by Ficoll density gradient centrifugation. Blood was layered onto Pancoll and centrifuged for 25 min at 20 °C, 1130 x g with deceleration set to 0. The PBMC-containing interphase was transferred to a new tube and washed two times with DPBS. Unless stated otherwise PBMCs were resuspended in FBS with 10% DMSO, gradually frozen to -80 °C and transferred to liquid nitrogen for long-term preservation.

Thawing of PBMCs for batched analysis and experiments

PBMCs were thawed in a 37 °C water bath until some ice in suspension remained. Cells were then directly transferred into 10 ml DPBS and centrifuged at 525 x g, RT for 5 min. Supernatant was aspirated and pellet was resuspended in defined volume of DPBS or medium for determination of cell number with the CASY TT cell counter.

Cell culture

Short-term stimulation of PBMCs

PBMCs were resuspended in PBMC culture medium and seeded at a density of $0.25*10^6$ cells per well of a 96-well round bottom plate. Cells were rested for 2 h at 37 °C, 5% CO_2. Either D_1-like receptor agonist A68930, D_2-like receptor agonist ropinirole or pan-DR agonist apomorphine was added directly to the wells at indicated concentrations. Concentrations were selected based on EC_{50} values for respective receptors. Stimulation with PMA/Iono (1.25 nM and 0.125 µg/ml, respectively) served as control. After 24 h cells were analyzed for CD69 expression and Annexin V binding by flow cytometry as described in subsequent section.

T cell-independent B cell stimulation

Protocol was adapted from Marasco et al. (2017). PBMCs were stained with CFDA-SE as described in subsequent section (see p. 38, 3rd paragraph). Afterwards cells were resuspended in B cell culture medium. $0.25*10^6$ CFSE-stained PBMCs were seeded per well of a 96-well round bottom plate and stimulated with CpG ODN 2006 (0.35 µM) and indicated concentrations of D_1-like receptor agonist A68930. Cells were cultured for 6 days at 37 °C, 5% CO_2. Supernatants were stored at -80 °C for further analysis. B cell proliferation and differentiation was analyzed by flow cytometry as described in subsequent section.

T cell-dependent B cell stimulation

Protocol was adapted from Gao et al. (2018). Freshly isolated PBMCs were counted and split into two. At least $22*10^6$ PBMCs were stained per sorting panel (listed in Table 5) for 20 min at 4 °C. Cells were sorted by Dr. Maren Claus on BD FACSJazz equipped with a blue (488 nm) and yellow-green (561 nm) laser. Numbers of sorted cells are depicted in Figure 5 A.

B cells were stained with CFDA-SE as described subsequently (see p. 38, 3rd paragraph). Cell numbers were determined thereafter and are shown in Figure 5 B. Naive $CD19^+TCR\alpha/\beta^-IgD^+CD27^-$ (20,000 cells/well) and memory $CD19^+TCR\alpha/\beta^-CD27^+$ B cells (15,000 cells/well) were either cultured alone or together with $TCR\alpha/\beta^+CD19^-CD45RA^-CXCR5^+$ T_{FH} cells (ratio 1:1) under stimulation with staphylococcal enterotoxin B (SEB, 100 ng/ml) and indicated concentrations of D_1-like receptor agonist A68930. Cells were cultured for 6 days at 37 °C, 5% CO_2. B cell proliferation and differentiation was analyzed by flow cytometry.

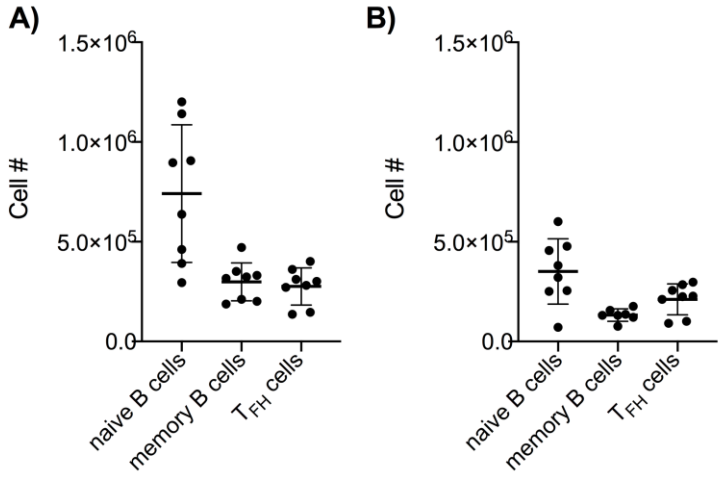

Figure 5: Obtained cell numbers for T cell-dependent B cell stimulation in vitro.
A) Cell yield after fluorescence associated cell sorting with BD FACSJazz. Within the first sort naive B cells were identified as $CD19^+TCR\alpha/\beta^-IgD^+CD27^-$, and memory B cells comprised both non-switched and class-switched memory B cells $CD19^+TCR\alpha/\beta^-CD27^+$. T_{FH} cells were identified as $TCR\alpha/\beta^+CD19^-CD45RA^-CXCR5^+$ within the second sort. B) Numbers of B and T_{FH} cells after CFDA-SE staining or resting on ice, respectively, used for culture. Both graphs show mean with SD, points represent individual samples.

3.2.3. Flow cytometric analysis

Samples were analyzed on a BD LSR Fortessa. At least 100,000 events were recorded in the live gate, whenever possible. Data were analyzed with FlowJo Version 10.3. FlowJo Version 887 was used for analysis of B cell proliferation. For analysis of these multi-color panels, gates were set based on appropriate fluorescence minus one (FMO) controls that include all antibodies of interest except one.

Extracellular staining

$0.5*10^6$ cells were dispensed per well of a 96-well V-bottom plate and kept on ice during the whole staining procedure. First, cells were incubated with Zombie NIR Fixable Viability dye in DPBS for 20 min at 4 °C. Cells were washed with DPBS and next, unspecific binding was blocked by incubation with FACS blocking buffer (2% BSA in DPBS) for 20 min at 4 °C. After washing, cells were stained with optimally diluted antibodies in FACS buffer (2% FBS in DPBS) for 20 min at 4 °C. Different panels are listed in Table 4. After another washing step with FACS buffer, cells were immediately analyzed or further processed.

Intracellular staining

For intracellular staining of D_2 DR, D_4 DR and TH, extracellularly stained samples were fixed with FACS fixation buffer (2% PFA in FACS buffer) for 10 min at RT. Afterwards cells were washed FACS buffer and then permeabilized with BD FACS Permeabilizing Solution 2 for 10 min at RT. Cells were again washed with FACS buffer. Unspecific intracellular binding was blocked in an additional blocking step with FACS blocking buffer for 20 min at RT. Cells were washed with FACS buffer and next stained intracellularly for 20 min at RT. After another washing step with FACS buffer, unconjugated primary antibodies were labeled with a secondary PE-labeled donkey anti-rabbit antibody during 20 min incubation at RT. Finally, cells were washed with FACS buffer and immediately analyzed.

Annexin V staining

To identify cells undergoing apoptosis, extracellularly stained PBMCs were resuspended in Annexin V binding buffer and stained with Annexin V-FITC for 15 min at RT. Equal volume Annexin V binding buffer was added afterwards. Cells were analyzed within 1 h.

CFDA-SE staining prior to culture

Proliferation was analyzed by CFSE-dye dilution. Cells were first stained with the Vybrant CFDA-SE Cell Tracer Dye. $1*10^6$ cells were resuspended in 1 ml DPBS / 0.5 µM CFDA-SE and stained for 30 min at 37 °C, 5% CO_2. Excess staining was blocked with medium containing 32.5% FBS. After centrifugation cells were resuspended in pre-warmed medium and used for culture in desired concentration.

Analysis of intracellular signaling

After stimulation with selective agonists or antagonists GPCRs respond within 5-30 min (Beaulieu 2016). To analyze intracellular signaling in B cells after D_1-like receptor stimulation of PBMCs, Phosflow technique was applied.

$0.5*10^6$ PBMCs were seeded per well of a 96-well round bottom plate. PBMCs were rested for 2 h at 37 °C, 5% CO_2. D_1-like receptor agonist A68930 was added directly to the wells at indicated concentrations and for indicated times. PMA (40 nM) served as positive control. Cells were fixed with 2% PFA in culture medium for 10 min at 37 °C 5% CO_2. Afterwards cells were transferred to a 96-well V-bottom plate and washed with FACS buffer. Subsequently, cells were stained extracellularly for 20 min at RT. Cells were again washed with FACS buffer and, depending on investigated (phospho-) protein, either permeabilized with BD FACS Permeabilizing Solution 2 for 10 min at RT or with methanol for 30 min at 4 °C. Cells were washed with FACS buffer and next, cells were stained intracellularly with antibodies against Ki-67 or pERK and pCREB for 20 min at RT. Both panels are listed in Table 4. Afterwards cells were washed with FACS buffer and immediately analyzed.

3.2.4. Immunological analysis

TriCat TM ELISA

For quantification of catecholamine content in immune cells, $1*10^6$ freshly isolated PBMCs were pelleted by centrifugation at 400 x g, 4 °C for 10 min and frozen at -80 °C. Further, Gabi Baumhoer from the department of analytical chemistry of the IfADo processed samples and performed TriCat ELISA. Briefly, cell pellets were lysed in 100 µl 0.1 M $HClO_4$ with 100 µM ascorbic acid. Thereafter, catecholamines were extracted and analyzed as described by the manufacturer. Limits of detection were 4, 8 and 20 pg/ml for DA, NE and Epi, respectively.

ELISA

To assess the effect of D_1-like receptor stimulation on IgG secretion of B cells the human IgG ELISA development kit from Mabtech was used. Assay was performed according to guidelines, except downscaling the volume in all steps to the half. Samples were measured in duplicate and optimal dilutions of supernatants were determined in preceding assays. pNPP was used as substrate. Absorbance at 405 nm was measured after 30 min.

For quantification of secreted IL-6 and IL-8 human IL-6 ELISA MAX™ and human IL-8 ELISA MAX™ Standard Sets from Biolegend were used, respectively. Assays were performed as described by the manufacturer, except downscaling of volume in all steps to the half. Samples were measured in duplicate and optimal dilutions of supernatants were determined in preliminary assays. TMB was used as substrate. Reactions were stopped with 2N H_2SO_4 after 20 min. Absorbance was determined at 570 and 450 nm. Absorbance at 570 nm was subtracted from absorbance at 450 nm.

Cytometric bead array

Supernatants from T cell-independent B cell stimulations were analyzed by cytometric bead arrays for secreted IL-6, IL-10, TNF-α and CCL3 within the LEGENDplex™ Human Th Cytokine Panel and Human Proinflammatory Chemokine Panel, respectively. Assays were performed as described in respective protocols, except downscaling of volume in all steps to a third. Samples were measured in duplicate and read on BD LSR Fortessa. Data was analyzed with BioLegend's LEGENDplex™ Software v8.

3.2.5. Statistical analysis

Statistical analysis was performed with Prism 8 software (GraphPad).

Numbers of investigated subjects are indicated in figures for each experiment. In descriptive analysis, outliers were identified and removed by iterative Grubbs' test ($\alpha = 0.05$). Two-way analysis of variance (ANOVA) or mixed-effects analysis with Geisser-Greenhouse correction was performed, depending on missing values, followed by Bonferroni's multiple comparisons

test. Pearson correlation with linear regression was used for correlation of expression data with clinical parameters. Data from *in vitro* stimulations are presented as absolute changes to control due to inter-individual differences. Here, unpaired T test was additionally used for analysis of control values between HC and RA. P values as indicated: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$, ****$p < 0.0001$.

4. Results

4.1. Study of dopamine receptor expression in peripheral immune cells

To study the expression of D_1-D_5 DR on protein level and directly compare the expression levels between peripheral T cells, B cells, NK cells and monocytes, PBMCs were investigated in a comprehensive approach by multi-color flow cytometry.

Since the homology between the different DRs is up to 80% (Beaulieu and Gainetdinov 2011) antibodies detecting epitopes specific for each DR are limited. Here, the five DR were either stained extracellularly with fluorochrome-conjugated antibodies (D_1, D_3, D_5) or intracellularly with unconjugated antibodies against cytoplasmic regions of the receptors (D_2, D_4) and detected via secondary PE-labeled antibodies.

To detect disease-related alterations in expression of D_1-D_5 DR, heterogeneous groups of rheumatic patients were recruited for cross-sectional study and compared with age- and sex-matched HC. Table 7 (see appendix) summarizes the characteristics of investigated HC and RA, PsA and SpA patients for cross-sectional study on expression of D_1-D_5 DR in peripheral immune cells.

4.1.1. T cells

Expression of D_1-D_5 DR in T cells was determined as shown in Figure 6 A. After doublets and dead cells were excluded, $CD3^+CD56^-$ T cells were gated in the lymphocyte population. All five DR were detected on peripheral $CD3^+$ T cells of HC and rheumatic patients (Figure 6 B). Within $CD3^+$ T cells, D_2-like receptors were expressed at higher frequencies than D_1-like receptors. More specifically, D_2 and D_4 DR showed the highest frequencies, followed by D_3, D_1 and D_5 DR. Donor-dependent variations in expression of each single DR were present. No differences in expression of D_1-D_5 DR were detected between HC and rheumatic patients.

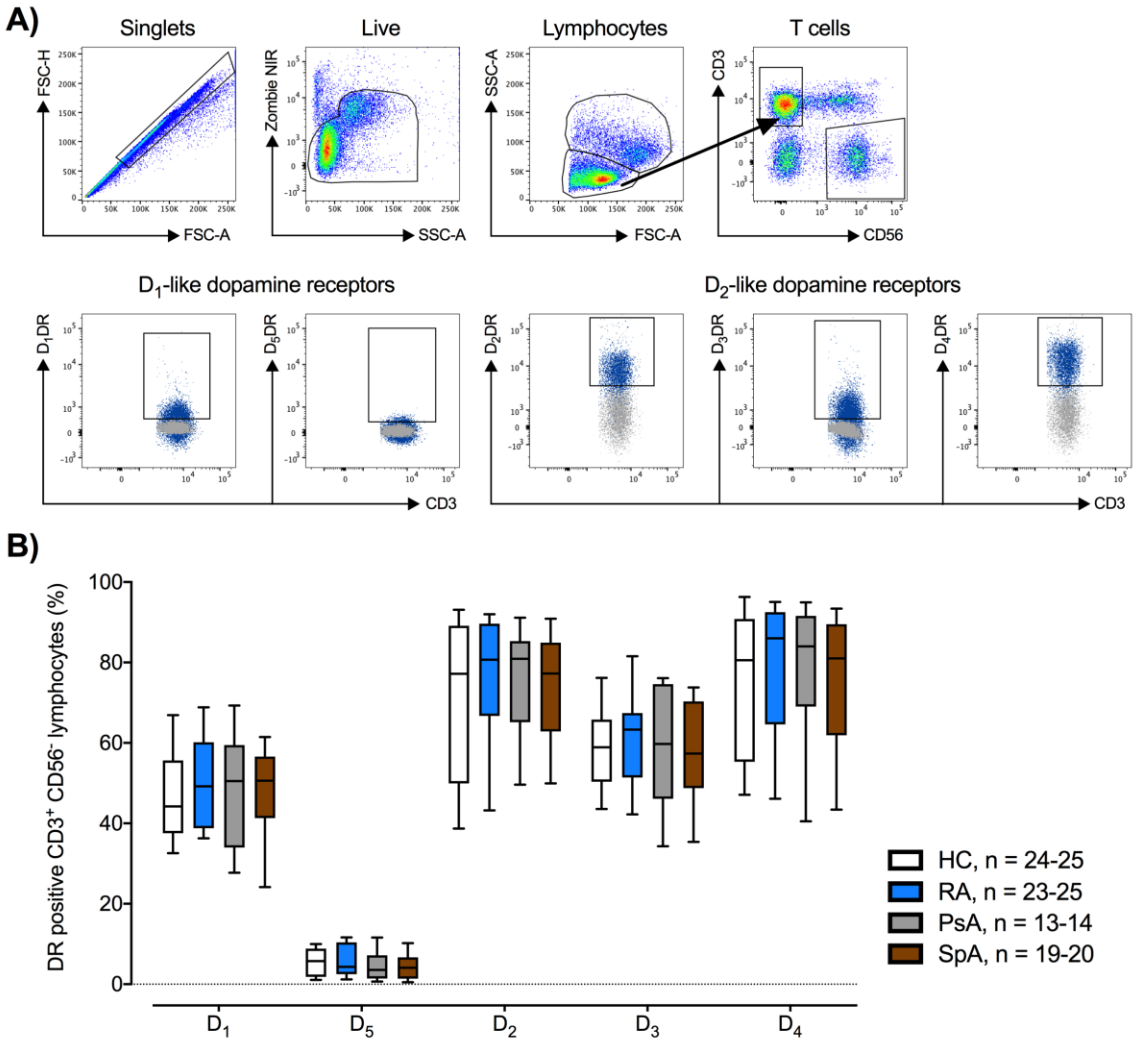

Figure 6: D_1-D_5 DR are expressed on peripheral $CD3^+CD56^-$ T cells.
PBMCs obtained from HC, RA, PsA and SpA patients were analyzed for D_1-D_5 DR expression by flow cytometry. A) Gating strategy to determine frequency of T cells expressing D_1-D_5 DR. First, doublets and dead cells were excluded in FSC-A/FSC-H plot and based on positivity of Zombie NIR staining, respectively. Lymphocytes were identified based on size and granularity in FSC-A/SSC-A plot. T cells were identified as $CD3^+CD56^-$ cells. Representative full staining of D_1-like and D_2-like DR in T cells with respective FMO controls in grey are shown below. B) Quantification of DR expression in T cells of HC, RA, PsA and SpA patients. Subjects were age- and sex-matched. Outliers were identified and removed by Iterative Grubbs' test ($\alpha = 0.05$) as indicated in the sample size. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Bonferroni's multiple comparisons test was used to compare D_1-D_5 DR expression between groups. DR: dopamine receptor, FMO: fluorescence minus one, HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis

4.1.2. B cells

B cells were identified as $CD19^+$ lymphocytes within the same staining panel (Figure 7 A). D_1-D_5 DR were expressed on peripheral B cells (Figure 7 B). As for T cells, $CD19^+$ B cells presented higher frequencies of cells expressing D_2-like receptors than D_1-like receptors. Again, frequencies of D_2 and D_4 DR expressing cells were highest, followed by D_3, D_1 and D_5 DR. Expression of D_1-D_5 DR varied between the investigated subjects. While the expression of D_2 and D_3 DR only tended to be elevated in peripheral B cells of RA compared to HC, the frequency of D_1 DR expressing $CD19^+$ B cells was significantly increased in RA ($p = 0.004$), but not in PsA or SpA.

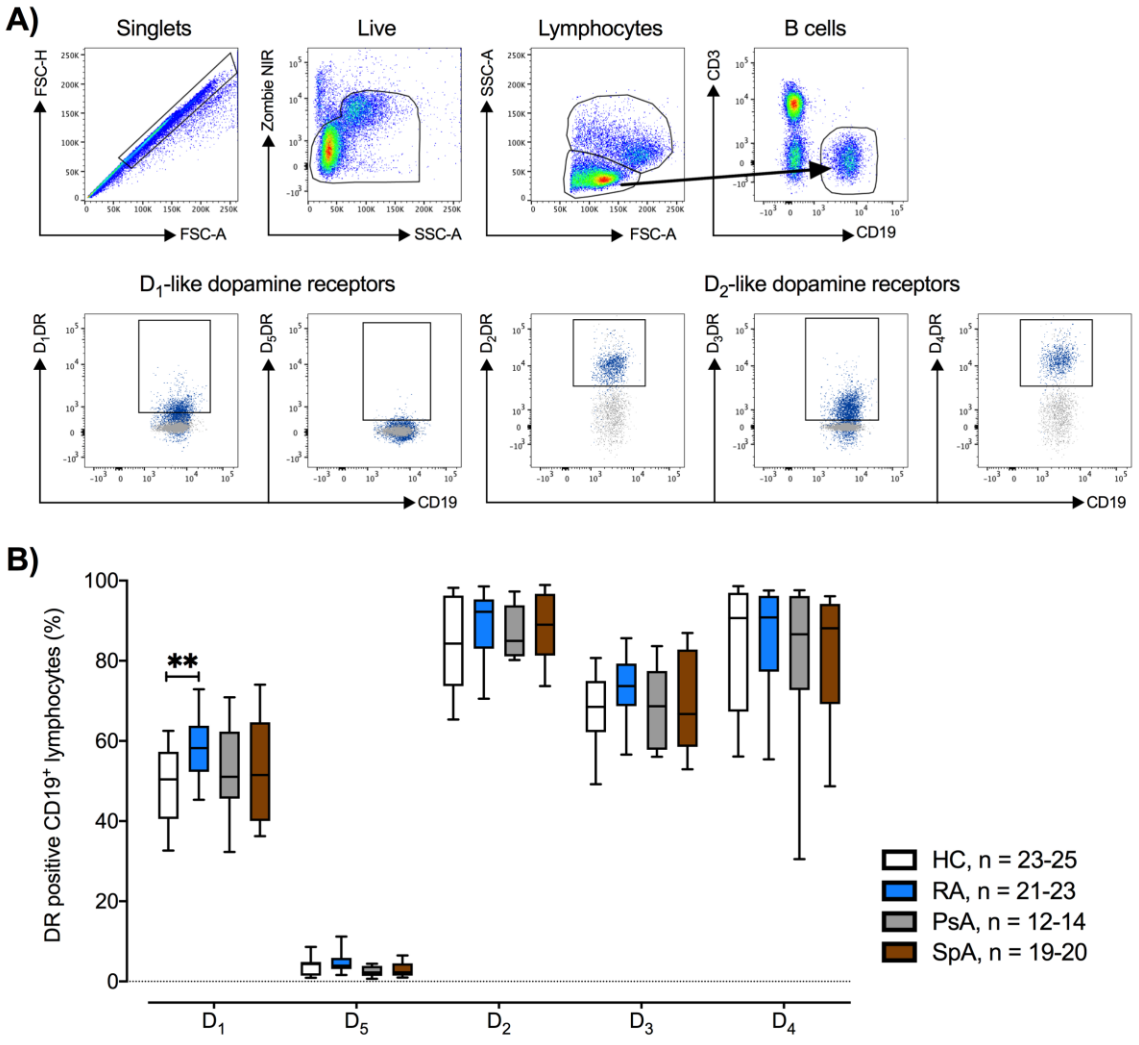

Figure 7: D_1-D_5 DR are expressed on peripheral $CD19^+$ B cells.
*PBMCs obtained from HC, RA, PsA and SpA patients were analyzed for D_1-D_5 DR expression by flow cytometry. A) Gating strategy to determine frequency of B cells expressing D_1-D_5 DR. As in the previous figure, doublets and dead cells were excluded in FSC-A/FSC-H plot and based on positivity of Zombie NIR staining, respectively. Lymphocytes were identified based on size and granularity in FSC-A/SSC-A plot. B cells were identified as $CD19^+CD3^-$ cells. Representative full staining of D_1-like and D_2-like DR in B cells with respective FMO controls in grey are shown below. B) Quantification of DR expression in B cells of HC, RA, PsA and SpA patients. Data from rituximab-treated patients were excluded herein. Subjects were age- and sex-matched. Outliers were identified and removed by Iterative Grubbs' test ($\alpha = 0.05$) as indicated in the sample size. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni's multiple comparisons test was used to compare D_1-D_5 DR expression between groups (**$p \leq 0.01$). DR: dopamine receptor, FMO: fluorescence minus one, HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis*

By analyzing the DR expression in peripheral B cells in relation to subject characteristics gender differences were detected (Figure 8 A). Particularly, female HC revealed lower frequencies of D_1 DR expressing peripheral B cells compared to male HC (p = 0.0109). Moreover, B cells of female RA patients presented a significantly elevated D_1 DR expression compared to female HC (p = 0.0001). The frequency of D_1 DR expressing B cells did not correlate with the age of female donors and patients (Figure 8 B). In contrast, frequency of D_1 DR expressing B cells was significantly rising with disease duration of female RA patients (Figure 8 C, p = 0.0035, r = 0.65). Notably, age and disease duration of female RA patients did not correlate. Since DAS28 and FFbH scores were not always available at date of blood sampling, some data could not be correlated and therefore n-numbers were limited. Within the available data, frequency of D_1 DR expressing B cells did not correlate with available DAS28 score (Figure 8 D). However, the frequency of D_1 DR expressing B cells tended to inversely correlate with functional capacity of female RA patients (Figure 8 E, p = 0.0934, r = -0.53). Within the study population, elevated D_1 DR expression was neither dependent on RF-seropositivity or erosiveness of disease, nor dependent on therapy (data not shown).

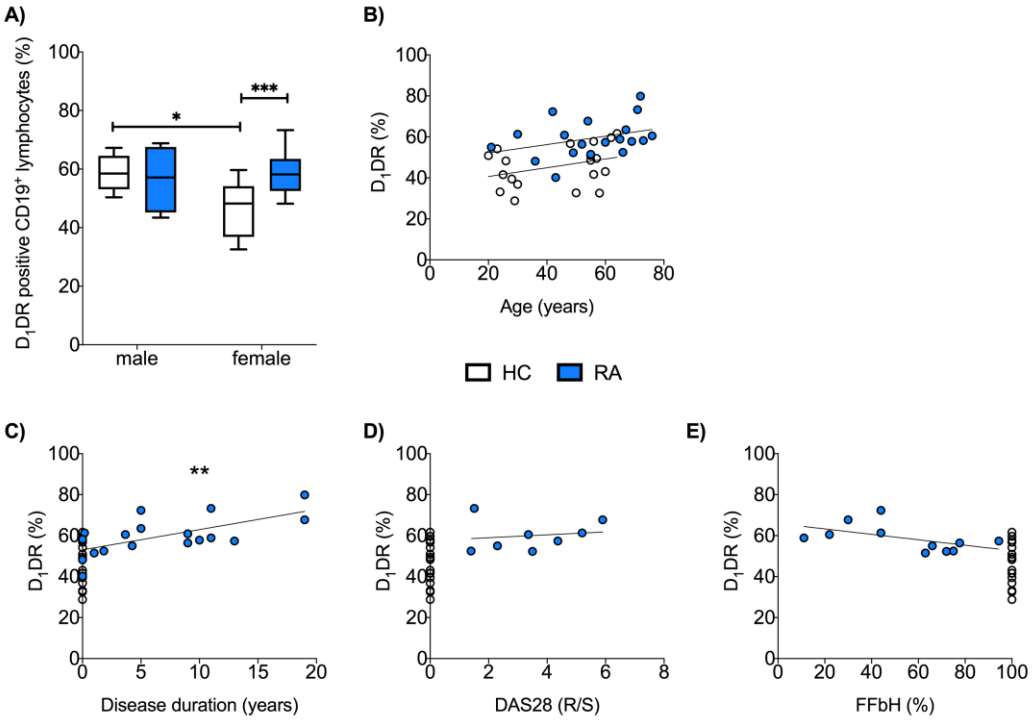

Figure 8: D_1 DR expression on CD19⁺ B cells is specifically altered in female RA patients correlating with disease duration.

*Expression data was correlated with provided patient information. Differential expression of D_1 DR on B cells was analyzed regarding sex (A). Data from female HC (white, n = 19) and RA patients (blue, n = 8-19) was further correlated with age (B), disease duration (C), DAS28 (D) and FFbH score (E). Data from rituximab-treated patients displaying an increased disease duration were excluded herein. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni's multiple comparison test was used to compare D_1 DR expression between sexes and groups (*P ≤ 0.05, ***P ≤ 0.001). Pearson correlation analysis with linear regression was used to analyze D_1 DR expression in relation to clinical parameters (**P ≤ 0.01). DAS28: disease activity score 28 (high score indicating severe disease, maximum score: 10), DR: dopamine receptor, FFbH: Funktionsfragebogen Hannover (measuring patients' functional capacity in percent), HC: healthy control, RA: rheumatoid arthritis*

4.1.3. NK cells

NK cells were identified as $CD56^{+}CD3^{-}$ cells within the lymphocyte population (Figure 9 A). Compared to T and B cells, NK cells expressed higher frequencies of D_1-D_5 DR (Figure 9 B). Additionally, the balance between D_1-like and D_2-like receptors was less distinct within $CD56^{+}$ NK cells. In general, D_3 DR was expressed at highest frequencies followed by D_1 DR, whereas expression levels of D_2 and D_4 DR were comparable. Only D_2 and D_4 DR presented variability between donors and patients investigated. While the frequency of NK cells expressing D_1 DR only tended to be decreased in RA compared to HC, the decrease in NK cells expressing D_3 DR was significant, yet small ($p = 0.0493$, mean difference = 1.164).

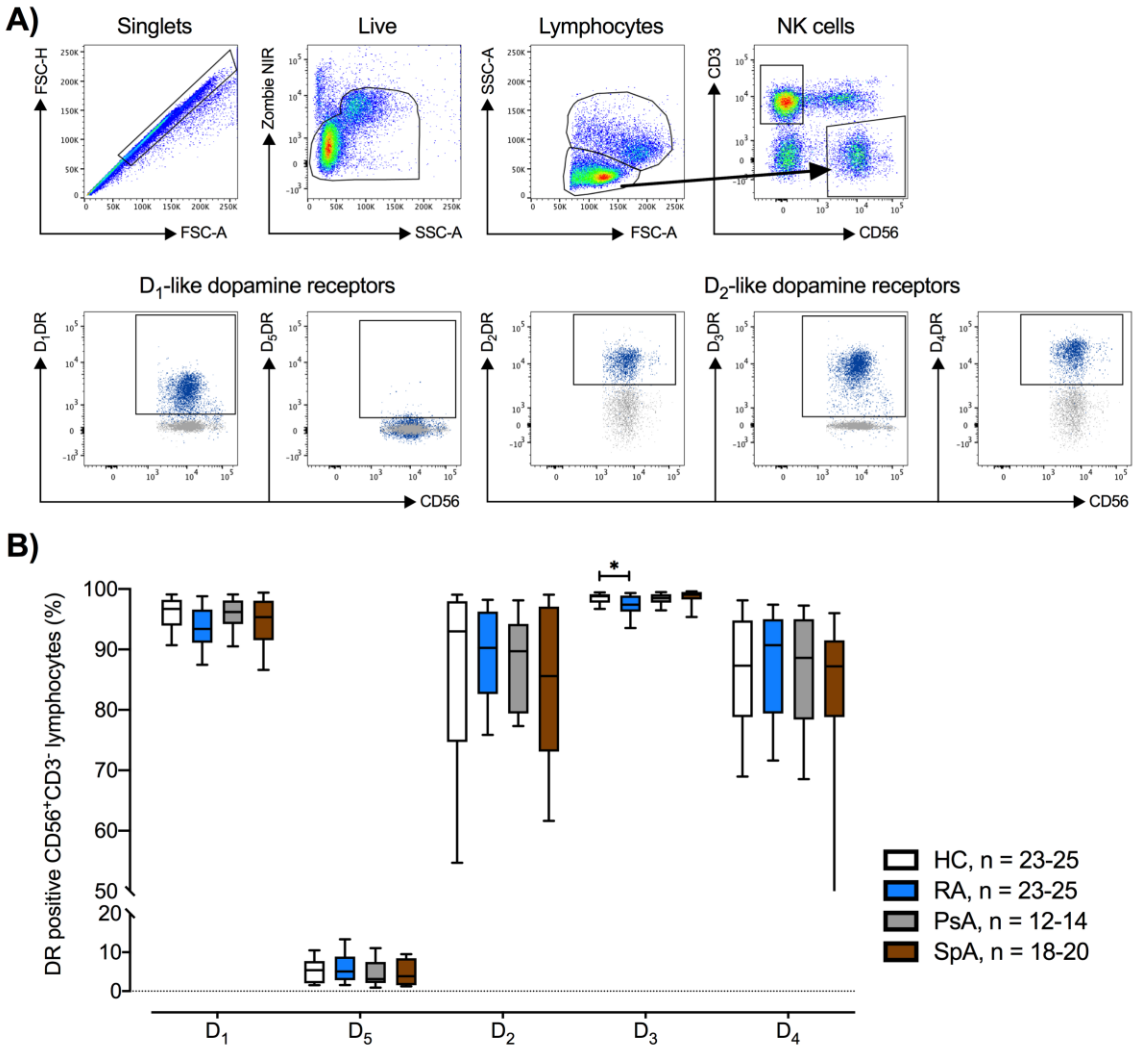

Figure 9: D_1-D_5 DR are expressed on peripheral $CD56^{+}CD3^{-}$ NK cells.
PBMCs obtained from HC, RA, PsA and SpA patients were analyzed for D_1-D_5 DR expression by flow cytometry. A) Gating strategy to determine frequency of NK cells expressing D_1-D_5 DR. As in the previous figures, doublets and dead cells were excluded in FSC-A/FSC-H plot and based on positivity of Zombie NIR staining, respectively. Lymphocytes were identified based on size and granularity in FSC-A/SSC-A plot. NK cells were identified as $CD56^{+}CD3^{-}$ cells. Representative full staining of D_1-like and D_2-like DR in NK cells with respective FMO controls in grey are shown below. B) Quantification of DR expression in NK cells of HC, RA, PsA and SpA patients. Subjects were age- and sex-matched. Outliers were identified and removed by Iterative Grubbs' test ($\alpha = 0.05$) as indicated in the sample size. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Bonferroni's multiple comparisons test was used to compare D_1-D_5 DR expression between groups ($p \leq 0.05$). DR: dopamine receptor, FMO: fluorescence minus one, HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis*

Moreover, as presented in the exemplary gating in Figure 9 A, expression of D_1 and D_3 DR differed between $CD56^{bright}$ and $CD56^{dim}$ NK cells. Further subgating of NK cells based on the surface density of CD56 (Figure 10 A) revealed a significant increase of both D_1 and D_3 DR with NK cell maturation from $CD56^{bright}$ to $CD56^{dim}$ (Figure 10 B and C, overall $p < 0.0001$). While $CD56^{dim}$ cells revealed overall high expression of D_1 and D_3 DR, $CD56^{bright}$ NK cells showed a more variable expression of D_1 and D_3 DR. $CD56^{bright}$ NK cells of SpA patients expressed significantly higher levels of D_1 DR than HC (Figure 10 B, $p = 0.0421$). No difference could be detected between HC and RA or PsA.

Nevertheless, data was correlated with disease parameters of RA patients. Frequency of D_1 DR expressing $CD56^{bright}$ NK cells did not correlate with disease duration (Figure 11 A), but with DAS28 score (Figure 11 B, $p = 0.0033$, $r = 0.826$). Additionally, D_1 DR expression correlated inversely with functional capacity of investigated RA patients (Figure 11 C, $p = 0.0024$, $r = -0.764$). Frequency of $CD56^{bright}$ NK cells expressing D_3 DR did not correlate with clinical parameters (data not shown).

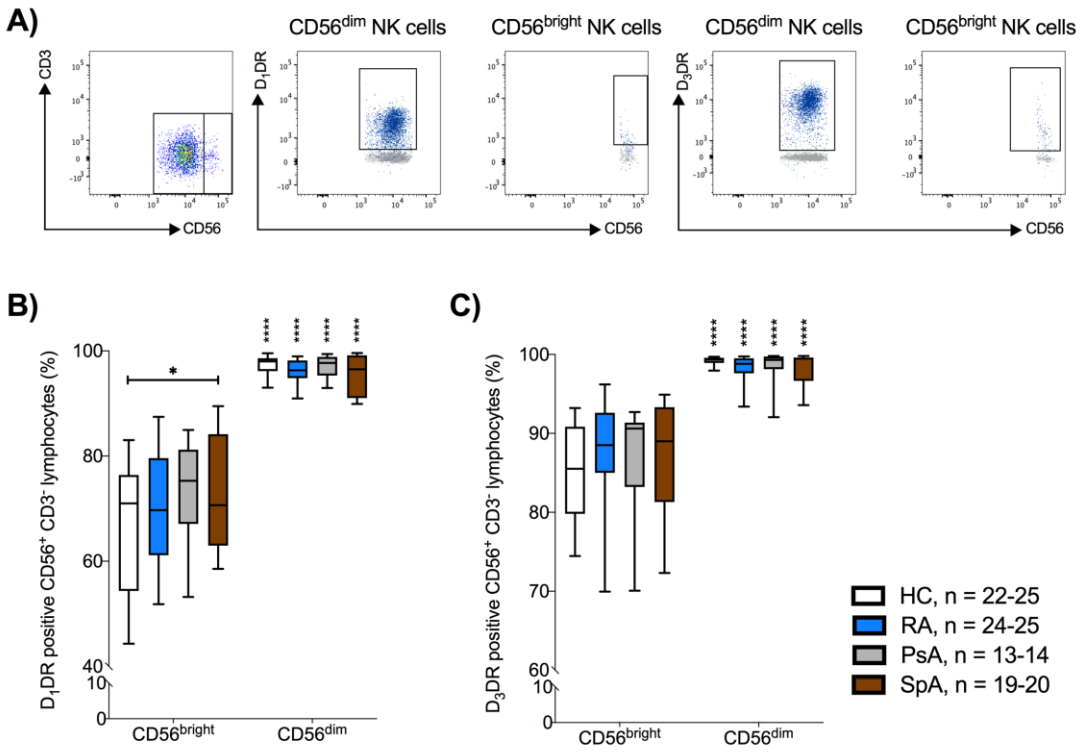

Figure 10: Expression of D_1 and D_3 DR differs between $CD56^{bright}$ and $CD56^{dim}$ NK cells.
A) Gating strategy. $CD56^{+}CD3^{-}$ NK cells were further divided based on surface density of CD56 expression. Representative full staining of D_1 and D_3 DR on $CD56^{dim}$ and $CD56^{bright}$ NK cells with respective FMO controls in grey. Quantification of D_1 (B) and D_3 DR (C) expression on $CD56^{bright}$ and $CD56^{dim}$ NK cells of HC, RA, PsA and SpA patients. Subjects were age- and sex-matched. Outliers were identified and removed by Iterative Grubbs' test ($\alpha = 0.05$) as indicated in the sample size. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Bonferroni's multiple comparisons test was used to compare D_1 and D_3 DR expression between groups and NK cell stages ($*p \leq 0.05$, $****p \leq 0.0001$). DR: dopamine receptor, FMO: fluorescence minus one, HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis

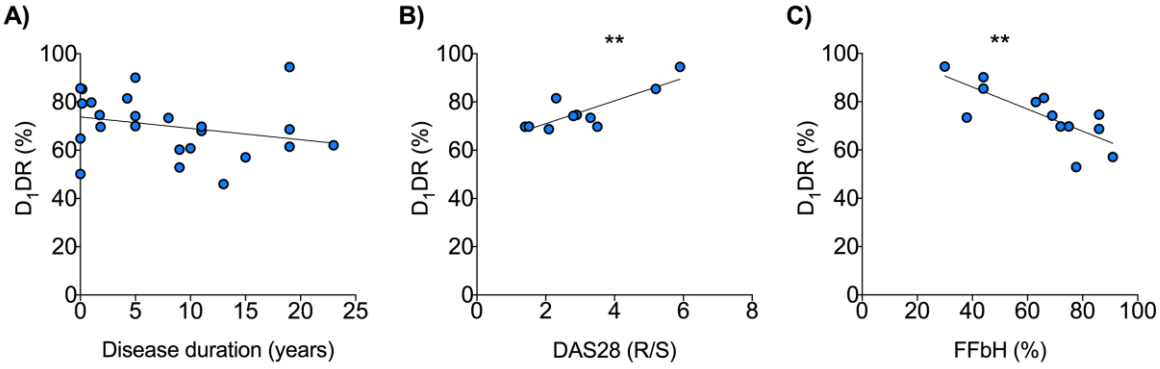

Figure 11: D_1 DR expression on CD56^{bright} NK cells correlates with disease severity and functional impairment in RA.
*Frequency of D_1 DR expressing CD56^{bright} NK cells of RA patients was correlated with disease duration (A), disease activity (B) and functional capacity (C) of RA patients (n = 10-24). Pearson correlation analysis with linear regression was used to analyze D_1 DR expression in relation to clinical parameters (**P ≤ 0.01). DAS28: disease activity score 28 (high score indicating severe disease, maximum score: 10), DR: dopamine receptor, FFbH: Funktionsfragebogen Hannover (measuring patients' functional capacity in percent), RA: rheumatoid arthritis*

4.1.4. Monocytes

As for identification of aforementioned immune cells, doublets and dead cells were first excluded in the gating strategy for monocytes (Figure 12 A). Here, monocytes presented generally higher background staining with Zombie NIR than the lymphocyte population due to their bigger size and thus amount of free amine groups. Further, monocytes were gated based on size and granularity in FSC/SSC plot and by expression of CD14. Expression of DR in CD14$^+$ monocytes was high, except for D_5 DR (Figure 12 B), similar to expression of NK cells and exceeding the frequencies of D_1-D_5 DR positive T and B cells. D_3 DR showed the highest expression in CD14$^+$ monocytes followed by D_1, D_2, D_4, and D_5 DR. Only the expression levels of D_2, D_4, and D_5 DR displayed some variability between subjects. Though, CD14$^+$ monocytes of SpA patients revealed a significant, yet small, increase in D_1 DR expression compared to HC (p = 0.0033, mean difference = 0.4471).

4.1.5. Correlation with subject characteristics and clinical data

The already demonstrated sex-specific difference of D_1 DR expression on B cells in HC as well as female HC and RA was the only sex-difference detected. Expression data did not correlate with the age of investigated subjects (data not shown). All disease-related differences in D_1-D_5 DR expression that were statistically significant were correlated with provided patient information as well as disease parameters as already shown for D_1 DR on B cells. Altered expression of D_3 DR on CD56$^+$ NK cells of RA patients did not correlate with disease duration, DAS28 or FFbH score and it was neither specific for the presence of RF-, ACPA-autoantibodies and erosiveness of disease, nor dependent on therapy (data not shown). Expression of D_1 DR on CD56^{bright} NK cells and CD14$^+$ monocytes of SpA patients was not correlating with disease duration, ASDAS, BASDAI or BASFI score (data not shown). Influence of therapy on D_1 DR expression on CD56^{bright} NK cells and CD14$^+$ monocytes of SpA patients

could not be evaluated statistically because the majority of investigated SpA patients received biologics targeting TNF-α and numbers of patients treated with anti-IL-17, anti-IL-12/23 biologic DMARD or conventional DMARD were limited.

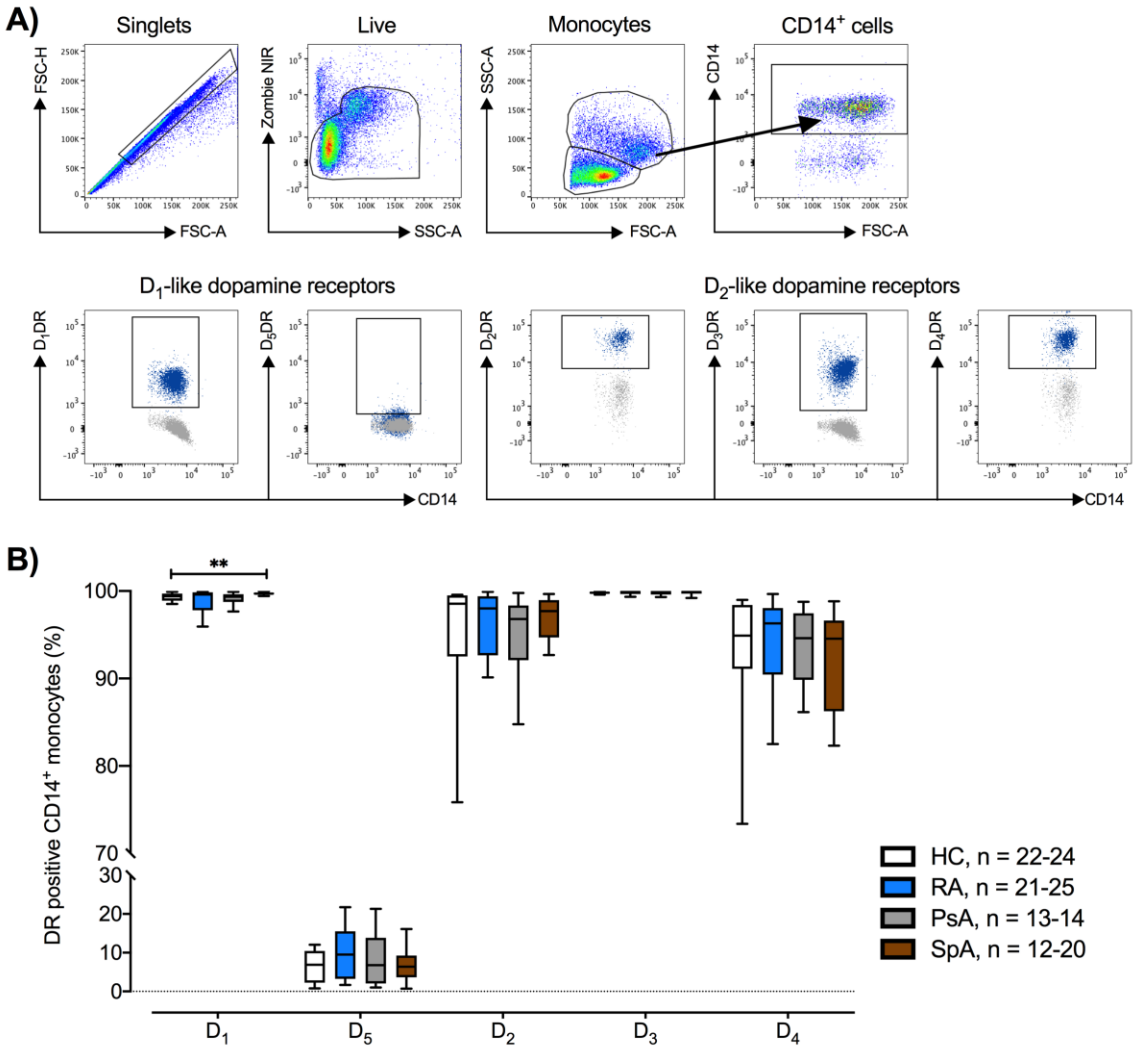

Figure 12: D_1-D_5 DR are expressed on peripheral CD14⁺ monocytes.
*PBMCs obtained from HC, RA, PsA and SpA patients were analyzed for D_1-D_5 DR expression by flow cytometry. A) Gating strategy to determine frequency of monocytes expressing D_1-D_5 DR. As in the previous figures, doublets and dead cells were excluded in FSC-A/FSC-H plot and based on positivity of Zombie NIR staining, respectively. Monocytes were identified based on size and granularity in FSC-A/SSC-A plot and further by surface expression of CD14. Representative full staining of D_1-like and D_2-like DR in monocytes with respective FMO controls in grey are showed below. B) Quantification of DR expression in monocytes of HC, RA, PsA and SpA patients. Subjects were age- and sex-matched. Outliers were identified and removed by Iterative Grubbs' test (α = 0.05) as indicated in the sample size. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni´s multiple comparisons test was used to compare D_1-D_5 DR expression between groups (**p ≤ 0.01). DR: dopamine receptor, FMO: fluorescence minus one, HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis*

4.2. Synthesis of catecholamines by immune cells

To measure the immune cells capability for dopamine synthesis, expression of TH, the rate-limiting enzyme in catecholamine synthesis, was studied by flow cytometry. For this purpose, a fluorochrome-conjugated antibody against TH was used for intracellular staining. Immune cell subsets were identified as shown in previous figures and subsequently TH^+ cells were gated based on FMO control (Figure 13 A). All investigated immune cell subpopulations expressed TH at high frequencies. Monocytes of PsA and SpA patients showed significantly increased expression levels compared to HC, although differences were small (p =0.0298, mean difference = 0.2006 and p = 0.0473, mean difference = 0.1833, respectively).

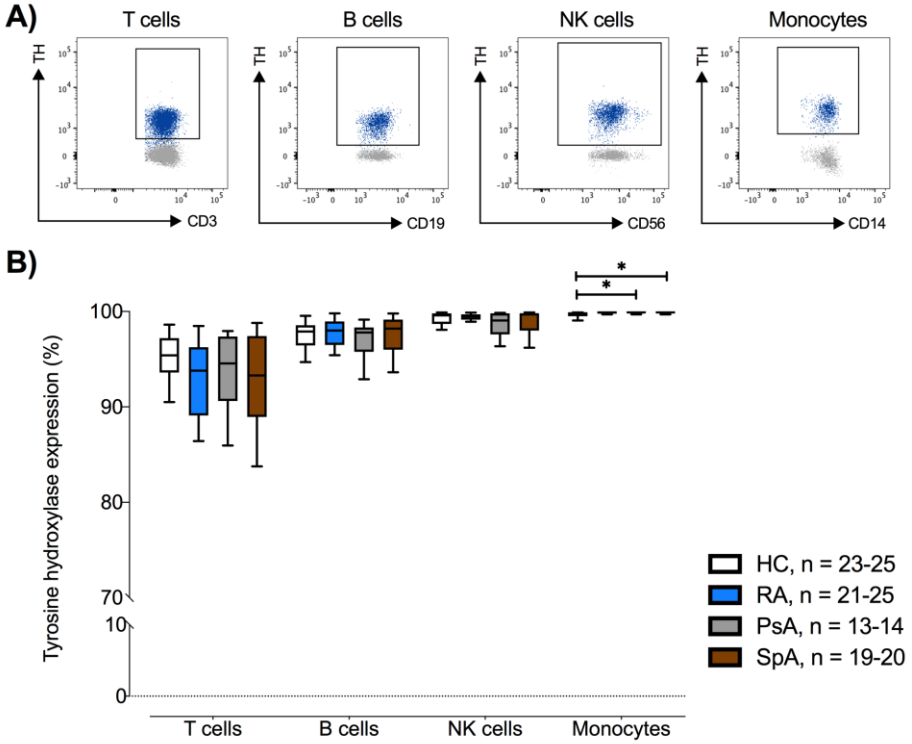

Figure 13: TH as the rate-limiting enzyme of catecholamine synthesis is expressed in peripheral immune cells.
*PBMCs obtained from HC, RA, PsA and SpA patients were analyzed for TH expression by flow cytometry. A) Gating to determine frequency of TH expressing cells. Expression of TH in defined immune cell subpopulations identified as previously shown with FMO controls in grey. B) Quantification of TH in T cells, B cells, NK cells and monocytes of HC, RA, PsA and SpA patients. Subjects were age- and sex-matched. Outliers were identified and removed by Iterative Grubbs' test (α = 0.05) as indicated in the sample size. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni's multiple comparisons test was used to compare TH expression between groups (*p ≤ 0.05). FMO: fluorescence minus one, HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis, TH: tyrosine hydroxylase*

To prove the results on TH expression in immune cells, freshly isolated PBMCs were also analyzed for catecholamine content by TriCat ELISA. Up to this point, analyses were limited to samples from HC and RA patients. Not all analyzed PBMCs exhibited detectable levels of DA, NE or Epi and therefore, individual values are presented in Figure 14. DA was detected exclusively in PBMCs of RA patients indicating a disease-specific difference. NE was detected in most samples analyzed and especially PBMCs of RA revealed huge differences in

concentration. Detectable Epi levels in PBMCs were comparable between HC and RA. Unfortunately, due to the limited number of investigated samples and partially undetectable levels, data was not quantifiable.

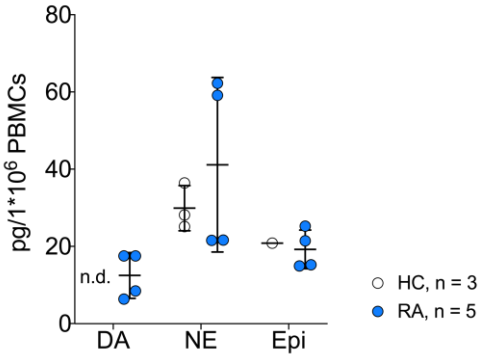

Figure 14: Freshly isolated PBMCs contain catecholamines.
Freshly isolated PBMCs of HC and RA patients were lysed and next, content of DA, NE and Epi was determined by TriCat ELISA. Graph shows individual values with mean and SD. Some values were below limit of detection and thus impeding statistical analysis. Preliminary data. DA: dopamine, Epi: epinephrine, HC: healthy control, n.d.: not detected, NE: norepinephrine, RA: rheumatoid arthritis

4.3. Functional impact of dopaminergic stimulation in peripheral immune cells

After detecting D_1- and D_2-like receptors in peripheral T cells, B cells, NK cells and monocytes and additionally showing their capacity to synthesize DA, functional effect of DR stimulation were investigated. Different studies already found that DA impacts activation and apoptosis of peripheral immune cells (Colombo et al. 2003; Zhu et al. 2017; Keren et al. 2019; Bergquist et al. 1997). However, contribution of D_1-like and D_2-like receptors is not fully understood. To study the respective impact of D_1- and D_2-like receptor stimulation on activation and apoptosis of peripheral immune cells, selective agonists were used. PBMCs from HC as well as RA, PsA and SpA patients (listed in Table 8, see appendix) were stimulated for 24 h *in vitro* with D_1-like receptor specific agonist A68930, D_2-like receptor specific agonist ropinirole or pan-DR agonist apomorphine and thereafter analyzed for CD69 expression and Annexin V binding.

4.3.1. Activation of peripheral immune cells

CD69 is a transmembrane C-type lectin being expressed by various immune cells and increases in response to activation (Ziegler et al. 1994). Its expression was investigated in peripheral T cells (Figure 15 A), B cells (Figure 15 B), NK cells (Figure 15 C) and monocytes (Figure 15 D) after 24 h stimulation of DR.

Generally, frequencies of CD69 expressing immune cells were low in basal state and did not differ between healthy and diseased subjects. Though, especially T cells but also NK cells and monocytes of some SpA patients tended towards higher CD69 expression than HC. Treatment with PMA/Iono induced strong increase in CD69 expression in all immune cell subsets investigated. Irrespective which class of DR was stimulated, expression of CD69 remained

unaltered in peripheral T cells and B cells of HC, RA, PsA and SpA patients. D_1-like receptor stimulation with specific agonist A68930 did also not induce any change of CD69 expression in NK cells and monocytes. Pan-DR stimulation with apomorphine decreased CD69 expression significantly in NK cells of RA patients at 10^{-6} M ($p = 0.0435$) and stimulation of D_2-like receptors with ropinirole increased CD69 expression significantly in monocytes of HC at 10^{-8} M compared to unstimulated control ($p = 0.0414$). However, observed changes in frequencies of CD69 positive cells were small and thus may be functionally irrelevant. Apart from that D_2-like receptor or pan-DR stimulation did not further affect CD69 expression.

4.3.2. Apoptosis of peripheral immune cells

Apoptotic cells can be identified by Annexin V binding (Vermes et al. 1995). Effect of DR stimulation on apoptosis of peripheral T cells (Figure 16 A), B cells (Figure 16 B), NK cells (Figure 16 C) and monocytes (Figure 16 D) was determined after 24 h of DR stimulation.

Frequencies of Annexin V^{++} cells were assessed in the $Zombie^-$ population. Thus, dead cells were already excluded facilitating evaluation of apoptotic cells at the time point of analysis. Frequencies of Annexin V^{++} NK cells and monocytes were generally low. B cells, especially of RA, showed higher rates of Annexin V binding, but frequency of Annexin V^{++} T cells was highest in basal state without any stimulus (note differentially segmented axis). Stimulation with PMA/Iono tended to increase the frequency of Annexin V^{++} cells in T cells, NK cells and monocytes. As PMA/Iono served as strong stimulus, decreased frequencies of Annexin V^{++} B cells may depend on the chosen time point for analysis and suggested earlier apoptosis. Stimulation of D_1-like receptors with A68930 increased frequency of Annexin V^{++} T cells from RA patients significantly at 10^{-9} M and 10^{-8} M ($p = 0.0127$ and $p = 0.0135$, respectively). D_2-like receptor stimulation with ropinirole led to increased frequencies of Annexin V^{++} T cells from HC at 10^{-8} M, 10^{-7} M, and 10^{-6} M ($p = 0.0048$, $p = 0.0373$ and $p = 0.0204$, respectively). RA T cells showed increased Annexin V binding only at 10^{-7} M ropinirole ($p = 0.0437$). Beside the observed effects of D_2-like receptor stimulation in T cells, ropinirole increased frequency of Annexin V^{++} NK cells of SpA patients at 10^{-6} M ($p = 0.0367$). Pan-DR stimulation with apomorphine increased Annexin V^{++} frequency in T cells from RA patients at 10^{-7} M and 10^{-6} M ($p = 0.0358$ and $p = 0.003$, respectively). For HC, a significantly elevated frequency of Annexin V^{++} T cells was only detected after apomorphine stimulation at 10^{-7} M ($p = 0.0185$). So especially T cells of HC and RA were prone to increased Annexin V^{++} binding after DR stimulation, independently which receptor(s) were targeted.

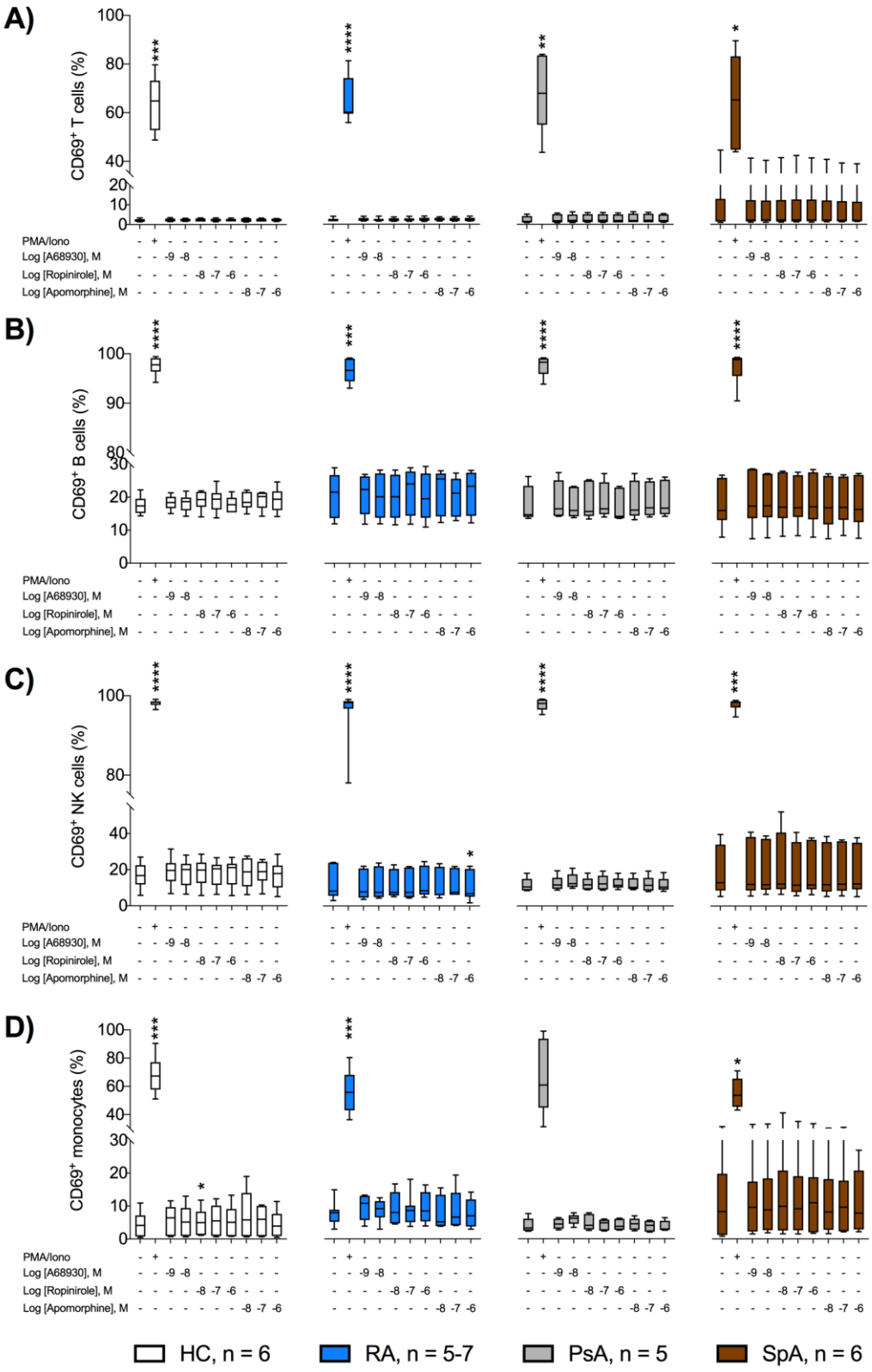

Figure 15: Short-term dopaminergic stimulation does not affect expression of the early activation marker CD69 in peripheral immune cells.

*PBMCs of HC, RA, PsA and SpA patients were stimulated for 24 h in vitro with either D1-like receptor agonist A68930, D2-like receptor agonist ropinirole or pan-DR agonist apomorphine at indicated concentrations. PMA/Iono served as positive control. Following short-term DR stimulation frequencies of CD69 expressing T cells (A), B cells (B), NK cells (C), and monocytes (D) were analyzed by flow cytometry. Immune cell subsets were gated as shown in chapter 4.1. Data from rituximab-treated patients were excluded herein. Subjects were not age- and sex-matched. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni's multiple comparisons test was used to determine impact of stimulation vs. unstimulated control within each group (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis*

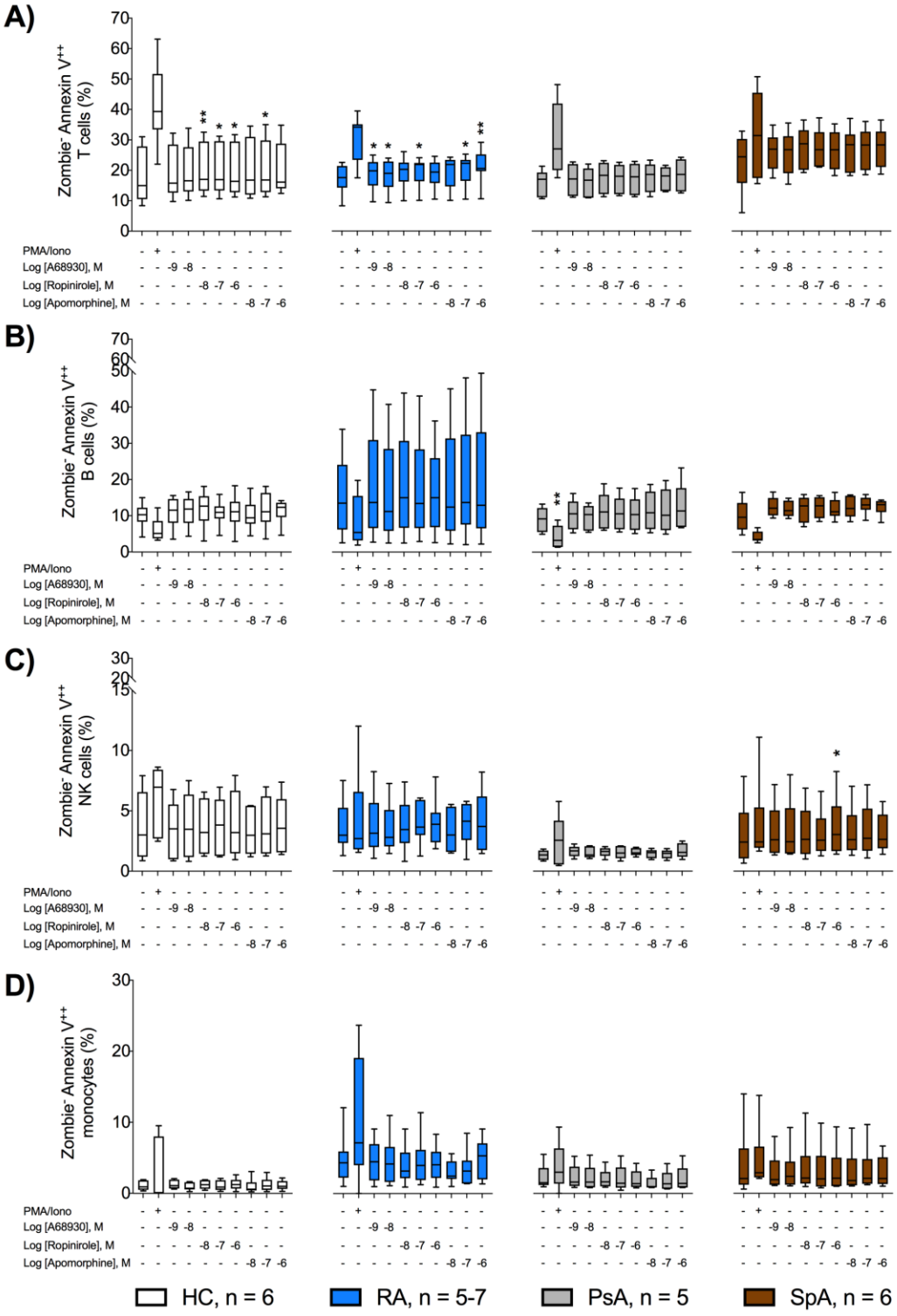

Figure 16: Short-term dopaminergic stimulation tends to increase apoptosis of peripheral immune cells, primarily T cells.

*PBMCs of HC, RA, PsA and SpA patients were stimulated for 24 h in vitro with either D_1-like receptor agonist A68930, D_2-like receptor agonist ropinirole or pan-DR agonist apomorphine at indicated concentrations. PMA/Iono served as control. Following short-term DR stimulation frequencies of apoptotic T cells (A), B cells (B), NK cells (C), and monocytes (D) were detected by flow cytometry as Annexin V^{++} in the Zombie$^-$ population. Immune cell subsets were gated as shown in chapter 4.1. Data from rituximab-treated patients were excluded herein. Subjects were not age- and sex-matched. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni's multiple comparisons test was used to determine impact of stimulation vs. unstimulated control within each group (*p ≤ 0.05, **p ≤ 0.01). HC: healthy control, PsA: psoriasis arthritis, RA: rheumatoid arthritis, SpA: ankylosing spondylitis*

4.3.3. Secretion of pro-inflammatory cytokines

D_1 DR was found to be exclusively upregulated in B cells of RA patients, not in those of PsA or SpA patients (Figure 7). To determine if this increased expression favors acute D_1-like receptor stimulation and consequently modulates IL-6 and IL-8 secretion, supernatants of 24 h stimulated PBMCs from HC and RA patients were analyzed by ELISA.

Generally, concentrations of IL-6 and IL-8 did not differ between HC and RA patients (Figure 17). Concentration of IL-6 was overall low and unaltered by D_1-like receptor agonist A68930 (Figure 17 A). On the contrary, basal concentration of IL-8 was higher and tended to decrease by D_1-like receptor stimulation in RA (Figure 17 B).

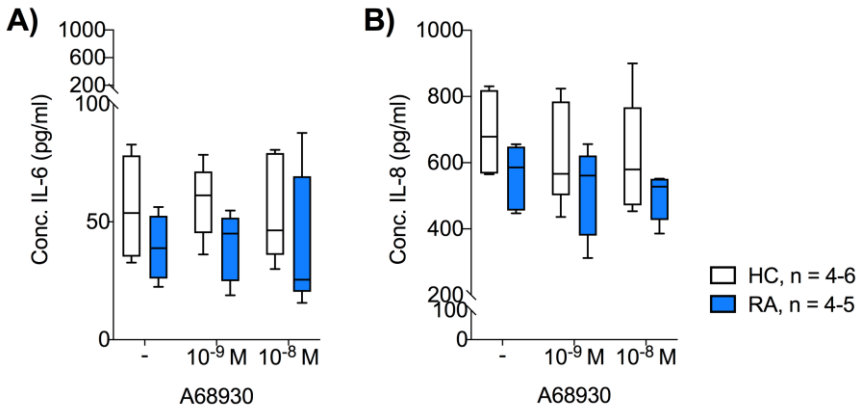

Figure 17: Short-term D_1-like receptor stimulation leaves IL-6 secretion unaltered but tends to decrease secretion of IL-8 in PBMCs of RA patients.

PBMCs of HC and RA were stimulated for 24 h in vitro with D_1-like receptor agonist A68930 at indicated concentrations. Concentrations of IL-6 and IL-8 in supernatants were quantified by ELISA. Subjects were not age- and sex-matched. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni's multiple comparisons test was used to determine impact of stimulation vs. unstimulated control within each group. HC: healthy control, RA: rheumatoid arthritis

4.4. Focus on the functional role of D_1 DR in B cells

4.4.1. Expression of D_1 DR during B cell maturation

As shown in Figure 7, only RA patients displayed a significantly increased frequency of D_1 DR expressing $CD19^+$ B cells. Next, interest was focused on D_1 DR expression during B cell development. Therefore, D_1 DR expression was analyzed in different B cell maturation stages based on characteristic antigen expression by flow cytometry. Characteristics of investigated HC and RA patients are listed in Table 9 (see appendix).

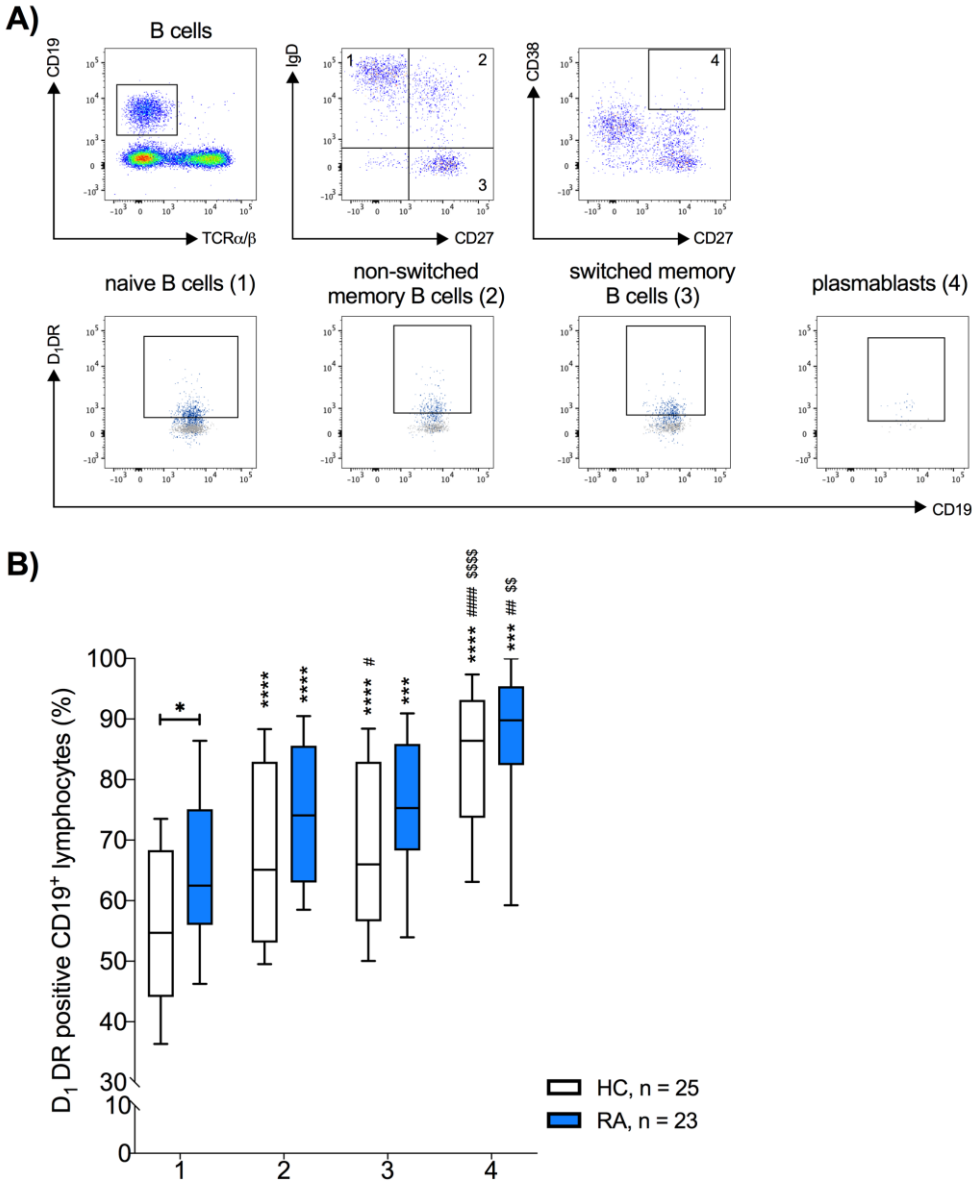

Figure 18: D_1 DR expression rises during B cell maturation and peripheral naive B cells of RA patients express higher D_1 DR levels than HC.

*D_1 DR expression was studied in defined B cell subpopulations of HC and RA patients by flow cytometry. A) Gating strategy to investigate frequency of D_1 DR expression in naive (1, $CD19^+TCR\alpha/\beta^-IgD^+CD27^-$), non-switched memory (2, $CD19^+TCR\alpha/\beta^-IgD^+CD27^+$), class-switched memory B cells (3, $CD19^+TCR\alpha/\beta^-IgD^-CD27^+$) and plasmablasts (4, $CD19^+TCR\alpha/\beta^-CD27^+CD38^-$), grey: FMO control. B) Quantification of D_1 DR on aforementioned B cell subsets of HC and RA. Subjects were age- and sex-matched. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Bonferroni's multiple comparisons test was used to analyze D_1 DR expression between groups and B cell stages (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p < 0.0001). Comparison within HC and RA groups: naive B *, non-switched memory B #, switched memory B $. DR: dopamine receptor, FMO: fluorescence minus one, HC: healthy control, RA: rheumatoid arthritis*

As introduced before, B cells were identified within the lymphocyte population. Here, B cells were gated as $CD19^{+}TCR\alpha/\beta^{-}$ cells (Figure 18 A). Naive B cells were further identified by surface expression of IgD and absence of CD27. CD27 is expressed on memory B cells (Agematsu et al. 1997), based on parallel presence or absence of IgD they are either non-switched or class-switched memory B cells, respectively. Moreover, plasmablasts were detected by high surface densities of CD27 and CD38 within B cells (Wei et al. 2011). Frequency of D_1 DR expression was analyzed within every B cell population.

As presented in Figure 18 B, D_1 DR expression increased during B cell maturation. Naive B cells displayed the lowest frequencies of D_1 DR, both in HC and RA patients. However, naive B cells of RA patients presented significantly increased expression of D_1 DR compared to HC ($p = 0.0273$). Non-switched, switched memory B cells and plasmablasts showed elevated D_1 DR frequencies compared to naive B cells of HC ($p < 0.0001$ overall) and RA patients ($p < 0.0001$, $p = 0.0002$ and $p = 0.0001$, respectively). Memory B cells of RA only tended to express higher D_1 DR levels than HC (non-switched: $p = 0.2383$, switched: $p = 0.3768$). In contrast to a significant increase of D_1 DR from non-switched to switched memory B cells in HC ($p = 0.0366$), no significant difference was detected in RA. Frequency of D_1 DR expressing plasmablasts was elevated compared to non-switched memory B cells both for HC ($p < 0.0001$) and RA ($p = 0.0057$). Increase of D_1 DR from switched memory B cells to plasmablasts was also present in investigated samples, but less distinct in RA (HC: $p < 0.0001$, RA: $p = 0.0093$). Here, D_1 DR expression did not correlate with subject characteristics and clinical data (data not shown).

4.4.2. Modelling classical germinal center interactions *in vitro*

Humoral immune responses develop dependent on T cells within lymphoid structures, which can also be detected ectopically in synovial tissues of 6 - 35% RA patients (Hutloff 2018). Papa et al. already demonstrated that DA is involved in GC interaction between B and T_{FH} cells within secondary lymphoid organs (Papa et al. 2017). Because of the increased D_1 DR expression detected in naive B cells of RA patients, the hypothesis emerged that dopaminergic signal transmission via D_1 DR is promoting B cell activation, maturation, and various functions during RA.

4.4.2.1. Expression of D_1 DR in T helper cell subsets

Prior to setting up *in vitro* co-cultures of B and T_{FH} cells, the capability of T_{FH} cells to sense stimulation via D_1 DR was studied by flow cytometry (for subject characteristics see appendix, Table 9). The stained surface antigens also permitted investigation of D_1 DR expression in naive and memory $CD4^{+}$ T cells. Figure 19 A describes the gating strategy. Within the lymphocyte population, T helper cells were identified as $CD4^{+}TCR\alpha/\beta^{+}$ cells. Naive $CD4^{+}$ T

cells were gated as CD45RA$^+$CXCR5$^-$, memory CD4$^+$ T cells as CD45RA$^-$CXCR5$^-$ and T_{FH} cells as CD45RA$^-$CXCR5$^+$ cells. Frequency of D_1 DR expression was analyzed within every T cell population. As depicted in Figure 19 B, D_1 DR expression also increased in more experienced CD4$^+$ T cells compared to naive CD4$^+$ T cells which displayed the lowest frequencies of D_1 DR positive cells analyzed here. Only memory CD4$^+$ T cells of RA patients revealed increased frequencies of D_1 DR expression compared to naive CD4$^+$ T cells ($p = 0.0003$). However, D_1 DR was strongly increased in T_{FH} cells compared to naive CD4$^+$ T cells (both HC and RA: $p < 0.0001$) as well as to memory CD4$^+$ T cells (HC: $p = 0.0014$, RA: $p < 0.0001$). Interestingly, frequency of peripheral D_1 DR expressing T_{FH} cells was significantly elevated in RA compared to HC ($p = 0.005$). D_1 DR expression did not correlate with subject characteristics and clinical data (data not shown).

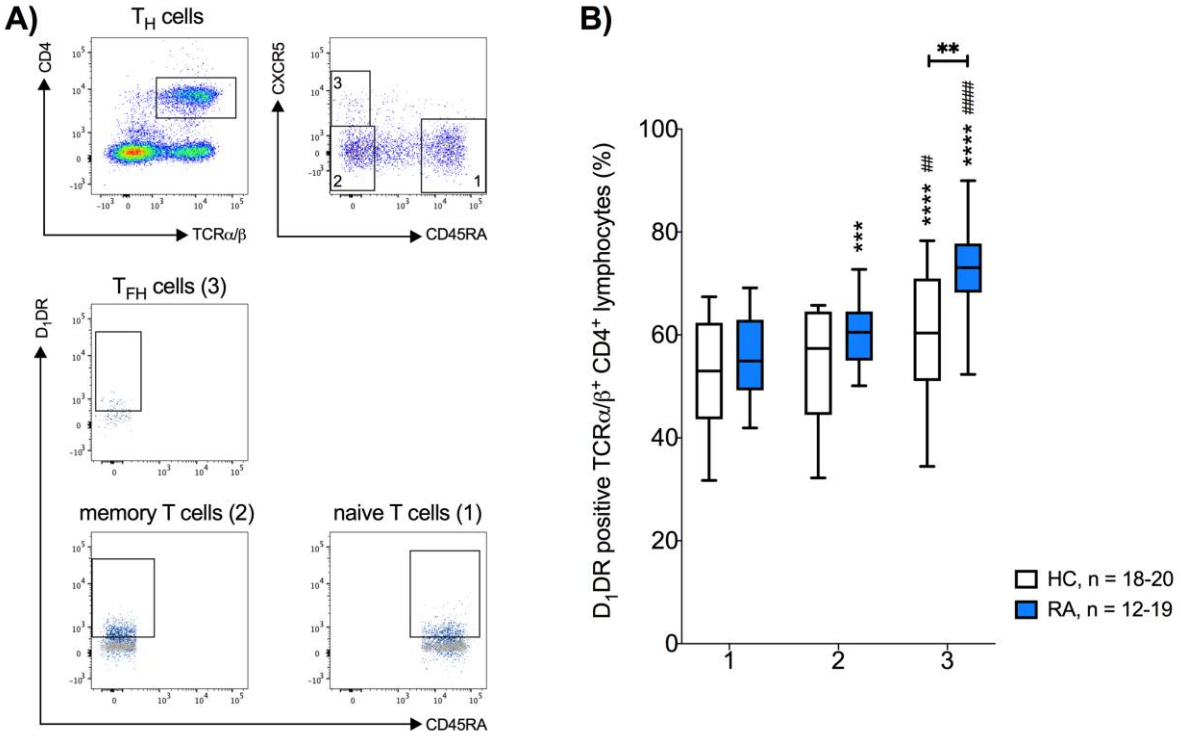

Figure 19: D_1 DR expression rises during T cell maturation and circulating T_{FH} cells of RA patients express higher D_1 DR levels than HC.
*D_1 DR expression was studied on CD4$^+$ T cell subpopulations of HC and RA patients by flow cytometry. A) Gating strategy to investigate frequency of D_1 DR expression in naive (1, CD4$^+$TCRα/β$^+$CD45RA$^+$CXCR5$^-$), memory T cells (2, CD4$^+$TCRα/β$^+$CD45RA$^-$CXCR5$^-$), and T_{FH} cells (3, CD4$^+$TCRα/β$^+$CD45RA$^-$CXCR5$^+$), grey: FMO control. B) Quantification of D_1 DR in aforementioned T cell subsets of HC and RA. Subjects were age- and sex-matched. Outliers were identified and removed by Iterative Grubbs' test (α = 0.05) as indicated in the sample size. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentile. Bonferroni's multiple comparisons test was used to analyze D_1 DR expression between groups and T cell stages (**p ≤ 0.01, ***p ≤ 0.001, ****p < 0.0001). Comparison within HC and RA groups: naive T *, memory T #. DR: dopamine receptor, FMO: fluorescence minus one, HC: healthy control, RA: rheumatoid arthritis, T_{FH}: follicular helper T cell*

4.4.2.2. Experimental setup

Next, the impact of D_1-like receptors in T cell-dependent B cell stimulation was determined (Figure 20 A). B cells and T_{FH} cells were isolated and co-cultivated under SEB stimulation as depicted in Figure 20 B. SEB as a superantigen binds to the α-chain of major histocompatibility complex (MHC) class II on B cells and a non-specific common variable region on the TCR of

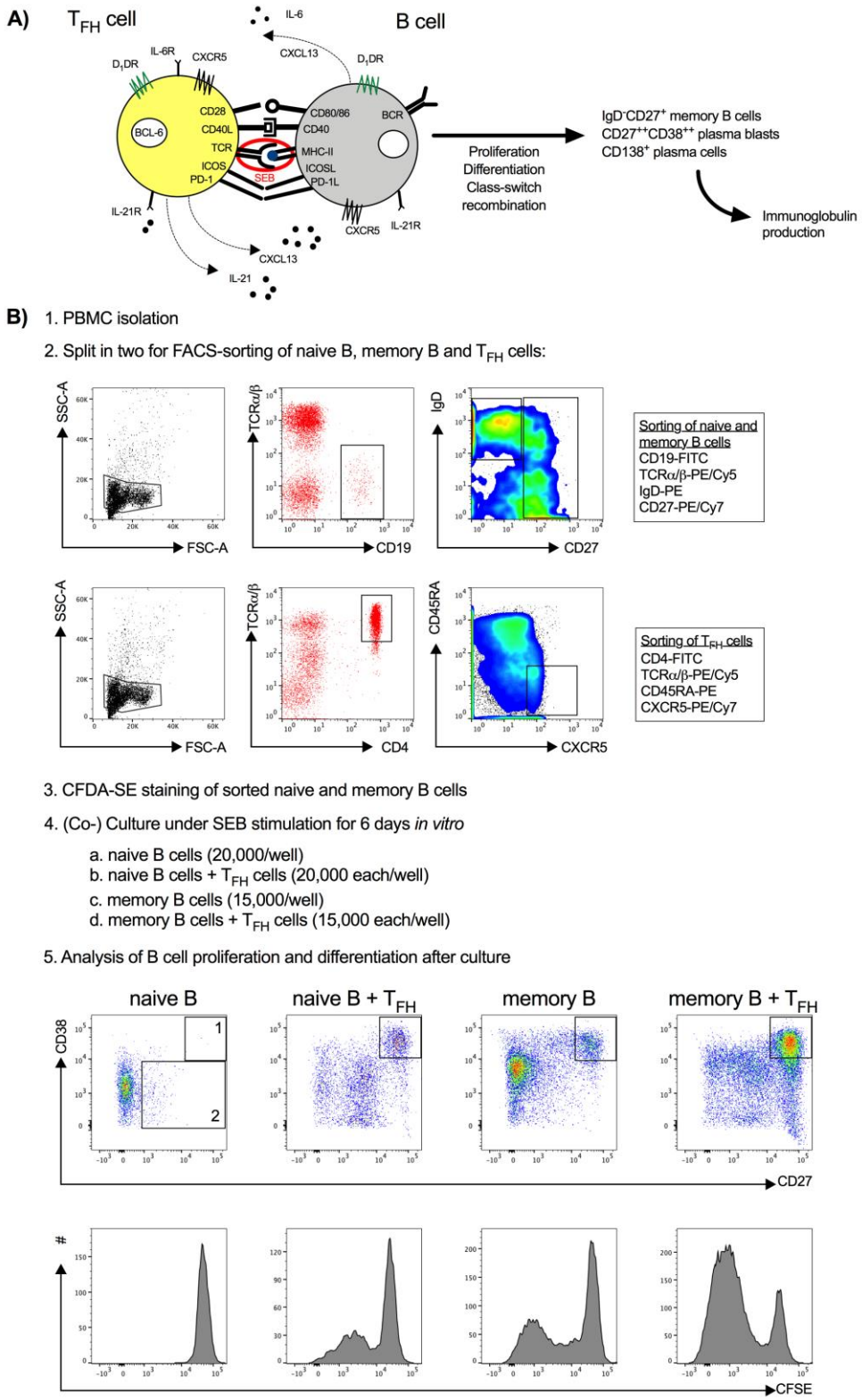

Figure 20: Experimental setup to investigate the influence of D_1-like receptor stimulation in T cell-dependent B cell stimulation.

A) Sketch about classical interaction of B and T_{FH} cells in GC of the lymph node. B) Experimental setup. PBMCs were freshly isolated and stained for sorting. Naive (TCRα/β$^-$CD19$^+$IgD$^+$CD27$^-$) and memory B cells (TCRα/β$^-$CD19$^+$ CD27$^+$) were sorted within the first panel, as well as T_{FH} cells (CD19$^-$TCRα/β$^+$CD4$^+$CD45RA$^-$CXCR5$^+$) within the second panel on a BD FACSJazz. B cells were stained with CFDA-SE and cultured either alone or in presence of T_{FH} cells (1:1) under stimulation with SEB (100 ng/ml) and indicated concentrations of D_1-like receptor agonist A68930. After 6 days in vitro B cell differentiation and proliferation were investigated by FACS staining and CFSE-dye dilution on BD LSR Fortessa. B cells were gated as single, live, CD4$^-$ cells to exclude also autofluorescent/dead cells in BV421 channel. Gate 1 quantifies plasmablasts (CD27^{++}CD38^{++}), and gate 2 memory B cells (CD27$^+$ CD38$^-$). GC: germinal center, SEB: staphylococcal enterotoxin B, T_{FH}: follicular helper T cell

$CD4^+$ T cells, mimicking physiological B-T_{FH} interaction and leading to an uncontrolled release of pro-inflammatory cytokines and immune cell activation (Pinchuk et al. 2010). After 6 days of stimulation with the superantigen SEB and D_1-like receptor agonist A68930, B cell differentiation and proliferation were investigated. To study possible dose-dependent effects of D_1-like receptor agonist A68930, concentrations for stimulation were expanded. Notably, A68930 exhibits a strong affinity for D_1-like receptors at 10^{-9} M and 10^{-8} M as already used for short-term stimulation of PBMCs. The additional applied concentrations of A68930 10^{-7} M and 10^{-6} M rather activate D_2-like receptors (Kebabian et al. 1990). As shown in the FACS plots of B cells (Figure 18) stimulation with SEB alone provided basis for survival, differentiation, and proliferation. Unfortunately, due to insufficient cell numbers isolated from RA patient blood and logistical issues experiments could only be performed with cells from HC (for characteristics see appendix Table 10). Similar to the expression of D_5 DR in total $CD19^+$ B and $CD3^+$ T cells, D_5 DR was almost absent in naive as well as memory B and in T_{FH} cells (data not shown).

4.4.2.3. Effect of D_1-like receptor stimulation on T cell-dependent B cell stimulation

Results of B cell proliferation and differentiation analysis are presented in Figure 21. Readouts were % divided B cells, i.e. frequency of B cells with diluted CFSE-dye (Figure 21 A), division index, i.e. the average number of cell divisions in the total B cell population (Figure 21 B), proliferation index, i.e. the number of cell divisions focusing only on the fraction of B cells undergoing at least one division (Figure 21 C), and plasmablast differentiation (Figure 21 D). Extent of these were in accordance with culture conditions. Particularly, memory B cells presented elevated proliferation as well as differentiation compared to naive B cells, and co-culture with T_{FH} cells boosted proliferation and differentiation compared to pure B cell cultures.

Percentage of divided B cells remained overall unaltered under D_1-like receptor stimulation with A68930 (Figure 21 A). However, memory B cells cultured alone seemed to be susceptible to stimulation of D_1-like receptors and were either supported or impeded in cell division, depending on donor and concentration of A68930. A similar picture was observed for division index of B cells stimulated with D_1-like receptor agonist (Figure 21 B). Overall, the proliferation index was not affected by D_1-like receptor stimulation (Figure 21 C), albeit naive B cells of some donors appeared responsive to A68930 stimulation triggering proliferation. Conversely, proliferation index of memory B cells cultured alone tended to be decreased by A68930 at 10^{-8} M ($p = 0.116$), as well as memory B cells in co-cultures with T_{FH} stimulated with A68930 at 10^{-9} M ($p = 0.0925$). Differentiation towards plasmablasts (Figure 21 D) was absent in cultures of naive B cells. Again, depending on donor and concentration of A68930, memory B cells cultured alone appeared to be sensitive for stimulation with D_1-like receptor agonist. In co-cultures of memory B and T_{FH} cells, stimulation with A68930 at 10^{-7} M decreased plasmablast maturation significantly ($p = 0.0094$). Stimulation of naive IgD^+CD27^- B cells with

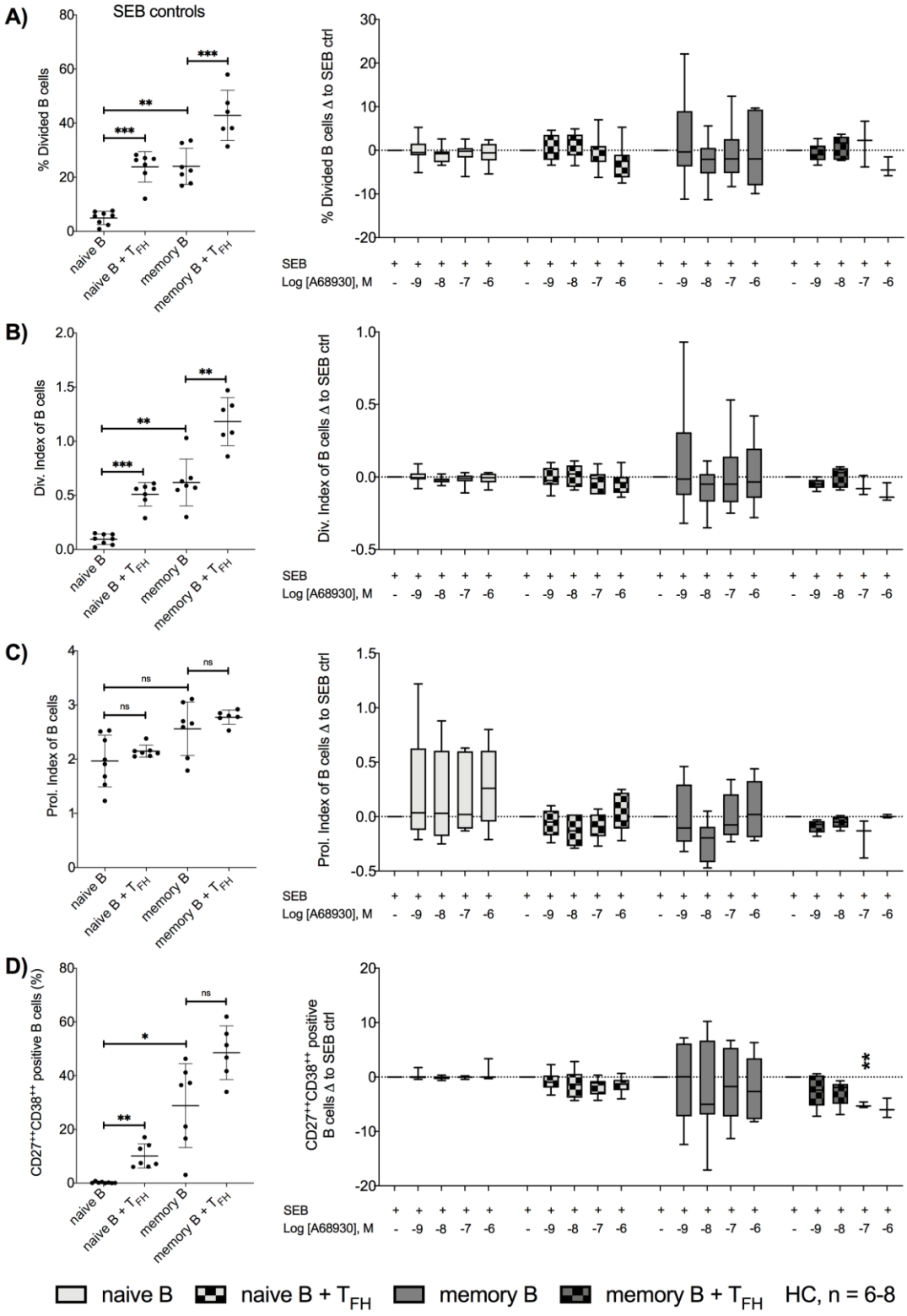

Figure 21: D_1-like receptor stimulation slightly affects B cell proliferation and maturation in the in vitro model for germinal center interactions.

*As illustrated in the previous figure, CFSE-stained naive or memory B cells were cultured either alone or with T_{FH} cells and stimulated with SEB and indicated concentrations of D_1-like receptor agonist A68930 for 6 days in vitro. Following long-term D_1-like receptor stimulation proliferation and differentiation of $CD19^+$ B cells were evaluated by flow cytometry. % Divided B cells (A), division index (B), proliferation index (C), and plasmablast frequency (D) of B cells under SEB stimulation are shown on the left for the respective cultures and presented as mean with SD, points represent individual samples. Effects of D_1-like receptor stimulation are presented as absolute changes to SEB controls on the right for the respective cultures. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Bonferroni's multiple comparisons tests was used to analyze the impact of cultivation and D_1-like stimulation vs. SEB control (*p ≤ 0.05, **p ≤ 0.01, ***p < 0.001). HC: healthy control, SEB: staphylococcal enterotoxin B, T_{FH}: follicular helper T cell*

SEB supported their maturation towards $CD27^+$ memory B cells (Figure 22). Overall, additional D_1-like receptor stimulation had no effect on maturation of naive B cells. However, depending again on donor and concentration, D_1-like agonist A68930 tended to in- or decrease naive B cell differentiation towards memory B cells.

As mentioned, effects occurred depending on the donor. However, due to the small sample size correlation of observed effects with age or sex of HC was not possible. Functional differences between immune cells of male and female could only be assumed (data not shown). Frequencies of D_1 DR expressing naive and memory B cells as well as T_{FH} cells were comparable between donors and did not determine observed effects (data not shown).

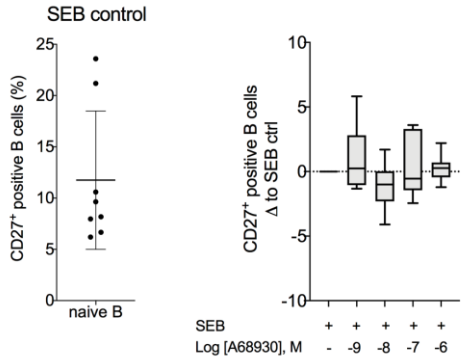

Figure 22: Maturation of naive towards memory B cells is hardly affected by D_1-like receptor stimulation. *As illustrated before, CFSE-stained naive B cells were cultured alone and stimulated with SEB and indicated concentrations of D_1-like receptor agonist A68930 for 6 days in vitro. Following long-term D_1-like receptor stimulation differentiation of B cells was evaluated by flow cytometry. Frequency of differentiated memory B cells under SEB stimulation is shown on the left and presented as mean with SD, points represent individual samples. Effects of D_1-like receptor stimulation are presented as absolute changes to SEB controls on the right. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Bonferroni's multiple comparisons test was used to analyze impact of D_1-like stimulation vs. SEB control. SEB: staphylococcal enterotoxin B*

4.4.3. Effect of D_1-like receptor stimulation on T cell-independent B cell stimulation

To study the role of D_1-like receptor stimulation in B cell activation independently of T cells, PBMCs were stimulated for 6 days *in vitro* with CpG and A68930 (for subject characteristics see appendix Table 11). CpG is a ligand for Toll-like receptor (TLR)-9 expressed by B cells and plasmacytoid dendritic cells in the blood. Especially Class B CpG ODN 2006 used here is known for its strong B cell activation but weak stimulation of plasmacytoid dendritic cells (Figure 23) (Krieg 2002).

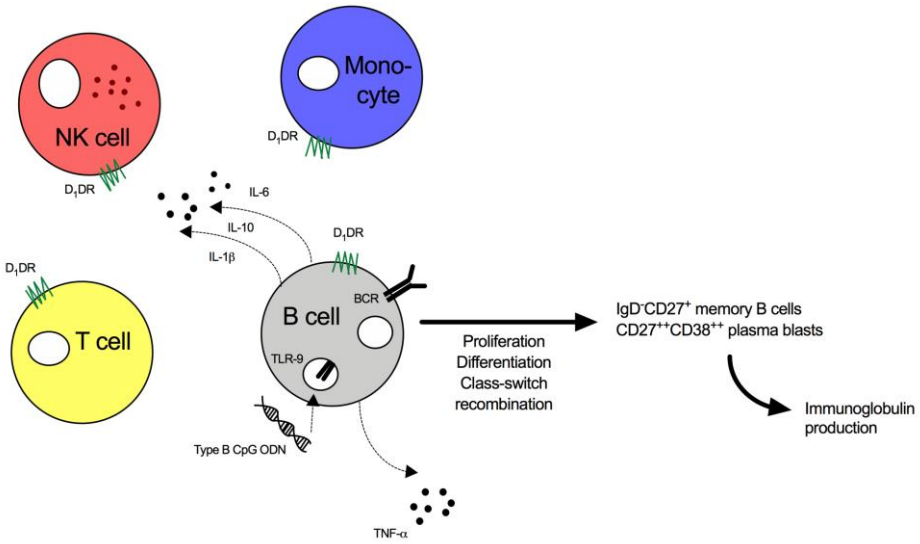

Figure 23: Model for T cell-independent B cell activation in vitro by Class B CpG ODN 2006 via TLR-9.
Sketch about T cell-independent B cell stimulation. Among PBMCs, B cells and less frequently found plasmacytoid dendritic cells express TLR-9 sensing unmethylated CpG rich DNA. Following such stimulus B cells undergo proliferation, differentiation as well as class-switch recombination and secrete immunoglobulins and various cytokines. ODN: oligodeoxynucleotide, TLR: Toll-like receptor

First, effects of D_1-like receptor stimulation on B cell proliferation in PBMCs were analyzed by flow cytometric analysis on CFSE-dye dilution. Stimulation with CpG alone led to enumerable amount of % divided B cells (Figure 24 A) and B cell proliferation (Figure 24 B) both in HC and RA. Further stimulation of D_1-like receptors had no effect on % divided B cells in both groups. However, the overall treatment effect of A68930 was significant for the proliferation index of B cells ($p = 0.0004$). Of note, different concentrations of A68930 directly compared to CpG control did not result in significant changes of proliferation index of B cells due to donor-dependent differences. Patient characteristics as well as disease stage and therapy did not impact the effect of D_1-like receptor stimulation of B cells (data not shown). Moreover, with respect to concentration of D_1-like receptor agonist, both HC and RA presented similar changes in proliferation indices. The lower concentrations of A68930 at 10^{-9} M and 10^{-8} M which display a higher affinity for D_1-like receptors increased B cell proliferation more than 10^{-7} M and 10^{-6} M that rather activate D_2-like receptors (Kebabian et al. 1990). However, this observation was not statistically significant. B cell proliferation was almost absent in unstimulated cultures (data not shown).

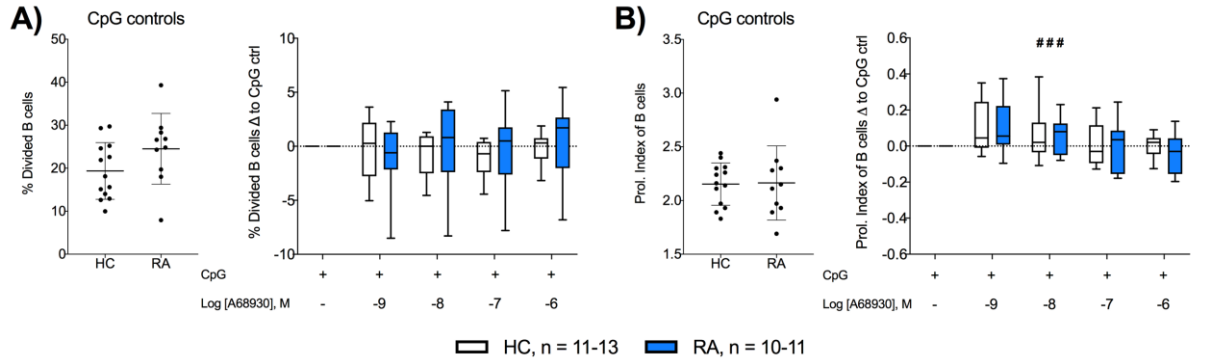

Figure 24: Proliferation of B cells, independent of T cells, is increased by D_1-like receptor stimulation.
PBMCs of HC and RA patients were stimulated with CpG (0.35 µM) and indicated concentrations of D_1-like receptor agonist A68930 for 6 days in vitro. Following long-term D_1-like receptor stimulation proliferation of $CD19^+$ B cells was analyzed by CFSE-dye dilution via flow cytometry. % Divided (A) and proliferation index (B) of B cells under CpG stimulation are shown on the left and presented as mean with SD, points represent individual samples. Use of unpaired T test for comparison of basal levels between groups. Effects of D_1-like receptor stimulation are presented as absolute changes to CpG controls on the right. Subjects were age- and sex-matched. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Use of mixed-effects analysis to determine the overall influence of disease and treatment ($^{###}p \leq 0.001$). Bonferroni's multiple comparisons test was used to determine impact of D_1-like receptor stimulation vs. CpG stimulated control within each group. HC: healthy control, RA: rheumatoid arthritis

To measure the influence of D_1-like receptor stimulation on B cells ability to mature and develop towards antibody secreting cells, plasmablast frequency was determined at the end of culture by flow cytometry. Additionally, secreted IgG was analyzed in supernatants by ELISA. Frequency of plasmablasts after CpG stimulation differed strongly between donors (Figure 25 A). Generally, maturation of B cells towards plasmablast was unaffected by additional D_1-like receptor stimulation, although RA B cells tended to be impeded in their maturation. Similarly, level of secreted IgG varied between subjects investigated (Figure 25 B). Some subjects responded to D_1-like receptor stimulation as presented in either in- or decreased IgG levels in supernatants, but overall D_1-like receptor stimulation had no impact. Again, effects were independent of patient characteristics, disease stage and therapy. Such as proliferation, B cell differentiation towards plasmablasts was almost absent in unstimulated cultures (data not shown).

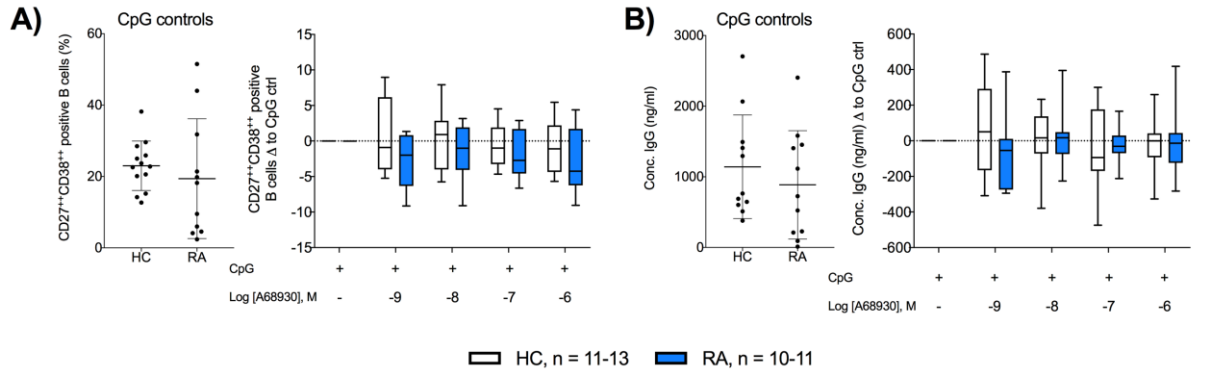

Figure 25: D_1-like receptor stimulation affects B cell maturation towards plasmablasts and secretion of IgG donor-dependently.

PBMCs of HC and RA patients were stimulated with CpG (0.35 µM) and indicated concentrations of D_1-like receptor agonist A68930 for 6 days in vitro. Following long-term D_1-like receptor stimulation plasmablast differentiation of $CD19^+$ B cells was analyzed by flow cytometry and secreted IgG in supernatants was quantified by ELISA. Plasmablast frequencies of B cells (A) and IgG concentrations (B) under CpG stimulation are shown on the left and presented as mean with SD, points represent individual samples. Use of unpaired T test for comparison of basal levels between groups. Effects of D_1-like receptor stimulation are presented as absolute changes to CpG controls on the right. Subjects were age- and sex-matched. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Use of mixed-effects analysis to determine the overall influence of disease and D_1-like receptor stimulation. Bonferroni's multiple comparisons test was used to determine impact of D_1-like receptor stimulation vs. CpG stimulated control within each group. HC: healthy control, RA: rheumatoid arthritis

Apart from B cell-derived autoantibodies, pro-inflammatory cytokines IL-6 and TNF-α are well known for their contribution to local joint inflammation. Also, recent findings suggest that IL-8 is involved in ACPA-driven osteoclastogenesis (Krishnamurthy et al. 2016) and secretion of CCL3 by B cells inhibits osteoblast formation further promoting bone damage (Sun et al. 2018). On the contrary, IL-10 secreted by e.g. regulatory B cells has a beneficial function in RA (Bankó et al. 2017). CpG as B cell-specific stimulus led to secretion of IL-6, TNF-α, IL-8, CCL3 and IL-10 in cultures of PBMCs from HC and RA patients. Concentration of IL-6 was significantly elevated in CpG-stimulated PBMCs of HC compared to RA patients (Figure 26 A, $p = 0.0356$). The effect of D_1-like receptor stimulation on secretion of IL-6 was dependent on investigated subjects ($p < 0.0001$). In general, cells of HC seemed more responsive for modulation of IL-6 secretion by A68930 stimulation than those of RA as indicated by large absolute in- or decreases compared to CpG control. After 6 days of CpG stimulation *in vitro*, levels of TNF-α were low in cultures of both HC and RA (Figure 26 B). As well, observed effects of D_1-like receptor stimulation were small. However, A68930 at 10^{-8} M tended to decrease TNF-α secretion in HC ($p = 0.0587$). In contrast, concentration of IL-8 was high after 6 days of CpG stimulation *in vitro* (Figure 26 C). Effects of D_1-like receptor stimulation on IL-8 secretion were dependent on subjects ($p < 0.0001$) and generally, cultures of RA PBMCs showed significantly increased IL-8 levels compared to HC ($p = 0.0091$). While analysis of single concentrations of D_1-like receptor agonist A68930 compared to CpG control revealed no significant effect on concentration of IL-8, overall treatment effect was significant in RA patients ($p = 0.0144$). Amount of CCL3 secreted in CpG stimulated cultures varied largely between subjects and D_1-like receptor stimulation with A68930 resulted in great changes of secreted CCL3 (Figure 26 D). As TLR-9 expression on peripheral B cells was not studied, differences between responders and non-responders to CpG stimulation could not be determined. Whereas effect of D_1-like receptor stimulation either enhanced or inhibited CCL3 secretion in HC responders, responding immune cells of RA seemed to be exclusively boosted in secretion of CCL3 by higher concentrations of A68930. CpG stimulation led to secretion of anti-inflammatory IL-10 in both cultures of HC and RA PBMCs (Figure 26 E) and here, HC tended towards higher IL-10 secretion than RA in CpG controls ($p = 0.0753$). D_1-like receptor stimulation had overall no effect on IL-10 secretion but significantly depended on subject ($p < 0.0001$) reflected in large variations.

Because effects largely depended on investigated subjects, data was correlated with their characteristics and available clinical data. Similar to B cell proliferation and differentiation, no correlation between observed effects and individual data could be determined (data not shown).

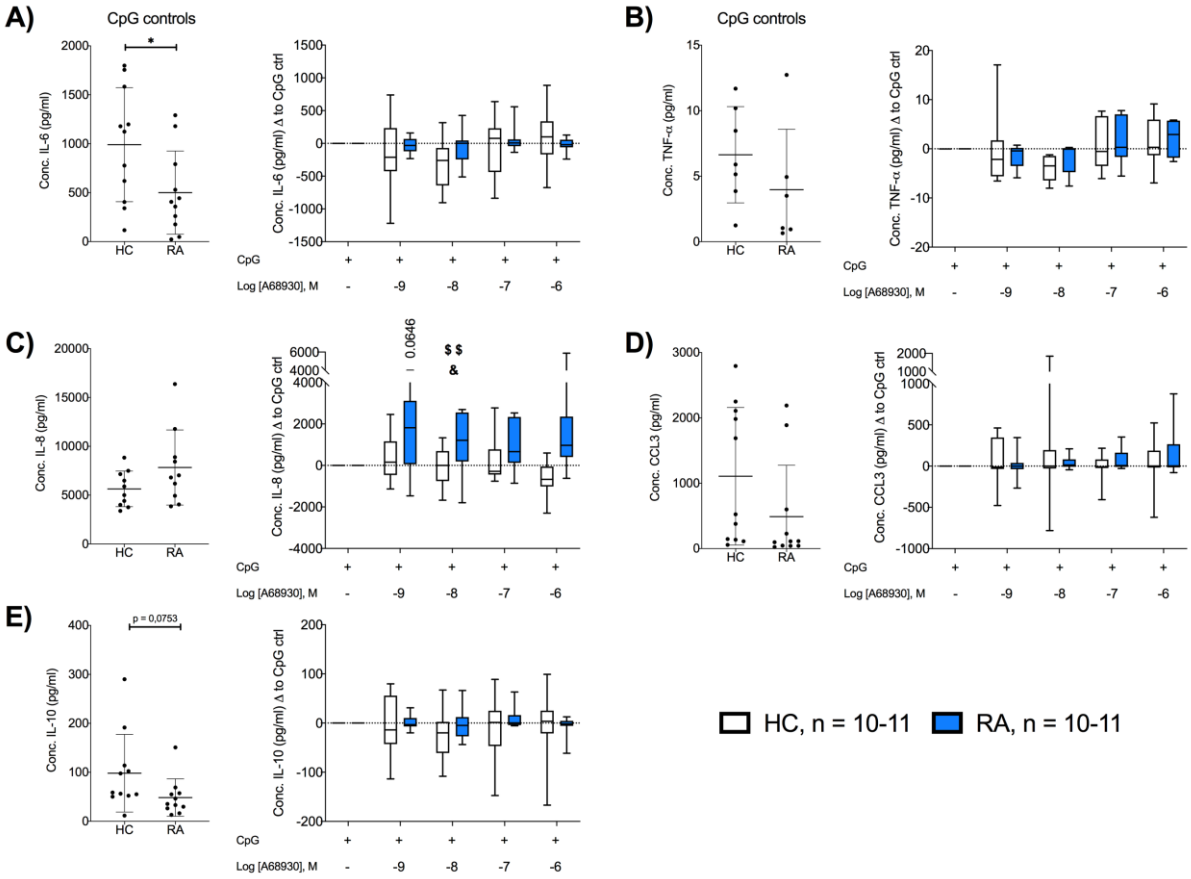

Figure 26: D_1-like receptor stimulation affects cytokine secretion in T cell-independent B cell stimulation donor-dependently and selectively increases IL-8 secretion in RA.
*PBMCs of HC and RA patients were stimulated with CpG (0.35 µM) and indicated concentrations of D_1-like receptor agonist A68930 for 6 days in vitro. Following long-term D_1-like receptor stimulation supernatants were stored at -80°C for subsequent analysis. Concentrations of IL-6, TNF-α, CCL3 and IL-10 in supernatants were quantified by cytometric bead array/LEGENDplex™ analysis. IL-8 concentration was determined by ELISA. Concentrations of IL-6 (A), TNF-α (B), IL-8 (C), CCL3 (D) and IL-10 (E) under CpG stimulation are shown on the left of each graph and presented as mean with SD, points represent individual samples. Use of unpaired T test for comparison of basal levels between groups (*p ≤ 0.05). Effects of D_1-like receptor stimulation are presented as absolute changes to CpG controls on the right. Subjects were age- and sex-matched. Some values were below limit of detection. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Use of 2way ANOVA or mixed-effects analysis, depending on missing values, to determine the overall influence of disease ($^{\$\$}p ≤ 0.01$) and D_1-like receptor stimulation (for interaction: $^{\&}p ≤ 0.05$). Bonferroni's multiple comparisons test was used to determine impact of D_1-like receptor stimulation vs. CpG stimulated control within each group. HC: healthy control, RA: rheumatoid arthritis*

4.4.4. Intracellular signaling after D_1-like receptor stimulation in B cells

Next, focus was set on the intracellular signaling after D_1-like receptor stimulation in B cells of HC and RA (for subject characteristics see appendix Table 12), to determine if there were disease-related differences in intracellular signaling as already described for SF in inflamed tissue (Jenei-Lanzl et al. 2015). Because isolated immune cells from patients were limited and

did not permit analysis in single subsets by e.g. western blot, intracellular flow cytometric staining with Ki-67 and phosflow antibodies for pERK and pCREB was applied.

D_1-like receptor agonist A68930 increased T cell-independent B cell proliferation *in vitro* (Figure 24), leading to analysis of possible changes of Ki-67 expression upon stimulation with A68930. Ki-67 is a nuclear protein expressed in G_1, S, G_2, and M phase of cell cycle, but not in G_0 phase, and thus identifies proliferating cells (Schwarting et al. 1986). Figure 27 A shows the gating strategy to investigate Ki-67 expression in B cells. Its expression could be detected in unstimulated B cells as well as in B cells stimulated with D_1-like receptor agonist (Figure 27 B). Extent of Ki-67 expression was significantly elevated in RA patients compared to HC after 10 min of stimulation ($p = 0.0364$) and only tendentially after 20 min ($p = 0.0765$). After 10 min overall effect of D_1-like receptor stimulation was significant ($p = 0.0002$). However, there was no interaction of disease and treatment effect, i.e. observed effects occurred independent of disease. Only stimulation with 10^{-8} M A68930 directly compared to unstimulated led to a significant decrease of Ki-67 after 10 min (HC: $p = 0.0201$, RA: $p = 0.0212$). Yet, observed effects were small and limited to analysis at this time. After 20 min stimulation with A68930 Ki-67 expression was unaltered compared to control.

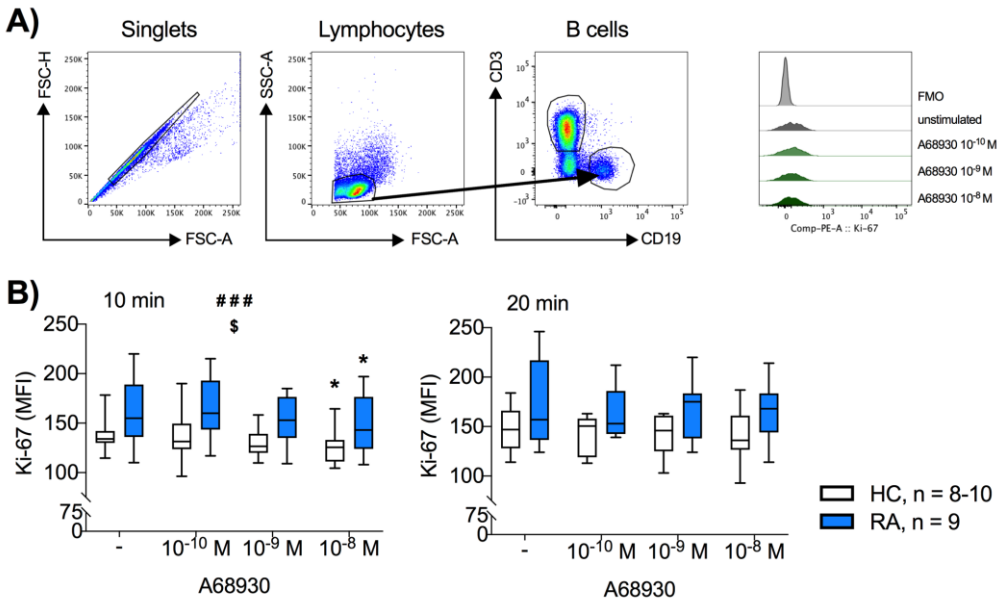

Figure 27: Ki-67 is expressed in peripheral B cells and only minimally affected by D_1-like receptor stimulation.
PBMCs of HC and RA patients were stimulated with D_1-like receptor agonist A68930 at indicated concentrations for indicated times in vitro. Following acute D_1-like receptor stimulation cells were fixed and permeabilized with 2% PFA and BD FACS Permeabilizing Solution 2, respectively, followed by intracellular staining for the proliferation marker Ki-67. A) Gating strategy to assess Ki-67 expression in B cells. First, doublets were excluded followed by gating on lymphocytes in FSC/SSC plot. B cells were identified as $CD19^+$ cells. Within the B cell population Ki-67 expression was evaluated by MFI. B) Quantification of Ki-67 expression in B cells of HC and RA patients after 10-20 min D_1-like receptor stimulation. Subjects were age- and sex-matched. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Use of mixed-effects analysis to determine overall influence of disease ($^{\$}p \leq 0.05$) and D_1-like receptor stimulation ($^{\#\#\#}p \leq 0.001$). Bonferroni's multiple comparisons test was used to determine impact of D_1-like receptor stimulation vs. unstimulated control within each group ($^{*}p \leq 0.05$). FMO: fluorescence minus one, HC: healthy control, MFI: mean fluorescence intensity, RA: rheumatoid arthritis

Canonical signaling of D_1-like receptors involves activation of adenylyl cyclase by $G\alpha_s$ protein and thus elevation of intracellular cAMP stimulating PKA. PKA in turn phosphorylates CREB as main target or DARPP-32, further able to phosphorylate ERK (Klein et al. 2019). B cells were analyzed for ERK and CREB phosphorylation after stimulation of D_1-like receptors by antibodies directed against phosphorylated epitopes. Figure 28 A presents the gating strategy to investigate pERK and pCREB in B cells. Increased levels of both were detected after unspecific stimulation with PMA (red histograms). However, levels of pERK (Figure 28 B) and pCREB (Figure 28 C) were low in unstimulated and D_1-like receptor stimulated B cells after 10 and 20 min. Additionally, pERK and pCREB expression in B cells of HC and RA remained unaltered after D_1-like receptor stimulation and did not diverge, although B cells of RA patients tended towards higher phosphorylation levels of pERK and especially pCREB. After 20 min of stimulation, B cells of RA patients presented overall higher levels of pCREB than those of HC (p = 0.0414).

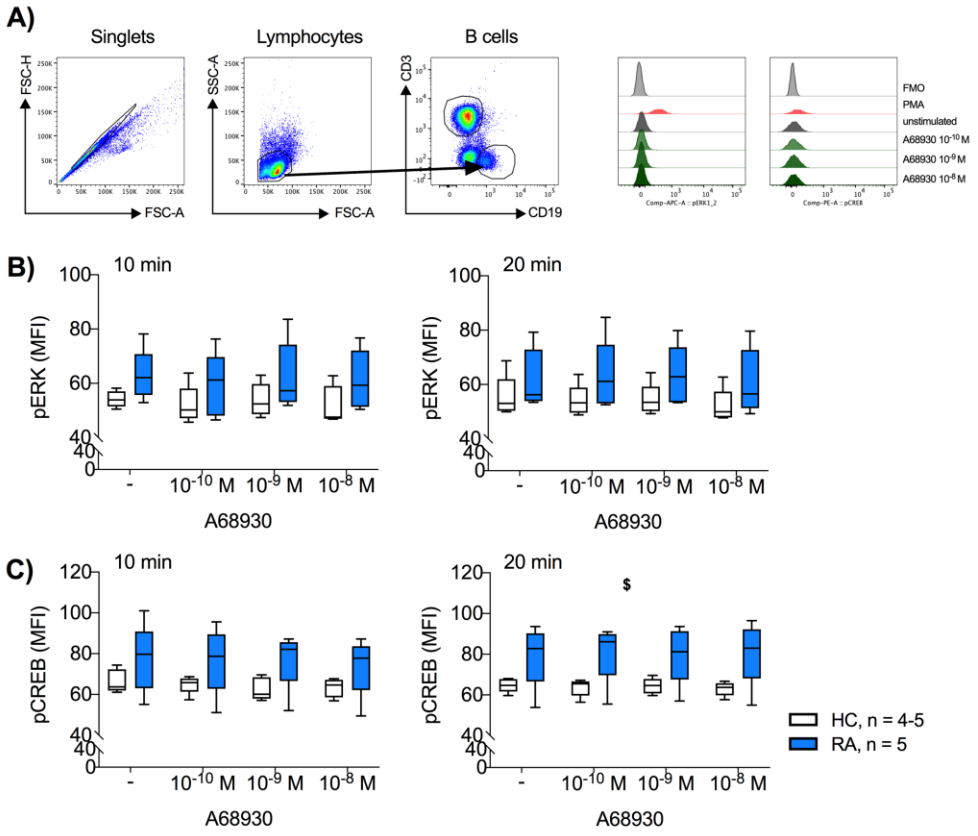

Figure 28: Phosphorylation of ERK and CREB in B cells is not affected by D_1-like receptor stimulation.
PBMCs of HC and RA patients were stimulated with D_1-like receptor agonist A68930 at indicated concentrations for indicated times in vitro. PMA served as positive control. Following acute D_1-like receptor stimulation cells were fixed and permeabilized with 2% PFA and methanol, respectively, followed by intracellular staining for phosphorylated ERK1/2 and CREB. A) Gating strategy to determine pERK1/2 and pCREB in B cells. First, doublets were excluded followed by gating on lymphocytes in FSC/SSC plot. B cells were identified as $CD19^+$ cells. Within the B cell population pERK1/2 and pCREB expression were evaluated by MFI of respective BD phosflow-antibodies. Quantification of pERK1/2 (B) and pCREB (C) in B cells of HC and RA patients after 10-20 min D_1-like receptor stimulation. Subjects were age- and sex-matched. Box plots show 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, and 90^{th} percentile. Use of mixed-effects analysis to determine overall influence of disease ($^{\$}p \leq 0.05$) and D_1-like stimulation. Bonferroni's multiple comparisons test was used to determine impact of D_1-like receptor stimulation vs. unstimulated control within each group. CREB: cAMP response element-binding protein, ERK: extracellular-signal regulated kinase, FMO: fluorescence minus one, HC: healthy control, MFI: mean fluorescence intensity, RA: rheumatoid arthritis

5. Discussion

5.1. TH and D_1-D_5 DR are present in peripheral immune cells under physiological conditions

Several studies suggested the involvement of the catecholamine DA in immunity. Here, TH, the rate-limiting enzyme in DA biosynthesis, and D_1-D_5 DR were detected in peripheral T cells, B cells, NK cells and monocytes. Peripheral immune cells are thus able to synthesize and respond to the catecholamine DA. Moreover, due to a comprehensive flow cytometric approach, expression levels between immune cell subsets could be directly compared.

TH was expressed at high frequencies in all investigated subpopulations meaning that cells are equipped with the rate-limiting enzyme for DA synthesis and thus are able to network with each other on alternative paths. Data on TH expression in human immune cells is limited. The finding on high frequency of monocytes expressing TH is in line with others (Gopinath et al. 2020). Gene expression of TH is demonstrated in $CD8^+$ T cells, T_{FH} cells and PBMCs (Nasi et al. 2019; Cosentino et al. 2002; Papa et al. 2017; Cosentino et al. 2002). Furthermore, analysis of catecholamines in freshly isolated PBMCs revealed the presence of DA, Epi and NE. This proves the high frequencies of TH expressing immune cells. Detectable levels of Epi and NE also implicate the presence and functionality of L-DOPA decarboxylase, DBH and PNMT in peripheral immune cells. Additional confirmation of TH presence in immune cells is given by rising concentrations of DA after stimulation of PBMCs *in vitro* (Cosentino et al. 2002). However, since the exact origin of DA is not given here, stimulation of single immune cell subsets and subsequent analysis for DA content by HPLC would be interesting to prove the functionality of TH in every immune cell subset.

Generally, cells belonging to the adaptive immune system presented a more variable expression of D_1-D_5 DR between donors than cells of the innate immune system. Frequency of D_5 DR expressing cells was low in every investigated cell type. Here, findings need to be confirmed with an appropriate positive control and therefore, D_5 DR expression levels are not included in the following considerations. The balance between low D_1-like and high D_2-like receptor expression in T and B cells shifted towards a comparable high D_1-like and D_2-like receptor expression in NK cells and monocytes. The DA system in adaptive immunity seems more flexible and could therefore be of relevance in immune cell homeostasis, while innate immune cells are able to respond promptly to DA stimulus. Generally, literature regarding D_1-D_5 DR expression in human peripheral immune cells is conflicting. The only study directly comparing different immune cell subsets by McKenna et al. (2002) suggests a more variable expression of D_2 and D_4 DR between donors in line with the findings presented. Although here, variability and overall high frequencies of D_2 and D_4 DR expressing cells may arise from the intracellular, i.e. total, staining and detection via secondary PE-labeled antibody. However, the reported high frequencies of D_3 and D_5 DR expressing cells compared to D_1, D_2 and D_5 DR are

not supported by the presented findings. In contrast to the presented data others even suggest the overall absence of D_1 DR but presence of D_5 DR (McKenna et al. 2002; Ricci et al. 1999). McKenna et al. also reported low DR expression in T cells and monocytes and higher expression in B cells and NK cells not revealed here. Regarding $CD3^+$ T cells, similar to stated results frequencies of DR expressing cells except D_5 DR are reported by others (Keren et al. 2019). Further studies focusing on $CD4^+$ and $CD8^+$ T cells reveal generally low frequencies of D_1-D_5 DR expressing cells (Nasi et al. 2019; Kustrimovic et al. 2014). However, since the experimental setup identified total T cells as $CD3^+CD56^-$ cells, it was not possible to discriminate between T helper and cytotoxic T cells; thus, the presented findings were not comparable with the above mentioned. Published data on B cells expressing D_1-D_5 DR is limited to genetic analysis and suggests in contrast to current findings presence of all DR except D_4 DR (Meredith et al. 2006). Also, expression of DR in NK cells was shown to be restricted on D_2-D_5 DR thus lacking D_1 DR and differing from the reported results (Mikulak et al. 2014). Overall discrepancies of published findings may be caused by the method used, such as the protein analysis with different (and often polyclonal) antibodies used to detect D_1-D_5 DR. Of course, presented results are based on the quality and reliability of antibodies too. For instance, intracellular staining of D_2 and D_4 DR after permeabilization and further detection via secondary PE-labeled antibody resulted in high frequencies of positive cells as total amount, i.e. also internalized, DR are stained. Especially the absence or presence of single DR in one or another study indicates the necessity for accredited methodology. Here, a sophisticated staining procedure and subsequent gating strategy was used to exclude several factors impeding correct analysis, i.e. doublets, dead cells, double positive cells, that none of the referred studies considered. The presented data on D_1-D_5 DR expression in peripheral immune cells is therefore of high significance.

Because of the heterogeneous group of subjects investigated, findings could be correlated with their age and sex. Here, sex-specific differences in the frequency of D_1 DR expressing peripheral B cells were identified. Frequency of D_1 DR expressing B cells was significantly lower in female compared to male healthy subjects. This detected sex-specific difference in D_1 DR expression could be caused by sex hormones and influence DA function. There is no clear link between DR expression in immune cells and sex hormones, especially estrogen to date. However, in cell-lines co-transfected with plasmids containing genes for both D_1 DR and steroid hormone receptors, the stimulation with estrogen resulted in upregulation of D_1 DR expression (Lee and Mouradian 1999). This is indicating binding sites for estrogen in the promotor region of the D_1 DR gene but contrasts with the observed lower frequency of D_1 DR expressing B cells in female than male HC. Opposite findings may depend on *in vitro/in vivo* system, on different hormonal levels as well as chronic vs. acute exposure. However, in our laboratory, acute *in vitro* stimulation of PBMCs with different estrogen receptor agonists hardly affected expression of D_1 DR in B cells. Of note, these were only preliminary experiments with

limited sample size. Further studies are necessary to identify the influence of sex on D_1 DR expression.

Deeper phenotyping of $CD4^+$ T cells, B cells and NK cells revealed an increase in D_1 DR expression during maturation. Since the increase is present in different cell types D_1 DR could represent a general lymphoid maturation marker. Additionally, the reported donor-dependent variations in frequency of D_1 DR expressing T cells and B cells could partly be explained by different compositions of populations between donors. Apart from that, mature cells emerge as more sensitive to D_1-like receptor stimulation. So far, literature on DR expression during maturation of immune cells is restricted to $CD4^+$ T cells. Although CD45RO is a more suitable marker for the identification of memory T cells, Kustrimovic et al. divided naive and memory T cells based on CD45RA expression as in the presented thesis. $CD45RA^-CD4^+$ T cells were further subdivided in central and effector memory T cells. In contrast to the presented data, frequencies of D_1 DR expressing naive and memory T cells are generally lower and central memory T cells show decreased D_1 DR expression compared to naive $CD4^+$ T cells not detected here (Kustrimovic et al. 2014). Also, activation of T cells and NK cells was shown to upregulate expression of DR (Keren et al. 2019; Kustrimovic et al. 2014; Mikulak et al. 2014). Hence, increased expression of D_1 DR could also represent immune cell status. More studies analyzing additional immune cell maturation markers in parallel to D_1 DR are necessary to validate these findings.

5.2. Disease-specific alterations of TH and D_1-D_5 DR in peripheral immune cells of RA, PsA and SpA patients

In addition to analysis of TH and D_1-D_5 DR expression in peripheral immune cells under physiological conditions, expression was investigated in patients with chronic inflammatory diseases of the joints. So far, DA was already shown to contribute to local joint inflammation in RA, but knowledge on the systemic immune-modulatory role of DA is limited. Additionally, to compare DA system in different diseases, PsA and SpA patients were included. The direct comparison of HC with RA, PsA and SpA patients uncovered disease-specific alterations. Notably, this is the first study on DR expression in peripheral immune cells of PsA and SpA patients and contributes additional information to the restricted findings of DR expression in B cells of RA.

As already noted, PBMCs contained catecholamines. Unfortunately, analysis of catecholamine content in PBMCs lacked samples of PsA and SpA patients. Interestingly, DA was exclusively detected in PBMCs of RA patients but not of HC, pointing towards a pivotal role in disease. Since NE and Epi were present in PBMCs of HC, DA must be present under physiological conditions as well, rather serving as a precursor for further conversion to NE and

Epi. However, as these findings are preliminary, further analysis of DA in both HC and RA samples is still required to fully prove the observed difference.

Compared to HC, a decreased frequency of D_3 DR expressing CD56 NK cells was detected in RA. Moreover, increased frequencies of D_1 DR expressing $CD56^{bright}$ NK cells and monocytes were found in SpA patients. Frequency of TH expressing monocytes was significantly increased in both PsA and SpA compared to HC. However, all findings listed above are based on high frequencies at around 100% implying small mean differences and are therefore most likely functionally irrelevant. Analysis of MFI was not conducted since cytometer settings were occasionally adjusted and thus did not permit direct comparison. Of note, most investigated patients herein had a longer disease duration and consequently extended drug intake. Stronger disease-related alterations prior to therapy could be present. Further analyses are needed to confirm the altered expression of DR and TH in NK cells and monocytes of rheumatic patients. Notably, all described alterations are based on findings in peripheral immune cells of RA, PsA and SpA patients. Within the environment of inflamed joints DA concentration is increased (Nakano et al. 2011) and hence expression of D_1-D_5 DR on immune cells may differ as DR are known to be internalized by regulatory phosphorylation of GRKs and β-arrestins after prolonged stimulation causing desensitization (Gainetdinov et al. 2004). Analysis of immune cells out of synovial fluid was not possible due to paucity of enrolled patients but is currently ongoing.

Although showing no difference to HC per se, the frequency of D_1 DR expression in peripheral $CD56^{bright}$ NK cells from RA patients correlated significantly with DAS28 score and functional impairment. $CD56^{bright}$ NK cells are greatly accumulated within synovial fluid (Yamin et al. 2019) and are decisive in triggering joint inflammation by secreting cytokines (Louis et al. 2020). Accordingly, these striking results suggest that D_1-like receptor signaling in $CD56^{bright}$ NK cells might be of relevance in the process of inflammation by triggering cytokine secretion also systemically. However, in contrast to the hypothesis in promoting inflammation, DA was shown to suppress proliferation and IFN-γ secretion of activated NK cells via D_1-like receptors (Mikulak et al. 2014). Yet this observation is based on *in vitro* experiments with NK cells of healthy donors and the effect of DA on NK cells may be different *in vivo*. Moreover, differences between NK cell origin should be considered here since response to DA may depend on chronic inflammation in RA.

A substantial increase of D_1 DR expression was discovered in peripheral B cells of RA patients, not in PsA and SpA, therefore alteration in D_1 DR expression was disease-specific. Among the investigated diseases, RA is the only one connected to autoantibodies. However, the finding of elevated D_1 DR in B cells was independent of autoantibody presence or absence (data not shown). Since B cells are substantially involved in pathogenesis of RA not only by producing autoantibodies (Volkov et al. 2020), but also promoting inflammation (Harre et al. 2012) and

bone destruction (Grötsch et al. 2019; Sun et al. 2018) the increased expression of D_1 DR in B cells could serve as a potential target for future therapy. Interestingly, considering the described sex-specific expression pattern in healthy subjects, the altered expression of D_1 DR in B cells originated solely from women affected by RA. As incidence of RA is three fold higher in women than in men (Deutsche Gesellschaft für Rheumatologie e.V.) this finding is of high impact and points to a novel sex-specific mechanism contributing to RA pathogenesis. Cause of increased D_1 DR expression in B cells of female RA patients is so far unclear. As introduced, estrogen was shown to increase expression of D_1 DR in cell-lines (Lee and Mouradian 1999). Since estrogens contribute to inflammation in RA and are elevated in synovial fluid (Cutolo et al. 2006) they may also contribute to the increased frequency of D_1 DR expressing B cells of female RA patients. In addition, early studies identified a decreased activity of GRKs in PBMCs of RA patients (Lombardi et al. 1999) that theoretically could result in defective DR internalization and increased D_1 DR levels. More studies are needed to identify the underlying cause in our patient cohort. Furthermore, the increased frequency of D_1 DR expressing B cells of female RA patients correlated significantly with disease duration and tended to also correlate with functional impairment. Hence, D_1-like receptor signaling appears to play a critical role in pathogenic progress of RA. For some recruited patients disease scores were not evaluated at the day of blood sampling (numbers of missing data are indicated in the respective tables with patient characteristics) and therefore expression data is missing in these analyses. Investigating additional samples of female RA patients with actual disease scores would further strengthen these results on D_1 DR expressing B cells in connection with RA severity. Limitations in B cell analysis also occurred in respect to therapy as RA patients with severe disease course and late stages were treated with rituximab and thus excluded herein. Future studies should as well consider D_1 DR expression on B cells as a potential biomarker in (pre-clinical) RA. Wei et al. already published findings on a decreased D_2 DR expression in B cells of RA patients negatively correlating with disease activity and changing after DMARD therapy (2015). However, findings from Wei et al. are not directly comparable with the herein presented data because of the already discussed methodological factors regarding flow cytometric staining and data analysis. Additionally, the representative dot plots in their publication do not at all illustrate the quantified data. In a subsequent publication Wei et al. linked the altered D_2 DR expression to TNF-α levels and bone destruction (2016). But data are equally questionable and quantification is missing. To directly investigate the influence of therapy we planned a longitudinal study. Samples from treatment-naive RA patients were already collected during summer 2019 but second sampling after one year is still ongoing. Future analysis will reveal possible effects of DMARD treatment on expression and function of D_1-D_5 DR and TH in immune cells. Besides this, inter-individual differences can be assessed separately with these samples.

Own results also uncover an increase of D_1 DR expression during B cell maturation. Nevertheless, first described findings of elevated D_1 DR expression in B cells of RA patients were not depending on increased frequency of memory B cells or plasmablasts. Instead, D_1 DR expression was significantly higher in naive B cells of RA patients and tended to be elevated also in memory B cell stages. Thus, especially early developmental B cell stages of RA patients seem to be susceptible for D_1-like stimulation that may promote their role in RA. Papa et al. clearly demonstrated the involvement of DA in GC interactions between B and T_{FH} cells. Here, T_{FH} cells were shown to release DA after interaction with B cells to augment the immunological synapse and further to promote B cell differentiation via D_1 DR (Papa et al. 2017). In this context, particularly naive B cells of RA patients appear more destined for maturation to memory B cells and plasmablasts via increased D_1 DR expression than HC. Additionally, it would be interesting to reveal if D_1 DR expression is limited to pathologic B cell clones (Tak et al. 2017) representing an even more desired target for therapy in early RA.

Interestingly, peripheral T_{FH} cells of RA patients also showed an increased D_1 DR expression. This is the first finding on DR expression in this exceedingly specific immune cell subset. As T_{FH} cells are able to synthesize and release DA (Papa et al. 2017) T_{FH}-derived DA could act in an autocrine fashion via D_1 DR. Effect of D_1-like receptor stimulation on T_{FH} cells is not described so far. For T cells in general, stimulation of D_1-like receptors reduces secretion of cytokines and proliferation (Cosentino et al. 2007; Besser et al. 2005; Saha et al. 2001). In line with these findings increased D_1 DR expression in RA could act as negative feedback for T_{FH} cells. However, activation of T cells was also shown to increase DR expression (Keren et al. 2019; Kustrimovic et al. 2014). Thus, increased D_1 DR expression in T_{FH} cells could simply represent their activation in RA. Further experiments are needed to define the role of D_1 DR in T_{FH} cells, especially in RA.

So far, all findings described are based on analysis of peripheral immune cells. Analysis of matched blood and synovial fluid samples of RA patients would make it possible to directly compare the dopaminergic system in peripheral and local immune cells. However, hitherto this analysis was hampered by the rarity of patients needed for this kind of analysis: patients with large swollen joints and clear RA diagnosis without DMARD treatment. Yet, disease-related alterations of the dopaminergic system were identified that may be even stronger in local inflammation similar to what has been described for SF within inflamed tissue (Capellino et al. 2014).

5.3. Functional relevance of DR expression in immune cells

5.3.1. Acute DR stimulation

Short-term stimulation of D_1-like receptors, D_2-like receptors or all DR with specific agonists had no major effect on CD69 expression in peripheral T cells, B cells, NK cells and monocytes.

Also, the described disease-specific alterations of DR expression did not result in altered responses. This indicates that acute DR stimulation alone is not sufficient for activation of resting peripheral immune cells, independent of expression levels. CD69 was chosen as early activation marker present in all immune cell populations of interest as indicated by unspecific stimulation with PMA/Iono. However, analysis of specific activation markers for individual immune cell subsets is still interesting. Morkawa et al. already determined that D_2-like receptor agonist bromocriptine suppresses B cell activation *in vitro* as indicated by decreased expression of other activation markers like HLA-DR (Morkawa et al. 1993). Further, effects of DA might depend on inflammatory environment and differ *in vivo*. Pre-stimulated $CD4^+$ and $CD8^+$ T cells slightly increase CD69 expression in response to DA (Torres et al. 2005). However, D_1-like receptor agonist fenoldopam reduces CD69 expression in activated $CD3^+$ T cells of psoriasis patients (Keren et al. 2019). Based on the above-mentioned findings, additional experiments simulating a chronic inflammation while stimulating DR would allow further conclusions on the general role of DA for immune cell activation in this context.

In contrast to B cells, NK cells and monocytes which are almost unaffected by DR stimulation, T cells, at least of HC and RA patients, showed increased Annexin V binding after short-term stimulation of D_1-like receptors, D_2-like receptors, or all DR. Yet, the higher frequencies of Annexin V positive $CD3^+$ T cells compared to unstimulated control occurred in an unclear pattern independent of agonist concentration. Since observed effects of DR stimulation were additionally small, their impact may not be of relevance. Annexin V binds to phosphatidylserine in the outer plasma membrane of apoptotic cells (Vermes et al. 1995). But especially for T cells increased Annexin V binding should be noted with caution as it is induced by activation in $CD8^+$ T cells (Fischer et al. 2006) and is increased as well on $CD4^+$ Tregs (Bollinger et al. 2020). Considering these facts, gating was strictly performed on Annexin V^{++} cells in the $Zombie^-$ population. As this gating identifies only early apoptotic cells and excludes late apoptotic cells, possible earlier effects on apoptosis during 24 h incubation could have been masked. However, this gating strategy was necessary to exclude general dead cells after thawing and culture. In line with the presented results, others report that resting PBMCs viability is hardly affected by short-term DA stimulation (Meredith et al. 2006). Described dose-dependent effects of DA on apoptosis of proliferating immune cells are independent of DR and may mostly be caused by oxidation of catecholamine DA being toxic (Colombo et al. 2003; Meredith et al. 2006; Mikulak et al. 2014). As stable agonists were used for *in vitro* stimulation, apoptosis by oxidation of DA can be excluded. The results thus suggest DR stimulation does not affect immune cell apoptosis. However, further experiments are necessary to prove the increased Annexin V binding in T cells after DR stimulation.

In line with the described data on activation and apoptosis of PBMCs, short-term D_1-like receptor stimulation did not alter IL-6 and IL-8 secretion. Thus, acute DR stimulation alone

does not affect secretion of those cytokines after 24 h. Again, effects may depend on the environment as in stimulated PBMCs DA alters cytokine secretion (Nakano et al. 2011; Keren et al. 2019). However, others have described increased expression of IL-8 mRNA after DA stimulation *in vitro* (Torres et al. 2005). Especially after this short-term DR stimulation, changes on mRNA level of cytokines appear as a more convincing readout to detect also small effects. Further experiments analyzing cytokine expression on gene level are needed to better evaluate effects of DR stimulation.

5.3.2. D_1-like receptor stimulation in T cell-dependent B cell stimulation

The *in vitro* modeling of B-T cell interactions resulted in B cell proliferation and differentiation and depended as expected on B cell stage and culture condition. Thus, the model worked and served as a reliable method to study the impact of D_1 DR stimulation. As D_5 DR was almost absent in B and T_{FH} cells, the observed effects of D_1-like receptor stimulation could be attributed primarily to action on D_1 DR. Overall, effects on proliferation and differentiation of B cells by D_1-like receptor stimulation were small and varied between donors. Limited number of investigated subjects did not permit analysis of causality for responders and non-responders or regarding D_1 DR expression. Nevertheless, as indicated in the bigger changes to SEB control and overall spread of data, D_1-like receptor stimulation had bigger impact on B cells cultured alone than on B cells in co-cultures with T_{FH} cells. B cell stimulation by T_{FH} cells is probably too strong and masks possible effects of the D_1-like receptor agonist A68930. As T_{FH} cells also express D_1 DR, observed effects on B cells could have originated indirectly from effects on co-cultured T_{FH} cells. As previously discussed, D_1-like receptor stimulation did not induce T or B cell apoptosis. But since secretion of some cytokines is downregulated in T cells by D_1-like receptor stimulation (Keren et al. 2019) it is possible that IL-21 secretion necessary for B cell activation, proliferation, and antibody secretion (Liu et al. 2012) is likewise decreased in T_{FH} cells. Specifically in this context, analysis of IL-21 as a critical cytokine for B cell help in co-culture supernatants is necessary to investigate this possibility. Apart from that, it would be interesting to stimulate D_1 DR in future experiments exclusively on B cells to eliminate indirect effects and draw clearer conclusions.

Naive B cells depend more on external triggers for proliferation and maturation which memory B cells intrinsically possess (Deenick et al. 2013; Tangye et al. 2003). Therefore, general proliferation and differentiation as well as effects of D_1-like receptor stimulation were smaller in naive B cells. However, *in vitro* stimulation with SEB also led to memory B cell formation in naive B cell cultures being slightly up- or downregulated by D_1-like receptor stimulation. Although significant effects of D_1-like receptor stimulation were not observed due to the large variability between donors, proliferation as well as maturation in pure B cell cultures to both memory B cells and plasmablasts occurred in a similar pattern respective to agonist concentration. While A68930 is highly specific for D_1-like receptors at lower concentrations

(EC_{50} 2.75 nM) higher concentrations activate preferably D_2-like receptors (EC_{50} 4.225 µM) (Kebabian et al. 1990). Despite the insignificant effects, the concentration-dependent trends indicate that DA signaling via D_1-like receptors is able to modulate B cell proliferation and differentiation. Stimulation with D_1-like agonist A68930 at 10^{-9} M presenting the highest affinity for D_1-like receptors, tended to upregulate B cell proliferation and differentiation. Further studies, especially with RA samples, are necessary to allow more reliable conclusions.

Yet, the trends agree with results from Papa et al. who already demonstrated DA signaling in classical GC interactions between T_{FH} and B cells. T_{FH} cells in secondary lymphoid organs store DA in vesicles and release it after contact with B cells. B cells respond via D_1 DR and upregulate ICOSL expression. In turn they induce a positive feedback in T_{FH} cells by ICOS and CD40 signaling resulting in prolonged interaction and improved B cell maturation (Papa et al. 2017). Dimitrijevic et al. investigated the influence of sex in GC reactions and antibody response after CIA induction in rats. While they did not focus on DA signaling within GC, they report stronger interactions between B and T_{FH} cells in female than male rats further resulting in greater IgG responses (Dimitrijević et al. 2020). Considering the elevated D_1 DR expression in B cells of solely female RA patients and the reported DA signal transmission in GC, it may relate to the stronger B-T_{FH} interaction observed in female CIA rats. Moreover, schizophrenia patients treated with DR antagonist clozapine suffer from antibody deficiency and reveal reduced concentrations of IgG, IgA and IgM (Ponsford et al. 2018). These findings highlight the importance of DA for B cell function. However, in contrast to the published *in vivo* findings D_1-like receptor stimulation did not promote B cell maturation and proliferation in this *in vitro* model. It should be considered to further improve this model because it may lack important factors of the interaction of B and T_{FH} cells *in vivo* in GC, especially in ectopic lymphoid structures within inflamed joints of RA patients. SF of RA patients were shown to increase numbers of $CD4^+$ $CXCR5^+$ $ICOS^+$ T_{FH} cells (Tang et al. 2017). Additionally, T_{PH} cells lacking CXCR5 and thus not included in our experiment are able to stimulate B cell responses in inflamed joints of RA patients and may signal via DA (Rao et al. 2017). Of note, pathogenic *in vivo* B-T interactions could also be promoted by increased DA levels in RA synovial joint (Nakano et al. 2011). Unfortunately, the limited availability of patient material as well as technical and logistical issues only allowed investigation of healthy donor cells. Due to the increased D_1 DR expression in naive B cells of RA patients it is likely that these are even more responsible for D_1-like receptor stimulation in a T cell-dependent framework. Since Papa et al. described that T_{FH} cells endogenously possess DA stored in vesicles it would be interesting to stimulate T_{FH} cells with reserpine, an uptake-inhibitor of catecholamines into chromaffin granules, to release DA (Cosentino et al. 2007) and evaluate the effect on B cell function in future studies.

5.3.3. D_1-like receptor stimulation in T cell-independent B cell stimulation

Independent of T cell help, D_1-like receptor stimulation increased proliferation index but not overall frequency of divided B cells. This finding suggests that D_1-like receptor stimulation is only promoting proliferation of D_1 DR expressing B cells in a T cell-independent manner while D_1 DR negative B cells are unresponsive. Again, D_5 DR expression was almost absent in B cells and thus did presumably not affected the readouts after D_1-like receptor stimulation. Moreover, this finding was independent of health condition, even when separating patients in early and late RA (data not shown). Considering the higher D_1 DR expression in B cells and the elevated concentration of DA in inflamed joints of RA patients (Nakano et al. 2011) this effect could represent an additional way to promote proliferation of pathogenic B cells *in vivo*. Up to now there are no comparable studies. Meredith et al. observed reduced proliferation in polyclonal stimulated B cells and PBMCs by DA. However, this effect was independent of DR and attributed to apoptosis induction by oxidation of DA (Meredith et al. 2006). Further experiments including selective antagonists for D_1-like receptors, e.g. SCH39166, are still pending to fully prove the suggested mode of action in increasing B cell proliferation via D_1-like receptors.

Overall, differentiation of B cells towards plasmablasts and IgG secretion *in vitro* were not affected by D_1-like receptor stimulation. However, absence of effects may be caused by donor-dependent differences. Even if data were normalized to the heterogeneous CpG controls, the occurring variations after D_1-like stimulation may be attributed to basal activation state or immunosuppressive therapy of RA patients. Also, effects of age or sex, as well as disease duration seem possible. Yet, the rather small sample size and missing clinical data made correlation of observed effects impossible. So far, the basis for the detected donor-dependent differences remains unclear. Furthermore, different intracellular signaling pathways could lead to divergent effects. Jenei-Lanzl et al. already identified a switch from $G\alpha_s$ to $G\alpha_i$ in synovial cells from RA patients (2015) leading to altered outcomes after stimulation of GPCRs. Further studies are necessary to explain the observed differences in plasmablast formation and IgG secretion between donors. Additionally, it could be of interest to analyze the effect of D_1-like receptor stimulation on IgA and IgM secretion of B cells as well, since RF and ACPA also comprise these classes of immunoglobulins and are relevant in disease (Volkov et al. 2020). Direct analysis of secreted ACPA and RF were not conducted as commercial kits used in the clinic are expensive and would not allow extensive testing. In addition, future analysis should include time-course analysis of major genes required in B cell development (e.g. *Pax5*, *BCL6*, *AID*, *Xbp1* and *Blimp1*) by qPCR. Hence, data could reveal a more precise picture of possible effects of D_1-like receptor stimulation.

Effect of D_1-like receptor stimulation on cytokine secretion varied largely between donors. Generally, levels of TNF-α and IL-10 were low and remained almost unchanged upon D_1-like

receptor stimulation. Also, levels of IL-6 and CCL3 were overall unaffected but RA patients tended towards increased CCL3 levels after D_1-like receptor stimulation. Levels of IL-8 increased in response to D_1-like receptor stimulation selectively in RA patients. Thus, IL-8 and CCL3 as mediators of bone destruction are increased by D_1-like receptor stimulation in a B cell-dependent manner especially in RA patients. As pure D_1-like receptor stimulation with A68930 for 24 h did rather downregulate IL-8 secretion, effects are probably dependent on the chronic inflammatory environment. Certainly, other PBMC subtypes possess D_1 DR as well and could have influenced level of cytokines, apparently also by indirect activation through B cell derived cytokines. To clearly link altered cytokine levels to B cells one could either isolate them for pure B cell cultures or stain cytokines intracellularly after treatment with protein trafficking inhibitor inducing intracellular accumulation of cytokines. The time point of analysis was initially chosen because the differentiation of plasmablasts and secretion of antibodies is expected with increasing proliferation cycles (Marasco et al. 2017). However, analysis at the end of culture could have masked effects on cytokines with shorter half-life. Therefore, future experiments should include time-course experiments to study the impact of DR stimulation on cytokines like TNF-α and IL-10. For instance, IL-10 producing B cells are induced by CpG after 48 h in culture and expanded with CD40 ligand, also from RA patients. Moreover, these regulatory B cells are able to suppress cytokine secretion of T cells (Bankó et al. 2017). Hence, it would be interesting to investigate the effect of D_1-like receptor stimulation on regulatory B cell formation especially for RA at an earlier time point. Literature regarding DA effects on cytokine secretion in human PBMCs is limited to anti-CD3/28 stimulated cells and moreover conflicting. While some report increased levels of IL-10, TNF-α, IL-6, and IL-8 after 18 h DA stimulation (Torres et al. 2005) others state impeding effect of D_1-like receptor agonist fenoldopam on TNF-α and IL-6 but not IL-10 and IL-8 secretion after 48 h (Keren et al. 2019). Observed differences might rely on stimulated DR but these findings also highlight the need to study the effects of D_1-like receptor stimulation on cytokine secretion earlier than conducted here. Additionally, other studies demonstrate that effects of DA on IL-6 and IL-8 secretion are clearly dependent on investigated cell types and context as contrary effects are observed for human SF and keratinocytes (Capellino et al. 2014; Parrado et al. 2012).

The findings of elevated CCL3 and IL-8 level after D_1-like receptor stimulation solely in PBMC cultures from RA patients add up to current knowledge. In RA, B cell-derived CCL3 inhibits osteoblast differentiation (Sun et al. 2018) and local IL-8 promotes osteoclastogenesis (Krishnamurthy et al. 2016). Although these are *in vitro* findings and may differ *in vivo*, increased levels of DA in inflamed joints of RA patients (Nakano et al. 2011) could contribute to bone damage by increasing CCL3 and IL-8 secretion of B cells via D_1-like receptor stimulation. This theory is in accordance with findings from Nakashioya et al. who reported that D_1-like receptor antagonism by SCH23390 prevents CIA induction in mice. Interestingly, they could neither detect effects on autoantibody secretion or $CD4^+$ T cell differentiation nor on

expression of IL-6 or TNF-α. Yet, osteoclastogenesis was inhibited by SCH23390 (Nakashioya et al. 2011). Similarly, Zhu et al. found that the peripheral blockade of L-DOPA conversion to DA by carbidopa decreases joint inflammation and more importantly inhibits osteoclastogenesis in CIA mouse model (Zhu et al. 2017). Although these findings are described in mice, they point to a role of D_1-like receptor signaling in bone remodeling. Further studies are necessary and should also focus on effects of DR stimulation on RANKL expression and secretion of B cells as this constitutes another important basis for bone destruction in RA (Meednu et al. 2016).

As D_1 DR in peripheral B cells is of functional relevance in RA, a targeted blockade via selective D_1-like receptor antagonists seems promising. Deeper study of the donor-dependent differences in D_1 DR expression reveals a spread in data, both for HC and RA women. It could be speculated that, depending on frequency of D_1 DR expressing B cells, female HC with frequencies in the upper quartile display a higher risk for developing RA whilst female RA with frequencies in the lower quartile may not respond to D_1 DR blockade. Further studies are necessary to assess effects of D_1 DR blockade for B cells proliferation and IL-8 secretion, optimally in a heterogeneous study population.

5.3.4. Signaling after D_1-like receptor stimulation

Overall, analysis of proliferation marker Ki-67 in B cells revealed no marked effects after D_1-like receptor stimulation. However, analysis of Ki-67 after 10-20 min stimulation was probably too short for B cells to escape G_o phase. Therefore, Ki-67 expression should be analyzed after longer stimulation and cells could be pre-stimulated to detect possible inhibitory effects. Nonetheless, RA patients presented overall higher Ki-67 expression in B cells than HC. This suggests an increased activation of RA B cells per se in line with the pro-inflammatory environment they derive from.

Similarly, phosphorylation levels of ERK or CREB in B cells remained unchanged after D_1-like receptor stimulation. B cells of RA patients presented higher levels of phosphorylated ERK and CREB in general, suggesting increased basal activation. Intracellular signaling of GPCRs in response to agonists occurs between 5-30 min (Beaulieu 2016). Due to limited numbers of patient PBMCs, time points within this range were specified in preceding assays. However, the results suggest that D_1-like receptor stimulation in B cells does not induce signals via the canonical pathway. Phosflow technique used to investigate intracellular signaling in B cells may not be sensitive enough to detect alterations in ERK and CREB phosphorylation. Unfortunately, the effect of D_1-like receptor stimulation on cAMP production in isolated B cells could not be investigated due to the limited number of patient material. Though, it is already demonstrated that neither stimulation with D_1- nor D_2-like receptor agonist induces alterations in cAMP level in EBV-transformed B cells (Natsukari et al. 1996). This finding further suggests

that DA signaling engages different pathways in immune cells. Interestingly, Mahendra et al. identified differentially regulated genes related to $G\alpha_q$ and cAMP signaling in autoreactive B cells of RA patients (Mahendra et al. 2019) suggesting that especially here DR signaling is different. Indeed, D_1 DR can also build heterodimers with D_2 DR and alternatively couple to $G\alpha_q$ to activate PLC (Rashid et al. 2007). Future studies should therefore also focus on alternative intracellular signaling pathways via $G\alpha_q$. In context with the presented findings, canonical D_1-like receptor signaling appears improbable as synthetic DMARD apremilast is elevating intracellular cAMP levels leading to anti-inflammatory effects in PsA patients. In RA patients, switching of G proteins is already described for synovial cells (Jenei-Lanzl et al. 2015) and could be present in other cells as well. Furthermore, DA was shown to induce non-canonical effects as oxidative stress and mitochondrial damage on immune cells (Elkashef et al. 2002; Colombo et al. 2003). Analyzing these metabolic parameters after DR stimulation in single immune cell subsets could contribute to decipher DA signaling in immune cells.

6. Conclusions and relevance for future treatment of rheumatic diseases

Here, the presence of D_1-D_5 DR and TH was demonstrated in peripheral T cells, B cells, NK cells and monocytes of HC and RA, PsA and SpA patients by comprehensive flow cytometry approaches. Importantly, disease-related alterations in peripheral immune cells of rheumatic patients were detected. Especially the increased expression of D_1 DR in peripheral B cells of solely female RA patients is of high significance as it correlated with disease parameters. *In vitro* stimulation of D_1-like receptors increased proliferation of B cells independent of T cell help and elevated IL-8 levels exclusively in RA. Thus, the neuroimmune-axis in peripheral B cells seems to contribute to RA pathogenesis and therefore targeted modulation of D_1 DR on B cells of RA patients constitutes a potential target for future therapy.

7. References

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8. Appendix

Table 7: Characteristics of investigated HC and rheumatic patients for cross-sectional study on D_1-D_5 DR expression in peripheral immune cells.

*Disease scores were transferred on actual basis, non-actual scores were stringently excluded, *[n] indicates the number of missing actual information at date of blood sampling; ACPA: anti-citrullinated protein antibody, ASDAS: Ankylosing Spondylitis Disease Activity Score, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, DAS28: disease activity score 28, DMARD: disease-modifying anti-rheumatic drug, FFbH: Funktionsfragebogen Hannover, HLA: human leukocyte antigen, SD: standard deviation, RF: rheumatoid factor*

	Healthy controls	*Rheumatoid Arthritis*	*Psoriasis Arthritis*	*Ankylosing Spondylitis*
Total, #	25	25	14	21
Age (years), mean ± SD	41.4 ± 16.3	54.6 ± 16	44 ± 12.8	44.5 ± 12.4
Female, # (%)	19 (76)	21 (84)	7 (50)	8 (38)
Years since first diagnosis, mean ± SD	-	7.9 ± 7.1	6 ± 4.7	13 ± 10.8
Patients with bone erosion, # (%)	-	6 (26) *[2]	1 (7)	-
RF positive patients, # (%)	-	13 (57) *[2]	-	-
ACPA positive patients, # (%)	-	7 (78) *[16]	-	-
HLA-B27 positive patients, # (%)	-	-	-	9 (90) *[11]
DAS28, mean ± SD	-	2.9 ± 1.1 *[17]	2.1 ± 0.8 *[7]	-
FFbH, mean ± SD	-	68 ± 24 *[14]	83 ± 20 *[6]	-
ASDAS, mean ± SD	-	-	1.7 ± 0.5 *[11]	2.2 ± 0.8 *[11]
BASDAI, mean ± SD	-	-	2.8 ± 1.7 *[11]	4.8 ± 2.1 *[11]
BASFI, mean ± SD	-	-	2 ± 1 *[11]	4.3 ± 2.7 *[11]
Receiving corticosteroids, # (%)	-	13 (59) *[3]	2 (14)	1 (5) *[1]
Receiving non-biologic DMARD, # (%)	-	14 (64) *[3]	12 (86)	2 (10) *[1]
Receiving biologic DMARD, # (%)	-	16 (73) *[3]	8 (57)	19 (95) *[1]

Table 8: Characteristics of HC and rheumatic patients for short-term DR stimulation on peripheral immune cells.

*Disease scores were transferred on actual basis, non-actual scores were stringently excluded, *[n] indicates the number of missing actual information at date of blood sampling; ACPA: anti-citrullinated protein antibody, ASDAS: Ankylosing Spondylitis Disease Activity Score, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, DAS28: disease activity score 28, DMARD: disease-modifying anti-rheumatic drug, FFbH: Funktionsfragebogen Hannover, HLA: human leukocyte antigen, SD: standard deviation, RF: rheumatoid factor*

	Healthy controls	*Rheumatoid Arthritis*	*Psoriasis Arthritis*	*Ankylosing Spondylitis*
Total, #	6	7	5	6
Age (years), mean ± SD	25.3 ± 3.6	62.4 ± 19.1	49 ± 10.6	43.7 ± 6.9
Female, # (%)	3 (50)	6 (86)	3 (60)	3 (50)
Years since first diagnosis, mean ± SD	-	10.8 ± 8.2	6.7 ± 2.7	13.3 ± 11.7
Patients with bone erosion, # (%)	-	5 (71)	-	-
RF positive patients, # (%)	-	5 (71)	-	-
ACPA positive patients, # (%)	-	6 (86)	-	-
HLA-B27 positive patients, # (%)	-	-	-	3 (50)

DAS28, mean ± SD	-	2.9 ± 0.7 *3	1.9 ± 0.7 *2	-
FFbH, mean ± SD	-	59.3 ± 34.5 *2	89.7 ± 17.9 *2	-
ASDAS, mean ± SD	-	-	2 *4	2 ± 0.6 *2
BASDAI, mean ± SD	-	-	4.8 *4	4.1 ±1.4 *2
BASFI, mean ± SD	-	-	1.4 *4	3.4 ± 3.1 *2
Receiving corticosteroids, # (%)	-	4	1	0
Receiving non-biologic DMARD, # (%)	-	3	5	0
Receiving biologic DMARD, # (%)	-	7	2	6

Table 9: Characteristics of HC and RA patients for D_1 DR analysis during B and T cell maturation.
*Disease scores were transferred on actual basis, non-actual scores were stringently excluded, *n indicates the number of missing actual information at date of blood sampling; ACPA: anti-citrullinated protein antibody, DAS28: disease activity score 28, DMARD: disease-modifying anti-rheumatic drug, FFbH: Funktionsfragebogen Hannover, SD: standard deviation, RF: rheumatoid factor*

	Healthy controls	*Rheumatoid Arthritis*
Total, #	25	23
Age (years), mean ± SD	44.6 ± 15.7	53.9 ± 14.4
Female, # (%)	19 (76)	20 (87)
Years since first diagnosis, mean ± SD	-	5.9 ± 6.9
Patients with bone erosion, # (%)	-	1 (4)
RF positive patients, # (%)	-	1 (5) *4
ACPA positive patients, # (%)	-	8 (42) *4
DAS28, mean ± SD	-	2.8 ± 1.3 *17
FFbH, mean ± SD	-	75 ± 16.5 *15
Receiving corticosteroids, # (%)	-	8 *1
Receiving non-biologic DMARD, # (%)	-	10 *1
Receiving biologic DMARD, # (%)	-	10 *1

Table 10: Characteristics of healthy donors for co-culture experiments.
SD: standard deviation

	Healthy controls
Total, #	8
Age (years), mean ± SD	49 ± 10.1
Female, # (%)	6 (75)

Table 11: Characteristics of HC and RA patients as donors for T cell-independent B cell stimulation.
*Disease scores were transferred on actual basis, non-actual scores were stringently excluded, *[n] indicates the number of missing actual information at date of blood sampling; ACPA: anti-citrullinated protein antibody, DAS28: disease activity score 28, DMARD: disease-modifying anti-rheumatic drug, FFbH: Funktionsfragebogen Hannover, SD: standard deviation, RF: rheumatoid factor*

	Healthy controls	*Rheumatoid Arthritis*
Total, #	13	11
Age (years), mean ± SD	43.2 ± 15	60.6 ± 11.4
Female, # (%)	10 (77)	10 (91)
Years since first diagnosis, mean ± SD	-	6.6 ± 8.1
Patients with bone erosion, # (%)	-	1 (9)
RF positive patients, # (%)	-	1 (13) *[3]
ACPA positive patients, # (%)	-	1 (14) *[4]
DAS28, mean ± SD	-	2.1 *[10]
FFbH, mean ± SD	-	92.5 ± 9.2 *[9]
Receiving corticosteroids, # (%)	-	3 *[3]
Receiving non-biologic DMARD, # (%)	-	2 *[3]
Receiving biologic DMARD, # (%)	-	5 *[3]

Table 12: Characteristics of HC and RA patients as donors for analysis of intracellular signaling after D_1-like receptor stimulation.
*Disease scores were transferred on actual basis, non-actual scores were stringently excluded, *[n] indicates the number of missing actual information at date of blood sampling; ACPA: anti-citrullinated protein antibody, DAS28: disease activity score 28, DMARD: disease-modifying anti-rheumatic drug, FFbH: Funktionsfragebogen Hannover, SD: standard deviation, RF: rheumatoid factor*

	Healthy controls	*Rheumatoid Arthritis*
Total, #	10	9
Age (years), mean ± SD	56.6 ± 4.9	55.7 ± 16.9
Female, # (%)	10 (100)	9 (100)
Years since first diagnosis, mean ± SD	-	6.7 ± 6.9
Patients with bone erosion, # (%)	-	2 (22)
RF positive patients, # (%)	-	2 (33) *[3]
ACPA positive patients, # (%)	-	4 (67) *[3]
DAS28, mean ± SD	-	2.2 ± 0.1 *[7]
FFbH, mean ± SD	-	83.3 ± 13.9 *[3]
Receiving corticosteroids, # (%)	-	3 *[3]
Receiving non-biologic DMARD, # (%)	-	3 *[3]
Receiving biologic DMARD, # (%)	-	5 *[3]

9. Curriculum vitae

The curriculum vitae is not included in the online version for reasons of data protection.

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10. Eidesstattliche Erklärungen

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Dortmund, den _____ _____

Karolin Wieber

Hiermit erkläre ich, gem. § 7 Abs. (2) e) + g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

Dortmund, den _____ _____

Karolin Wieber

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema *„The dopaminergic pathway: A potential approach to target specific leukocyte subpopulations in chronic inflammatory joint diseases“* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Karolin Wieber befürworte.

Dortmund, den _____ _____

Silvia Capellino