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Influence of the Cannabinoids Anandamide and Cannabidiol on Cytokine Production by CD4+ T Cells and Th17 Differentiation in Patients with Rheumatoid Arthritis

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Die Konzeption der Experimente zur Expression der Cannabinoidrezeptoren CB1 und CB2 sowie dem Einfluss von Anandamid und Cannabidiol auf Zytokin-Positivität erfolgte in Zusammenarbeit mit Herrn Professor Dr. med. David Kofler, Iris Waqué und Thom Haak. Die Idee zur Erweiterung dieser Untersuchungen um den Rezeptor GPR55 sowie der Korrelationsanalysen wurde ausschließlich von mir entwickelt und konzeptualisiert. Die praktische Durchführung aller durchflusszytometrischen, ELISA und qPCR Experimente sowie die dazugehörige Datenerhebung, anschließende statistische Auswertung und Interpretation der Resultate wurden von mir eigenständig und ohne fremde Beteiligung durchgeführt. Lediglich der initiale Schritt der Zellisolierung aus Patientenblut wurde gemeinschaftlich von den beteiligten Mitarbeiterinnen und Mitarbeitern des Labors für Molekulare Immunologie durchgeführt, da die isolierten Zellen anschließend für verschiedene experimentelle Vorhaben unter den an der Isolation beteiligten Laboranten aufgeteilt wurden. Eine Einweisung in die experimentellen Methoden erfolgte durch Frau Dr. Anja Meyer. Die Erhebung klinischer Informationen sowie die Entnahme und Bereitstellung des Patientenbluts erfolgte durch die Kolleginnen und Kollegen der Klinik für Innere Medizin II – Nephrologie, Rheumatologie, Diabetologie und Allgemeine Innere Medizin.

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Contents

Abbreviations

2-AG	2-Arachidonoylglycerol
5-HT1A	5-Hydroxytryptamine Receptor 1A (Serotonin Receptor 1A)
5-HT2A	5-Hydroxytryptamine Receptor 2A (Serotonin Receptor 2A)
A2A	Adenosine A2A receptor
ACPA	Anti-Citrullinated Protein Antibodies
ACR	American College of Rheumatology
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
AEA	Anandamide
AHR	Aryl Hydrocarbon Receptor
Akt	Protein Kinase B
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
B2M	β-2-Microglobulin
cAMP	Cyclic Adenosine Monophosphate
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CBC	Cannabichromene
CBD	Cannabidiol
CBG	Cannabigerol
CCL2	CC-Chemokine Ligand 2
CCL5	CC-Chemokine Ligand 5
CCL20	CC-Chemokine Ligand 20
CCR6	C-C Motif Chemokine Receptor 6
CD	Cluster of Differentiation
CD4+	Cluster of Differentiation 4 Positive
CIA	Collagen Induced Arthritis
COX	Cyclooxygenase
cPLA2	Cytoplasmic Phospholipase A2
CRP	C-Reactive Protein
CSF2	Colony Stimulating Factor 2
DAS28-CRP	Disease Activity Score of 28 Joints with C-Reactive Protein
DC	Dendritic Cell
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
EAE	Experimental Autoimmune Encephalomyelitis
ECS	Endocannabinoid System
EDTA	Ethylenediaminetetraacetic Acid

ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Erythrocyte Sedimentation Rate
EULAR	European League Against Rheumatism
FAAH	Fatty Acid Amide Hydrolase
FLS	Fibroblast-Like Synoviocyte
FoxP3	Forkhead-Box-Protein P3
FSC	Forward Scatter
G-CSF	Granulocyte Colony-Stimulating Factor
GATA3	GATA Binding Protein 3
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPCR	G-Protein Coupled Receptor
GPR55	G-Protein Coupled Receptor 55
HC	Healthy Control
HDL	High-Density Lipoprotein
HLA	Human Leukocyte Antigen
ICOS	Inducible T Cell Costimulator
IFN-γ	Interferon-Gamma
IKZF3	Ikaros Family Zinc Finger 3
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-17	Interleukin-17
IL-17A	Interleukin-17A
IL-18	Interleukin-18
IL-21	Interleukin-21
IL-22	Interleukin-22
IL-23	Interleukin-23
IL-1β	Interleukin-1-Beta
iNOS	Inducible Nitric Oxide Synthase
JAK-STAT	Janus Kinase/Signal Transducer and Activator of Transcription
Lck	Lymphocyte-Specific Protein Tyrosine Kinase
LOX	Lipoxygenase

LPS	Lipopolysaccharide
MACS	Magnetic-Activated Cell Sorting
MAGL	Monoacylglycerol Lipase
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MMP	Matrix-Metalloproteinase
MOG	Myelin Oligodendrocyte Glycoprotein
NET	Neutrophil Extracellular Trap
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK	Natural Killer
NLRP3	NLR Family Pyrin Domain Containing 3
NO	Nitric Oxide
NICD	Notch Intracellular Domain
OPG	Osteoprotegerin
OSM	Oncostatin M
PBMCs	Peripheral Blood Mononuclear Cells
PDGF	Platelet-Derived Growth Factor
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-Kinase
PMA	Phorbol Myristate Acetate
PPAR	Peroxisome Proliferator-Activated Receptor
PsA	Psoriatic Arthritis
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of NF-κB Ligand
RCT	Randomized Controlled Trial
RelA	REL-Associated Protein A
RF	Rheumatoid Factor
RORα	Retinoic acid receptor-related orphan receptor alpha
RORC	Retinoic Acid Receptor-Related Orphan Receptor C
RORγt	Retinoic Acid Receptor-Related Orphan Receptor Gamma T
ROS	Reactive Oxygen Species
qPCR	Quantitative Polymerase Chain Reaction
SGK1	Serum Glucocorticoid Regulated Kinase-1
SLE	Systemic Lupus Erythematosus
SSC	Side Scatter
STAT3	Signal Transducer and Activator of Transcription 3

STAT5	Signal Transducer and Activator of Transcription 5
T-bet	T-box Expressed in T Cells
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor Beta
Th1	T Helper 1
Th2	T Helper 2
Th17	T Helper 17
THC	Δ9-Tetrahydrocannabinol
THCV	Tetrahydrocannabivarin
TIMP-1	Tissue Inhibitor of Metalloproteinases-1
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
Treg	Regulatory T Cell
TRP	Transient Receptor Potential
TRPV1	Transient Receptor Potential Cation Channel Subfamily V Member 1
VAV1	Vav Guanine Nucleotide Exchange Factor 1
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
ZAP-70	Zeta-Chain-Associated Protein Kinase 70

1. German Summary

Diese Arbeit basiert auf Untersuchungen zu den Auswirkungen der Cannabinoide Cannabidiol (CBD) und Anandamid (AEA) auf phänotypische Veränderungen in Cluster of Differentiation 4-positiven (CD4+) T-Zellen von Patienten, die an rheumatischen Autoimmunerkrankungen, insbesondere rheumatoider Arthritis (RA), leiden. Cannabinoide wie CBD werden in der Gesellschaft immer präsenter und oft pauschal zur Behandlung verschiedener Erkrankungen, einschließlich der RA, empfohlen. Dies wird durch das neue Cannabisgesetz (CanG) unterstrichen, das zum 1. April 2024 den Umgang mit Cannabis in Deutschland neu regelte und unter anderem den Zugang zu medizinischem Cannabis erleichterte. Hierdurch ist eine weitere Zunahme der Akzeptanz und des Konsums von Cannabis und Cannabisderivaten ohne medizinische Aufsicht zu erwarten. Zusätzlich haben die erhofften analgetischen Eigenschaften von CBD zu einer vermehrten Nutzung unter RA-Patienten geführt, die nach Alternativen oder Ergänzungen zu ihrer bestehenden Schmerzmedikation suchen. Angesichts dieser zunehmenden Verbreitung der CBD-Nutzung, oft ohne medizinische Aufsicht, ist es klar, dass ein besseres Verständnis des gesamten Spektrums der CBD-Effekte notwendig ist. Nur so können Patienten ausreichend über die Vorteile und Risiken informiert werden, die mit der Aufnahme von CBD in ihre bestehenden Medikationspläne einhergehen.

Neben der zunehmenden Verbreitung des Cannabinoidkonsums schreitet auch unser Verständnis der RA als Krankheit voran. Während der Schwerpunkt der RA-Forschung ursprünglich auf T-Helfer 1 (Th1)-Zellen lag, gewinnen T-Helfer 17 (Th17)-Zellen und ihr Zusammenspiel mit regulatorischen T (Treg)-Zellen als mögliche zentrale Akteure in der Pathogenese der RA an Bedeutung. Dies ermöglicht eine präzisere Untersuchung potenzieller Therapeutika wie CBD und deren Auswirkungen auf RA-spezifische Pathomechanismen. Unser Wissen über den Einfluss von CBD auf Th17-Zellen und dessen Bedeutung im Kontext der RA ist noch unvollständig. Ziel dieser Arbeit ist es, ein tieferes Verständnis der Auswirkungen von den Cannabinoiden CBD und AEA auf die Th17-Differenzierung und Interleukin-17A (IL-17A) Positivität zu erlangen.

Mit diesem Ziel wurden umfassende *in vitro* Studien durchgeführt, um direkte Zusammenhänge zwischen Cannabinoidexposition und CD4+ T-Zell-Eigenschaften herzustellen. Die Analyse von Cannabinoid-Rezeptoren 1 und 2 (CB1, CB2) sowie des G-Protein-gekoppelten Rezeptors 55 (GPR55) zeigte keine signifikanten Unterschiede zwischen RA-Patienten und gesunden Kontrollen, obwohl eine bemerkenswerte Tendenz zu erhöhter GPR55 Expression bei RA- und Psoriasis-Arthritis-Patienten beobachtet wurde. Die Ergebnisse zeigen, dass CBD die Lebensfähigkeit von CD4+ T-Zellen signifikant reduzierte, während paradoxerweise der Anteil IL-17A-positiver Zellen, insbesondere bei RA-

Patienten, erhöht wurde. Dieser Effekt blieb auch unter Th17-polarisierenden Bedingungen bestehen. Die Genexpressionsanalyse zeigte, dass CBD bei RA-Patienten Serum-Glucocorticoid-Kinase 1 (*SGK1)* signifikant hochregulierte und Kolonie-stimulierenden Faktor 2 (*CSF2)* herunterregulierte, was auf eine komplexe Modulation entzündungsbezogener Signalwege hindeutet. Trotz erhöhter zellulärer IL-17A-Positivität zeigte die ELISA-Analyse eine reduzierte Sekretion von IL-17A, Interferon-Gamma (IFN-γ) und Tumornekrosefaktor-alpha (TNF-α) im Zellkulturmedium, möglicherweise aufgrund der beobachteten erheblichen zytotoxischen Effekte. Vorläufige klinische Beobachtungen von Patienten, die über nicht-standardisierten eigenen CBD-Konsum berichteten, zeigten sowohl einen Anstieg des Anteils IL-17A-positiver CD4+ T-Zellen als auch erhöhte Krankheitsaktivitätswerte, was mit unseren *in vitro* Ergebnissen übereinstimmt.

Obwohl CBD gleichzeitig den Anteil an TNF-α und IFN-γ positiven CD4+ Zellen reduzieren konnte, deutet unser erweitertes Verständnis der RA als eine überwiegend von Th17-Zellen vermittelte Erkrankung darauf hin, dass der Konsum von CBD bei RA-Patienten aus Sicht der Autoimmunität nachteilig sein könnte. Diese Vermutung ist hauptsächlich auf die Erkenntnis zurückzuführen, dass CBD zu einem erhöhten Anteil an IL-17A-positiver Zellen unter CD4+ T-Zellen geführt hat, was bei der Empfehlung von CBD als Therapieergänzung berücksichtigt werden sollte. Allerdings sind weitere Studien erforderlich, um den Wirkmechanismus, der zu den hier beobachteten Effekten führt, zu verstehen. Zusätzlich sollten In-vivo-Mäusestudien durchgeführt werden, um die Auswirkungen des CBD-Konsums im Hinblick auf die vielfältigen und komplexen Wechselwirkungen im Körper besser zu verstehen. Außerdem ist es wichtig zu analysieren, inwieweit sich die hier dargestellten *in vitro* Ergebnisse direkt auf Patienten übertragen lassen, da in diesen Experimenten hohe Dosen von CBD verwendet wurden.

Bisher scheinen die Daten nicht ausreichend zu sein, um CBD uneingeschränkt als Behandlungsansatz für RA-Patienten zu empfehlen. In Zukunft sind weitere Studien notwendig, um das Potenzial und die Risiken von Cannabinoiden in der RA-Therapie zu bewerten und eine Empfehlung für die RA-Therapie auszusprechen.

2. English Summary

This work is based on investigations into the effects of cannabinoids such as cannabidiol (CBD) and anandamide (AEA) on phenotypic changes in cluster of differentiation 4-positive (CD4+) T cells from patients suffering from rheumatic autoimmune diseases, particularly rheumatoid arthritis (RA). Cannabinoids such as CBD are becoming increasingly prevalent in society and are often recommended for the treatment of various conditions, including RA. This is further underscored by the new German Cannabis Act (CanG), which regulates the handling of cannabis as of April 1, 2024, and, among other changes, eased access to medicinal cannabis. This is expected to further increase public acceptance and unsupervised use of cannabis and its derivatives. Additionally, the anticipated analgesic properties of CBD have led to increased use among RA patients seeking alternatives or supplements to their existing pain management plans. Given this growing prevalence of CBD use, often without medical supervision, it is clear that a better understanding of the full spectrum of CBD effects is necessary. Only in this way can patients be adequately informed about the benefits and risks associated with incorporating CBD into their existing medication plans.

Alongside the increasing prevalence of cannabinoid use, our understanding of RA as a disease is also advancing. While the focus of RA research was initially on T helper 1 (Th1) cells, T helper 17 (Th17) cells and their interplay with regulatory T (Treg) cells are gaining importance as potential key players in the pathogenesis of RA. This enables a more precise investigation of potential therapeutics like CBD and their effects on RA-specific pathomechanisms. Our knowledge regarding the influence of CBD on Th17 cells and its significance in the context of RA is still incomplete. The aim of this work is to gain a deeper understanding of the effects of cannabinoids on Th17 differentiation and Interleukin-17A (IL-17A) positivity.

With this objective, comprehensive *in vitro* studies were conducted to establish direct relationships between cannabinoid exposure and CD4+ T cell properties. Analysis of cannabinoid receptors 1 and 2 (CB1, CB2) showed no significant differences between RA patients and healthy controls, though a notable trend toward increased G-protein coupled receptor 55 (GPR55) expression was observed in RA and psoriatic arthritis patients. The results demonstrate that CBD significantly reduced CD4+ T cell viability while paradoxically increasing the proportion of IL-17A-positive cells, particularly in RA patients. This effect persisted even under Th17-polarizing conditions. Gene expression analysis revealed that CBD significantly upregulated serum glucocorticoid-regulated kinase 1 (*SGK1)* while downregulating colony stimulating factor 2 (*CSF2)* in RA patients, suggesting complex modulation of inflammation-related pathways. Despite increased cellular IL-17A positivity, ELISA analysis showed reduced secretion of IL-17A, interferon gamma (IFN-γ), and tumor

necrosis factor alpha (TNF-α) in culture supernatants, potentially due to the substantial cytotoxic effects observed. Importantly, preliminary observational data from patients self-reporting non-standardized CBD use showed both increased IL-17A-positive CD4+ T cell percentages and elevated disease activity scores, aligning with our *in vitro* findings.

Although CBD was able to reduce TNF-α- and IFN-γ-positive CD4+ T cells, our understanding of RA as an increasingly Th17 cell-mediated disease suggests that CBD use in RA patients could be detrimental from an autoimmunity perspective. This is primarily due to the finding that CBD led to an increased proportion of IL-17A-positive cells among CD4+ T cells, which should be taken into account when considering recommending CBD as a therapy supplement. However, further studies are required to understand the mechanism of action leading to the effects observed here. *In vivo* mouse studies should also be conducted to better understand the effects of CBD consumption in relation to the multiple and complex interactions within the body. It is also essential to analyze the extent to which the *in vitro* results presented here can be translated directly to patients given the high doses of CBD used in these experiments.

So far, the data is not sufficient to unreservedly recommend CBD as a treatment approach for RA patients. Further studies are necessary to evaluate the potential and risks of cannabinoids in RA therapy and to make a recommendation for RA therapy.

3. Introduction

3.1. Overview of Rheumatoid Arthritis

3.1.1. Etiology and Genetic Predispositions

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disease characterized by persistent inflammation, synovial hyperplasia, and the progressive deterioration and destruction of joints. Understanding the pathophysiology of RA is essential to applying knowledge about disease onset, progression and exacerbation in enhancing preventative and therapeutic measures. The complex etiology of RA remains partially undiscovered. However, a large body of evidence points to a significant interplay among several factors in RA development: genetic predisposition, environmental triggers, and intrinsic determinants such as sex and age.[1]

The genetic component of RA susceptibility is pronounced. A family history of RA is associated with a 3- to 5-fold increased disease risk and heritability estimates attribute roughly 40-60% of disease susceptibility to genetic factors.[2-4] The Human Leukocyte Antigen (*HLA*) region, particularly alleles within the *HLA-DRB1* locus, strongly correlates with RA development.[5,6]

Environmental variables also play a role in the risk of RA development. Among these, smoking confers the highest risk, especially in genetically predisposed individuals.[7-10] Other environmental risk factors such as viral or bacterial infections, hormonal transition states in females, and even gut microbiota have also been investigated. However, there is insufficient definitive evidence for determining the exact role environmental factors play in RA etiology.[11-13]

The autoimmune characteristic of RA is defined by the immune system targeting self-antigens, leading to the chronic inflammation that is characteristic of this disease. RA often develops within a wider range of autoimmune dysregulation. For example, it is not uncommon for RA patients to also develop features characteristic of Systemic Lupus Erythematosus (SLE) or suffer from Sjögren's syndrome.[14,15]

Gender and age disparities are prevalent in the RA patient population. The disease disproportionately affects females, with the female-to-male ratio being between 2:1 and 5:1 depending on the age group studied.[16] This indicates a potential role of age- and sex-related hormonal factors in disease susceptibility.[17] The median age at onset of RA symptoms is 45 years in females and 50 years in males. However, the disease is not age-bound and can in theory appear at any time.[18]

3.1.2. Pathophysiology of RA

RA's pathophysiology is marked by persistent inflammation in the synovial membrane of affected joints. This inflammation drives several pathological processes. These include cellular infiltration, excessive cytokine production, and tissue breakdown.[19] The chronic inflammation leads to the formation of hyperplastic, invasive tissue referred to as pannus. The pannus becomes a focal point for destructive mechanisms. These mechanisms affect nearby cartilage, subchondral bone, and soft tissue.[20]

The pannus is made up of several cell types, each contributing to RA pathology. Fibroblast-like synoviocytes (FLS) in the pannus become aggressive. They invade and degrade cartilage by secreting matrix metalloproteinases (MMPs).[21] Macrophages produce proinflammatory cytokines like Tumor Necrosis Factor alpha (TNF-α) and Interleukin-1 (IL-1).[22] T and B cells contribute to local autoimmunity and cytokine production. Endothelial cells promote new blood vessel growth and inflammatory cell migration.[23,24] Chondrocytes and osteoclasts, activated by signaling molecules like receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) ligand (RANKL) and TNF-α, play crucial roles in cartilage and bone erosion, respectively.[25,26]

The inflammatory milieu in RA is modulated by a diverse array of cytokines that are integral to the initiation, perpetuation, and escalation of inflammation. TNF-α, primarily produced by macrophages and T helper 1 (Th1) cells, is a fundamental cytokine in initiating the inflammatory cascade.[27] It upregulates adhesion molecule expression on endothelial cells, facilitating leukocyte infiltration into the synovium, and acts synergistically with other cytokines to amplify their proinflammatory effects.[28-30] Interleukin-6 (IL-6), secreted by various cells including macrophages, T lymphocytes, and fibroblasts, exhibits pleiotropic actions impacting both local joint and systemic physiology. It promotes neutrophil migration, osteoclast maturation, plasma cell differentiation, and T helper 17 (Th17) differentiation.[31,32] Additional cytokines such as IL-1, Interleukin-15 (IL-15), Interleukin-18 (IL-18), and Interleukin-23 (IL-23) also contribute to the inflammatory landscape in RA, each with their distinct roles and interactions within the complex cytokine network.[33]

The cellular landscape in the RA synovium is a complex microenvironment comprising diverse cells from both innate and adaptive immune systems.[34] Th1 cells, secreting Interferon-gamma (IFN-γ), activate macrophages and sustain the inflammatory environment.[35] Regulatory T cells (Tregs), responsible for maintaining self-tolerance, exhibit compromised function in RA, contributing to immune dysregulation.[36] Macrophages and dendritic cells (DC) present antigens to T cells and produce proinflammatory cytokines like TNF-α and IL-6.[37,38] Neutrophils

release reactive oxygen species (ROS), proteolytic enzymes, and Neutrophil Extracellular Traps (NET), further contributing to inflammation.[39,40]

The inflamed synovial tissue is rich in non-physiologic molecular mediators and activated signaling pathways, including NF-κB, Janus kinase/signal transducer and activator of transcription (JAK-STAT), and Mitogen-activated protein kinase (MAPK). These orchestrate cellular functions such as proliferation, survival, and cytokine production.[41]

3.1.3. Autoimmunity and Joint Destruction in RA

Autoimmunity plays a central role in the pathogenesis of RA, with a critical point of failure in immunological tolerance occurring both centrally in the thymus and peripherally. Compromised function of Tregs and other immune checkpoints leads to the survival of autoreactive T cells that escape thymic selection.[42] Autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) form immune complexes that deposit in joints, triggering complement activation and subsequent inflammation.[43,44]

The systemic inflammation in RA, driven by elevated levels of proinflammatory cytokines such as IL-6, not only contributes to joint destruction but also has relevance to various other organ systems, leading to multiple comorbidities.[32,45] Cardiovascular complications, such as accelerated atherosclerosis, myocardial infarction, heart failure, and arrhythmias, are associated with the chronic inflammatory state and endothelial dysfunction experienced by RA patients.[46-49] Metabolic disturbances, including increased fat mass, muscle wasting (rheumatoid cachexia), impaired insulin signaling, and dysregulated lipid metabolism, further contribute to the disease burden.[50-52] Pulmonary complications, such as interstitial lung disease and pleural effusions, as well as psychological comorbidities, potentially caused by cytokine-mediated effects on neurotransmitter levels, are also prevalent in RA patients.[53,54,55] Other comorbidities include osteoporosis and anemia of chronic disease.[56,57]

In RA, bone erosion contributes to the destruction of joints. This is a consequence of the imbalance between bone formation and resorption, with the RANKL/osteoprotegerin (OPG) ratio skewed towards RANKL, thereby promoting osteoclast differentiation and activation.[58] Proinflammatory cytokines, such as TNF-α and Interleukin-1 beta (IL-1β), act synergistically with RANKL to enhance osteoclastogenesis.[59,60] Overexpression of Cathepsin K, an enzyme produced by osteoclasts, further contributes to bone matrix degradation in RA patients.[61,62]

Cartilage degradation in RA is driven by phenotypic changes in FLS cells, driven by autocrine loops involving cytokines and growth factors such as platelet-derived growth factor (PDGF).[63]

Matrix-degrading enzymes, such as MMPs and A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS), also play a significant role in cartilage breakdown.[64] Elevated levels of nitric oxide (NO) in the synovium induce cellular proliferation and synovial opacity formation, exacerbating cartilage damage and bone destruction.[65]

Increased levels of Interleukin-17A (IL-17A) in RA joints also contribute to matrix and cartilage degradation with subsequent bone erosion. IL-17A is able to upregulate various MMPs such as MMP-1, MMP-2, MMP-9 and MMP-13, which in conjunction with TNF-α and Oncostatin M (OSM), shift the MMP:Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) ratio in favor of MMPs, leading to proteoglycan depletion, increased matrix turnover as well as cartilage and bone degradation.[66,67] IL-17A is also able to promote bone degradation twofold through the direct induction of osteoclastogenesis from monocytes independent of exogenous RANKL, as well as through the upregulation of RANKL production in RA FLS.[68] Another possible mechanism by which Th17 cells are involved in bone destruction is through the upregulation of B cell antibody production via Interleukin-21 (IL-21) and Interleukin-22 (IL-22) signaling. The resulting antibody excess and immune complexes are involved in the promotion of osteoclastogenesis.[69]

Muscle atrophy, resulting from chronic inflammation and disease-related reduction in physical activity, contributes to functional impairment in RA patients.[50] Pain mechanisms often involve a neuropathic component, attributed to the release of neuropeptides like substance P.[70] The combined effects of joint destruction, inflammation, and pain significantly impact the quality of life of RA patients, limiting their ability to perform daily activities and maintain self-care.[71]

Despite advances in understanding the complex pathophysiology of RA and the development of targeted therapeutics, substantial challenges remain in treating this disease effectively. Current therapies often fail to achieve remission in approximately half of all patients, and many treatments are associated with adverse effects that might limit their long-term use.[72,73] This therapeutic gap highlights the need to identify novel immunomodulatory compounds that target specific pathological immune pathways in RA. While Th17 cells have emerged as significant drivers of RA pathology, approaches to selectively modulate this pathway remain understudied. Understanding how potential immunomodulatory compounds affect Th17 cell function in the specific context of RA represents a critical research need.

3.2. Differentiation and Functioning of CD4+ T Cells

3.2.1. CD4+ T Cells: Overview and Role in Adaptive Immunity

Cluster of Differentiation 4 positive (CD4+) T cells are central players in the adaptive immune

system, and are characterized by the expression of the CD4 co-receptor. These cells recognize antigens presented via major histocompatibility complex (MHC) class II molecules and coordinate an array of immune responses, including the activation of other immune cells and the regulation of antibody production.[74]

Originating from hematopoietic stem cells, CD4+ T cells undergo maturation in the thymus, where positive and negative selection refine their antigen recognition abilities, ensuring self-tolerance while preserving responsiveness to foreign antigens.[74] The remarkable diversity of their T cell receptors (TCR), generated through somatic recombination, allows CD4+ T cells to recognize an extensive array of antigens, equipping the adaptive immune system with specificity and adaptability.[75]

The activation of CD4+ T cells is a two-step process initiated by the binding of the TCR to an MHC class II-antigen complex. The TCR-MHC interaction is reinforced by co-stimulatory signals provided by molecules such as CD28. This interaction triggers intracellular signaling cascades, including the MAPK and NF-κB pathways, leading to the activation of transcription factors that regulate gene expression and facilitate T cell proliferation and differentiation. Co-stimulatory signals are crucial for complete T cell activation and the prevention of anergy.[74,76]

Upon activation, naïve CD4+ T cells differentiate into distinct subsets of effector cells, each with unique cytokine profiles and functions. These cell subsets include Th1, T helper 2 (Th2), Th17, and Tregs. The differentiation process is guided by transcription factors responsive to environmental cues such as cytokines. T-box Expressed in T Cells (T-bet) governs Th1 differentiation, GATA Binding Protein 3 (GATA3) is essential for the Th2 lineage, Retinoic Acid Receptor-Related Orphan Receptor Gamma t (RORγt) directs Th17 differentiation, and Forkhead-Box-Protein P3 (FoxP3) acts as the master regulator for Treg differentiation.[74]

The core principles of adaptive immunity—antigen specificity, immune memory, and self-tolerance—are significantly influenced by the functional attributes of CD4+ T cells.[77] Th1 cells, which predominantly secrete IFN-γ, are crucial for cell-mediated immunity against intracellular pathogens. Th2 cells, characterized by the secretion of Interleukin-4 (IL-4), Interleukin-5 (IL-5), and Interleukin-13 (IL-13), are vital for targeting extracellular pathogens and parasites and play a key role in humoral immunity. Th17 cells, major producers of the Interleukin-17 (IL-17) family of cytokines, are important for host defense against extracellular bacteria and fungi but are also implicated in autoimmune and inflammatory diseases.[74]

Tregs play a pivotal role in maintaining immune homeostasis and self-tolerance. They secrete

immunosuppressive cytokines such as Interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β). Tregs also engage in direct cell-cell interactions to regulate the activity of other immune cells. The regulatory function of Tregs is critical for preventing autoimmune disorders and limiting chronic inflammation.[78]

CD4+ T cells also interact with B cells, providing essential signals for B cell maturation and antibody class switching, thereby shaping the humoral arm of the adaptive immune response.[79]

3.2.2. CD4+ T Cells: Activation and Co-Stimulatory Signals

The activation of CD4+ T cells is a tightly regulated process that requires two distinct signals: antigen recognition through the TCR and co-stimulatory signals provided by antigen-presenting cells (APCs). The primary activation signal is initiated when the TCR recognizes and binds to a specific peptide-MHC class II complex on the surface of APCs, such as dendritic cells, macrophages, and B cells.[74,80]

APCs capture, process, and present antigens as peptides loaded onto MHC class II molecules. The processing of antigens occurs within endosomal compartments, and the resulting peptide-MHC class II complexes are then transported to the cell surface for recognition by the TCR of CD4+ T cells.[83]

While the TCR-peptide-MHC class II interaction ensures specificity, co-stimulatory signals are essential for complete T cell activation and the prevention of anergy. The most well-characterized co-stimulatory pathway is the CD28-B7 axis, which involves the interaction between CD28 on T cells and its ligands B7-1 (CD80) and B7-2 (CD86) on APCs. This interaction enhances T cell activation, promotes survival, and prevents anergy.[81] Other co-stimulatory molecules, such as Inducible T Cell Costimulator (ICOS) and OX40 (CD134), are upregulated following initial activation and contribute to the sustained T cell response and enhanced survival.[76]

The engagement of the TCR and co-stimulatory receptors triggers intracellular signaling cascades that lead to T cell activation, proliferation, and differentiation. These signaling pathways involve the activation of tyrosine kinases, such as lymphocyte-specific protein tyrosine kinase (Lck) and zeta-chain-associated protein kinase 70 (ZAP-70), which catalyze the phosphorylation of downstream molecules.[82] The Phosphoinositide 3-Kinase (PI3K)/Protein Kinase B (Akt) pathway, activated by co-stimulatory signals, promotes T cell survival, growth, and differentiation by serving as a central node for transducing extracellular signals into coordinated intracellular responses.[83]

Transcription factors, including NF-κB, activator protein 1 (AP-1), and Nuclear Factor of Activated T cells (NFAT), are activated downstream of these signaling cascades. Upon activation, these transcription factors translocate to the nucleus, where they regulate the expression of genes crucial for T cell functioning, such as those involved in cytokine production, cell cycle progression, and effector functions.[84-86]

Co-stimulatory signals are directly involved in determining cytokine production behavior for CD4+ T cells. CD28 has been shown to upregulate human IL-17A expression by promoting the recruitment of REL-associated protein A (RelA)/NF-κB and signal transducer and activator of transcription 3 (STAT3) on the proximal promoter.[87] In addition to STAT3, the cytokines TGF-β and IL-6 are activated, which in conjunction with STAT3 induce the expression of RORγt, thus shifting the cell towards the Th17 lineage.[88-91] Interestingly, CD28 signaling is also vital for Treg homeostasis and functioning in the periphery, thereby possibly contributing to immune homeostasis as a factor in the Th17/Treg balance.[92] The co-stimulatory molecule CD226 is able to promote IL-17A production through guanine nucleotide exchange factor Vav Guanine Nucleotide Exchange Factor 1 (VAV1)-mediated signaling, which is required for T cell activation.[93] However, not all co-stimulatory signals lead to increased IL-17A levels, with activation of OX40 leading to a methylation-based “closing” of the chromatin structure at the locus required for IL-17A production thus inhibiting it.[94] Beyond IL-17A, IFN-γ production is also modulated through co-stimulation, with cross-linking of CD28 molecules resulting not only in enhanced T cell proliferation, but also a strong increase in IFN-γ, and Interleukin-2 (IL-2) RNA levels and secretion.[95] Furthermore, a toll-like receptor (TLR) 7/8 ligand has been shown to increase IFN-γ production in γδ T cells upon co-stimulation with IL-2 and Interleukin-12 (IL-12).[96] As is the case with IL-17A, not all co-stimulatory signals result in increased IFN-γ levels. CD46, another co-stimulatory receptor, is able to promote the differentiation of Th1 cells into a Treg phenotype causing a reduction in IFN-γ production along with an increase in IL-10 production. In contrast, CD46 is a potent driver of IFN-γ production in CD8+ T cells.[97]

Following activation, CD4+ T cells transition away from a quiescent state and re-enter the cell cycle, a process driven by the upregulation of cyclins and cyclin-dependent kinases.[98] Activated T cells undergo metabolic reprogramming, shifting from oxidative phosphorylation to aerobic glycolysis to meet the increased bioenergetic and biosynthetic demands resulting from rapid proliferation.[99]

In the context of autoimmune diseases like RA, co-stimulatory signals can contribute to the activation of autoreactive T cells.[100] Therapeutic interventions, such as abatacept, which disrupt the CD28-B7 interaction, have been developed to modulate abnormal T cell activation

and have shown promising results in RA clinical trials.[101]

3.2.3. Th17 Cell Differentiation: Overview and Molecular Mechanisms

The differentiation of naïve CD4+ T cells into Th17 cells is a critical process in adaptive immunity, particularly in the context of autoimmune diseases such as RA. Th17 cells are characterized by the production of IL-17A and IL-17F. These cytokines play an essential role in host defense against extracellular pathogens but can also contribute to autoimmune pathology when dysregulated.[102,103]

The differentiation of Th17 cells is initiated by the engagement of the TCR with specific antigenic peptides presented by MHC class II molecules on APCs.[80] Following this initial activation, the fate of a naïve CD4+ T cell is largely determined by the surrounding cytokine milieu. The presence of TGF-β, IL-23, IL-6, and IL-1β is crucial for driving the activated T cell towards a Th17 phenotype.[104] Interactions with other immune cells, such as Tregs, have been shown to promote or inhibit Th17 differentiation, depending on the specific regulatory molecules and cytokines involved.[104]

The molecular mechanisms driving Th17 differentiation involve a network of transcription factors and signaling pathways. The master regulator of Th17 differentiation is RORγt, which binds to the promoter regions of Th17-specific genes and initiates the transcription of IL-17A and IL-17F.[104] A close relative, retinoic acid receptor-related orphan receptor alpha (RORα) has been shown to induce the expression of genes that define Th17 cells.[105] Another crucial transcription factor is STAT3, which is activated by cytokines such as IL-6 and IL-21. Upon activation, STAT3 dimerizes and translocates to the nucleus, where it enhances the expression of RORγt and subsequently upregulates the production of IL-17A and other proinflammatory cytokines characteristic of Th17 cells.[106]

The stability and maintenance of the Th17 phenotype as well as the pathogenicity are dependent on the continued presence of IL-21 and IL-23, which provide essential autocrine and paracrine signals, respectively.[104,106] The expression of the IL-23 receptor (IL-23R) on Th17 cells is regulated by serum glucocorticoid-regulated kinase 1 (*SGK1*), which inhibits the transcription factor Foxo1, a direct repressor of IL-23R expression.[107,108]

Several other genes and signaling pathways have been implicated in the regulation of Th17 differentiation and function. For example, colony stimulating factor 2 (*CSF2*), which encodes granulocyte-macrophage colony-stimulating factor (GM-CSF), has been shown to enhance IL-6 dependent Th17 development and survival.[109] In addition, *CSF2*-deficient mice suffered from less severe arthritis when compared to wild-type mice.[110] Another relevant gene is Ikaros family

zinc finger 3 (*IKZF3*), which encodes the zinc finger protein Aiolos, a hematopoietic-specific transcription factor. Th17 cells express higher levels of *IKZF3* compared to other CD4+ T cell subsets, and Aiolos-deficient mice exhibit impaired Th17 differentiation, as evidenced by reduced expression of IL-17A and other Th17-associated genes.[111]

The Notch signaling pathway also plays a significant role in Th17 differentiation. This pathway consists of four receptors (Notch1-4) and their corresponding ligands, such as Delta-like and Jagged. Upon activation, Notch receptors undergo a series of proteolytic cleavages, releasing the Notch intracellular domain (NICD). The NICD then translocates to the nucleus, where it interacts with transcription factors like RORγt and STAT3 to modulate the expression of Th17-specific genes.[112] Aberrant activation of Notch signaling has been linked to autoimmune diseases, including RA, and the overactivation of this pathway contributes to the pathogenicity of Th17 cells in RA.[113] Preliminary evidence suggests that cannabinoids, such as cannabidiol (CBD), may influence Notch signaling, although the specific mechanisms in the context of RA require further investigation.[114]

The JAK-STAT pathway is another crucial signaling cascade involved in cytokine-driven Th17 differentiation. Dysregulation of this pathway in RA has been shown to favor the excessive differentiation of pathogenic Th17 cells, contributing to the proinflammatory environment characteristic of the disease.[115,116] Cannabinoids, including anandamide (AEA) and CBD, have demonstrated the potential to modulate these pathways. For instance, CBD has been found to inhibit STAT3 activation, which may lead to the suppression of Th17 differentiation.[117-119]

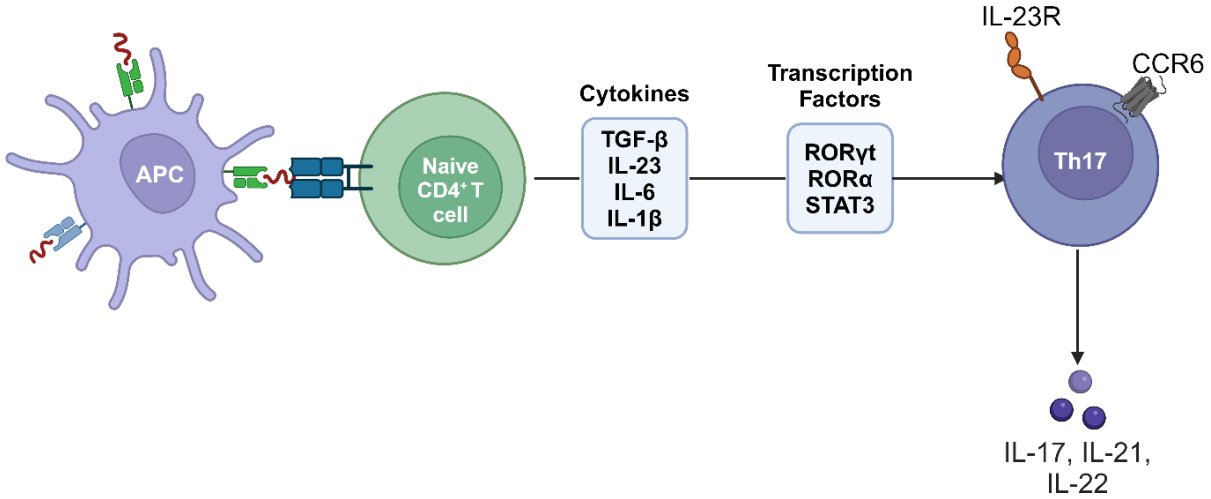

Figure 1. Th17 cell differentiation pathway This diagram illustrates the key steps in the differentiation of naïve CD4+ T cells into Th17 cells. The process begins with antigen presentation by an antigen-presenting cell (APC) to a naïve CD4+ T cell. Specific cytokines (TGF-β, IL-23, IL-6, IL-1β) drive the differentiation, activating transcription factors (RORγt, RORα, STAT3) that are crucial for Th17 cell development.[104,105] The mature Th17 cell is characterized by the expression of IL-23R and CCR6

receptors, and produces signature cytokines IL-17A, IL-21, and IL-22.[107,108,120,121] This pathway plays a significant role in the pathogenesis of RA and other autoimmune diseases.

3.2.4. Physiologic Functions and Interactions of Th17 Cells

Th17 cells play a crucial role in maintaining mucosal homeostasis and protecting against extracellular pathogens, particularly at mucosal surfaces such as the gastrointestinal tract, respiratory system, and integumentary system. Three of the cytokines produced by Th17 cells, IL-17A, IL-17F, and IL-22, are essential for the recruitment of neutrophils and the induction of antimicrobial peptide synthesis, providing a first line of defense against bacterial and fungal infections.[121]

IL-17A also exerts a significant influence on granulopoiesis by interacting with bone marrow stromal cells to stimulate the production of granulocyte colony-stimulating factor (G-CSF). This interaction promotes the differentiation and mobilization of neutrophils into circulation, further enhancing the immune response against extracellular pathogens. In addition to their role in host defense, Th17 cells contribute to the maintenance of epithelial barrier integrity. IL-22, in particular, promotes the proliferation and regeneration of epithelial cells and enhances mucus production, thereby reinforcing the barrier function and preventing the entry of potential pathogens.[121]

Recent studies have also implicated Th17 cells in metabolic processes and adipose tissue inflammation, although the precise mechanisms underlying these functions remain to be fully uncovered. It has been suggested that Th17 cells may also affect systemic insulin sensitivity, highlighting their potential involvement in metabolic disorders.[122]

Th17 cells engage in complex interactions with various other cell types within the immune system. They modulate macrophage functionality primarily through the secretion of IL-17A and IL-22, influencing their activation status and cytokine production.[123] Dendritic cells also play a pivotal role in the Th17 cellular network, as certain DC subsets produce cytokines such as IL-6 and TGF-β, which are essential for driving Th17 cell differentiation. In turn, Th17 cells can influence the maturation and antigen-presenting capabilities of DCs, creating a feedback loop that perpetuates the inflammatory response.[124,125]

The relationship between Th17 cells and Tregs is of particular importance, as these two cell types often have opposing roles in immunological homeostasis. The balance between Th17 cells and Tregs is crucial for maintaining an appropriate level of immune response while preventing excessive inflammation and autoimmunity. Disruption of this equilibrium has been strongly implicated in the pathogenesis of autoimmune diseases like RA.[126] Various factors can skew the differentiation of Th17 cells towards a regulatory phenotype, highlighting the

plasticity and dynamic nature of these T cell subsets.[127,128]

Th17 cells also interact with B cells and play a role in germinal center reactions. Through the secretion of IL-17A and IL-21, Th17 cells facilitate antibody class switching and promote the differentiation of B cells into plasma cells and memory B cells. This Th17 function contributes to the humoral arm of the adaptive immune response.[129]

3.3. Th17 Cells and Rheumatoid Arthritis

The diversity of CD4+ T cell subsets and their complex regulatory mechanisms present both challenges and opportunities for therapeutic intervention in autoimmune diseases. While the general biology of these cells is well-characterized, how specific immunomodulatory compounds affect CD4+ T cell differentiation and function in disease-specific contexts remains incompletely understood. Particularly in RA, where Th17 cells play a prominent pathogenic role, there is limited knowledge about how potential therapeutic agents might selectively modulate this subset while preserving protective immune functions. This is especially relevant when considering novel compounds with pleiotropic immunomodulatory effects, such as cannabinoids, whose actions may vary depending on the specific inflammatory environment.

3.3.1. Th17 Cells in RA Inflammation and Autoimmunity

Th17 cells play a central role in the pathophysiology of RA, contributing to both inflammation and autoimmunity. The imbalance between Th17 cells and Tregs is a key feature of RA, with elevated Th17 activity and impaired Treg function exacerbating the inflammatory process.[126] In the RA milieu, the differentiation of naïve CD4+ T cells is skewed towards the Th17 lineage, largely due to an aberrant cytokine environment characterized by high levels of IL-6.[130]

In a clinical setting, RA patients frequently exhibit elevated levels of Th17 cells and IL-17A positivity among peripheral blood mononuclear cells (PBMCs). Interestingly, the median percentage of Th17 cells was higher in active RA when compared to inactive disease states. The percentage of Th17 cells among PBMCs was also positively correlated with Disease Activity Score of 28 joints with C-reactive Protein (DAS28-CRP), Erythrocyte Sedimentation Rate (ESR) and C-reactive Protein (CRP) levels.[131] In addition to the peripheral blood, Th17 cells are abundant in the synovial fluid where the level of Th17 cells correlates with disease severity.[102]

Th17 cell signature cytokines act as potent mediators of proinflammatory responses and are closely linked to RA pathophysiology.[132] The IL-17 family of cytokines, collaborates with TNF-α and IL-6 to potentiate the inflammatory response and directly contributes to bone erosion

through osteoclast activation as well as neutrophil recruitment, thereby further perpetuating inflammation.[133,134]

In the context of autoimmunity, aberrant activation of CD4+ T cells in RA often arises from interactions with self-antigens presented by MHC class II molecules. Citrullinated proteins are a notable example of such self-antigens and are abundant in the inflamed synovial tissues of RA patients.[135] Th17 cells play a crucial role in amplifying this autoimmune response by promoting the recruitment of additional immune cells to the synovial fluid and tissue thereby contributing to the inflammatory microenvironment.[136]

Th17 cells contribute to the pathogenic neovascularization observed in RA by secreting vascular endothelial growth factor (VEGF), which promotes angiogenesis and pannus formation. Th17 cells also stimulate synovial fibroblasts to release proinflammatory cytokines such as IL-6 and IL-8, as well as MMP-1 and MMP-3, which contribute to tissue destruction and remodeling.[136]

The chemokine CC-chemokine ligand 20 (CCL20), produced by Th17 cells, plays a pivotal role in recruiting additional immune cells, including dendritic cells and more Th17 cells, to the inflamed synovial regions. This chemokine-cytokine network sustains and escalates the inflammatory cycle, contributing to the persistent autoimmunity characteristic of RA.[102,136,137]

Advancements in the understanding of Th17 cells and their role in RA have informed the development of targeted therapies. For example, secukinumab, a monoclonal antibody that specifically inhibits IL-17A, has been developed as a targeted intervention for RA. By neutralizing IL-17A, secukinumab aims to disrupt the inflammatory cascade driven by Th17 cells and alleviate the symptoms of RA.[138]

3.3.2. Dysregulation of Th17 Cells and Th17/Treg Balance in RA

In RA, the differentiation and function of Th17 cells appear to be dysregulated, leading to an excessive accumulation and activation of these cells within affected tissues. This dysregulation is influenced by both intrinsic and extrinsic factors, resulting in a self-sustaining loop of inflammation and autoimmunity.

Intrinsic factors contributing to aberrant Th17 differentiation in RA include altered gene expression and epigenetic modifications, which enhance the responsiveness of precursor cells and other helper T cell phenotypes to Th17-polarizing cytokines.[139,140] Genetic loci such as the locus for C-C Motif Chemokine Receptor 6 (CCR6), which is involved in Th17 cell migration, is associated with an increased risk

for RA.[120,141] Cell-intrinsic dysregulation of cytokine signaling pathways, particularly those involving IL-2 and IL-3, have also been shown to directly promote Th17 differentiation.[142,143]

Extrinsic factors encompass the inflammatory milieu in RA, which is enriched with cytokines such as IL-1β, IL-6, IL-23, and TGF-β. These cytokines not only promote Th17 cell differentiation but also enhance their survival and pathogenicity. Transcriptional changes, including altered expression and activation of critical transcription factors like RORγt and STAT3, further skew differentiation towards the Th17 lineage.[136,144,145] Moreover, emerging research suggests that gut microbiota may modulate Th17 differentiation, potentially contributing to RA pathogenesis.[146]

RA is closely linked to the imbalance between Th17 cells and Tregs. In healthy individuals, the Th17/Treg balance is tightly regulated to maintain immune homeostasis. However, in RA, this balance is skewed towards Th17 cells, with patients displaying elevated Th17 cell levels and simultaneous reductions in Tregs. Functional changes also occur, with Th17 cells adopting a more pathogenic phenotype and Tregs exhibiting diminished suppressive abilities.[126]

Several mechanisms contribute to the altered Th17/Treg balance in RA. Th17 and Treg cells originate from a shared differentiation pathway, which is modulated by the availability of specific cytokines such as TGF-β, IL-2 and IL-6. In the RA cytokine milieu, Th17 differentiation is favored at the expense of Tregs. The transcription factors RORγt and FoxP3, which guide the differentiation of Th17 and Treg cells, respectively, compete for binding sites and co-factors which further exacerbates the imbalance. Proinflammatory cytokines secreted by Th17 cells can also inhibit Treg differentiation and functionality, creating a feedback loop that amplifies the disequilibrium.[107,126,130,147]

The consequences of elevated Th17 cell counts and reduced Treg function in RA are significant. Increased production of proinflammatory cytokines, such as IL-17A and IL-22 by Th17 cells intensifies synovial inflammation.[148] Simultaneously, the decline in Tregs compromises immune tolerance mechanisms, leading to sustained autoimmune responses.[149,150]

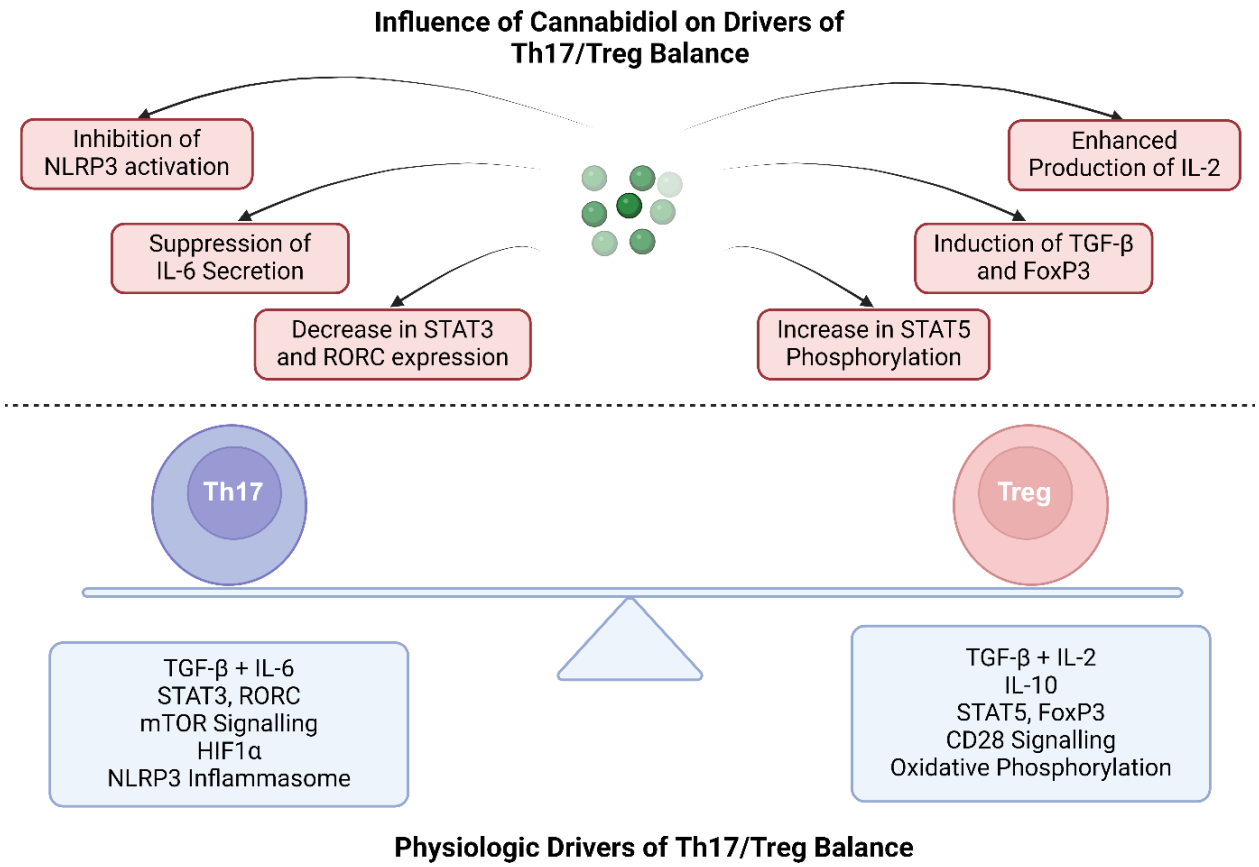

Figure 2. Cannabidiol's modulation of the Th17/Treg balance in autoimmune conditions This figure illustrates the multifaceted effects of CBD on the drivers of Th17/Treg balance, crucial in autoimmune pathologies. CBD inhibits NLR family pyrin domain-containing 3 (NLRP3) inflammasome activation, which has been shown to restore the Treg/Th17 balance in RA.[151,152] It suppresses IL-6 secretion, a key cytokine in Th17 differentiation, while enhancing IL-2 production, which is essential for Treg development and function through signal transducer and activator of transcription 5 (STAT5) and subsequent FoxP3 induction.[88,153-156] Beyond IL-2, CBD has also been shown to increase IL-10 production, which is vital for Treg generation.[157] The induction of TGF-β by CBD further supports Treg development.[157] CBD decreases STAT3 and Retinoic Acid Receptor-Related Orphan Receptor C (*RORC)* expression, critical for Th17 differentiation.[158] These actions collectively shift the balance toward a more immunoregulatory Treg phenotype, potentially alleviating autoimmune inflammation.

The complex dysregulation of Th17 cells in RA, influenced by both intrinsic and extrinsic factors, presents a significant therapeutic target. However, current approaches to modulating the Th17/Treg balance, such as IL-6 receptor blockade, often affect multiple immune pathways with unintended consequences and may not selectively target pathological Th17 functions but function via the Treg axis instead.[159,160] This highlights the need to identify compounds that can normalize this imbalance with greater specificity. While cannabinoids have shown immunomodulatory effects in other disease contexts, their specific impact on the Th17/Treg

axis in RA remains largely unexplored. Addressing this knowledge gap is particularly important given the increasing interest in cannabinoids as potential therapeutic agents for RA and other inflammatory conditions.

3.4. Cannabinoids and the Immune System

3.4.1. Cannabinoids: Definition, Classification and Types

Cannabinoids are a diverse class of chemical compounds that interact with the endocannabinoid system (ECS), a cell-signaling network involved in various physiological processes. The definition and our understanding of cannabinoids has evolved significantly since the isolation of CBD as one of the first phytocannabinoids from the *Cannabis sativa* plant in the early 20th century.[161]

Cannabinoids can be classified based on their origin into three primary types: phytocannabinoids, endocannabinoids, and synthetic cannabinoids. Phytocannabinoids are naturally occurring compounds found primarily in the *Cannabis sativa* and *Cannabis indica* plants. The most well-known phytocannabinoids are Δ9-tetrahydrocannabinol (THC) and CBD. THC serves as the primary psychoactive component of cannabis, interacting predominantly with the cannabinoid receptor 1 (CB1) in the central nervous system to induce a range of effects such as euphoria, altered perception, and increased appetite.[162] In contrast, CBD is non-psychoactive and is noted for its diverse pharmacological activities, including anti-inflammatory, neuroprotective, and anti-cancer effects.[163] Other phytocannabinoids, such as cannabigerol (CBG), cannabichromene (CBC), and tetrahydrocannabivarin (THCV), are also starting to gain scientific interest for their unique biological activities.[164]

Endocannabinoids are endogenous lipids synthesized on demand within various cell types, including activated T and B cells. They are integral components of the ECS, which encompasses the enzymes responsible for their synthesis and degradation, as well as the CB1 receptor and cannabinoid receptor 2 (CB2). The two primary endocannabinoids are AEA and 2-arachidonoylglycerol (2-AG). AEA and 2-AG serve as endogenous agonists for both CB1 and CB2 receptors and are involved in a myriad of physiological processes, such as mood regulation, immune function, neuroprotection, and immunomodulation.[161,164,165]

Pharmacologically, cannabinoids exert a wide array of effects. Immunomodulatory cannabinoids, such as CBD, have been shown to significantly regulate T cell function and cytokine production.[166] Neuromodulatory cannabinoids, exemplified by THC, are most notable for their central nervous system effects, which include pain modulation and psychoactivity.[162]

Metabolic cannabinoids like THCV have demonstrated potential in modulating metabolic processes and are gaining attention for their anti-obesity and anti-diabetic properties.[167]

3.4.2. Endogenous Cannabinoid System and Exogenous Cannabinoid Interactions

The ECS consists of three core components: endocannabinoids, cannabinoid receptors, and metabolic enzymes. Endocannabinoids, such as AEA and 2-AG, are endogenously produced lipids that interact with cannabinoid receptors. The two primary cannabinoid receptors are CB1 and CB2, both of which are G-protein coupled receptors (GPCRs). CB1 receptors are predominantly located in the central nervous system, while CB2 receptors are more abundant in peripheral tissues and immune cells. Metabolic enzymes, including fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), are responsible for the synthesis and degradation of endocannabinoids.[161,168]

CB1 receptors, encoded by the *CNR1* gene, are primarily expressed in the brain, where they modulate neurotransmitter release and are involved in various physiological processes such as appetite regulation, pain perception, and synaptic plasticity. In contrast, CB2 receptors, encoded by the *CNR2* gene, are predominantly expressed in immune cells, including macrophages, B cells, and T cells, where they play a vital role in regulating inflammation and immune responses. Both CB1 and CB2 receptors couple with Gi/o proteins, and their activation generally leads to the inhibition of adenylyl cyclase and a decrease in cyclic AMP (cAMP) levels.[169-172] Cannabinoid receptor activation can modulate the functionality of ion channels and activate MAPKs, which are involved in gene expression and cellular proliferation.[173]

In the context of neuroprotection, the ECS regulates neuronal excitability and guards against oxidative stress. As an immunomodulator, the ECS affects cytokine production, cell migration, and cellular proliferation, which are particularly relevant in autoimmune diseases such as RA. Moreover, the ECS contributes to the regulation of appetite, sleep, and pain perception, helping to maintain internal equilibrium.[174-177]

Exogenous cannabinoids interact with the ECS by acting as agonists, antagonists, or allosteric modulators at cannabinoid receptors. THC exhibits a high affinity for CB1 receptors and mediates various effects, including analgesia, euphoria, and altered cognition.[162,178] In contrast, CBD possesses low affinity for CB1 and CB2 receptors but can influence their activity through indirect mechanisms, such as allosteric modulation. CBD also interacts with other receptors, including Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1), opioid receptors, and the serotonin receptor 1A (5-HT1A) and serotonin receptor 2A (5-HT2A).[178-181]

Cannabinoids can also exert indirect effects on the ECS, with CBD inhibiting FAAH and thereby preventing the physiologic breakdown of the CB1 and CB2 receptor ligand AEA.[182]

Research-focused synthetic cannabinoids, such as AM-1220, AM-2232, CP 55,940, and WIN 55,212-2 have been synthesized to investigate the structure and function of cannabinoid receptors.[183] These synthetic variants are often more potent and selective compared to their natural counterparts making them more effective in research to investigate the specific functions of cannabinoid receptors.[184] Exogenous cannabinoids can modulate endocannabinoid signaling by acting as agonists or antagonists at cannabinoid receptors or by altering the conformation of these receptors through allosteric modulation, thereby changing the binding affinity and efficacy of endogenous ligands.

In the context of RA, the ECS has been implicated in the pathophysiology of the disease. Elevated expression of CB2 receptors has been observed in the synovial tissues of RA patients, suggesting a potential role in the inflammatory process.[185,186] Activation of the ECS has been shown to inhibit the production of proinflammatory cytokines involved in RA, and modulation of the ECS may present novel opportunities for pain management in RA patients.[187,188]

The binding affinities of cannabinoids to their receptors and the subsequent downstream signaling events are crucial factors in determining their pharmacological effects. THC exhibits a high affinity for CB1 receptors, while AEA engages CB1 receptors with a lower affinity. CBD and some synthetic cannabinoids display elevated affinity for CB2 over CB1 receptors, which are principally located in immune cells.[159] Cannabinoids can also interact with non-canonical receptors, such as the G-protein coupled receptor 55 (GPR55), although with variable affinities.[189]

Upon activation, CB1 and CB2 receptors typically inhibit adenylyl cyclase, leading to a decrease in cAMP levels. Cannabinoid receptor activation can also modulate the functionality of ion channels, primarily calcium and inwardly-rectifying potassium channels, thereby affecting cellular excitability. Activation of these receptors can also trigger the phosphorylation and subsequent activation of MAPKs, which are involved in gene expression and cellular proliferation.[173]

Some cannabinoids exhibit a phenomenon known as "biased agonism" or "ligand bias," where they selectively activate one signaling pathway over another. For example, certain

cannabinoids may preferentially activate the β-arrestin signaling pathway rather than G-protein coupling, resulting in receptor internalization and desensitization.[173,190]

3.4.3. Anandamide: Biological Functions and Immunomodulatory Effects

AEA is an endogenous cannabinoid that exerts a wide range of biological functions through its interactions with cannabinoid receptors and other signaling pathways. Through the CB1 receptor, AEA influences various neurotransmitter systems, impacting synaptic plasticity and cognitive functions, including learning and memory.[191] AEA also engages in the regulation of multiple cellular processes such as apoptosis, proliferation, and migration, demonstrating the ability to inhibit cancer cell proliferation via CB1 receptors.[192,193] In the cardiovascular space, AEA contributes to vasodilation and holds implications for metabolic processes, including insulin resistance.[194,195]

On the immunological front, AEA primarily exerts its effects through the CB2 receptor, which is predominantly expressed on immune cells. Activation of CB2 receptors by AEA reduces inflammation by inhibiting the release of cytokines and chemokines, and suppressing immune cell proliferation and activation.[196] AEA has been shown to inhibit cytokine secretion in Th17 cells of healthy individuals and thus holds potential significance in the pathogenesis and treatment of RA.[197] Extending beyond the canonical CB1 and CB2 receptors, AEA interacts with a range of other receptors such as GPR55, Peroxisome Proliferator-Activated Receptors (PPARs) and transient receptor potential (TRP) channels thereby broadening its functional spectrum.[164] For example, AEA-induced activation of PPAR-γ leads to anti-inflammatory effects, including the inhibition of NF-κB signaling, which is vital for Th17 functioning.[198]

AEA has a significant influence on the differentiation of naïve T cells into specialized effector and regulatory subsets, including the Th17 and Treg cell populations. In a mouse model of neutrophilic asthma, selective activation of the CB2 receptor was able to regulate Th17/Treg balance, indicating that AEA might serve as a potential angle for doing so in RA patients.[199]

The impact of AEA extends to macrophage function. After initial AEA administration, macrophages become immobile, whereas long-term administration not only mobilizes this cell type, but also enhances endothelial adherence and transmigration. AEA is able to inhibit NO release from lipopolysaccharide (LPS)-activated macrophages in a dose-dependent manner. It has also been shown to induce apoptosis in dendritic cells. In terms of cytokine regulation, AEA can alter the expression and release of key cytokines and chemokines like IFN-α, IL-6, and IL-12, thereby fulfilling anti-inflammatory and immunosuppressive functions.[200] Inhibitors of FAAH, the enzyme responsible for AEA degradation, such as CBD have demonstrated potential in ameliorating inflammation and autoimmune responses.[201,202]

3.4.4. Anandamide's Effects on T Cell Activation, Cytokines and Inflammation

AEA strongly suppresses anti-CD3/anti-CD28 induced CD4+ and CD8+ T cell proliferation in a dose-dependent manner, an effect mediated through interaction with the CB2 receptor, as demonstrated by the ability of a CB2 receptor inhibitor to counteract this effect, whereas a CB1 receptor inhibitor showed no effect.[197]

In addition to its direct effects on T cell proliferation, AEA has been shown to inhibit keratinocyte-dependent induction of Th1 and Th17 responses via CB1 receptor interaction. Naïve T cells cultured with AEA showed a CB1-dependent 5-fold and 2-fold reduction in IFN-γ and IL-17A production, respectively, compared to those cultured without AEA.[203] AEA can also directly modulate cytokine production, suppressing proinflammatory cytokines such as IL-2, IFN-γ, and TNF-α, while favoring the secretion of anti-inflammatory cytokines like IL-10.[197,204]

AEA exerts its anti-inflammatory effects through multiple signaling pathways. One of the most well-characterized pathways is the NF-κB pathway, wherein AEA inhibits the activation of NF-κB, consequently dampening the transcription of proinflammatory genes.[205] AEA also targets the MAPK pathway, leading to increased phosphorylation and activation of arachidonate-specific cytoplasmic phospholipase A2 (cPLA2). Interestingly, inhibition of cPLA2 has been shown to ameliorate inflammation in experimental autoimmune encephalomyelitis (EAE) by modulating Th1 and Th17 responses and by promoting Treg activation and cytokine signaling in rats.[206,207]

3.4.5. Cannabidiol: Source and Classification

CBD is a phytocannabinoid found in its highest concentration in the indica variety of cannabis plants. It is distinct from other cannabinoids such as THC due to its non-psychoactive properties. CBD can be sourced from both marijuana and hemp variants of *Cannabis indica* and *Cannabis sativa*. While marijuana-derived CBD extracts feature a mixture of cannabinoids and may contain variable levels of THC, hemp-derived CBD extracts are characterized by their minimal THC content, typically less than 0.3%.[208,209]

Synthetically produced CBD is another source that offers the advantage of controlled purity and concentration, circumventing the variability inherent in plant extracts. Study results suggest that there is no pharmacological difference *in vitro* in the antiproliferative, anti-inflammatory or permeability effects of synthetic CBD when compared to naturally occurring CBD.[210] Of note, the terpenes contained within the cannabis plant can be responsible for an

“entourage effect”, enhancing cannabinoid activity, which would be missing in the synthetically produced variant.[211]

Molecularly, CBD is a 21-carbon terpenophenolic compound with the chemical formula $C_{21}H_{30}O_2$. It shares structural similarities with other phytocannabinoids but features unique functional groups that contribute to its distinct pharmacological profile. CBD exists in several isomeric forms, with the most common being (-)-CBD, the naturally occurring isomer. Other isomers like (+)-CBD and various diastereomers also exist, and their bioactivity can differ, potentially leading to distinct pharmacological effects.[212]

3.4.6. Cannabidiol’s Immunomodulatory Effects and Mechanisms

CBD plays a significant role in modulating immune responses through a complex network of mechanisms, exerting both immunosuppressive and anti-inflammatory effects. Unlike its psychoactive counterpart THC, CBD affects a broader spectrum of targets within the innate and adaptive arms of the immune system.[213] Despite its low affinity for the canonical cannabinoid receptors CB1 and CB2, CBD functions as a negative allosteric modulator of the CB1 receptor and can still exert immunomodulatory effects via the CB2 receptor, which is mainly expressed in immune cells such as macrophages, B lymphocytes, and T lymphocytes.[214-216]

CBD also engages with a diverse set of receptors beyond the CB1 and CB2 receptors, including serotonin receptors, TRPV1, the dopamine D2 receptor, and orphan GPCRs such as GPR55.[179] Upon receptor engagement, CBD activates multiple intracellular signaling cascades, such as the inhibition of adenylyl cyclase, modulation of calcium and sodium ion channels, and regulation of transcription factors like NF-κB, AP-1, STAT1, STAT3, and STAT5 thereby influencing the expression of proinflammatory cytokines.[217,218]

The immunomodulatory effects of CBD extend to various immune cell types. It directly inhibits the release of proinflammatory cytokines, such as TNF-α, IFN-γ, IL-17, IL-6, and IL-1β, from macrophages and fibroblasts while inducing apoptosis in activated immune cells.[153,158,219,220] CBD also modulates the balance between M1 and M2 macrophage phenotypes, although the research in this regard has yielded inconsistent and conflicting results.[221] In the context of T cell-mediated immunity, CBD inhibits the proliferation of activated T cells, triggers their apoptosis, and modulates the Th1/Th2 cytokine equilibrium by downregulating Th1-related cytokines like IFN-γ and upregulating Th2-related cytokines such as IL-4 and IL-10, albeit with conflicting results regarding its role in IL-10 production.[158,218,222,223]

CBD has been shown to decrease the secretion of IL-17A in T cell/antigen-presenting cell co-cultures while promoting the differentiation of Tregs, which is of particular interest given the skewed Th17/Treg ratio in RA.[154,158,224] CBD also diminishes the cytotoxic activity of activated CD8+ T cells, potentially mitigating tissue damage in autoimmune conditions.[225]

In addition to its effects on T cells, CBD influences B cell function by downregulating immunoglobulin production, although the underlying mechanisms remain poorly understood.[226] In addition, CBD causes an increase in the number of early apoptotic B cells at the expense of viable cells while also reducing IL-10 and TNF production.[227] CBD also modulates chemotaxis by inhibiting the release of chemokines like CC-chemokine ligand 2 (CCL2) and CC-chemokine ligand 5 (CCL5) and the expression of adhesion molecules such as Vascular Cell Adhesion Molecule 1 (VCAM-1), potentially limiting the recruitment of inflammatory cells to damaged tissues.[228]

Beyond its direct effects on immune cells, CBD enhances epithelial barrier function by increasing the expression of tight junction proteins, thus fortifying the initial defense against pathogens.[229] Moreover, CBD possesses anti-oxidative properties, attenuating oxidative stress, which is a known inducer of inflammation.[230]

A key aspect of CBD's immunomodulatory role is its ability to maintain immune homeostasis by exerting a balancing influence, mitigating hyperactive immune responses, and enhancing immunosuppressive mechanisms, making it particularly relevant for potential therapeutic applications in autoimmune diseases like RA.[231] CBD may also indirectly modulate immune responses through feedback mechanisms by regulating endocannabinoid levels, such as preventing AEA degradation, via FAAH inhibition.[182] CBD's interactions with the nervous system through serotonin and vanilloid receptors, may also be the source of secondary immunomodulatory effects.[232,233]

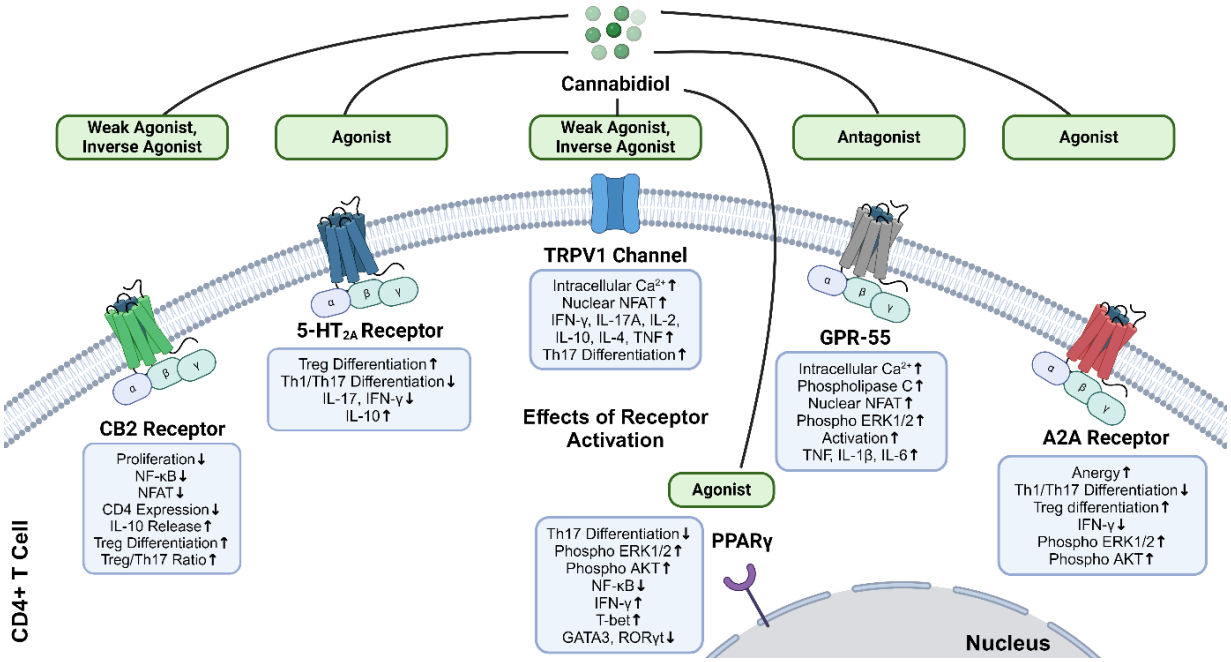

Figure 3. A selection of cannabidiol's multifaceted effects on CD4+ T cell receptors in rheumatoid arthritis This figure illustrates the complex interactions between CBD and various receptors on CD4+ T cells, with implications for RA pathology. CBD acts as a weak agonist and inverse agonist on the CB2 receptor, potentially reducing T cell proliferation, NF-κB activation, and promoting Treg differentiation with an associated increase in the Treg/Th17 ratio—effects that could alleviate inflammation in RA.[180,199,215,234] As an agonist of the 5-HT2A receptor, CBD may enhance Treg differentiation while suppressing Th1/Th17 differentiation and reducing proinflammatory cytokines IL-17A and IFN-γ, potentially beneficial in managing RA's autoimmune response.[180,181] CBD's weak agonistic and inverse agonistic effect on TRPV1 channels could modulate intracellular Ca2+ and influence cytokine production, including IL-17A, which is crucial in RA pathogenesis.[233,235-237] As an antagonist of GPR55, CBD might reduce T cell activation and proinflammatory cytokine production, potentially beneficial in managing RA inflammation.[230,238,239] CBD's agonistic action on Adenosine A2A receptors (A2A) could suppress Th1/Th17 differentiation and IFN-γ production, processes typically overactive in RA.[240-242] CBD's effects on PPARγ activation may further modulate Th17 differentiation and inflammatory pathways, offering another avenue for potential therapeutic intervention in RA.[243-246]

3.4.7. Inhibition of Proinflammatory Mediators through Cannabidiol

Beyond cytokines, CBD also demonstrates potential in suppressing the activity of proinflammatory enzymes including cyclooxygenase (COX) and lipoxygenase (LOX).[247,248] These enzymes are instrumental in synthesizing prostaglandins and leukotrienes, respectively, which act as lipid mediators in the inflammatory cascade.[249,250] The inhibition of these enzymes offers an additional dimension to CBD's anti-inflammatory properties.

Another avenue of research focuses on CBD's role in attenuating inducible nitric oxide synthase (iNOS) activity, thereby mitigating the production of NO. However, further research

is needed regarding this effect in RA patients.[251] NO, a free radical, is implicated in both inflammation and tissue damage associated with RA.[252]

The expansive inhibition of proinflammatory mediators by CBD also lays a foundation for potential combination therapies. When co-administered with traditional disease-modifying anti-rheumatic drugs (DMARDs), CBD may enhance their therapeutic efficacy and potentially facilitate dose-reductions, thus minimizing adverse effects.

3.4.8. Cannabidiol's Relevance in Th17 Differentiation

CBD's influence extends to the regulatory machinery governing Th17 differentiation. It has been shown to interfere with the activity of STAT3 while increasing STAT5 phosphorylation in T_{MOG} cells in a mouse EAE model of multiple sclerosis.[156] This interference may lead to the attenuation of Th17 cell development and a corresponding reduction in the production of proinflammatory cytokines like IL-17. In addition to its effects on STAT3 activity, CBD decreases *RORC* expression, which is critical for Th17 differentiation.[158]

NLRP3 inflammasome activation has been shown to regulate Th17 differentiation in RA. When compared to healthy controls, CD4+ T cells from RA patients showed higher levels of NLRP3 activation. This activation of the NLRP3 inflammasome was correlated with RA disease activity and IL-17A concentration in the sera of RA patients. The knockdown of NLRP3 has been shown to inhibit Th17 differentiation.[253] This is interesting in the context of RA given the ability of CBD to inhibit NLRP3 activation, thus potentially exerting favorable effects on the Th17 status of RA patients.[152]

Research on EAE, a model of multiple sclerosis, has demonstrated that CBD dose-dependently suppresses the production and secretion of IL-17 from activated myelin oligodendrocyte glycoprotein (MOG)35-55-specific encephalitogenic T cells.[224] Gene profiling in this model revealed that CBD treatment suppresses transcription of numerous proinflammatory genes, with "IL-17 differentiation" and "IL-6 signaling" identified among the top processes affected.[254] However, whether these immunomodulatory effects translate to Th17 cells in RA remains unknown, representing a critical knowledge gap given the differences in pathophysiological mechanisms between these autoimmune conditions.

The modulatory capacity of CBD extends to the cytokine environment essential for Th17 differentiation. CBD has been found to downregulate proinflammatory cytokines such as IL-6 while upregulating the production of TGF-β. Although TGF-β can support both Treg and Th17 differentiation, IL-6 is more adept at orienting naïve CD4+ T cells towards the Th17

lineage.[154,255,256] This effect potentially establishes a less conducive milieu for Th17 cell differentiation.

3.5. Potential Implications and Use-Cases of Cannabinoids in RA

3.5.1. Previous Studies on Cannabinoids in RA

Accumulating evidence suggests that cannabinoids, particularly CBD and THC, may modulate inflammatory and immune pathways integral to RA pathogenesis.[187] However, the positive effects that can be derived for RA patients from cannabinoid use always must be weighed against the potential side effects and possible adverse events.[257,258] In preclinical studies, CBD has been shown to reduce joint pain, synovial inflammation, and the production of proinflammatory cytokines.[259] Studies involving collagen-induced arthritis (CIA), the prototypical RA animal model, in rats have also highlighted the beneficial disease-modifying effects of THC.[260]

In animal experiments, using CIA mice, oral CBD administration has shown both an anti-inflammatory effect as well as the inhibition of joint damage.[261,262] Synthetic cannabinoids such as JWH-133 and HU-320 have also shown efficacy in the treatment of CIA mice.[185,263]

On the clinical front, trials and observational studies have presented a mixed picture. While there is much anecdotal evidence for the efficacy of cannabinoids in alleviating pain associated with RA, the evidence to substantiate this is lacking.[264] Not only does the scarcity of robust and reproducible evidence need to be taken into account when considering the viability of cannabinoids in the treatment of RA, but also our ever expanding repertoire of classical pharmacological interventions with improvements over time in both efficacy and safety, reducing the necessity for alternative treatment modalities.

Safety profiles have generally been favorable for cannabinoids, with reported adverse events being mostly mild to moderate, although long-term safety data still needs to be established.[265] In contrast to randomized controlled trials (RCTs), observational studies, often reliant on self-reported data, present limitations including susceptibility to recall and self-selection biases but potentially offer pragmatic insights into patient preferences and real-world applications.

3.5.2. Therapeutic Potential of Cannabinoids in RA

The immunomodulatory role of cannabinoids, especially their influence on T cell activation and differentiation, also warrants consideration in the context of RA, where dysregulated T cell responses contribute to the disease pathology.[266-268] Studies indicate that activation of CB2 receptors could inhibit osteoclast differentiation and function thereby offering protective effects against bone erosion, a hallmark of advanced RA.[185]

While cannabinoids demonstrate a range of therapeutic possibilities, the distinction between symptomatic relief and disease modification remains an important evaluation criterion. Symptomatic relief primarily focuses on the analgesic properties of compounds like CBD and THC and can provide value to patients independent of actual disease modification. In contrast, disease modification involves targeting the underlying pathophysiological mechanisms driving RA such as a pathogenic expanded Th17 population and an elevated Th17/Treg ratio.

Further research is crucial to better understand a potential role of cannabinoids in RA management. Not only do we need to better grasp the molecular mechanisms by which cannabinoids influence RA-relevant aspects of the immune system, but also how these fare in the broader context of clinical use beyond *in vitro* experimentation.

3.6. Aims of This Study

This study was designed to address critical knowledge gaps regarding cannabinoid effects on CD4+ T cells in rheumatic autoimmune diseases. While cannabinoids have demonstrated immunomodulatory effects in other autoimmune contexts, including suppression of IL-17 production in experimental models of multiple sclerosis, their effects specifically in the context of RA remain largely unexplored.[224,254] This represents a significant knowledge gap for several reasons. First, the distinct pathophysiology of RA may lead to disease-specific responses to cannabinoid treatment. Additionally, the growing trend of patients with rheumatic diseases self-administering cannabinoids highlights the need to understand the potential immunological consequences of such use. An example of this is Canada, where after cannabis legalization, the percentage of RA patients engaging in cannabis use almost tripled (4.3% to almost 12.6%), with only about 20% of consumed cannabis being obtained through medicinal outlets.[269] Furthermore, given the central role of Th17 cells in the pathogenesis of RA, it is crucial to determine whether cannabinoids influence this pathway in ways that could impact disease activity. This necessity is further supplemented by the limitations of current treatment approaches, both in terms of efficacy and side effect profiles.[72,73] Identification of compounds that could augment current treatment would drive significant value for patients.

Cannabis and its associated compounds are enjoying increased societal acceptance and less regulatory scrutiny. Considering the increased prevalence of CBD use, oftentimes without medical supervision, it is important to fully understand the effects these compounds can have on the immunologic function and dysfunction in RA patients. While much research on cannabinoids and their effect on our immune system has been conducted in the past, there is still a lot to be uncovered. As our understanding of the RA pathophysiology continues to

expand, so too does our ability to characterize compounds in terms of their potential benefits and risks to patients.

This study sought to investigate whether patients with rheumatic autoimmune diseases exhibit alterations in cannabinoid receptor expression on CD4+ T cells compared to healthy controls under the hypothesis that an altered cannabinoid receptor profile might play a role in immune dysregulation, especially given previous findings indicating an overexpression of CB2 in synovial tissues from rheumatic joints compared to osteoarthritic joints.[185] The hypothesis was that the overexpression of CB2 may extend to CD4+ T cells from RA patients and play a role in altered immune regulation in the context of the disease. Understanding such differences could provide insight into whether altered endocannabinoid signaling might contribute to disease pathogenesis or influence responses to exogenous cannabinoids.

A central aim was to determine how CBD and AEA affect proinflammatory cytokine production in CD4+ T cells from RA patients compared to healthy controls, focusing on key cytokines implicated in RA pathogenesis: IL-17A, IFN-γ, and TNF-α. Given the widely reported anti-inflammatory properties of cannabinoids in other immune contexts, it was hypothesized that both CBD and AEA treatment would suppress the production of the proinflammatory cytokines IFN-γ and TNF-α in activated CD4+ T cells from both RA patients and healthy controls. Consistent with findings in other models and proposed mechanisms involving pathways like STAT3, it was further anticipated that CBD and AEA would exert similar suppressive effects on IL-17A production.[156]

To identify potential mechanisms underlying these anticipated immunomodulatory actions, we examined the effects of CBD and AEA on the expression of genes involved in T cell differentiation and function. Specifically, it was hypothesized that the expected cannabinoid-mediated cytokine suppression, particularly of IL-17A, would be reflected in the downregulation of genes promoting Th17 pathogenicity, such as *CSF2*, and potentially involve modulation of key regulators like *SGK1*, *IKZF3*, and the Aryl Hydrocarbon Receptor (*AHR)*.

Finally, to bridge laboratory findings with clinical relevance, the research sought to determine whether the immunomodulatory effects observed *in vitro* might correlate with clinical parameters in RA patients using CBD therapeutically. This translational component was assessed to provide preliminary insights, in full acknowledgment of the translational gap, into how experimental observations might manifest in a clinical setting with the initial hypothesis being that self-administration of CBD would lead to a reduction in IL-17A positivity and improve disease activity as measured by the DAS28-CRP score.

The intended overarching outcome of this study was to enable greater insight into the immunomodulatory effects of both CBD and AEA as they relate to RA pathogenesis, primarily through the assessment of their effects on cytokine production, CD4+ T cell differentiation, and cell survival. By establishing these fundamental immunological responses to cannabinoids in the context of RA, this research aimed to provide evidence-based guidance regarding the potential benefits or risks associated with cannabinoid use in patients with rheumatic autoimmune diseases, particularly given their increasing self-administration for symptom management without medical supervision.

4. Materials and Methods

4.1. Study Population

All patients included in the scope of this study were, at the time of selection, undergoing treatment in the Department of Internal Medicine I at the University Hospital of Cologne. The study population consisted of adult patients with RA who fulfilled the 2010 American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) classification criteria. Additional cohorts of patients with SLE, Psoriatic Arthritis (PsA) were also included. Additional test subjects without known autoimmune conditions were recruited to serve as a control group. All patients provided written informed consent prior to inclusion.

A total of 114 participants were recruited for this study. The patient cohort included 74 individuals with RA, 16 patients with PsA, and 7 patients with SLE. Additionally, 17 individuals without known autoimmune conditions were recruited as a healthy control (HC) group. The number of samples reported for individual experiments varies due to factors including sample availability and the technical requirements of the conducted experiment. At the time of blood withdrawal, none of the subjects were known to be suffering from additional chronic autoimmune diseases, nor were any experiencing unrelated acute onset diseases. Current treatment regimen was not considered in patient selection.

All test subjects were educated about study participation and provided their informed consent. Blood was drawn by the outpatient division of the department for immunology and rheumatology at the university hospital of Cologne. For the purpose of the study, 15-18 mL of blood was drawn from each patient into an ethylenediaminetetraacetic acid (EDTA) S-Monovette®. After the blood was drawn it was stored in a dry, dark place at room temperature and processed within 24 hours.

In order to maintain the anonymity of study participants, blood samples were assigned a number to reference in the course of experiments and analysis.

4.2. Materials and Laboratory Equipment

4.2.1. Buffer

Hanks Salt Solution 1x, phosphate buffered saline (PBS)	Biochrom AG Berlin, Germany
autoMACS® Pro Running Buffer	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

autoMACS® Pro Washing Buffer	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

4.2.2. Medium

X-VIVO™ 15	Lonza, Verviers, Belgium
Human Serum Albumin	Sigma-Aldrich, Saint Louis, USA
Penicillin-Streptomycin	Sigma-Aldrich, Saint Louis, USA

4.2.3. Chemicals and Reagents

Ethanol 96%, DAB, reinst.	Carl Roth, Karlsruhe, Germany
β-Mercaptoethanol	BioChemica, AppliChem Darmstadt/Panreac Quimica SLU, Barcelona, Spain
Ionomycin, Calcium Salt	Cell Signaling Technology®, Denver, USA
Phorbol Myristate Acetate (PMA)	Cell Signaling Technology®, Denver, USA
Brefeldin A (1000x Solution)	eBioscience, San Diego, USA
Pancoll®	PAN™-Biotech GmbH, Aidenbach, Germany
TaqMan® Fast Advanced Master Mix	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
RNase-free water	Qiagen, Hilden, Germany
FlowClean Cleaning Agent, 500 mL	Beckman Coulter, Krefeld, Germany
FlowCheck Pro Fluorospheres	Beckman Coulter, Krefeld, Germany
Trypan Blue stain 0.4%	Invitrogen, ThermoFisher Scientific, Carlsbad, USA

4.2.4. Antibodies

T Cell Stimulation

Recombinant Human IL-23	PeproTech, Rocky Hill, USA
Recombinant Human IL-6	Miltenyi Biotec, Bergisch Gladbach, Germany
Recombinant Human TGF-β	PAN™-Biotech GmbH, Aidenbach, Germany

Recombinant Human IL-2	Miltenyi Biotec, Bergisch Gladbach, Germany

Flow Cytometry Antibodies

Species	Target	Fluorophore	Isotype	Company
Anti-human	IL-17A	Brilliant-Violet 421	Mouse IgG1, kappa	BioLegend
Anti-human	IL-17A	PE	Mouse IgG1, kappa	eBioScience
Anti-human	IFN-γ	APC	Mouse IgG1, kappa	BioLegend
Anti-human	TNF-α	APC	Mouse IgG1, kappa	BioLegend
Anti-human	CB1 Receptor	unconjugated	Rabbit IgG	Abcam plc
Anti-human	CB2 Receptor	unconjugated	Rabbit IgG	Abcam plc
Anti-human	GPR55	unconjugated	Rabbit IgG	Abcam plc
Anti-rabbit	IgG1, kappa	Brilliant-Violet 421	Donkey Polyclonal Ig	BioLegend

5.2.5. Cannabinoids

Anandamide (ethanol solution)	Abcam plc, Cambridge, United Kingdom
(-)-Cannabidiol	Abcam plc, Cambridge, United Kingdom

5.2.6. TaqMan Quantitative PCR-Primer

Target	Company
β-2-Microglobulin	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
AHR	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
CSF2	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
IKZF3	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA

RORC	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
SGK1	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
TBX21	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA

5.2.7. Kits

CD4+ T Cell Isolation Kit	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
T cell Activation/Expansion Kit	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
LIVE/DEAD™ Fixable Green Dead Cell Stain Kit	Invitrogen, ThermoFisher Scientific, Carlsbad, USA
BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit	BD Bioscience, Heidelberg, Germany
RNeasy® Mini Kit	Qiagen®, Hilden, Germany
Quantitect Reverse Transcription Kit	Qiagen®, Hilden, Germany
ELISA MAX™ Deluxe Set Human IFN-γ	BioLegend, San Diego, USA
ELISA MAX™ Deluxe Set Human TNF-α	BioLegend, San Diego, USA
ELISA MAX™ Deluxe Set Human IL-17A	BioLegend, San Diego, USA

5.2.8. Consumables

Autoclavable Bag 5 L	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
Classic Nitrile PowderFree Gloves	ABENA®, Aabenraa, Denmark
Reagent Tube 1,5 mL	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
SafeSeal MicroTubes	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
Sarstedt Serological Pipette 5 mL, 10 mL, 25 mL	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
96 Biosphere Filter Tips, Biosphere® Plus (0,1 µL - 10 µL, 2 µL - 100 µL, 100 µL - 1000 µL)	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
BD Falcon™ 15 mL Polypropylen Conical Tubes	BD Bioscience, Heidelberg, Germany

BD Falcon™ 50 mL Polypropylen Conical Tubes	BD Bioscience, Heidelberg, Germany
Tubes	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
TC Plate 96 well, Standard	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
TC Plate 24 well, Standard	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
TC Plate 12 well, Standard	SARSTEDT Aktiengesellschaft & Co, Nümbrecht, Germany
Micro Amp® Fast Optical 96 well reaction plate with Barcode 0,1 mL	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
Countess™ cell counting chamber slides	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
LS Columns	Miltenyi Biotec, Bergisch Gladbach, Germany

5.2.9. Laboratory Machines

Megafuge 1.0R	Heraeus Instruments Düsseldorf, Germany
AutoMACS® Pro Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
QuadroMACS Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS MultiStand	Miltenyi Biotec, Bergisch Gladbach, Germany
CO_2 Incubator	Binder GMBH, New York, USA
Nanodrop 1000 Spectrophotometer	ThermoFisher Scientific, Carlsbad, USA
7500 Fast Real Time PCR System	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
ZX3 Advanced Vortex Mixer	VELP Scientifica, Usmate Velate MB, Italy
Mars Sterilbank Safety Class 2	Labogene, Denmark
Gallios™ Flow Cytometer	Beckman Coulter, Krefeld, Germany
Countess II FL Automated Cell Counter	Applied Biosystems, ThermoFisher Scientific, Carlsbad, USA
Vi-CELL XR Cell Counter	Beckman Coulter, Krefeld, Germany
ThermoStat™ 5320	Eppendorf, Wesseling, Germany

Temperature Controlled Waterbath	GFL®, Burgwedel, Germany
-80°C Freezer	Panasonic Corporation, Kadoma, Japan
-20°C Freezer	Liebherr Premium, Bulle FR, Switzerland
4°C Fridge	Liebherr Premium, Bulle FR, Switzerland

5.2.10. Software

GraphPad Prism	GraphPad Software, San Diego, USA
Adobe Illustrator CC	Adobe, San José, USA
Microsoft® Excel®	Microsoft, Redmond, USA
Microsoft® Powerpoint®	Microsoft, Redmond, USA
Microsoft® Word®	Microsoft, Redmond, USA
FlowJo Software	Tree Star Inc., Ashland, USA
Kaluza Analysis Software	Beckman Coulter, Krefeld, Germany
Nanodrop 1000 Operating Software 3.8.1	ThermoFisher Scientific, Carlsbad, USA

4.3. Methods

S1 Safety protocols were followed for all cell culture work performed in the scope of this study. Cell culture work was performed under a “Sterilbank Mars Safety Class 2”

4.3.1. Cell Isolation

Separation of PBMCs from patient whole blood was performed by density gradient centrifugation. The separation medium used for PBMC isolation was Ficoll-Hypaque, a high-molecular-mass, neutral, highly branched, hydrophilic polysaccharide.

Whole blood was diluted with phosphate buffered saline (PBS) in a ratio of 1:1. The diluted whole blood was then slowly layered over the Ficoll-Hypaque in a 50 mL conical tube in a ratio of 2:1. The 50 mL conical tube was then placed in a centrifuge for 25 minutes at 460 rcf at room temperature with disengaged brake.

The PBMC layer was collected with a 5 mL serological pipette and transferred into a 50 mL conical tube. The cells were then washed with MACS Running Buffer and centrifuged at 460 rcf for 20 minutes after which the supernatant was discarded.

CD4+ T cells were isolated from PBMCs by means of negative selection magnetic-activated cell sorting (MACS), both manual and automated separation methods were used.

In the case of manual separation, LS Columns and the QuadroMACS Separator were used. After incubation with the CD4+ T Cell Isolation Kit according to manufacturer's instructions, the PBMCs were suspended in autoMACS® Pro Running Buffer, and separated using the MACS MultiStand and appropriate LS Columns according to the manufacturer's instructions. The CD4+ T cells were collected in a 15 mL conical tube, washed with autoMACS® Pro Running Buffer at 460 rcf for 10 minutes after which the supernatant was discarded. CD4+ T cells were then resuspended in X-Vivo 15 medium at 36.6°C.

Automatic cell separation was performed using the AutoMACS® Pro Separator. After incubation with the CD4+ T Cell Isolation Kit, the PBMCs were suspended in AutoMACS® Pro Running Buffer. CD4+ T Cell isolation was performed by means of negative selection according to manufacturer's instructions using the program setting "Depletes" in the AutoMACS® Pro Separator. The CD4+ T cells were collected in a 15 mL conical tube, washed with AutoMACS® Pro Running Buffer at 460 rcf for 10 minutes after which the supernatant was discarded. CD4+ T cells were then resuspended in X-Vivo 15 medium at 36.6°C.

4.3.2. Viable Cell Count

The Vi-CELL XR cell counter and the Countess II FL Automated cell counter were used to determine the viable number of CD4+ T cells for each sample. The isolated CD4+ T cells were diluted 1:1 with a trypan blue stain, and cell counts were determined according to the manufacturer's instructions.

4.3.3. Cell Culture

Upon cell count completion, isolated CD4+ T cells were cultured under different conditions depending on the specific experimental set-up.

4.3.3.1 General Cell Culture Conditions

After completion of the cell count, 1 x 10^6 cells were placed in a total cell culture medium volume of 800 µL in 24-well suspension plates for the purpose of cell culture**.** Culture medium was composed of X-VIVO 15™ Medium + 1% Human Serum + 1% Penicillin/Streptomycin. Cells were incubated at 37°C and 5% CO_2.

4.3.3.2 In Vitro Cannabinoid Stimulation

Isolated CD4+ T cells were cultured in 24-well suspension plates with 1 x 10^6 cells in 800 µL of cell culture medium per well. In order to assess the effects of cannabinoids on these cells *in vitro*, (-)-CBD or AEA were added to the culture medium in concentrations of 15 µM and 25 µM, respectively. As the cannabinoids were added in form of an ethanol solution, vehicle control groups were established for both CBD and AEA. For vehicle controls, an equivalent volume of ethanol was added to cell cultures to match the final ethanol concentration in the cannabinoid-treated conditions. The final ethanol concentration in all

cultures was maintained below 0.1% v/v to avoid non-specific effects on T cell function. Cells were incubated at 37°C and 5% CO2 for 48 hours. After 48 hours, cells were removed from the incubator for analysis.

4.3.3.3 In Vitro Th17 Polarization in the Presence of Cannabinoids

Isolated CD4+ T cells were cultured in 24-well suspension plates with 1 x 10^6 cells in 800 µL of cell culture medium per well. The cytokines IL-1β, TGF-β, IL-6 and IL-23 were added to the cell culture medium in concentrations of 12.5 ng/mL, 5 ng/mL, 25 ng/mL, and 25 ng/mL respectively, to induce the differentiation of CD4+ T cells to the Th17 phenotype. In order to induce T Cell activation and expansion, the T Cell Activation/Expansion Kit was used according to the manufacturer's instructions.

In order to assess the effects of cannabinoids on the differentiation of CD4+ T cells under Th17 polarizing conditions *in vitro*, (-)-CBD or AEA were added to the culture medium after 24 hours of culture in concentrations of 15 µM and 25 µM, respectively. After the addition of either of the two cannabinoids and ethanol for vehicle controls, the cells were incubated at 37°C and 5% CO_2 for an additional 72 hours with the cell medium and cytokines being refreshed after the first 48 hours. Cells were subsequently removed from the incubator for analysis.

4.3.4. Flow Cytometry

Flow cytometric analyses were performed to assess cannabinoid receptor expression and cytokine positivity in CD4+ T cells. Two distinct protocols were employed: (1) *ex vivo* cannabinoid receptor staining and (2) intracellular cytokine staining with integrated viability assessment.

4.3.4.1 Ex Vivo Cannabinoid Receptor Staining

Upon isolation of CD4+ T cells, the expression levels of CB1 and CB2 as well as GPR55 were investigated. For each analysis, 5 x 10^5 cells were washed with PBS for 3 minutes at 460 rcf prior to staining for the individual receptors.

Prior to antibody incubation, cells were fixed and permeabilized by means of the BD Cytofix/Cytoperm Fixation/Permeabilization Kit. In order to achieve this, cells were incubated with the fixation and permeabilization solution Cytofix/Cytoperm for 20 minutes at 4°C in the dark.

The Permeabilization buffer PermWash was diluted 1:10 with RNase-free water. Cells were washed with 1 mL of PermWash 1:10 for 5 minutes at 240 rcf after the fixation step, the supernatant was then discarded. All subsequent washes were performed in the same manner with PermWash 1:10.

Cells were incubated with the primary antibody for 20 minutes at 4°C in the dark, after which the cells were washed with PermWash 1:10. Cells were then incubated with a secondary antibody bound to the fluorochrome Brilliant-Violet 421. This secondary antibody was specific for the isotype of the primary antibody used. Incubation was performed again 20 minutes at 4°C in the dark, after which the cells were washed with PermWash 1:10. Cells were then suspended in PermWash 1:10 for flow cytometric analysis using the Gallios Flow Cytometer.

4.3.4.2 Flow Cytometric Cell Viability Assessment and Intracellular Cytokine Staining

For the assessment of cytokine positivity, both after standard and Th17 skewing cell culture conditions, a sequential protocol was established that integrated viability assessment with intracellular cytokine staining to ensure that analyses were performed exclusively on viable cells.

Prior to processing the cells for flow cytometric cytokine analysis, Phorbol Myristate Acetate (PMA; 100 ng/mL) and Ionomycin (1.5 µM) were added to the cell culture medium for a total of 3 hours. PMA and Ionomycin are used to activate CD4+ T cells independent of T-cell receptor complex-mediated activation. Brefeldin A was also added to the culture supernatant in a concentration of 3 µg/mL to inhibit protein transport, leading to intracellular accumulation of cytokines.

After the incubation period, cells and cell culture supernatant were separated by means of centrifugation at 460 rcf for 5 minutes. Cell culture supernatant was frozen at -20°C for further use in ELISA experiments. Cells were washed with PBS for 3 minutes at 460 rcf prior to further use.

Prior to intracellular cytokine staining, cell viability was assessed using the LIVE/DEAD™ Fixable Green Dead Cell Stain Kit according to manufacturer's instructions. This step was essential to ensure that subsequent cytokine analyses were performed only on viable cells. Cells were incubated with the LIVE/DEAD™ Fixable Green Dead Cell Stain Kit for 30 minutes

in the dark at room temperature. Cells were then washed twice with PBS for 3 minutes at 460 rcf, the supernatant was discarded after each washing step.

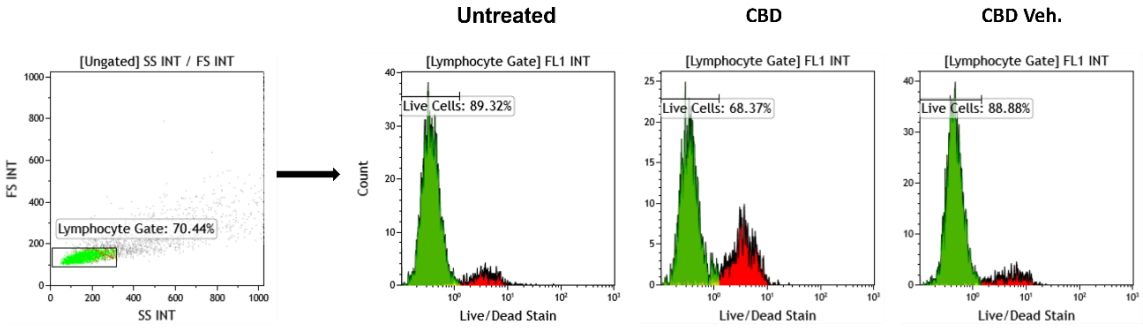

Figure 4. Flow cytometric gating strategy for viability assessment using LIVE/DEAD™ Fixable Green Dead Cell Stain Representative dot plots and histogram showing the sequential gating approach. Initial gating on the dominant lymphocyte population based on forward scatter (FSC) and side scatter (SSC) characteristics. Histogram of LIVE/DEAD™ fluorescence intensity showing distinct peaks for viable cells (left peak, lower fluorescence intensity) and non-viable cells (right peak, higher fluorescence intensity). The clear separation between peaks allows for accurate quantification of cell survival following cannabinoid treatment.

Following viability staining, cells were fixed and permeabilized by means of the BD Cytofix/Cytoperm Fixation/Permeabilization kit according to the manufacturer's instructions. In order to achieve this, cells were incubated with the fixation and permeabilization solution Cytofix/Cytoperm for 20 minutes at 4°C in the dark.

The Permeabilization buffer PermWash was diluted 1:10 with RNase-free water. Cells were washed with 1 mL of PermWash 1:10 for 5 minutes at 240 rcf after the fixation step, the supernatant was discarded. All subsequent washes were performed in the same manner with PermWash 1:10.

Cell samples were incubated with the appropriate cytokine-specific antibodies for 20 minutes at 4°C in the dark, after which the cells were washed with PermWash 1:10. Cells were then suspended in PermWash 1:10 for flow cytometric analysis using the Gallios Flow Cytometer.

For analysis, a sequential gating strategy was employed. First, cells were gated based on forward scatter and side scatter properties to identify the dominant lymphocyte population. Within this gate, viable cells were identified based on LIVE/DEAD fluorescence intensity. The percentage of viable cells was determined by gating on the population with lower fluorescence intensity up to the midpoint of the trough between the viable and non-viable cell population

peaks. The LIVE/DEAD gate was used as a filter for the subsequent cytokine positivity analysis. This methodology allowed for both the assessment of cannabinoid-induced cytotoxicity and the measurement of cytokine positivity specifically in viable CD4+ T cells.

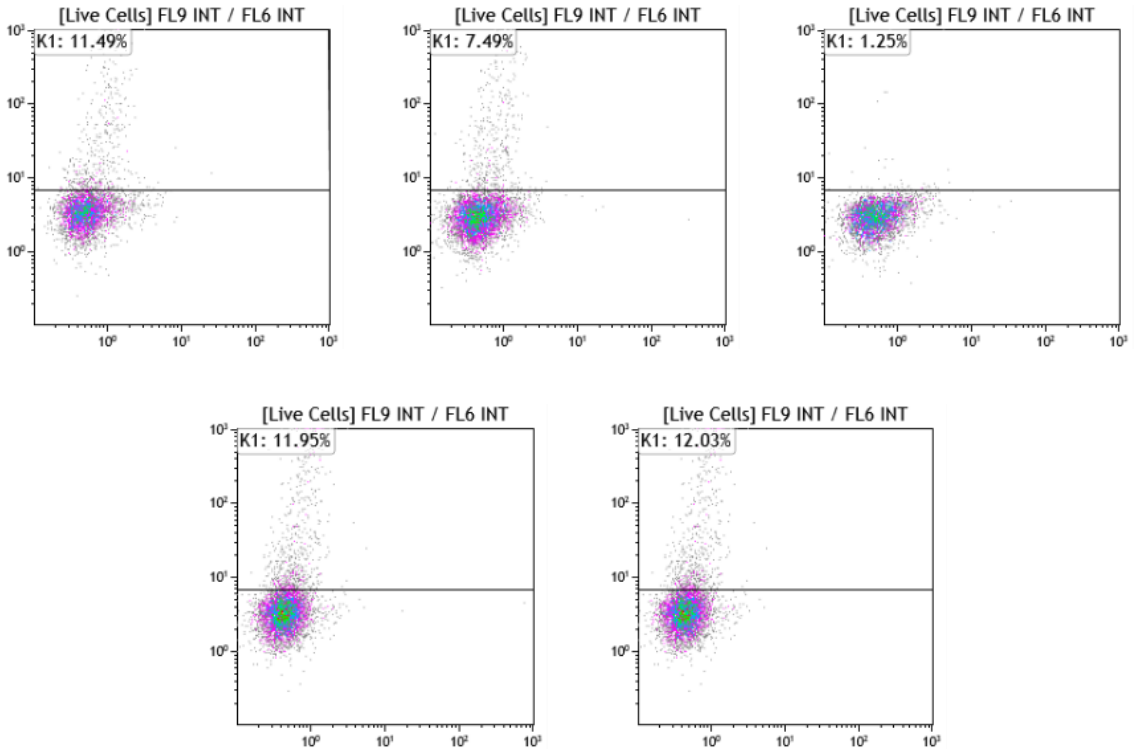

Figure 5. Flow cytometric analysis of TNF-α production in viable CD4+ T cells following cannabinoid treatment Representative dot plots showing TNF-α positivity in CD4+ T cells from patients with rheumatic autoimmune diseases under different treatment conditions. The y-axis represents the fluorescence intensity of the TNF-α-specific fluorophore (APC in channel 6), while the x-axis represents an unrelated fluorescence channel used to optimize population visualization. Only viable cells (as determined by prior LIVE/DEAD™ staining) are included in these plots. The quadrant gate (K1) was established based on the untreated control sample and maintained in the identical position across all treatment conditions for consistent analysis. The percentage values indicate the proportion of viable CD4+ T cells positive for TNF-α. This gating approach was applied consistently for all cytokines assessed.

4.3.5. Molecular Biology

In order to investigate the effects of AEA and CBD on the gene expression in CD4+ T cells from patients with RA, quantitative PCR (qPCR) was performed with synthesized cDNA.

4.3.5.1 RNA Isolation

Upon conclusion of cell culturing, cells were isolated from cell culture supernatant by means of centrifugation for 5 minutes at 460 rcf. Cells were washed with PBS at 4°C for 5 minutes at 460 rcf and frozen at -80°C for a minimum of 30 minutes prior to RNA isolation.

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Hilden) according to the manufacturer's instructions.

Isolated RNA concentration was analyzed with the spectrophotometer Nanodrop 1000 at a wavelength of 260nm. RNA was then stored at -80°C prior to further use.

4.3.5.2 cDNA Synthesis

For analysis by means of qPCR, the isolated RNA was converted to complementary DNA (cDNA). This was achieved by using the QuantiTect Reverse Transcription Kit which was used according to the manufacturer's instructions. Due to variations among subjects, the previously isolated RNA was present in varying concentrations which was accounted for in the cDNA synthesis to ensure a consistent amount of cDNA in each well for each reaction.

4.3.6. Quantitative PCR

Identical Master mixes were created for all experimental conditions

Table 1. Quantitative PCR master mix For the amplification of the individual molecular targets, identical master mixes were created.

Mastermix qPCR 1x	**Amount**
TaqMan Fast Advanced Master Mix	10 µL
RNase-free water	8 µL
Oligonucleotide primer (Applied Biosystems)	1 µL

The master mix was pipetted into a 96-well Micro Amp® Fast Optical 96 well reaction plate and 1 µL of cDNA was added. The qPCR was then performed using a 7500 Fast Real Time PCR System (Applied Biosystems, Carlsbad) using the reaction protocol as seen in Table 2.

Table 2. Reaction conditions for quantitative PCR Upon completion of a 20 second initialization phase at 95°C to denature cDNA, 45 amplification cycles were run consisting of a 3 second denaturing phase at 95°C, and an annealing / extending phase at 60°C

Description	Reaction	Temp	Time (mins)	Cycles
Polymerase Activation	Activation	95°C	00:20	1x
PCR Amplification	Denaturing	95°C	00:03	45 x
2.	Annealing / Extending	60°C	00:30	45 x
3.	Cooling			

After initialization of the cDNA amplification process through a 20 second polymerase activation phase at 95°C, 45 amplification cycles were run, each starting with a 3 second denaturing phase at 95°C followed by a 30 second annealing / extending phase at 60°C.

Analysis of the data obtained from the qPCR was performed using Microsoft Excel. Gene expression was normalized to the housekeeping gene β-2-microglobulin (*B2M)*, which was selected as the reference gene due to its stable expression across experimental conditions in CD4+ T cells. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with untreated controls normalized to 1.0. For each target gene, expression levels following cannabinoid treatment were expressed as fold-change relative to the untreated control.

4.3.7. Sandwich-ELISA (Enzyme-Linked Immunosorbent Assay)

In order to assess the effects of cannabinoids on the release of cytokines from CD4+ T cells into cell culture supernatant, Sandwich-ELISAs were performed for TNF-α, IFN-γ and IL-17A.

Cell culture supernatant was stored at -20°C and thawed in the fridge at 4°C prior to use. Samples were diluted 1:1 with 1X Assay Diluent A before performing the ELISA.

The ELISAs for TNF-α, IFN-γ, and IL-17A were performed using the ELISA MAX™ Deluxe Sets according to manufacturer's instructions. Analysis of the data obtained from the Sandwich-ELISAs was performed using Microsoft Excel.

4.3.8. Statistical Analysis

Statistical Analysis and graphing were performed using the GraphPad Prism 5.0, 6.0, 7.0 and 10.0 software. For data where a normal distribution of the underlying population could be assumed, parametric tests (paired or unpaired Student's t-test) were applied. For data where normality could not be assumed, non-parametric alternatives (Mann-Whitney U test for unpaired comparisons, Wilcoxon signed-rank test for paired comparisons) were used. A *p*-value < 0.05 was considered statistically significant.

Correlation analysis between cannabinoid receptor expression and IL-17A induction in CD4+ T cells was performed using linear regression. The baseline expression of CB1, CB2, and GPR55 was correlated with the percentage increase in IL-17A-positive CD4+ T cells following cannabinoid treatment. Coefficient of determination (R^2) values were calculated to assess the strength of these relationships, with 95% confidence intervals of the slope to determine statistical significance. A confidence interval not crossing zero was considered indicative of a statistically significant correlation.

Data are presented as mean ± standard deviation (SD) unless otherwise specified. Statistical significance was defined as $p < 0.05$, with significance levels indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

5. Results

5.1. Expression of Cannabinoid Receptors in CD4+ T Cells from Patients with Rheumatic Autoimmune Diseases

Cannabinoid receptor expression in CD4+ T cells was investigated to determine potential differences between healthy controls and patients with rheumatic autoimmune diseases. Flow cytometry was used to measure the expression of CB1, CB2, and GPR55 receptors across subject groups to establish baseline receptor profiles.

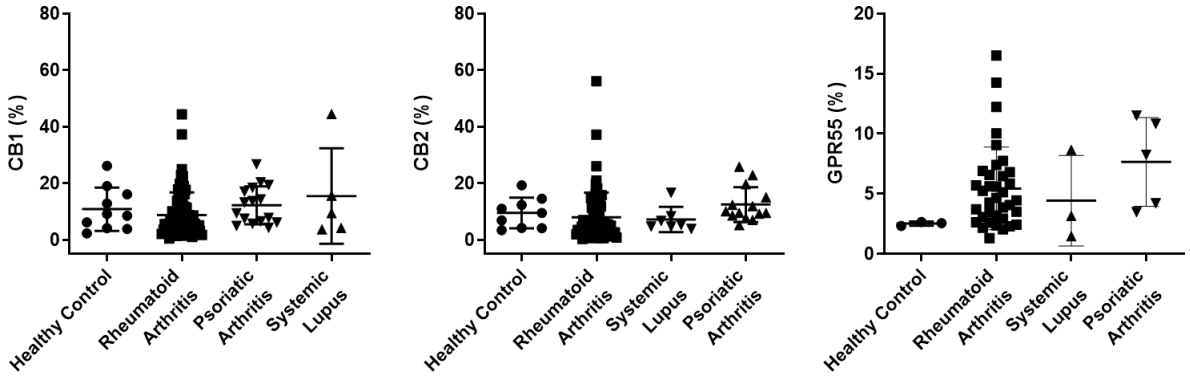

Figure 6. Expression of cannabinoid receptors CB1, CB2, and GPR55 on CD4+ T cells Dot plots depicting the percentage of CD4+ T cells expressing (left) CB1, (middle) CB2, and (right) GPR55, as determined by flow cytometry. Each symbol represents an individual subject; horizontal lines indicate mean ± standard deviation. No statistically significant differences were observed between groups for any receptor ($p > 0.05$). See Supplementary Table 1 for detailed statistics.

Flow cytometric analysis revealed no statistically significant differences in the expression of CB1 in RA patients as compared to healthy controls. Similarly, SLE patients and PsA patients showed no significant differences in CB1 expression when compared to healthy controls. For CB2 receptor expression, no statistically significant differences between healthy controls and patients with RA, PsA, or SLE were observed. While a trend towards higher expression of the GPR55 receptor in rheumatic autoimmune diseases was identified, especially in RA patients (5.45 ± 3.44%) and PsA patients (7.65 ± 3.69%) compared to healthy controls (2.51 ± 0.17%), the results failed to reach statistical significance with $p = 0.154$ and $p = 0.059$, respectively.

5.2. Effects of CBD and AEA on CD4+ T Cell Survival in RA Patients and Healthy Controls

Cell viability assays were conducted to evaluate the cytotoxic effects of cannabinoids on CD4+ T cells from RA patients. CD4+ T cells were exposed to CBD (15μM) or AEA (25μM) for 48 hours and 96 hours, under standard and Th17-polarizing conditions respectively, to assess cannabinoid impact on cell survival.

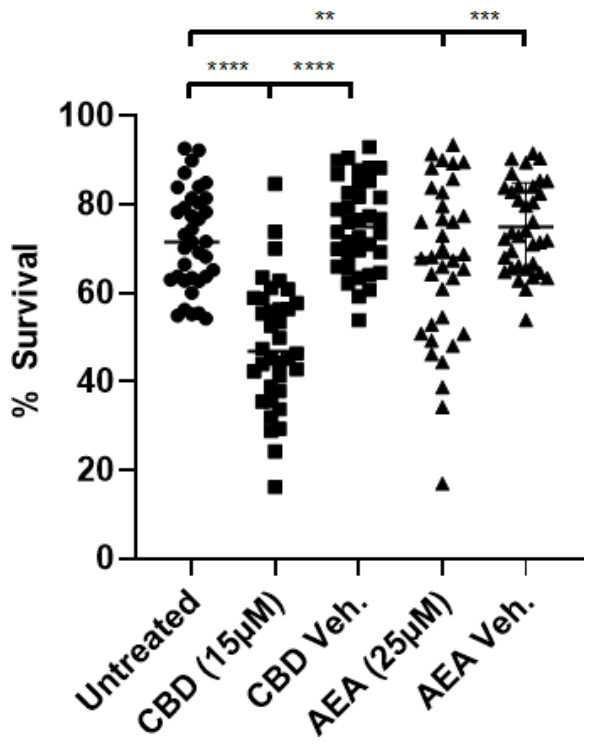

Figure 7. Cytotoxic effects of cannabinoids on CD4+ T cells from rheumatoid arthritis patients
Dot plot showing percent survival of primary CD4+ T cells isolated from RA patients following 48-hour exposure to CBD (15 μM), CBD vehicle, AEA (25 μM), or AEA vehicle compared to untreated controls under standard cell culture conditions. Each data point represents an individual patient sample. CBD significantly reduced cell survival compared to untreated and vehicle treated control groups (****p < 0.0001). AEA also reduced cell survival compared to untreated and vehicle treated control groups, albeit with weaker statistical significance than CBD (**p < 0.01, ***p < 0.001). Mean ± SD: Untreated (71.97 ± 11.21), CBD (15 μM) (48.34 ± 14.47), CBD Veh. (75.05 ± 10.14), AEA (25 μM) (66.67 ± 18.26), AEA Veh. (74.85 ± 9.99). n = 36.

As shown in Figure 7, CBD exposure significantly reduced CD4+ T cell survival compared to untreated conditions (48.34 ± 14.47 % vs. 71.97 ± 11.21 %, p < 0.0001). The CBD vehicle control showed no significant effect on cell survival. AEA also demonstrated a modest but statistically significant reduction in CD4+ T cell survival compared to untreated cells (66.67 ± 18.26 % vs. 71.97 ± 11.21 %, p < 0.01), though this effect was less pronounced than that observed with CBD. The AEA vehicle had no significant impact on cell survival.

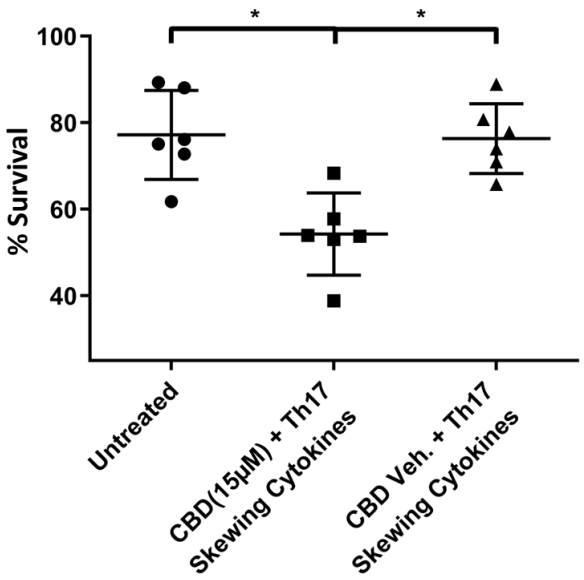

Figure 8. CBD reduces CD4+ T cell viability under Th17-polarizing conditions in RA patients Dot plot depicting the percentage of live CD4+ T cells isolated from RA patients under standard culture conditions (Control), Th17-polarizing conditions with CBD treatment (Th17 Skewing + CBD), or Th17-polarizing conditions with vehicle (Th17 Skewing + Vehicle) after 96 hours of total culture time. Each symbol represents an individual patient sample. CBD significantly reduced cell survival under Th17-polarizing conditions as compared to untreated and vehicle treated control groups (*$p < 0.05$). Mean ± SD: Control (77.21 ± 10.28), Th17 Skewing + CBD (54.3 ± 9.5), Th17 Skewing + Vehicle (76.37 ± 8.06). n = 6.

To determine whether the cytotoxic effects of CBD persisted under Th17-polarizing conditions, cell survival in CD4+ T cells from RA patients cultured with TGF-β, IL-1β, and IL-23 in the presence or absence of CBD was assessed. As shown in Figure 8, CBD significantly reduced cell survival under Th17-skewing conditions compared to the vehicle control and untreated control groups (54.3 ± 9.5 % vs. 76.37 ± 8.06 %, $p < 0.05$). Notably, the Th17-skewing conditions themselves did not significantly affect cell viability compared to standard culture conditions, as evidenced by the similar survival rates between the control (77.21 ± 10.28 %) and the Th17-skewing with vehicle conditions (76.37 ± 8.06 %).

5.3. Effects of Cannabinoids on Proinflammatory Cytokine Production in CD4+ T Cells

Flow cytometric analysis was conducted to evaluate how CBD and AEA exposure influences the positivity of key proinflammatory cytokines in CD4+ T cells across different patient groups. Cells were treated with cannabinoids for 48 hours to determine disease-specific effects on IL-17A, IFN-γ, and TNF-α positivity, providing insights into their immunomodulatory potential.

5.3.1. Impact of CBD and AEA on IL-17A Positivity among CD4+ T Cells

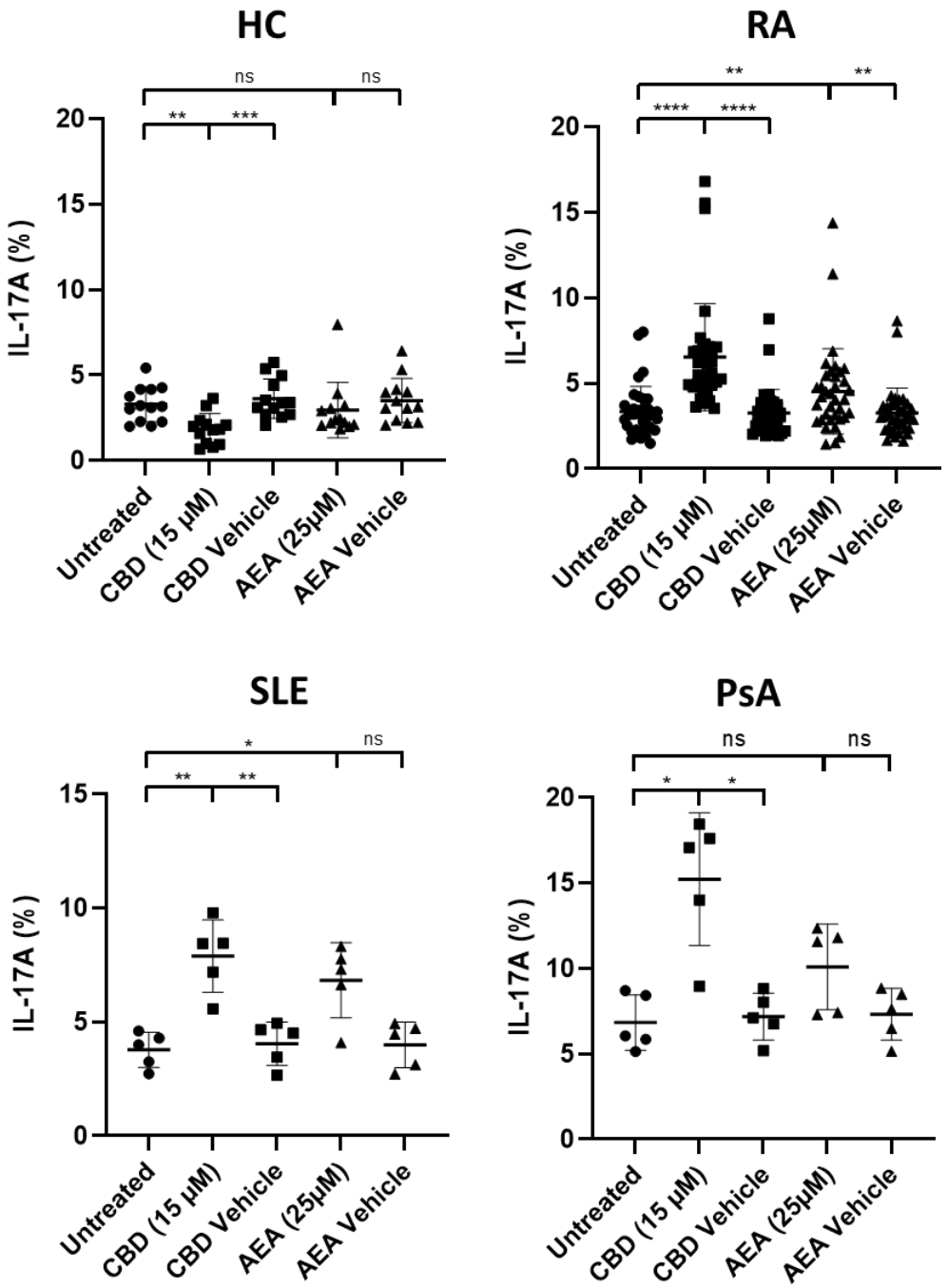

Figure 9. Disease-specific effects of cannabinoids on IL-17A expression in CD4+ T cells Dot plots showing the percentage of IL-17A-positive CD4+ T cells following 48-hour exposure to CBD (15 μM), CBD vehicle, AEA (25 μM), or AEA vehicle compared to untreated controls across different patient groups: HC (n = 13), RA (n = 36), SLE (n = 5), and PsA (n = 5). In healthy controls, CBD significantly reduced IL-17A expression, while in RA patients, both CBD and AEA significantly increased IL-17A expression. SLE patients showed significant increases in IL-17A expression with CBD treatment, but not with AEA treatment. PsA patients demonstrated significant IL-17A positivity increases with CBD. Each symbol represents an individual subject; horizontal lines indicate mean ± standard deviation. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = non-significant. See Supplementary Table 2 for detailed statistics.

The effects of cannabinoids on IL-17A positivity in CD4+ T cells revealed differences between healthy controls and patients with rheumatic autoimmune diseases. In healthy controls, CBD treatment significantly reduced the percentage of IL-17A-positive CD4+ T cells compared to untreated conditions (1.86 ± 0.89 % vs. 3.30 ± 1.03 %, p < 0.01).

Both CBD and AEA treatments significantly increased the percentage of IL-17A-positive CD4+ T cells in RA patients compared to untreated conditions. This effect was particularly pronounced with CBD treatment, which showed a substantial and statistically significant elevation in IL-17A positivity (6.54 ± 3.12 % vs. 3.36 ± 1.46 %, p < 0.0001). The AEA-mediated increase in IL-17A-positive cells in RA patients (4.53 ± 2.50 % vs. 3.36 ± 1.46 %, p < 0.01), while less dramatic than the CBD effect, was still statistically significant when compared to untreated and vehicle controls.

In SLE patients, both CBD and AEA treatments increased the percentage of IL-17A-positive CD4+ T cells compared to the untreated group. CBD effects showed statistical significance when compared to both untreated and vehicle-treated groups (7.89 ± 1.59 % vs. 3.77 ± 0.77 %, p < 0.01), while AEA failed to produce statistically significant results when compared to the vehicle control group. For PsA patients, CBD treatment showed statistical significance in comparison to untreated and vehicle-treated control groups (15.22 ± 3.88 % vs. 6.83 ± 1.62 %, p < 0.05). AEA did not produce statistically significant results in the PsA patient group, though a trend toward higher IL-17 positivity was observed.

5.3.2. Impact of CBD and AEA on IFN-γ Positivity among CD4+ T Cells

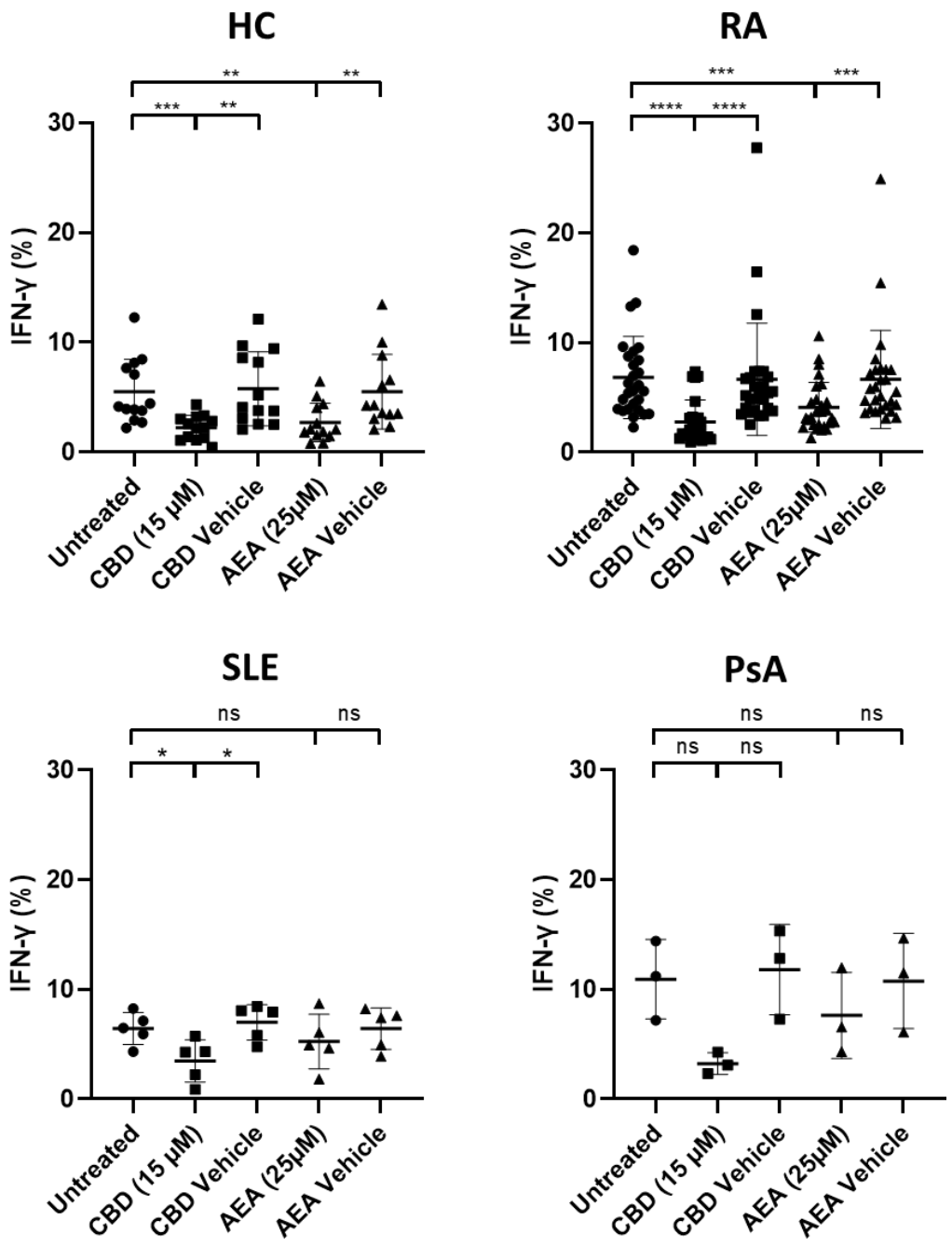

Figure 10. Disease-specific effects of cannabinoids on IFN-γ expression in CD4+ T cells Dot plots showing the percentage of IFN-γ-positive CD4+ T cells following 48-hour exposure to CBD (15 μM), CBD vehicle, AEA (25 μM), or AEA vehicle compared to untreated controls across different patient groups: HC (n = 13), RA (n = 27), SLE (n = 5), and PsA (n = 3). Each symbol represents an individual subject; horizontal lines indicate mean ± standard deviation. Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = non-significant. See Supplementary Table 3 for detailed statistics.

Treatment of CD4+ T cells with CBD significantly reduced the percentage of IFN-γ-positive cells in both healthy controls (untreated: 5.51 ± 2.95 % vs. CBD: 2.25 ± 1.12 %; $p < 0.001$) and RA patients (untreated: 6.83 ± 3.73 % vs. CBD: 2.79 ± 1.99 %; $p < 0.0001$) compared to untreated conditions. This suppressive effect had the highest statistical significance in RA patients followed by healthy controls. The suppressive effect was also present for SLE patients (untreated: 6.43 ± 1.45 % vs. CBD: 3.49 ± 1.92 %; $p < 0.05$), however to a far lesser degree of significance. While the trend in PsA patients is indicative of a similar suppressive potential for CBD as was the case in RA patients, the low sample size does not allow for the determination of statistical significance.

In contrast, AEA treatment demonstrated a more variable effect on IFN-γ production. While there was a significant reduction in IFN-γ-positive cells in both HC (untreated: 5.51 ± 2.95 % vs. AEA: 2.72 ± 1.75 %; $p < 0.01$) and RA groups (untreated: 6.83 ± 3.73 % vs. AEA: 4.12 ± 2.28 %; $p < 0.001$) compared to untreated controls, the effect was less pronounced than that observed with CBD treatment. There was no statistical significance in reduction of IFN-γ-positive cells for SLE and PsA patients. In contrast to CBD treatment, there was a less pronounced trend to reduction in these two patient groups, indicating that this may not be solely an effect of the low sample size, but also possibly due to an overall less pronounced effect of AEA in these two patient groups as compared to RA patients.

5.3.3. Impact of CBD and AEA on TNF-α Positivity among CD4+ T Cells

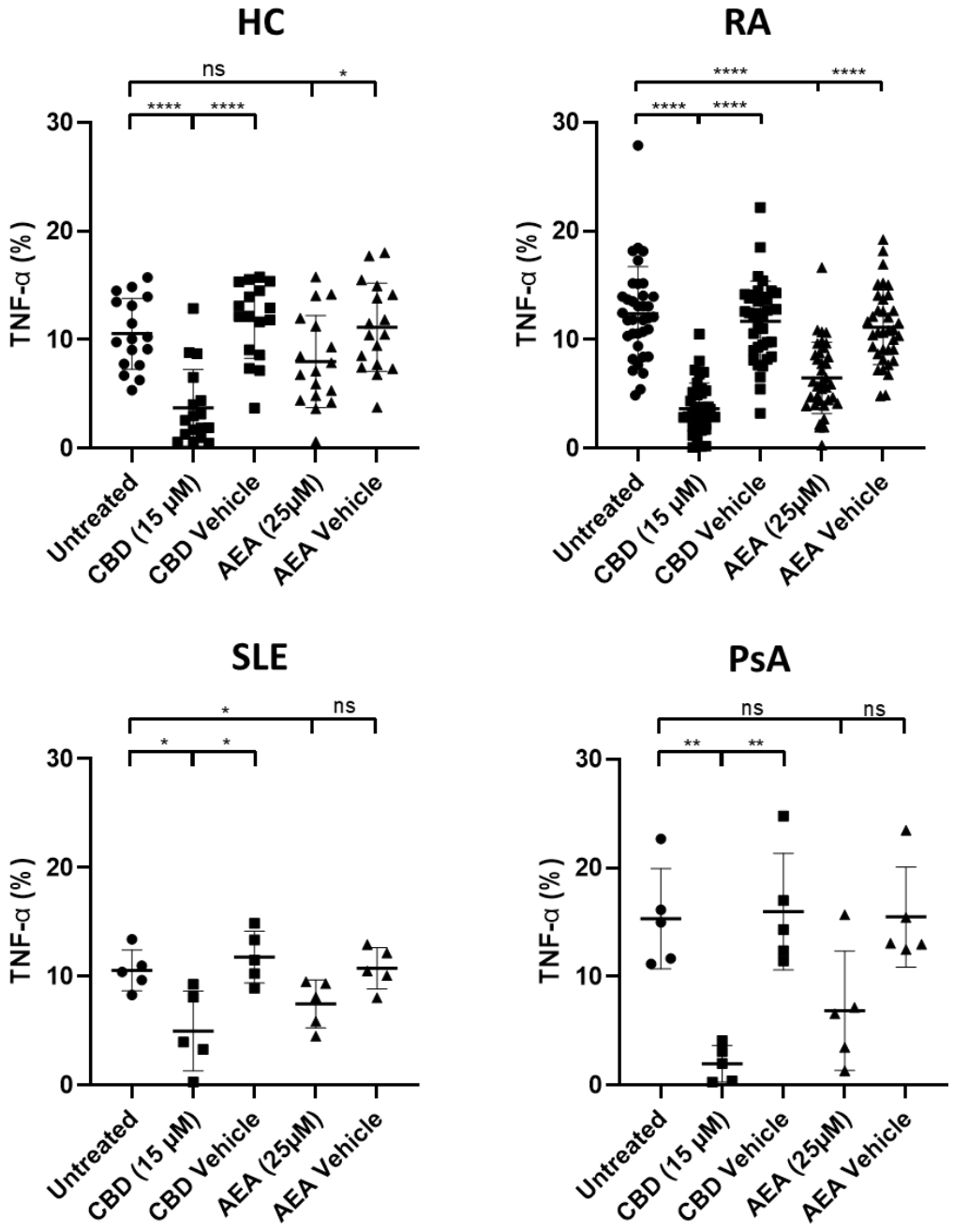

Figure 11. Disease-specific effects of cannabinoids on TNF-α expression in CD4+ T cells Dot plots showing the percentage of TNF-α-positive CD4+ T cells following 48-hour exposure to CBD (15 μM), CBD vehicle, AEA (25 μM), or AEA vehicle compared to untreated controls across different patient groups: HC (n = 17), RA (n = 36), SLE (n = 5), and PsA (n = 5). Each symbol represents an individual subject; horizontal lines indicate mean ± standard deviation. Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = non-significant. See Supplementary Table 4 for detailed statistical data.

CBD treatment suppressed TNF-α production in CD4+ T cells across all patient groups. The statistical significance was strongest among healthy controls and RA patients. AEA treatment also significantly reduced TNF-α production in RA patients, though to a lesser extent than CBD. In the SLE group, AEA treatment showed a modest but statistically significant reduction in TNF-α-positive cells when compared to the untreated group, but not when compared to the vehicle control group. No significant effect was observed in HC or PsA patients following AEA treatment.

5.4. Correlation Between Cannabinoid Receptor Expression and IL-17A Induction in CD4+ T Cells

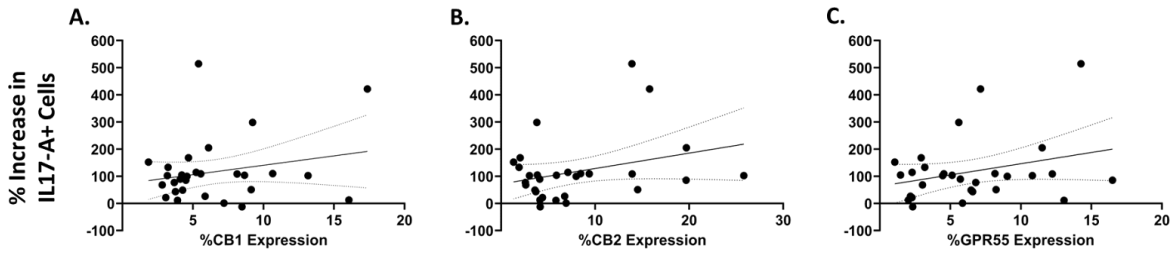

Figure 12. Relationship between cannabinoid receptor expression and IL-17A induction in CD4+ T cells from RA patients after CBD treatment Scatter plots depicting the correlation between baseline expression of cannabinoid receptors (A. CB1, B. CB2, and C. GPR55) and the percentage increase in IL-17A-positive CD4+ T cells following 48 hours of CBD treatment. Solid lines represent linear regression, with dotted lines indicating 95% confidence intervals. Each point represents an individual patient sample. Despite weak positive trends observed across all three receptors (R^2 values: CB1 = 0.052, CB2 = 0.092, GPR55 = 0.086), the wide confidence intervals crossing zero (Slope values: CB1 = -4.754 to 18.60, CB2 = -1.347 to 12.70, GPR55 = -2.37 to 18.92) indicate the absence of statistically significant correlations. N = 29.

To investigate whether the varying responses to CBD treatment might be influenced by the baseline expression of cannabinoid receptors, correlation analyses were performed between receptor expression as determined after initial cell isolation (CB1, CB2, and GPR55) and the percentage increase in IL-17A-positive cells following 48 hours of cannabinoid treatment for patient samples in which all data points were available.

As shown in Figure 12, linear regression analysis revealed weak positive trends between the expression of each cannabinoid receptor and the increase in IL-17A positivity, although none reached statistical significance. For CB1 receptor expression, the 95% confidence interval for the slope ranged from -4.754 to 18.60, with an R^2 value of 0.052. Similarly, for CB2 receptor expression, the 95% confidence interval for the slope was -1.347 to 12.70, with an R^2 value of 0.092. For GPR55 expression, the 95% confidence interval was -2.37 to 18.92, with an R^2 value of 0.086.

The low R^2 values indicate that only a small proportion of the variability in IL-17A induction (approximately 5-9 %) could be explained by differences in receptor expression. Furthermore, the fact that all confidence intervals for the slopes crossed zero suggests that a positive or negative relationship between receptor expression and the cannabinoid-induced increase in IL-17A positivity cannot definitively be established.

5.5. Impact of CBD on Th17 Differentiation Under Th17 Skewing Conditions

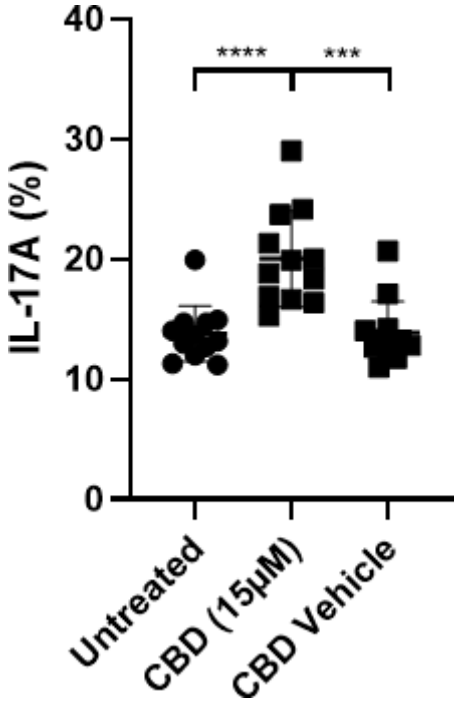

Figure 13. CBD increases IL-17A expression in CD4+ T cells from RA patients under Th17-polarizing conditions Scatter plot showing the percentage of IL-17A-positive CD4+ T cells isolated from RA patients and cultured under Th17-skewing conditions for 24 hours (TGF-β, IL-1β, and IL-23) followed by addition of CBD (15 μM) or a vehicle control for an additional 72 hours. Each symbol represents an individual patient sample; horizontal lines indicate mean ± standard deviation. Significance levels: ***$p < 0.001$, ****$p < 0.0001$. n = 12.

To specifically examine the effect of CBD under Th17-skewing conditions, CD4+ T cells from RA patients were cultured under Th17-skewing conditions (TGF-β, IL-1β, and IL-23) for 24 hours after which CBD was added to the cell culture for an additional 72 hours. CD4+ T cells cultured under these Th17-polarizing conditions showed a statistically significant increase in IL-17A positivity following CBD treatment compared to untreated controls (from 13.80 ± 2.32 % to 20.04 ± 3.98 %, $p < 0.0001$). Importantly, the CBD vehicle control had no significant effect on IL-17A positivity compared to untreated cells (13.87 ± 2.62 % vs. 13.80 ± 2.32 %, ns).

5.6. Quantitative Analysis of Cytokine Secretion by CD4+ T Cells Following Cannabinoid Treatment

ELISA assays were performed to quantify cytokine secretion in cell culture supernatants after cannabinoid treatment. This complementary approach to flow cytometry measured secreted IL-17A, IFN-γ, and TNF-α levels to provide a more comprehensive understanding of the functional impact of cannabinoids on CD4+ T cell cytokine production.

5.6.1. IL-17A Secretion

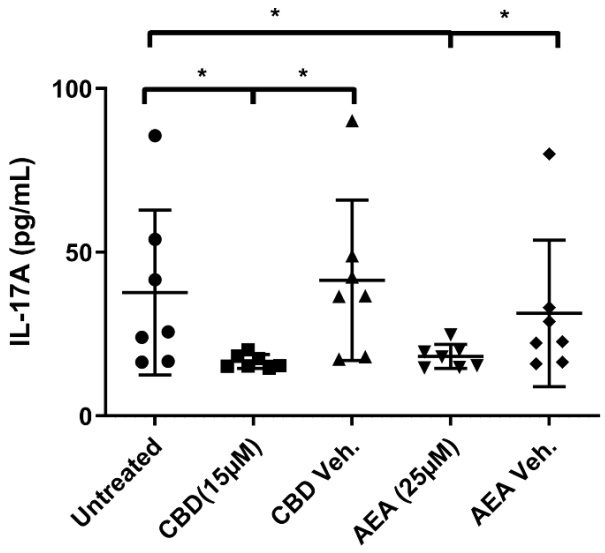

Figure 14. CBD and AEA reduce IL-17A secretion in CD4+ T cell cultures from RA patients Dot plot depicting ELISA quantification of IL-17A in cell culture supernatants of untreated samples incubated for 48 hours and samples after 48-hour exposure to cannabinoids or vehicle controls. Data points represent individual patient samples (n = 7). Both CBD (15 µM) and AEA (25 µM) significantly decreased IL-17A concentration compared to untreated and vehicle controls (*$p < 0.05$).

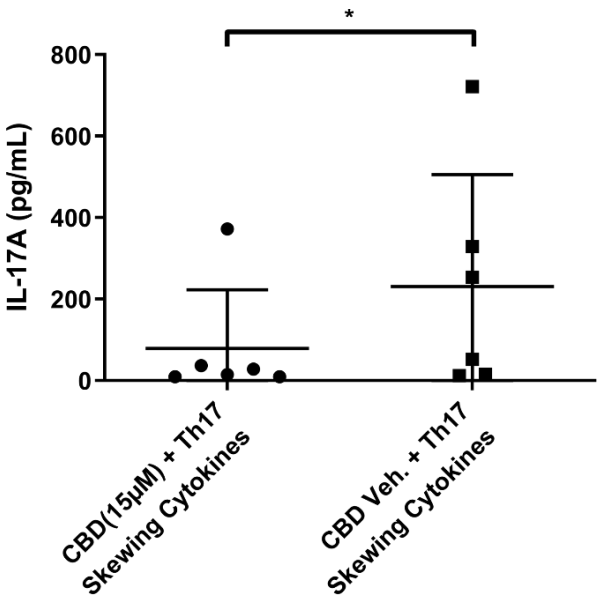

Figure 15. IL-17A concentration in supernatants from CD4+ T cells cultured under Th17-polarizing conditions Dot plot depicting ELISA quantification of IL-17A in cell culture supernatants of samples after 48-hour exposure to CBD (15 μM) and vehicle controls under Th17 skewing conditions. Individual data points represent separate patient samples (n = 6). CBD treatment significantly reduced IL-17A secretion relative to the vehicle control group (*$p < 0.05$).

As shown in Figure 14, analysis of IL-17A concentration in culture supernatants from RA patients revealed a significant reduction following cannabinoid treatment. Compared to the untreated condition (37.72 ± 25.16 pg/mL), IL-17A concentration was significantly lower after treatment with both CBD (16.67 ± 2.09 pg/mL; $p < 0.05$) and AEA (18.21 ± 3.66 pg/mL; $p < 0.05$). In contrast, the respective vehicle controls showed no significant effect on secretion (CBD Veh.: 41.48 ± 24.52 pg/mL; AEA Veh.: 31.35 ± 22.36 pg/mL). This suppressive effect was also observed under Th17-polarizing conditions (Figure 15), where CBD treatment significantly reduced IL-17A concentration from 230.9 ± 274.7 pg/mL in the vehicle control group to 78.72 ± 144.1 pg/mL ($p < 0.05$).

5.6.2. IFN-γ Secretion

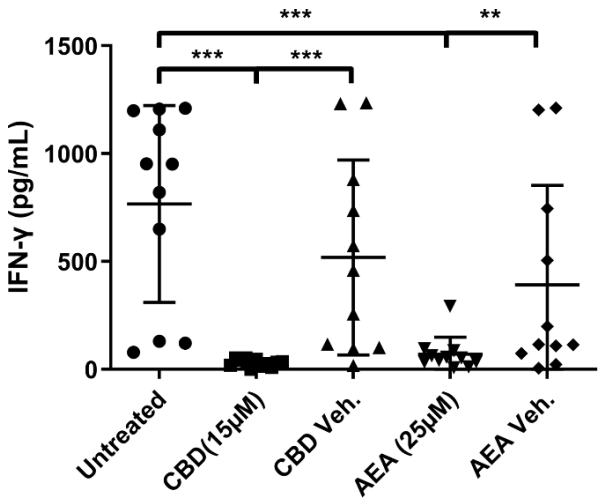

Figure 16. Cannabinoids suppress IFN-γ secretion by CD4+ T cells from RA patients Dot plot depicting ELISA quantification of IFN-γ in cell culture supernatants of untreated samples and samples after 48-hour exposure to cannabinoids or vehicle controls. Data points represent individual patient samples (n = 11). Both CBD (15 μM) and AEA (25 μM) significantly decreased IFN-γ concentration compared to untreated and vehicle controls (***$p < 0.001$, **$p < 0.01$).

For IFN-γ, both CBD and AEA treatments significantly reduced the concentration in CD4+ T cell culture supernatant from RA patients. CBD treatment led to a profound decrease from 766.9 ± 455.7 pg/mL to 30.33 ± 17.82 pg/mL ($p < 0.001$), while AEA reduced levels to 71.15 ± 78.73 pg/mL ($p < 0.01$). Vehicle controls showed some reduction but remained significantly higher than cannabinoid treatments (CBD Veh.: 518.9 ± 451.8 pg/mL, AEA Veh.: 391.6 ± 461.0 pg/mL).

5.6.3. TNF-α Secretion

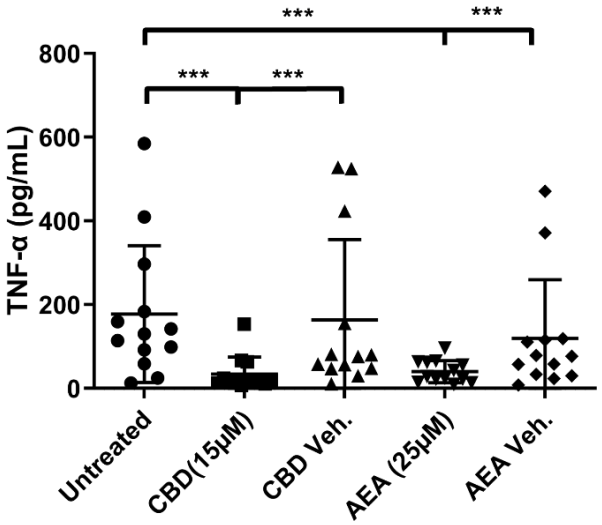

Figure 17. Strong suppression of TNF-α secretion in CD4+ T cells from RA patients by cannabinoids Dot plot depicting ELISA quantification of TNF-α levels in cell culture supernatants of untreated samples and samples after 48-hour exposure to cannabinoids or vehicle controls. Data points represent individual patient samples (n = 13). Both CBD (15 μM) and AEA (25 μM) significantly decreased TNF-α concentration compared to untreated and vehicle controls (***$p < 0.001$).

Similarly, the TNF-α concentration in supernatant was markedly decreased following both CBD and AEA treatment (Figure 17), with reductions from 177.7 ± 163.3 pg/mL (untreated) to 34.25 ± 40.53 pg/mL (CBD) and 40.06 ± 26.47 pg/mL (AEA) (both $p < 0.001$). Treated groups also showed statistically significant decreases in TNF-α concentration when compared to their respective vehicle controls (CBD Veh.: 163.4 ± 192.1 pg/mL, AEA Veh.: 119.8 ± 140.1 pg/mL), confirming that the observed suppression was attributable to the cannabinoids themselves rather than their delivery vehicles.

5.7. Cannabinoid Treatment Effect on Gene Expression in CD4+ T Cells

Reverse Transcription with subsequent qPCR was performed to examine how cannabinoids affect the expression of genes involved in T cell function and inflammatory responses. CD4+ T cells from both healthy controls and RA patients were treated with CBD or AEA for 48 hours to assess changes in *SGK1*, *IKZF3*, *CSF2*, and *AHR* expression, revealing differential effects between patient populations.

5.7.1. Impact of CBD and AEA on Gene Expression in Healthy Controls

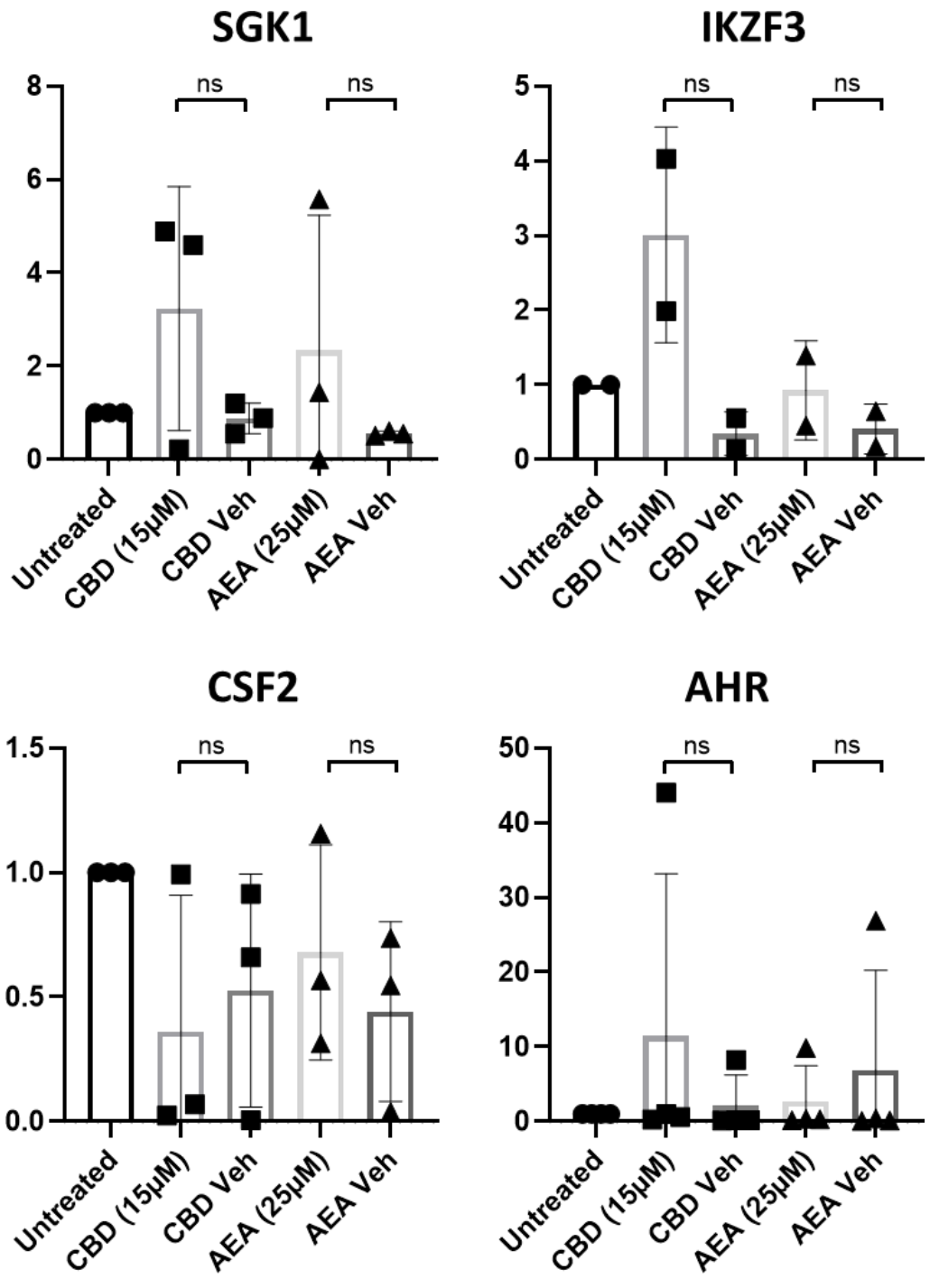

Figure 18. Effects of cannabinoids on gene expression in CD4+ T cells from healthy controls Bar graphs with individual data points showing relative expression of *SGK1* (n = 3), *IKZF3* (n = 2), *CSF2* (n = 3), and *AHR* (n = 4) genes in CD4+ T cells from healthy controls following 48-hour exposure to CBD (15 μM), CBD vehicle, AEA (25 μM), or AEA vehicle compared to untreated controls (normalized to 1.0 using $2^{-\Delta\Delta Ct}$ method). Each symbol represents an individual subject; bars indicate mean ± standard deviation. ns = non-significant ($p > 0.05$). See Supplementary Table 5 for detailed statistical data.

Gene expression analysis in CD4+ T cells from healthy controls revealed no statistically significant changes in any of the examined genes following cannabinoid treatment when compared to their respective vehicle controls. As shown in Figure 18, where gene expression was normalized to untreated controls (set to 1.0) using the $2^{-\Delta\Delta Ct}$ method, neither CBD nor AEA treatment resulted in significant alterations in the expression of *SGK1*, *IKZF3*, *CSF2*, or *AHR* genes. While some trends toward increased expression were observed, particularly for *SGK1* (3.24 ± 2.61 vs. 0.88 ± 0.33) and *IKZF3* (3.01 ± 1.44 vs. 0.35 ± 0.29) following CBD treatment, these changes did not reach statistical significance when compared to the CBD vehicle control.

5.7.2. Impact of CBD and AEA on Gene Expression in Rheumatoid Arthritis Patients

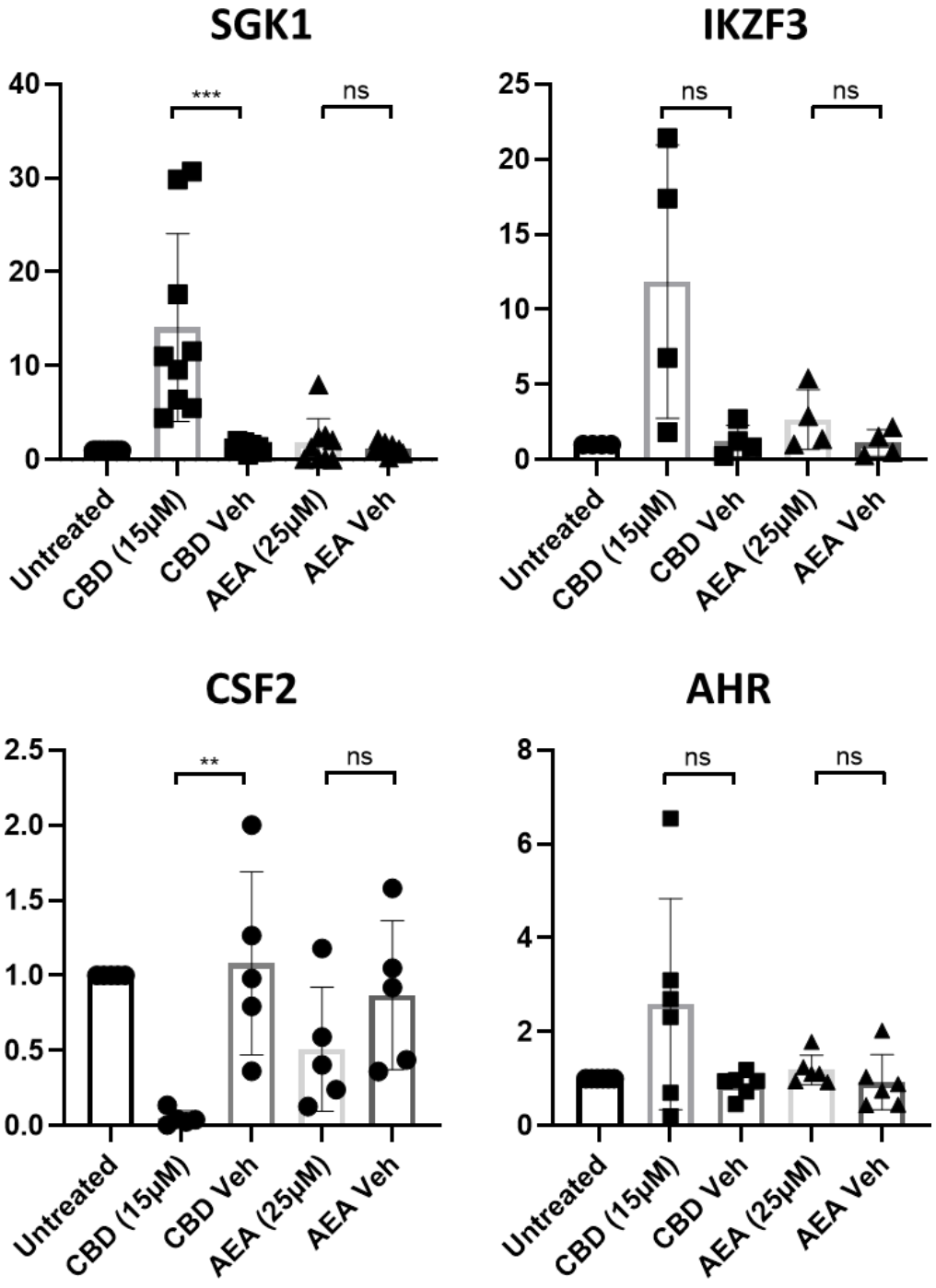

Figure 19. Effects of cannabinoids on gene expression in CD4+ T cells from RA patients Bar graphs with individual data points showing relative expression of *SGK1*, *IKZF3*, *CSF2*, and *AHR* genes in CD4+ T cells from RA patients following 48-hour exposure to CBD (15 μM), CBD vehicle, AEA (25 μM), or AEA vehicle compared to untreated controls (normalized to 1.0 using $2^{-\Delta\Delta Ct}$ method). Each symbol represents an individual RA patient; bars indicate mean ± standard deviation. Significance levels: $^{**}p < 0.01$, $^{***}p < 0.001$, ns = non-significant ($p > 0.05$). See Supplementary Table 6 for detailed statistical data.

In CD4+ T cells isolated from RA patients, CBD treatment induced significant changes in the expression of specific genes when compared to CBD vehicle control, as illustrated in Figure 19. Using the $2^{-\Delta\Delta Ct}$ method with untreated controls normalized to 1.0, CBD significantly upregulated *SGK1* expression compared to CBD vehicle control (14.07 ± 10.0 vs. 1.31 ± 0.49, $p < 0.001$), representing a substantial increase in relative expression levels. Conversely, CBD treatment significantly downregulated *CSF2* expression (0.05 ± 0.05 vs. 1.08 ± 0.61, $p < 0.01$) compared to CBD vehicle control.

The expression of *IKZF3* and *AHR* genes showed no statistically significant changes following CBD treatment compared to CBD vehicle, though a trend toward both increased *IKZF3 and AHR* expression was observed (11.85 ± 9.09 vs. 1.26 ± 1.05 and 2.59 ± 2.25 vs. 0.88 ± 0.25).

In contrast to CBD, AEA treatment did not significantly alter the expression of any of the examined genes in RA patients when compared to AEA vehicle control. No statistically significant differences were observed for *SGK1*, *IKZF3*, *CSF2*, or *AHR* expression following AEA treatment when compared to AEA vehicle controls.

5.8. Observational Clinical Data on CBD Use in Rheumatoid Arthritis Patients

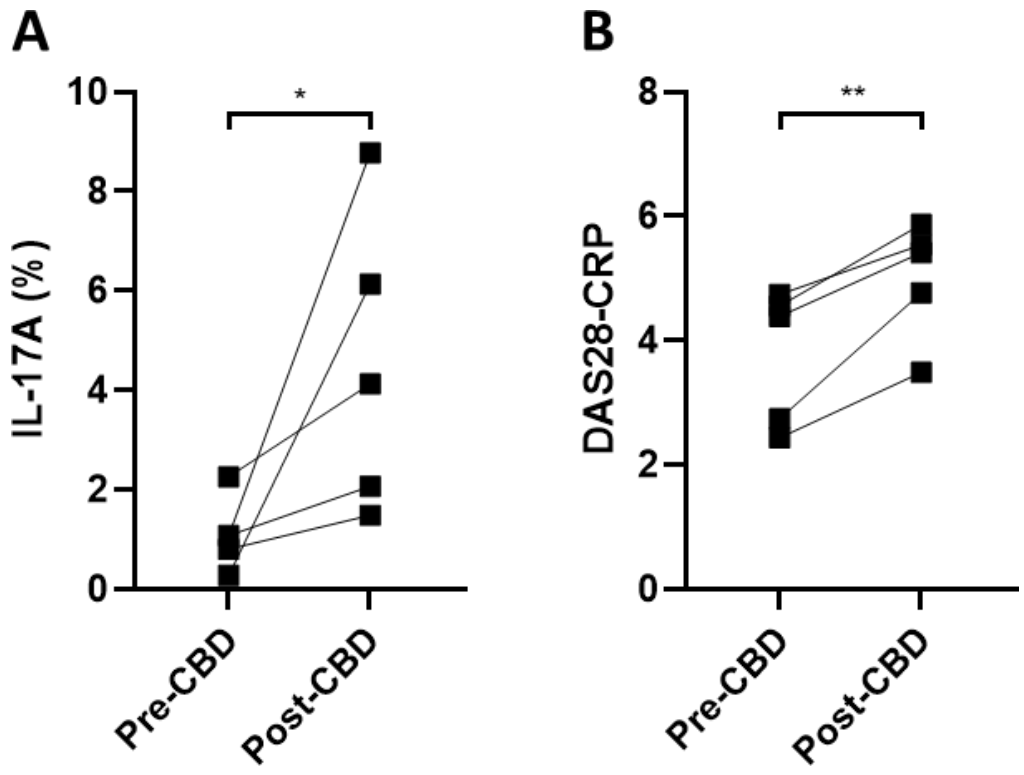

Figure 20. Changes in IL-17A expression and disease activity following self-reported CBD use in RA patients Line graphs showing paired measurements from individual RA patients (n = 5) before and after a period of self-reported CBD use (4-8 weeks). (A) Percentage of IL-17A-positive CD4+ T cells following CBD use. (B) Disease Activity Score 28 with C-Reactive Protein (DAS28-CRP). Each line connects paired measurements from the same individual.

In addition to our *in vitro* experiments, observational data from a small cohort of RA patients who self-reported voluntary CBD use over a period of 4-8 weeks was obtained. Blood samples were collected prior to CBD treatment initiation (Pre-CBD) and after the reported consumption period (post-CBD). The percentage of IL-17A-positive CD4+ T cells increased significantly in all patients following the reported CBD use period, rising from 1.1 ± 0.73% (Pre-CBD) to 4.52 ± 3.00% (post-CBD) ($p < 0.05$). This increase in IL-17A positivity was accompanied by a concurrent significant elevation in DAS28-CRP scores, which increased from 3.77 ± 1.09 (Pre-CBD) to 5.02 ± 0.94 (post-CBD) ($p < 0.01$), indicating a worsening of disease state. These clinical observations align with our *in vitro* findings regarding CBD's effects on IL-17A expression in CD4+ T cells from RA patients.

6. Discussion

6.1. Cannabinoid Receptor Expression in Rheumatic Autoimmune Disease

In an attempt to better understand the endocannabinoid system under rheumatic conditions, the expression of the classical endocannabinoid system receptors CB1 and CB2, along with the putative third cannabinoid receptor GPR55 was assessed in healthy controls as well as patients afflicted primarily by RA as well as PsA and SLE.

With the CB2 receptor being implicated in the modulation of immune function, the expression of these receptors, in addition to the CB1 receptor, in CD4+ T cells was measured to better understand possible differences in the healthy and diseased state that might affect the observations when treating these cells with AEA and CBD. There was no statistically significant difference in expression of the CB1 and CB2 receptors in healthy controls as compared to patients suffering from the aforementioned diseases. In the context of this insight, it became clear that any effects observed during *in vitro* experiments would not be the result of a differing cannabinoid receptor expression in our target cells occurring as a result of disease specific alterations to cannabinoid receptor expression.

Given that AEA via the CB2 receptor can cause a suppression of cellular proliferation as well as a reduction in the production and release of IL-17A, TNF-α, and IFN-γ, the possibility of altered CB2 receptor expression being part of the pathophysiologic cascade in RA was considered.[197,199,234,270] The hypothesis that a possible reduction in CB2 expression and a resulting inability of AEA to inhibit overarching immune function plays a role in RA pathology was rejected on the basis of the aforementioned flow cytometric analysis of receptor expression. While the CB1 receptor is less implicated in immune modulation, the observations also enable a rejection of an altered CB1 receptor expression being implicated in RA development given the lack of a statistically significant difference observable in RA patients when compared to healthy controls.

While the results bear no statistical significance, the expression profile of the putative third cannabinoid receptor GPR55, proved to be interesting. While the collected data does not allow for the conclusion that GPR55 is overexpressed in CD4+ T cells of RA patients ($p = 0.154$), it was notable that a trend towards higher expression was observed. Furthermore, a similar trend in PsA patients approached statistical significance ($p = 0.059$), with these patients showing the highest mean expression at 7.65 ± 3.69% compared to 2.51 ± 0.17% in healthy controls. This near-significant difference and underlying expression trend suggests that with increased sample sizes, a statistically significant difference might emerge. While the sample size is far too small to draw definitive conclusions, it does raise the question of whether GPR55 expression may in fact be altered in rheumatic autoimmune diseases, especially those with a strong Th17 pathology component like PsA, where the trend was most pronounced and RA.

Given the lack of broad research on the GPR55 receptor, these results need to be viewed critically. Due to the observational nature of data collected, there is a possibility of confounding variables leading to alterations in GPR55 expression and thus the observed trend that are inherently not related to RA and PsA and their respective pathogenesis. Beyond an increased sample size, further experiments in an attempt to derive conclusions regarding the causal effect between RA affliction and GPR55 expression levels would be of significant value. Considering that the expression and activation of this receptor have been linked to pathologically overshooting immune responses, further exploration of its significance in RA is warranted.[239,271,272] The results obtained in this study provoke an initial suspicion that an increased expression of the GPR55 receptor in CD4+ T cells from RA patients might contribute to the immune dysregulation that underlies this disease. Further experiments aiming to isolate GPR55 mediated effects on cytokine production, such as through use of a selective agonist or antagonist and correlating treatment to changes in clinical severity in appropriate mouse models, could be valuable in assessing the role of this receptor plays in RA.

The correlation analysis between cannabinoid receptor expression and IL-17A induction in CD4+ T cells from RA patients provided additional insights into the relationship between receptor positivity and functional outcomes. This analysis was conducted to determine whether the variable responses to cannabinoid treatment observed across patients could be attributed to differences in baseline receptor expression. Despite trends suggesting weak positive correlations between the expression of cannabinoid receptors (CB1, CB2, and GPR55) and the magnitude of IL-17A induction following cannabinoid treatment, none of these correlations reached statistical significance. The relatively low R^2 values (ranging from 0.052 to 0.092) indicated that only a small fraction of the variability in IL-17A induction could potentially be explained by differences in receptor expression.

This lack of significant correlation suggests that while receptor expression may contribute to the response variability, other factors likely play more substantial roles in determining the magnitude of IL-17A induction following cannabinoid treatment. These factors could include variations in downstream signaling pathways, differences in receptor functionality rather than mere expression levels, or patient-specific inflammatory environments that influence cellular responsiveness to cannabinoids. The absence of a strong correlation between receptor expression and functional outcomes reinforces the conclusion that the differential effects of cannabinoids on CD4+ T cells from RA patients compared to healthy controls are unlikely to be attributable to altered expression of cannabinoid receptors. Instead, these findings point toward disease-specific alterations in post-receptor signaling mechanisms or inflammatory contexts that modify cellular responses to cannabinoid stimulation. Further investigation into these potential mechanisms, such as analysis of signaling pathway activation or receptor functionality assays, would be valuable for uncovering the precise mechanisms underlying the disease-specific effects of cannabinoids on IL-17A production in rheumatic autoimmune diseases.

6.2. The Effect of Cannabidiol and Anandamide on CD4+ T Cell Survival

Assessment of cell survival following cannabinoid treatment was crucial to provide context for interpreting the complex immunomodulatory effects observed in the conducted experiments. This analysis was particularly important given the apparent paradox between increased IL-17A positivity detected by flow cytometry and decreased IL-17A secretion measured by ELISA in supernatants from CD4+ T cells of RA patients.

The findings demonstrated significant cannabinoid-induced cytotoxicity in CD4+ T cells from RA patients, with CBD exerting more pronounced effects than AEA. This differential cytotoxicity has substantial implications for interpreting the immunomodulatory effects of these compounds. The reduction in cell viability following cannabinoid treatment suggests that the overall immune response modulation observed may result from a combination of direct effects on cytokine production and selective effects on cell survival.

The seemingly contradictory observations of increased IL-17A-positive cell percentages despite decreased total IL-17A secretion might be explained by the significant reduction in total viable cells. A possible explanation may be that while the proportion of IL-17A-positive cells among surviving cells increases following cannabinoid treatment, the substantial decrease in overall cell numbers could result in fewer IL-17A-producing cells in absolute terms, explaining the reduced accumulation of IL-17A in supernatants. However, this remains a hypothesis that requires experimental verification. Time-course experiments measuring both cell viability and

IL-17A secretion at multiple time points would help establish whether the kinetics of cell death correlate with the reduction in total secreted IL-17A, thereby testing this hypothesis directly. This finding highlights the importance of assessing both relative cellular phenotypes and absolute secreted cytokine levels when evaluating immunomodulatory compounds.

An intriguing possibility raised by these observations is that cannabinoids may exhibit differential cytotoxicity across CD4+ T cell subsets. The increased proportion of IL-17A-positive cells despite overall reduced viability suggests that Th17-committed cells might be more resistant to cannabinoid-induced cell death compared to other CD4+ T cell subpopulations. The survival rate in CBD-treated cultures dropped to 48.34 ± 14.47% (compared to 71.97 ± 11.21% in untreated conditions), yet the proportion of IL-17A-positive cells increased rather than remaining constant. This strongly suggests subset-specific survival advantages among Th17 cells under exposure to CBD.

The cytotoxicity data also provide important context for interpreting the gene expression findings. Changes in expression profiles following cannabinoid treatment likely reflect both direct transcriptional effects and the altered cellular composition resulting from differential survival of specific CD4+ T cell subsets. Future experiments employing single-cell approaches could help disentangle these confounding factors and provide clearer insights into cannabinoid-mediated transcriptional regulation.

6.3. The Effect of Cannabidiol and Anandamide on Cytokine Production and Secretion in CD4+ T Cells

To comprehensively understand how cannabinoids modulate immune function in the context of rheumatic autoimmune diseases, the effects of AEA and CBD on both the intracellular presence and extracellular secretion of key inflammatory cytokines in CD4+ T cells were investigated.

Flow cytometric analysis revealed a complex pattern of cannabinoid effects on cytokine-positive cell populations that varied markedly between healthy controls and patients with rheumatic autoimmune diseases. Notably, both CBD and AEA consistently suppressed the percentage of TNF-α and IFN-γ positive CD4+ T cells across all groups, with CBD demonstrating stronger suppressive effects. This anti-inflammatory action aligns with previous reports documenting cannabinoid-mediated inhibition of cell activation and cytokine production in various immune cell populations.[158,218,273,274]

However, a striking and unexpected finding emerged regarding IL-17A. While CBD reduced the percentage of IL-17A-positive CD4+ T cells in healthy controls, consistent with its traditionally understood anti-inflammatory properties and existing literature, it significantly increased IL-17A positivity in RA patients against expectations derived from other work showing a reduction in IL-17 production and secretion, albeit in non-RA settings.[158,224] This paradoxical effect was replicated under Th17-polarizing conditions, where CBD further enhanced IL-17A positivity. Given the central role of the Th17/IL-17A axis in RA pathogenesis, this observation raises important considerations regarding the therapeutic application of cannabinoids in this disease context and is unexpected given CBD's previously outlined interference in STAT3 activity and suppressive effect on *RORC* expression, both critical for Th17 differentiation.[158,275] Furthermore, CBD has been shown to reduce the production and secretion of IL-17 in other autoimmune contexts such as multiple sclerosis.[224] Taken together, these insights may be indicative of disease specific alterations to cannabinoid functioning in rheumatic autoimmune diseases that may override these expected immunomodulatory effects. This observation is of particular relevance to RA patients given the growing role attributed to the Th17/IL-17A axis in RA pathogenesis, which could be aggravated through therapeutic application of cannabinoids such as CBD.

When extending the analysis to examine cytokine secretion via ELISA, a discrepancy that warrants careful interpretation was observed. Despite the increased proportion of IL-17A-positive cells detected by flow cytometry, both CBD and AEA significantly reduced the total amount of IL-17A secreted into culture supernatants. A similar reduction was observed under Th17-polarizing conditions. In contrast, the secretion patterns for IFN-γ and TNF-α aligned with the flow cytometry findings, with both cannabinoids significantly reducing their levels in culture supernatants.

This apparent contradiction between increased IL-17A-positive cell percentages and decreased IL-17A secretion could potentially be explained by considering the cytotoxicity findings. The substantial reduction in cell viability following cannabinoid treatment, particularly with CBD, might result in fewer total viable cells in culture. While the proportion of IL-17A-positive cells may increase among surviving cells, the absolute number of IL-17A-producing cells could be diminished, explaining the reduced accumulation of IL-17A in supernatants. However, it must be emphasized that this hypothesis requires rigorous experimental verification before acceptance. Alternative explanations that warrant equal consideration include possible cannabinoid effects on secretory pathways as has been shown for other cytokines such as IL-1, impaired cellular function in surviving cells, or post-transcriptional regulation of IL-17A production.[276] Time-course experiments measuring both cell viability and

IL-17A secretion at multiple time points, coupled with absolute cell counting, would be necessary to test this hypothesis directly.

The differential effects of cannabinoids on cytokine production across healthy and diseased states suggest disease-specific alterations in how CD4+ T cells respond to cannabinoid signaling. This differential response is particularly noteworthy given the finding that cannabinoid receptor expression remains largely unchanged between healthy controls and patients with rheumatic autoimmune diseases. Notably, while RA patients demonstrated a pronounced IL-17A increases in response to CBD, similar trends were observed in SLE and PsA patients, albeit with varying magnitudes. The stronger effects observed in PsA patients (with IL-17A increases reaching 15.22 ± 3.88% compared to 6.54 ± 3.12% in RA and 7.89 ± 1.59% in SLE following CBD treatment) suggest potential disease-specific sensitivity patterns that may correlate with the underlying pathophysiology of each condition, particularly given the prominent role of Th17 cells in PsA pathogenesis. The mechanisms underlying these disease-specific response patterns remain to be uncovered but may involve alterations in downstream signaling pathways or the inflammatory milieu characteristic of each rheumatic autoimmune condition.

A particularly noteworthy finding was the differential effect of CBD under Th17-polarizing conditions. Even when CD4+ T cells were already receiving strong Th17-differentiation signals, CBD treatment still significantly increased IL-17A positivity (from 13.80 ± 2.32% to 20.04 ± 3.98%, $p < 0.0001$). Interestingly, the proportional increase under Th17-polarizing conditions was less pronounced than under standard conditions, suggesting some context-dependency in CBD's effects. This observation is particularly relevant when considering that inflammatory environments in RA may already contain Th17-polarizing cytokines, and indicates that CBD's pro-IL-17A effect persists even in contexts where Th17 differentiation is already being actively promoted.

While these findings provide valuable insights into cannabinoid-mediated immunomodulation in rheumatic autoimmune diseases, several limitations must be acknowledged. First, the *in vitro* experimental model, while allowing for controlled administration of cannabinoids, cannot fully replicate the complex *in vivo* environment where CD4+ T cells encounter varied and continuous stimuli. Second, the cannabinoid concentrations used in the experimental setup (15µM for CBD and 25µM for AEA) were relatively high for CBD and very high for AEA. These concentrations were selected to ensure measurable effects guided by previous *in vitro* work.[154,277,278] However, the translation to clinical contexts requires caution when considering that Epidiolex®, a CBD-based therapeutic for treatment-resistant seizures typically leads to

blood concentrations of ~1μM, indicating that the concentrations used in this work go beyond what is likely achievable *in vivo*.[158] Lower concentrations might yield different or even opposing effects, as cannabinoids are known to exhibit biphasic dose-dependent responses in various biological systems.[276]

To address these limitations, future studies should explore cannabinoid effects using more physiologically relevant concentrations across a dose spectrum. Additionally, time-course experiments measuring both cell viability and cytokine secretion at multiple time points, would help establish the relationship between cannabinoid-induced cytotoxicity and changes in cytokine production. Single-cell approaches could provide further insights as to whether cannabinoids exhibit differential effects across CD4+ T cell subsets. Furthermore, investigations should be extended to animal models of RA, with collagen-induced arthritis mouse models being particularly valuable to evaluate the effects of cannabinoids on disease progression, joint pathology, and systemic immune parameters in a controlled *in vivo* setting.

6.4. The Effect of Cannabidiol and Anandamide on Gene Expression in CD4+ T Cells

The genes selected for this analysis—*SGK1*, *IKZF3*, *CSF2*, and *AHR*—were chosen strategically based on their established roles in T cell differentiation pathways and inflammation as relevant to rheumatic autoimmune diseases. *SGK1* was selected for its pivotal role in the reciprocal regulation of Th17 and Treg development, with implications for maintaining immunological balance. *IKZF3* (Aiolos) was included due to its function in lymphocyte development and autoimmunity. *CSF2*, encoding GM-CSF, was chosen for its critical contribution to Th17 cell pathogenicity and its established role in arthritic disease models. *AHR* was selected given its involvement in Th17/Treg balance and its potential as a target for cannabinoid interaction.

Additionally, RORγt, the protein encoded by the *RORC* gene was targeted as the master transcription factor for Th17 differentiation, though technical limitations hampered the measurement of its expression.[279] A specific challenge was encountered with the CBD treatment group, where difficulties were faced in isolating sufficient mRNA, likely due to the cytotoxic effects of CBD, to generate adequate cDNA for qPCR-based quantification of *RORC* mRNA expression. *RORC* mRNA expression levels fell below reliable detection thresholds, preventing conclusive assessment of cannabinoid effects on this critical Th17 regulator. This limitation is particularly significant given the observed changes in IL-17A production, as *RORC* directly regulates IL-17A transcription. The inability to quantify *RORC* expression leaves an important gap in the mechanistic understanding of how cannabinoids influence Th17

differentiation in rheumatic autoimmune diseases on a gene level. Future experiments employing higher cell numbers per sample, alternative detection methods such as protein-level assessment via Western blot, or single-cell approaches could help overcome this technical limitation and provide crucial insights into cannabinoid effects on the master regulator of Th17 differentiation.

The paradoxical relationship between gene expression changes and observed cellular phenotypes merits particular attention. The CBD-mediated upregulation of *SGK1*—a gene involved in restraining Th17 cell development—stands in contrast to the increased IL-17A positivity observed following treatment. This discrepancy suggests that cannabinoids may simultaneously engage multiple, potentially opposing pathways. The concurrent downregulation of *CSF2* by CBD treatment would typically predict reduced Th17 differentiation, yet the flow cytometry data indicates otherwise. These seemingly contradictory findings highlight the complex, multifaceted nature of cannabinoid signaling in the immunological context of RA.

The divergent effects between CBD and AEA on gene expression patterns, with CBD inducing more pronounced changes than AEA, further suggests distinct signaling mechanisms. While both compounds can interact with classical cannabinoid receptors, CBD's broader pharmacological profile—including interactions with non-cannabinoid receptors and ion channels—likely contributes to its more diverse transcriptional effects. Future mechanistic studies employing receptor-specific antagonists or gene silencing approaches would help delineate the specific pathways mediating these transcriptional changes.

These gene expression findings must be interpreted with consideration of the cytotoxicity data, as the observed changes may reflect both direct transcriptional effects and altered cellular composition due to differential survival of CD4+ T cell subsets. Single-cell transcriptomic approaches would be valuable in distinguishing these possibilities and providing greater resolution of cannabinoid effects across heterogeneous T cell populations in the context of rheumatic autoimmune diseases.

6.5. The Impact of Treatment Heterogeneity on Study Interpretation

A significant consideration in interpreting the results of this study is the heterogeneity of treatment regimens among the patient cohort. Patients with RA, PsA, and SLE included in this investigation were undergoing various therapeutic interventions, including conventional DMARDs such as methotrexate, as well as different biological agents targeting specific immune pathways. These medications have distinct mechanisms of action that may

differentially modulate immune cell function, potentially influencing the observed responses to cannabinoid treatment in our experimental system.
The immunomodulatory effects of medications such as methotrexate, TNF-α inhibitors, IL-6 receptor antagonists, and other biologicals could potentially alter CD4+ T cell responses to cannabinoids by affecting baseline cytokine production, receptor expression, or downstream signaling pathways. This treatment heterogeneity represents a potential confounding variable that might contribute to the variability observed in cellular responses across patient samples and could partially explain some of the divergent effects observed between patient groups.

Future studies should ideally stratify patients according to treatment regimens or, where ethically possible, include treatment-naïve patients to more precisely delineate cannabinoid effects in the absence of confounding pharmaceutical interventions. Alternatively, larger sample sizes would permit subgroup analysis to evaluate whether specific treatment modalities influence cannabinoid responsiveness.

6.6. Clinical Implications of Cannabinoid Use in Rheumatoid Arthritis

Given the complexity of translating *in vitro* findings to clinical relevance, observational clinical data from a small cohort of RA patients who self-reported CBD use over a 4-8 week period was included. This preliminary investigation into observational clinical data was undertaken to assess whether the concerning proinflammatory effects of CBD observed in the cellular experiments might manifest in a clinical context. The significant increase in IL-17A-positive CD4+ T cells following the reported CBD use period, accompanied by concurrent elevation in DAS28-CRP scores, suggests potential alignment between the laboratory observations and clinical outcomes.

These findings, while preliminary and subject to significant limitations, contribute an important translational dimension to the study. The concordance between the controlled *in vitro* experiments demonstrating increased IL-17A positivity following CBD treatment and the observed increase in IL-17A-positive cells in patients reporting CBD use warrants serious consideration. This is particularly noteworthy given the established pathogenic role of the Th17/IL-17A axis in RA and the corresponding worsening of disease activity as measured by DAS28-CRP.

Several methodological limitations must be acknowledged when interpreting these clinical observations. The self-reported nature of CBD consumption without standardization of dosage, product composition, or administration protocol introduces considerable variability. Additionally, the absence of a control group prevents definitive attribution of the observed changes exclusively to CBD use, as they may reflect natural disease fluctuations,

environmental factors, or changes in medication adherence. Furthermore, the small sample size limits statistical power and generalizability.

Despite these limitations, these preliminary clinical findings raise legitimate concerns regarding CBD use in RA patients that merit further investigation. The parallel increase in IL-17A positivity and disease activity suggests that despite CBD's established anti-inflammatory properties in other contexts, its effects in RA may be more complex and potentially detrimental. These observations underscore the need for controlled animal studies using well-established mouse models of arthritis. Mouse models would allow for systematic evaluation of dose-dependent effects and comprehensive immunological profiling in a controlled *in vivo* environment. Such preclinical research is essential to thoroughly characterize cannabinoid effects in inflammatory arthritis before any further recommendations regarding cannabinoid use in RA can be formulated.

The clinical implications of these findings extend beyond RA to potentially other Th17-mediated autoimmune conditions. Given the increasing popularity and accessibility of CBD products, coupled with perceptions of their anti-inflammatory benefits, the observations highlight the importance of disease-specific research into cannabinoid effects rather than generalizing anti-inflammatory properties across diverse pathological contexts. This cautionary perspective is particularly relevant for patients with rheumatic autoimmune diseases who may consider cannabinoid use for symptom management without medical supervision.

7. References

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8. Appendix

8.1. Expanded Results Statistics

Supplementary Table 1. Expression of cannabinoid receptors in CD4+ T cells across patient groups.

Patient Group	CB1 (Mean ± SD, %)	n	CB2 (Mean ± SD, %)	n	GPR55 (Mean ± SD, %)	n
HC	10.80 ± 7.68	10	9.45 ± 5.39	9	2.51 ± 0.17	3
RA	8.69 ± 8.06	74	7.90 ± 8.78	73	5.45 ± 3.44	36
SLE	15.47 ± 16.92	5	7.14 ± 4.45	7	4.42 ± 3.76	3
PsA	12.18 ± 6.72	16	12.45 ± 6.29	14	7.65 ± 3.69	5

HC: Healthy controls; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; PsA: Psoriatic arthritis; SD: Standard deviation; n: Number of subjects.

Supplementary Table 2. Percentage of IL-17A-positive CD4+ T cells across treatment conditions and patient groups

Group	Untreated (Mean ± SD %)	CBD (15 µM) (Mean ± SD %)	CBD Veh. (Mean ± SD %)	AEA (25 µM) (Mean ± SD %)	AEA Veh. (Mean ± SD %)	n
HC	3.30 ± 1.03	1.86 ± 0.89	3.62 ± 1.15	2.96 ± 1.62	3.51 ± 1.29	13
RA	3.36 ± 1.46	6.54 ± 3.12	3.27 ± 1.36	4.53 ± 2.50	3.28 ± 1.45	36
SLE	3.77 ± 0.77	7.89 ± 1.59	4.04 ± 0.95	6.83 ± 1.65	3.99 ± 1.00	5
PsA	6.83 ± 1.62	15.22 ± 3.88	7.18 ± 1.38	10.09 ± 2.51	7.33 ± 1.52	5

HC: Healthy controls; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; PsA: Psoriatic arthritis; SD: Standard deviation; n: Number of subjects.

Supplementary Table 3. Percentage of IFN-γ-positive CD4+ T cells across treatment conditions and patient groups

Group	Untreated (Mean ± SD %)	CBD (15 µM) (Mean ± SD %)	CBD Veh. (Mean ± SD %)	AEA (25 µM) (Mean ± SD %)	AEA Veh. (Mean ± SD %)	n
HC	5.51 ± 2.95	2.25 ± 1.12	5.79 ± 3.36	2.72 ± 1.75	5.51 ± 3.40	13
RA	6.83 ± 3.73	2.79 ± 1.99	6.66 ± 5.12	4.12 ± 2.28	6.67 ± 4.48	27
SLE	6.43 ± 1.45	3.49 ± 1.92	7.00 ± 1.61	5.25 ± 2.49	6.43 ± 1.88	5
PsA	10.94 ± 3.62	3.25 ± 0.99	11.82 ± 4.12	7.64 ± 3.93	10.77 ± 4.33	3

HC: Healthy controls; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; PsA: Psoriatic arthritis; SD: Standard deviation; n: Number of subjects.

Supplementary Table 4. Percentage of TNF-α-positive CD4+ T cells across treatment conditions and patient groups

Group	Untreated (Mean ± SD %)	CBD (15 µM) (Mean ± SD %)	CBD Veh. (Mean ± SD %)	AEA (25 µM) (Mean ± SD %)	AEA Veh. (Mean ± SD %)	n
HC	10.55 ± 3.26	3.74 ± 3.53	11.78 ± 3.49	7.80 ± 4.24	11.15 ± 4.06	17
RA	12.38 ± 4.35	3.67 ± 2.34	11.70 ± 3.68	6.50 ± 3.27	11.14 ± 3.43	36
SLE	10.55 ± 1.89	4.97 ± 3.67	11.78 ± 2.38	7.46 ± 2.20	10.75 ± 1.91	5
PsA	15.32 ± 4.62	1.99 ± 1.67	15.97 ± 5.36	6.85 ± 5.48	15.48 ± 4.61	5

HC: Healthy controls; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; PsA: Psoriatic arthritis; SD: Standard deviation; n: Number of subjects.

Supplementary Table 5. Mean ± SD values of relative gene expression in CD4+ T cells from healthy controls following cannabinoid treatment

Treatment	*SGK1* (n=3)	*IKZF3* (n=2)	*CSF2* (n=3)	*AHR* (n=4)
CBD (15 μM)	3.24 ± 2.61	3.01 ± 1.44	0.36 ± 0.55	11.48 ± 21.70
CBD Veh.	0.88 ± 0.33	0.35 ± 0.29	0.53 ± 0.47	2.19 ± 4.02
AEA (25 μM)	2.34 ± 2.89	0.93 ± 0.66	0.68 ± 0.43	2.69 ± 4.78
AEA Veh.	0.56 ± 0.05	0.41 ± 0.33	0.44 ± 0.36	6.90 ± 13.34

Supplementary Table 6. Mean ± SD values of relative gene expression in CD4+ T cells from RA patients following cannabinoid treatment

Treatment	*SGK1* (n=9)	*IKZF3* (n=4)	*CSF2* (n=5)	*AHR* (n=6)
CBD (15 μM)	14.07 ± 10.0	11.85 ± 9.09	0.05 ± 0.05	2.59 ± 2.25
CBD Veh.	1.31 ± 0.49	1.26 ± 1.05	1.08 ± 0.61	0.88 ± 0.25
AEA (25 μM)	1.86 ± 2.53	2.69 ± 2.00	0.51 ± 0.41	1.19 ± 0.32
AEA Veh.	1.19 ± 0.60	1.14 ± 0.88	0.87 ± 0.50	0.93 ± 0.59

9.2. List of Figures

9.3. List of Tables

9. Pre-Publication of Results

Please find enclosed the published article which resulted from the work conducted for this thesis:

Kotschenreuther K, Waqué I, Yan S, et al. Cannabinoids drive Th17 cell differentiation in patients with rheumatic autoimmune diseases. Cell Mol Immunol 2021; 18(3): 764-6.

Further peer-reviewed publications resulting from work completed in the Laboratory for Molecular Immunology are:

Yan S, Kotschenreuther K, Deng S, Kofler DM. Regulatory T cells in rheumatoid arthritis: functions, development, regulation, and therapeutic potential. Cell Mol Life Sci 2022; 79(10): 533.

Kotschenreuther K, Yan S, Kofler DM. Migration and homeostasis of regulatory T cells in rheumatoid arthritis. Front Immunol 2022; 13: 947636.

Dittrich-Salamon M, Meyer A, Yan S, et al. Regulatory T Cells from Patients with Rheumatoid Arthritis Are Characterized by Reduced Expression of Ikaros Zinc Finger Transcription Factors. Cells 2022; 11(14).

Yan S, Golumba-Nagy V, Kotschenreuther K, et al. Membrane-bound IL-6R is upregulated on Th17 cells and inhibits Treg cell migration by regulating post-translational modification of VASP in autoimmune arthritis. Cell Mol Life Sci 2021; 79(1): 3.

Meyer A, Wittekind PS, Kotschenreuther K, Schiller J, von Tresckow J, Haak TH, Kofler DM. Regulatory T cell frequencies in patients with rheumatoid arthritis are increased by conventional and biological DMARDs but not by JAK inhibitors. Ann Rheum Dis 2021; 80(12): e196.

CORRESPONDENCE OPEN

Cannabinoids drive Th17 cell differentiation in patients with rheumatic autoimmune diseases

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The legalization of cannabinoids for medical use has reinforced their emerging role as a treatment of chronic pain in patients with cancer or rheumatic diseases.[1,2] In addition to their role as pain relievers, evidence obtained from animal models suggests that cannabinoids have immunosuppressive properties.[3] However, a definite immunosuppressive function of cannabinoids has not yet been confirmed in clinical trials.[4] We therefore analyzed the influence of the cannabis derivative cannabidiol (CBD) and the endogenous cannabinoid anandamide (AEA) on T helper type 17 (Th17) cells from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and psoriatic arthritis (PsA). Interestingly, in vitro culture in the presence of CBD significantly increased Th17 cell differentiation in CD4+ T cells from the peripheral blood of patients with RA, SLE, or PsA, while Th17 cell differentiation was suppressed in healthy individuals (Fig. 1a and Supplementary Fig. S1A). In RA patients, the median Th17 cell frequency in CBD-treated cells was 6.54 ± 0.52 vs. 3.27 ± 0.23 in the vehicle control group ($p < 0.0001$), and in healthy controls, the frequency was 1.86 ± 0.25 in CBD-treated cells vs. 3.62 ± 0.32 in the vehicle control group ($p = 0.0002$). AEA showed similar effects on CD4+ T cells from patients but did not affect CD4+ T cells from healthy controls (Fig. 1a). The addition of the Th17 skewing cytokines transforming growth factor-β, interleukin (IL)-1β, IL-6, and IL-23 further increased the Th17-inducing properties of CBD (Fig. 1b). As shown previously in experimental autoimmune encephalomyelitis (EAE) mice, the production of interferon-γ and tumor necrosis factor-α was reduced in the presence of CBD in patients with rheumatic diseases, as well as in healthy individuals (Supplementary Fig. S1B, C). During our study, some of our RA patients reported the use or planned use of CBD oil as a pain reliever. In these cases, we compared Th17 cell frequencies before and after treatment initialization and found that treatment with CBD oil for 4–8 weeks drove Th17 cell expansion in vivo (1.10 ± 0.32 before vs. 4.52 ± 1.34 after CBD treatment; Fig. 1c). Interestingly, disease activity measured by Disease Activity Score 28-joint count C reactive protein significantly increased during CBD treatment (Fig. 1d). In accordance with previous reports, this immunomodulatory effect of CBD was not mediated by the receptors CB1, CB2, or GPR55 (Supplementary Fig. S2A).[5] To further assess the characteristics of the CBD-induced Th17 cells, we analyzed their gene expression profiles and discovered a CBD-mediated increase in *SGK1* expression (Fig. 1e, Supplementary Fig. S2C). This is remarkable, as SGK1 is an important regulator of the reciprocal development of proinflammatory Th17 cells.[6] In addition, the expression of *CSF2* was decreased and the expression of *AHR* was increased by CBD (Fig. 1f–g and Supplementary Fig. S2B, C).

Th17 cells play a central role in the pathogenesis of PsA and ankylosing spondylitis. In addition, they have been linked at least partly to the pathogenesis of various other rheumatic autoimmune diseases. We observed an increase in Th17 cell frequencies induced by CBD in vitro, as well as in some patients with RA receiving CBD treatment. These results are in contrast to observations made in mice with EAE, in which cannabinoids ameliorated disease activity.[3] However, CB2-selective agonists are often used in these animal studies.[3] The CB2 receptor is known to mediate immunosuppressive effects, while immune-activating effects have been attributed to other receptors.[3] We used cannabinoids that activate various receptors and pathways. Variations in these receptors and pathways between patients with rheumatic autoimmune diseases and healthy individuals could explain differences between patients and healthy subjects. Moreover, the variety of CBD receptors could be responsible for the discrepancy between animal studies and findings in humans, including our study. In conclusion, our data show that cannabinoids increase Th17 cell frequencies and suggest that they may therefore be used with caution in patients with rheumatic autoimmune diseases.

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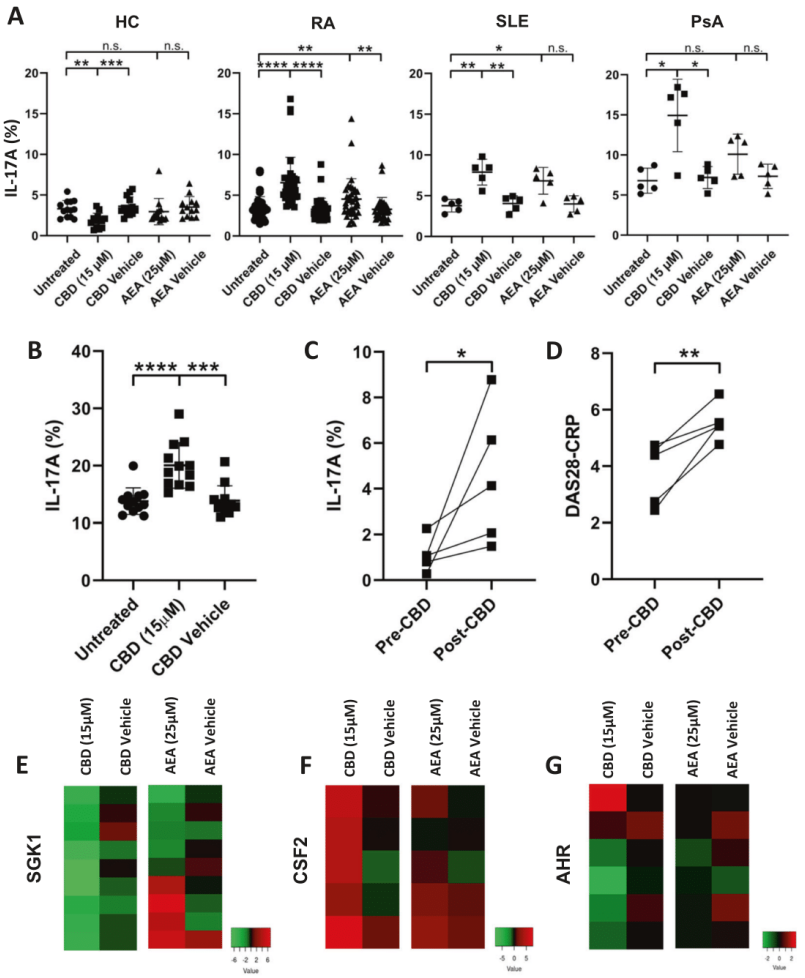

Fig. 1 Cannabinoids induce Th17 cell differentiation in patients with rheumatic diseases. **a** Expression of IL-17A in CD4+ T cells from healthy controls and patients was analyzed by flow cytometry (HC, $n = 13$; RA, $n = 36$; SLE, $n = 5$, PsA, $n = 5$). **b** CBD-mediated induction of IL-17A expression in CD4+ T cells from RA patients in the presence of TGFβ, IL-1β, IL-6, and IL-23 ($n = 12$). **c** Increase in IL-17A-positive Th17 cells in patients receiving cannabidiol oil for 4–8 weeks ($n = 5$). **d** DAS28-CRP in patients receiving cannabidiol oil for 4–8 weeks ($n = 5$). **e–g** Heat maps showing gene expression in RA patients treated with CBD or AEA. Gene expression was analyzed by RT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; the data are presented as the mean ± SEM; significant differences were determined using the unpaired Mann–Whitney test and Student's *t* test

AUTHOR CONTRIBUTIONS

K.K., I.W., S.Y., A.M., and D.M.K. made substantial contributions to the study concept and design. K.K., I.W., S.Y., A.M., T.H., J.v.T., J.S., L.G., and M.D.-S. made substantial contributions to the acquisition of the data. All authors drafted the article or revised it critically for important intellectual content, reviewed the draft, and approved the submission of the manuscript. Open access funding provided by Projekt DEAL.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41423-020-0437-4) contains supplementary material.

Competing interests: The authors declare no competing interests.

Ethics approval: This study was approved by the Ethics Committee of the University Hospital Cologne (no. 13-091).

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Supplementary Methods

Study subjects. All patients with RA fulfilled the 2010 ACR/EULAR classification criteria. Blood samples from age- and sex-matched healthy individuals were used as controls. Blood was drawn after written informed consent was obtained in accordance with the Declaration of Helsinki following a study protocol approved by the Ethics Committee of the University of Cologne (4).

Human T cell isolation. Primary human lymphocytes were isolated from peripheral blood from patients and healthy controls by Pancoll® density gradient centrifugation (PAN™-Biotech GmbH, Aidenbach, Germany). $CD4^+$ T cells were purified by negative selection using the $CD4^+$ T cell isolation Kit. The purity of the CD4+ T cell populations was verified by flow cytometry and was at least 96%. Viable cells were counted using the Vi-CELL XR cell counter (Beckman Coulter, Krefeld, Germany) or the automated cell counter CellCountess (Life Technologies GmbH, Darmstadt, Germany).

Th17 polarization. $CD45RA^+RO^-$ naïve $CD4^+$ T cells were cultured in X-Vivo 15 (Lonza, Cologne, Germany) media supplemented with 1% human serum and 1% Penicillin-Streptomycin (both Sigma-Aldrich, Saint Louis, U.S.). Cells were cultured for 4 days with the T cell Activation/Expansion Kit (Miltenyi Biotec) and recombinant human TGF-β (5 ng/µl; PAN™-Biotech GmbH, Aidenbach, Germany), IL-1β (12.5 ng/µl), IL-6 (25 ng/µl; both Miltenyi Biotec) and IL-23 (25 ng/µl; PeproTech, Rocky Hill, U.S.). Medium and cytokines were refreshed after 3 days.

Flow cytometry. $CD4^+$ T cells were stimulated with PMA (100 ng/ml) and ionomycin (1.5 µM; both Cell Signaling Technology®, Danvers, U.S.) in the presence of Brefeldin A (eBioscience, San Diego, U.S.) for 3 hours. To exclude dead cells, cells

were stained by the LIVE/DEAD™ Fixable Dead Cell Stain Kit (Invitrogen, ThermoFisher Scientific, Carlsbad, U.S.). For intracellular staining, cells were fixed and made permeable by the BD Cytofix/Cytoperm Kit (BD Bioscience, Heidelberg, Germany) according to the manufacturer´s instructions and stained with anti-IL17A, anti-IFN-γ (both affymetrix eBioscience). Data were acquired on the Gallios 10/3 flow cytometer (Beckman Coulter, Krefeld, Germany).

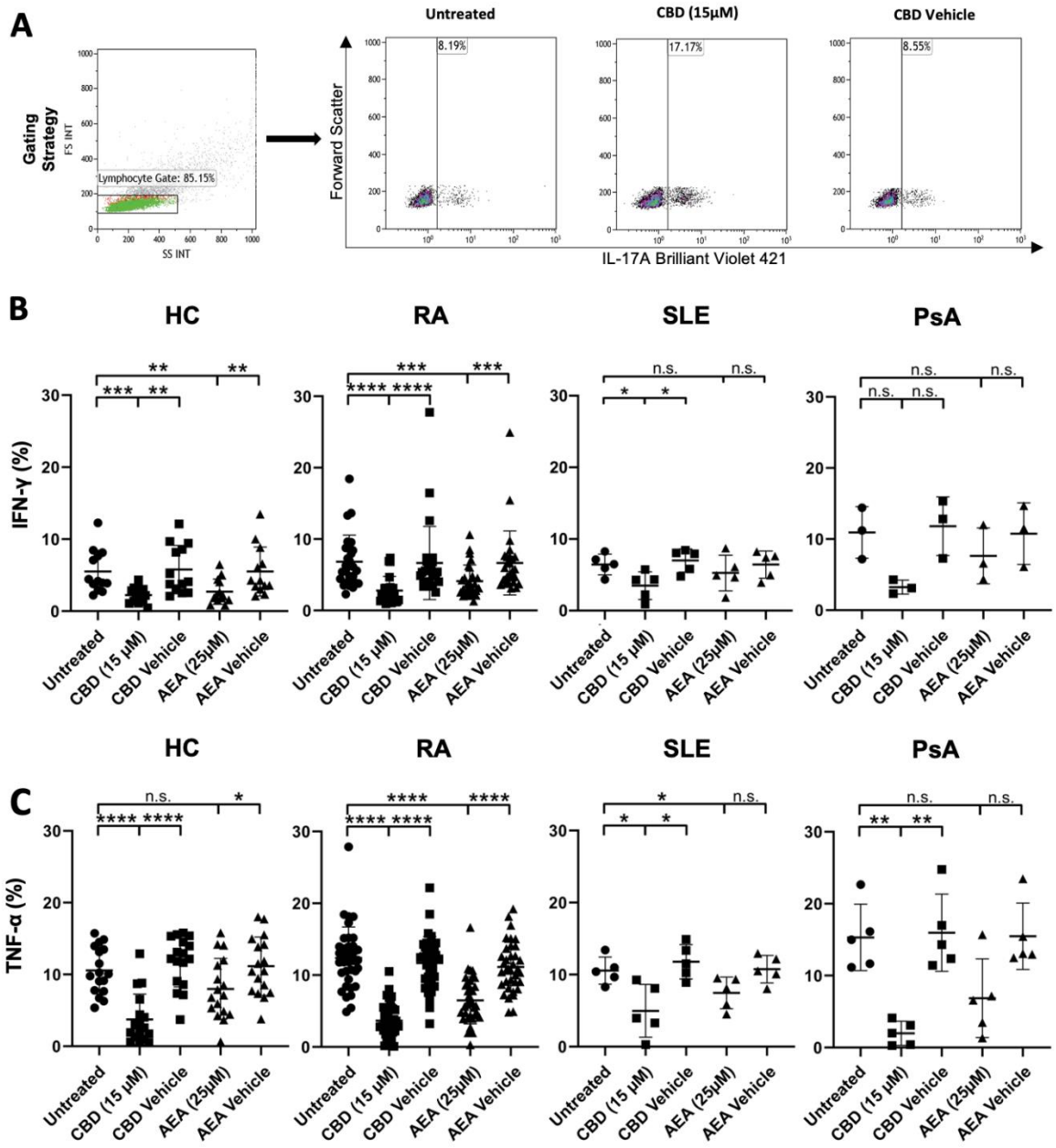
Quantitative Real Time PCR. RNA was isolated from $CD45RA^{+}RO^{-}$ T cells using the RNeasy Mini Kit and converted into cDNA using the QuantiTectReverse Transcription Kit (both Qiagen, Hilden, Germany). All primers were purchased from Applied Biosystems. All reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). The values are represented as the difference in C_t values normalized to β2-microglobulin for each sample using the following formula: relative RNA expression = $(2^{-dCt}) \times 10^3$.

Statistics. Statistical analysis was performed using GraphPad Prism. Where indicated, data were analyzed by non-parametric Mann-Whitney test or student's t-test and are presented as the mean ± SEM. $p < 0.05$ was considered as statistically significant.

Supplementary Table S1: Characteristics of patients with RA

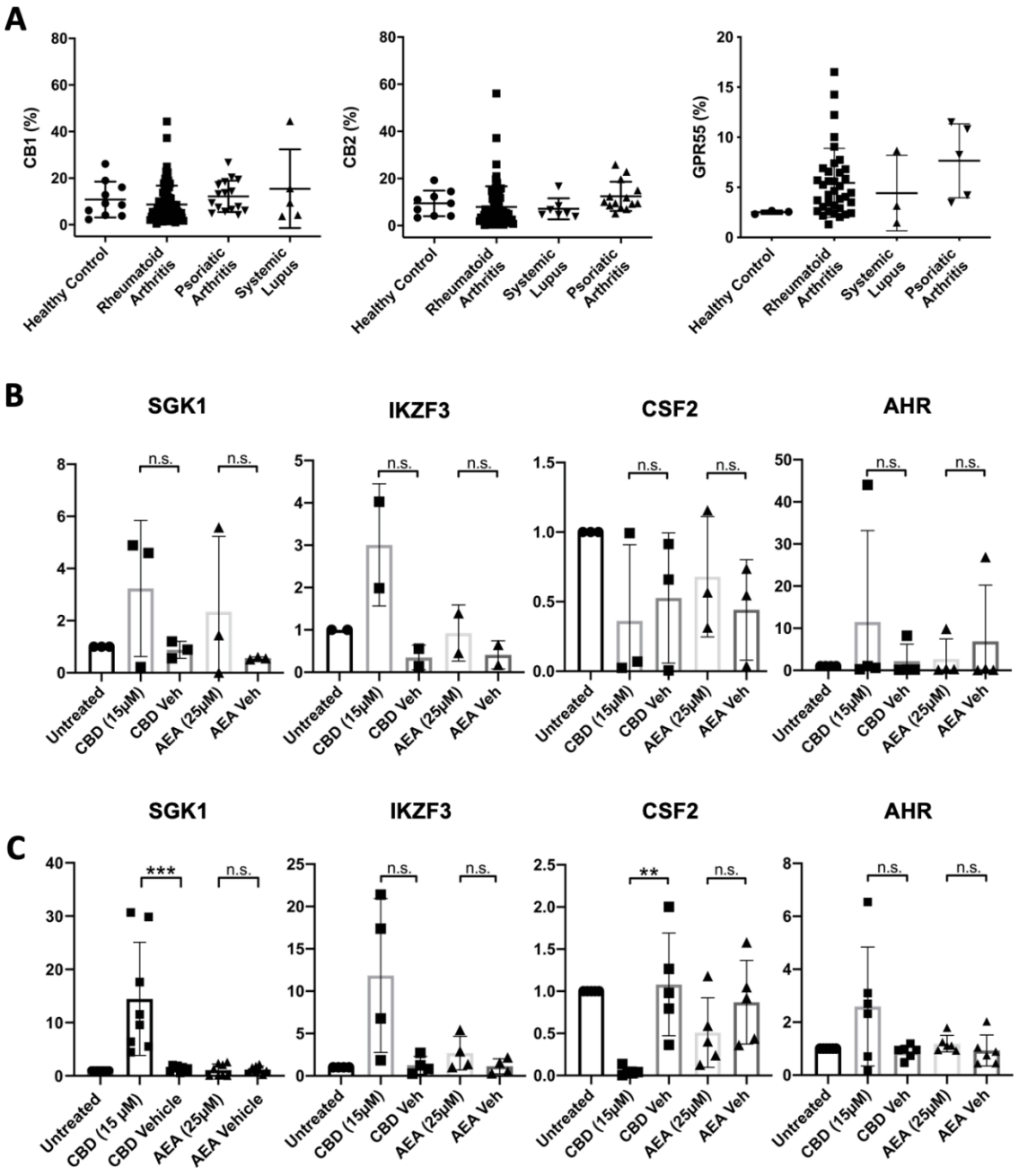
Patient characteristics	average	range
Age (years)	58	31-84
Sex (f : m)	3.8:1	
Time since disease onset (years)	11.14	0 - 39
Number of previous treatments	3.62	1-10
DAS-28(BSG)	2.85	1.11 – 6.05
DAS-28 (CRP)	2.58	0.96 - 6
CRP [mg/l]	6.12	06 – 62.7
BSG [mm/h]	16	2 - 71
Rheumatoid factor (% positive)	51	
ACPA (% positive)	56	

Supplementary Figure S1



(A) Representative example of flow cytometry analysis of Th17 cells. (B) IFNg and (C) TNFa expression in CD4+ T cells from the peripheral blood of healthy controls or from patients with RA, SLE or PsA. *p<0.05, **p<0.01, ***p<0.001,****p<0.0001; data is presented as mean±SEM; significant differences were determined using the unpaired Mann Whitney test.

Supplementary Figure S2



(A) Expression of the cannabinoid receptors CB1, CB2 and GPR55 on CD4+ T cells from the peripheral blood of healthy controls or from patients with RA, SLE or PsA. (B) Gene expression of specific genes in healthy controls and in patients with RA (C) was analyzed by RT-PCR. *p<0.05, **p<0.01, ***p<0.001,****p<0.0001; data is presented as mean±SEM; significant differences were determined using the unpaired Mann Whitney test.