

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS QUÍMICAS



TESIS DOCTORAL

Cannabinoides e inmunomodulación: nuevas estrategias terapéuticas dirigidas frente a células dendríticas para enfermedades inflamatorias

Cannabinoids and immunomodulation: novel therapeutic strategies targeting dendritic cells for inflammatory diseases

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

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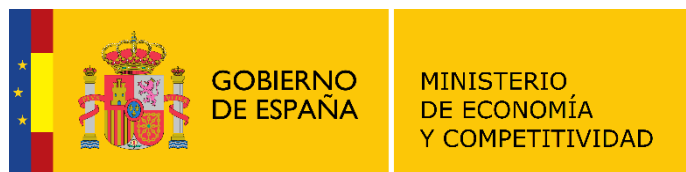
Oscar Palomares Gracia

Madrid, 2020

La investigación presentada en esta Tesis Doctoral se ha realizado en el laboratorio de Vacunas y Células Dendríticas en el departamento de Bioquímica y Biología Molecular de la Facultad de Ciencias Químicas de la Universidad Complutense de Madrid (UCM), bajo la dirección del Dr. Oscar Palomares Gracia.

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A mis padres y mis hermanos

A Arturo

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“Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.”

Antonio Machado

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Abbreviations

2-AG: 2-arachidonoylglycerol
2-AGE: 2-arachidonoylglycerol ether
3-MA: 3-methyladenine
4EBP: eIF4E binding protein
ACADM: acyl-coenzyme A dehydrogenase
Acetyl-CoA: acetyl coenzyme A
AEA: anandamide
Ahr: aryl hydrocarbon receptor
AIT: allergen-specific immunotherapy
Akt: protein kinase B
ALI: air-liquid interphase
AMPK: AMP-activated protein kinase
AP-1: activation protein 1
APC: allophycocyanine
ATF4: activating transcription factor 4
ATG: autophagy-related protein
ATP: adenosine triphosphate
ATP5A1: ATP synthase subunit α
BALF: bronchoalveolar fluid
BDCA: blood dendritic cell antigen
BSA: bovine serum albumin
BV: brilliant violet
cAMP: cyclic adenosine monophosphate
CB1: cannabinoid receptor 1
CB2: cannabinoid receptor 2
CBD: cannabidiol
CBN: cannabinol
CBRs: cannabinoid receptors
cDCs: conventional dendritic cells
CFSE: carboxyfluorescein succinimidyl ester

CNS: central nervous system
COX-2: cyclooxygenase 2
CPE: crude peanut extract
CPT1A: carnitine O-palmitoyltransferase 1
CRSwNP: chronic rhinosinusitis with nasal polyposis
CTLA-4: cytotoxic T lymphocyte antigen 4
DAGL: diacylglycerol lipase
DAPI: 4',6-diamidino-2-phenylindole
DCs: dendritic cells
DNA: deoxyribonucleic acid
ECS: endocannabinoid system
EDTA: ethylenediaminetetraacetic acid
EF1 α : elongation factor 1 alpha
ELISA: enzyme-linked immunosorbent assay
EPIT: epicutaneous immunotherapy
ERK: extracellular signal regulated kinase
FAAH: fatty acid amide hydrolase
FADH: flavin adenine dinucleotide
FcR γ : Fc gamma receptor
FGF: fibroblast growth factor
FITC: fluorescein isothiocyanate
FLT3L: FMS-like tyrosine kinase ligand
FOXP3: forkhead box P3
GATA-3: GATA binding protein 3
G-CSF: granulocyte colony-stimulating factor
GLS: glutaminase
GLUT1: glucose transporter 1
GM-CSF: granulocyte-macrophage stimulating factor
GPCRs: G protein-coupled receptors
HBEC: human bronchial epithelial cells
HIF-1 α : hypoxia-inducible factor 1 alpha
HK2: hexokinase 2

HLA-DR: human leukocyte antigen DR
hmoDCs: human monocyte-derived dendritic cells
HRV-16: human rhinovirus 16
ICOSL: inducible costimulatory molecule ligand
IDH3A: isocitrate dehydrogenase subunit alpha
IDO: indoleamine 2,3-dioxygenase
IFN: interferon
Ig: immunoglobulin
IKK: I κ B kinase complex
IL: interleukin
ILCs: innate lymphoid cells
ILN: inguinal lymph node
IRF: interferon response factor
I κ B: inhibitor of NF- κ B
JNK: c-Jun N-terminal kinase
LC3: microtubule-associated protein 1A/1B-light chain 3
LDHA: lactate dehydrogenase
LPS: lipopolysaccharide
mAb: monoclonal antibody
MAGL: monoacylglycerol lipase
MAPKs: mitogen-activated protein kinases
MCP-1: monocyte chemoattractant protein 1
MDA5: melanoma differentiation-associated gene
mDCs: myeloid dendritic cells
MDP: monocyte and dendritic cell precursor
MFI: mean fluorescence intensity
MHC: major histocompatibility complex
MOI: multiplicity of infection
mTOR: mammalian target of rapamycin
NAD: nicotinamide adenine dinucleotide
NADA: N-arachidonoyldopamine
NADP: nicotinamide adenine dinucleotide phosphate

NAPE: N-acyl-phosphatidylethanolamine
NAPE-PLD: NAPE-hydrolysing phospholipase D
NF- κ B: nuclear factor kappa B
NK cells: natural killer cells
NLRs: NOD-like receptors
OIT: oral immunotherapy
P70S6k: p70S6 kinase
PAMPs: pathogen-associated molecular patterns
PBMC: peripheral blood mononuclear cells
PBS: phosphate-buffered saline
PD-1: Programmed death 1
pDCs: plasmacytoid dendritic cells
PDHA: pyruvate dehydrogenase
PD-L1: programmed death-ligand 1
PE: phycoerythrin
PEA: N-palmitoylethanolamine
PerCP: peridinin chlorophyll protein
PFA: paraformaldehyde
PFKFB3: phosphofructokinase 3
PI3K: phosphatidylinositol 3-kinase
PINK1: PTEN-induced kinase 1
PLC: phospholipase C
PPAR: peroxisome proliferator-activated receptor
PPP: pentose phosphate pathway
Pre-cDC: conventional dendritic cell precursor
Pre-pDC: plasmacytoid dendritic cells precursor
PRRs: pattern recognition receptors
RIG-1: retinoic acid-inducible gene 1
RLRs: RIG-like receptors
RNA: ribonucleic acid
RORC2: retinoic acid receptor-related orphan receptor 2
ROS: reactive oxygen species

RUBCN: Rubicon
RV: rhinovirus
RXR: retinoic acid receptor
SAMPs: self-associated molecular patterns
SDHA: succinate dehydrogenase subunit alpha
SEAP: secreted embryonic alkaline phosphatase
SLC1A3: amino acid transporter 1
SLIT: sublingual immunotherapy
SREBP: sterol regulatory element-binding protein
T bet: t-box transcription factor
TCA cycle: tricarboxylic acid cycle
TCR: T-cell receptor
TER: transepithelial electrical resistance
Tfh: T follicular helper cell
TGF β : transforming growth factor beta
Th cell: T helper cell
THC: Δ^9 -tetrahydrocannabinol
TJ: tight junction
TLR2L: TLR2 ligand
TLRs: toll-like receptors
TMC: tonsil mononuclear cells
TNF α : tumor necrosis factor alpha
Treg cells: regulatory T cells
TRPV1: transient receptor potential vanilloid 1
ULK1: Unc-51 like kinase
UV: ultraviolet
VEGF: vascular endothelial growth factor
ZO-1: zonula occludens 1

RESUMEN/ABSTRACT

Resumen

Las células dendríticas (DCs) son células presentadoras de antígeno profesionales que tienen un papel clave en la inducción de las respuestas inmunes conectando el sistema inmune innato con el adaptativo. La principal función de las DCs es reconocer antígenos en la periferia y transportarlos a los órganos linfoides más próximos. Allí, las DCs presentan los antígenos procesados a las células T vírgenes, induciendo su activación y diferenciación hacia distintos fenotipos dependiendo del antígeno, de las señales encontradas en el ambiente, de las señales co-estimuladoras y de las citoquinas. Cambios en el metabolismo energético de las DCs son esenciales en la regulación de su activación y función. Varios estudios han demostrado la implicación de la autofagia, un proceso catabólico esencial para las células, en el control de la función de las DCs.

El sistema endocannabinoide (ECS) es una red compleja de señalización implicada en varios procesos fisiológicos como desarrollo neuronal, plasticidad cerebral, memoria, supervivencia celular, metabolismo e inmunidad. Los principales componentes del ECS son los ligandos endocannabinoides, los receptores de cannabinoides (CBRs) y las enzimas implicadas en la síntesis y degradación de los ligandos. Varios estudios han mostrado que los compuestos cannabinoides presentan propiedades anti-inflamatorias en diferentes enfermedades mediadas por el sistema inmune. Sin embargo, su capacidad para regular las respuestas inmunes no se conoce completamente, especialmente en humanos. Las DCs humanas expresan todos los componentes del ECS. La habilidad de los cannabinoides de inmunomodular el fenotipo y la función de las DCs se desconoce. Teniendo en cuenta estos aspectos, el objetivo de esta Tesis Doctoral es investigar el papel de los cannabinoides sintéticos en la regulación de la función de las DCs humanas, los mecanismos subyacentes y sus posibles implicaciones en modelos preclínicos *in vitro* e *in vivo* de enfermedades mediadas por el sistema inmune como posibles nuevas estrategias terapéuticas para la prevención y tratamiento de trastornos inflamatorios.

En este trabajo hemos desarrollado y validado la sonda fluorescente HU210-Alexa488 para visualizar y cuantificar células que expresan CB1 en sangre periférica y amígdalas mediante citometría de flujo y microscopía confocal. Además, hemos demostrado por primera vez que el cannabinoide sintético WIN55212-2 induce DCs tolerogénicas con capacidad de generar células T reguladoras FOXP3⁺ funcionales mediante mecanismos dependientes de la inducción de autofagia vía CB1 y PPAR α . La posible relevancia clínica de nuestros resultados fue estudiada en diferentes modelos preclínicos humanos y murinos tanto *in vivo* como *in vitro*. El cannabinoide sintético WIN55212-2 ejerce efectos protectores y anti-inflamatorios *in vivo* en un modelo de

sepsis inducida por LPS mediante mecanismos que dependen de la inducción de autofagia vía activación de CB1 y PPAR α . En un modelo de alergia a cacahuete, la administración de WIN55212-2 durante la provocación con cacahuete protege a los ratones alérgicos de la reacción anafiláctica inmediata. Además, la administración de WIN55212-2 induce la generación de células T reguladoras FOXP3⁺ específicas de alérgeno en la fase efectora tardía. Finalmente, WIN55212-2 repara la alteración del epitelio de las vías respiratorias inducida por rinovirus restaurando la integridad de las proteínas de las uniones estrechas sin afectar a la respuesta inmune innata inducida por las células epiteliales.

En resumen, en esta Tesis Doctoral demostramos la capacidad del cannabinoide sintético WIN55212-2 de inmunomodular el fenotipo y la función de las DCs humanas. Hemos demostrado por primera vez los mecanismos moleculares mediante los cuales WIN55212-2 induce propiedades tolerogénicas en DCs humanas, que incluyen la inducción de autofagia mediante la activación de CB1 y PPAR α y reprogramación metabólica. Además, hemos demostrado potenciales aplicaciones clínicas de WIN55212-2 en diferentes modelos preclínicos humanos y de ratón como sepsis, alergia al cacahuete o inflamación inducida por rinovirus. Los resultados presentados en esta Tesis Doctoral podrían sentar las bases que contribuyan en el desarrollo de futuras estrategias basadas en cannabinoides para la prevención y el tratamiento de enfermedades mediadas por el sistema inmune.

Abstract

Dendritic cells (DCs) are professional antigen presenting cells that play a key role in the orchestration of immune responses by linking innate and adaptive immunity. The main function of DCs is to capture antigens in the periphery and transport them to the closer lymph nodes. There, they present the processed antigens to naïve T cells, inducing T cell activation and polarization into different phenotypes depending on the antigen, the environmental cues, costimulatory signals and cytokines. Changes in the energetic metabolism of DCs are essential for the regulation of their functional properties. At this regard, several studies have reported the involvement of autophagy, an essential catabolic process, in the control of DC functions.

The endocannabinoid system (ECS) is a complex signalling network involved in many physiological processes such as neuronal development, brain plasticity, memory, cell survival, metabolism and immunity. The main components of the ECS include the endocannabinoid ligands, the cannabinoid receptors (CBRs) and the enzymes related to the ligand synthesis or degradation. Several studies showed that cannabinoid compounds display anti-inflammatory properties in different immune-mediated diseases. However, their potential capacity to regulate the orchestration of immune responses is not well understood, specifically in humans. Human DCs express all the components of the ECS. The ability of cannabinoids to immunomodulate the phenotype and function of DCs remain elusive. Considering all these aspects, the main objective of this Doctoral Thesis is to investigate the role and the underlying molecular mechanisms of synthetic cannabinoids in the control of human DC function and their potential implications in preclinical *in vitro* and *in vivo* models of immune-mediated diseases as potential novel strategies for the prevention and treatment of inflammatory disorders.

In this work, we have developed and validated a novel HU210-Alexa488 fluorescence probe to visualize and quantify CB1-expressing cells in peripheral blood and tonsils by flow cytometry and confocal microscopy. We have also demonstrated for the first time that the synthetic cannabinoid WIN55212-2 promotes the generation of tolerogenic DCs with the capacity to generate functional FOXP3⁺ regulatory T (Treg) cells by mechanisms depending on CB1- and PPAR α -mediated autophagy induction. We have validated the potential relevance of our findings in different preclinical human and mouse models. The synthetic cannabinoid WIN55212-2 exhibits *in vivo* protective and anti-inflammatory effects in LPS-induced sepsis model by mechanisms that rely on autophagy induction via activation of CB1 and PPAR α . In a mouse model of peanut food allergy, the administration of WIN55212-2 during peanut challenge protects peanut-allergic mice from the immediate anaphylactic reaction. Moreover, WIN55212-2

administration promotes the generation of allergen-specific FOXP3⁺ regulatory T cells during late-phase responses. Finally, we have demonstrated that WIN55212-2 repairs rhinovirus-induced airway epithelial disruption by restoring the integrity of tight junction proteins without affecting epithelial cell-mediated innate immune response.

In summary, in this Doctoral Thesis we uncover the previously unknown capacity of the synthetic cannabinoid WIN55212-2 to immunomodulate the phenotype and function of human DCs. We show for the first time the molecular mechanisms by which WIN55212-2 imprints tolerogenic features in human DCs, which include CB1- and PPAR α -mediated autophagy induction and metabolic reprogramming. In addition, we have demonstrated the potential clinical applications of WIN55212-2 in different *in vitro* and *in vivo* preclinical human and mouse models such as sepsis, peanut allergy or rhinovirus-induced inflammation. The data presented in this Doctoral Thesis might well contribute to pave the way for the future development of novel cannabinoid-based strategies for the prevention and treatment of immune-mediated diseases.

INTRODUCTION

Introduction

1. The immune system

1.1. Overview of the immune response

The immune system is a complex network of molecules, cells and organs that synergistically cooperate to develop appropriate responses against potentially dangerous pathogens while maintaining a state of tolerance to self- and non-self-innocuous antigens. To perform this essential function, the immune system has multiple tightly regulated mechanisms, whose dysregulation leads to immune-mediated diseases such as allergy, autoimmune diseases, organ rejection and cancer (1).

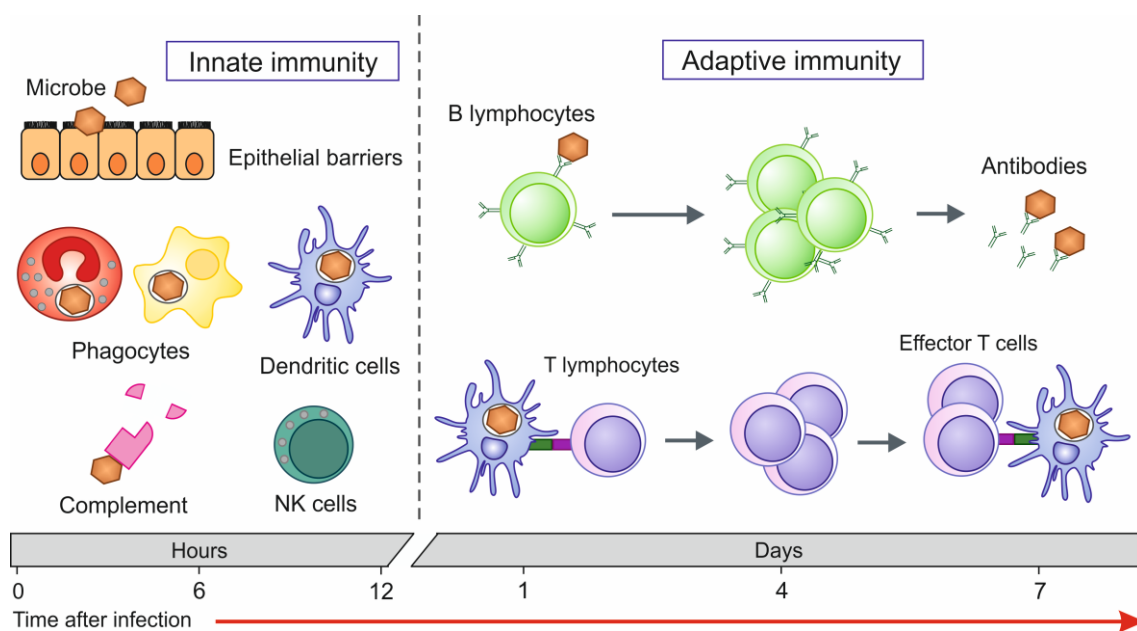


Figure I.1. Mechanisms of innate and adaptive immunity. Adapted from *Cellular and Molecular Immunology* (A.K.Abbas).

The coordinated response of the components of the immune system to foreign substances is called the immune response. Host immune response is classically divided into two different arms, the innate immune and the adaptive immune responses (1, 2). The innate immune response provides the first and early line of defence in a non-specific manner. The main components of the innate response are the physical and chemical barriers, such as epithelia and antimicrobial chemicals produced at epithelial surfaces; phagocytic cells, dendritic cells and natural killer (NK) cells; and proteins, including members of the complement system and cytokines (3). In contrast, the adaptive immune response is a later response characterized by high specificity for distinct molecules and immunological memory. The adaptive immune response is mediated by T cells, B cells, and their secreted products such as antibodies. T and B cells express specific T- and B-

cell receptors for a very large number of antigens. Thus, after the exposure to a specific antigen, lymphocytes undergo considerable proliferation, termed clonal expansion, and then develop antigen-specific, long-lasting memory cells (**Figure I.1**) (4).

For a long time it was assumed that immunological memory was an exclusive hallmark of the adaptive immune response. However, several studies have reported that the innate immune system also exhibits adaptive characteristics, a feature that has been termed trained immunity (5). This concept describes the long-term functional reprogramming of innate immune cells after the encounter with certain pathogens, which leads an increased response to a second infection against the same or unrelated pathogens. Trained immunity is defined by immunological mechanisms, metabolic reprogramming and epigenetic changes (6-8) (**Figure I.2**).

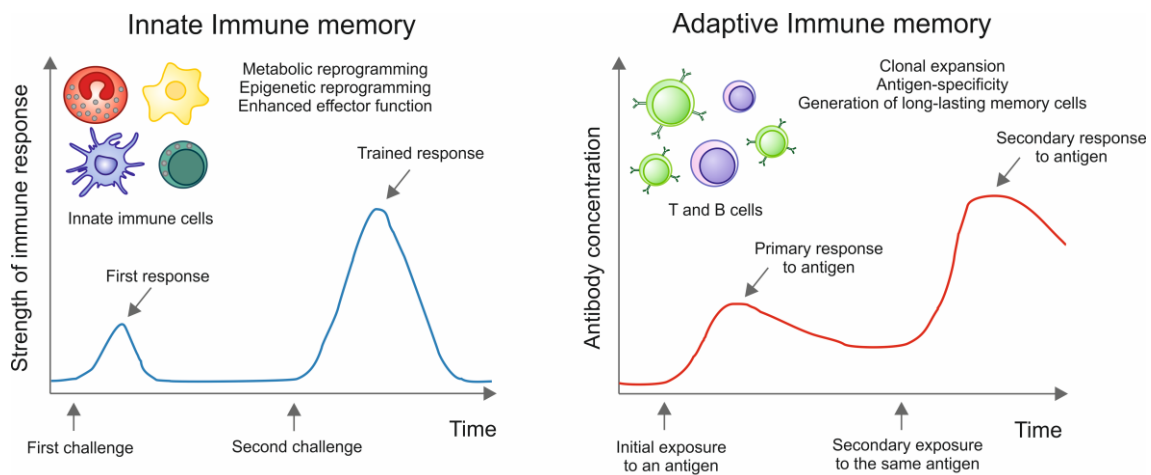


Figure I.2. Immunological memory in innate and adaptive immune response. Adapted from *Induction of innate immune memory: the role of cellular metabolism*. *Curr Opin Immunol*, 2019; 56:10-16.

1.2. Dendritic cells as the link of innate and adaptive immune responses

Dendritic cells (DCs) were discovered by Steinman and Cohn in 1973 (9). DCs are professional antigen presenting cells linking the innate and adaptive immune responses (10, 11). DCs are located in all peripheral tissues, blood and lymphoid organs (12). Depending on the encountered antigens and the received signals, DCs display the dual capacity to induce proper immune responses against pathogens or to generate tolerance against self-antigens and innocuous substances (13-15).

To perform this vital function DCs are able to process encountered antigens and present them to T cells. DCs recognize antigens in the periphery and migrate through afferent lymphatic vessels to lymphoid nodes, where they present processed antigens to naïve T cells to induce different T cell populations (16). The antigen presentation is mediated by class I and class II major histocompatibility complex (MHC) molecules, which are expressed in the surface of DCs. Class I MHC molecules bind peptides derived

from endogenous antigens and are recognized by CD8⁺ T cells (cytotoxic). However, class II MHC molecules present peptides derived from exogenous antigens for recognition by CD4⁺ T cells (helper). Under certain conditions, DCs also have the ability to capture extracellular antigens and present them to CD8⁺ T cells by class I MHC molecules, a process called cross-presentation (16, 17).

Under homeostatic conditions, DCs are found in peripheral tissues in an immature stage, characterized by reduced expression of mature and co-stimulatory markers CD40, CD80, CD86 and CD83, reduced secretion of cytokines, high phagocytic and processing antigen capacity and limited capacity to stimulate T cells (18). The presence of microbial stimuli triggers phenotypic and functional changes in DCs, inducing a process called maturation. In this process, DCs increase the expression of MHC and co-stimulatory molecules such as CD40, CD80, CD86 and CD83, and secrete high levels of pro-inflammatory cytokines. Mature DCs migrate to the lymph nodes, where they present the processed antigens to naïve T cells, inducing T cell differentiation (18-20). For long time, it has been accepted that immature DCs mainly induce tolerance through the generation of regulatory T (Treg) cells, whereas mature DCs prime different subsets of pro-inflammatory effector T cells depending on the context. However, recent studies have demonstrated that there might be specific conditions in which mature DCs are also able to generate Treg cells (20, 21).

DCs are equipped with a long battery of different pattern recognition receptors (PRRs) that allow them to recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Moreover, it has been proposed the existence of self-PRRs that recognize self-associated molecular patterns (SAMPs) that might well play a role in keeping homeostasis (22, 23). The recognition of PAMPs by PRRs triggers an intracellular signalling pathway that usually induces the activation of the

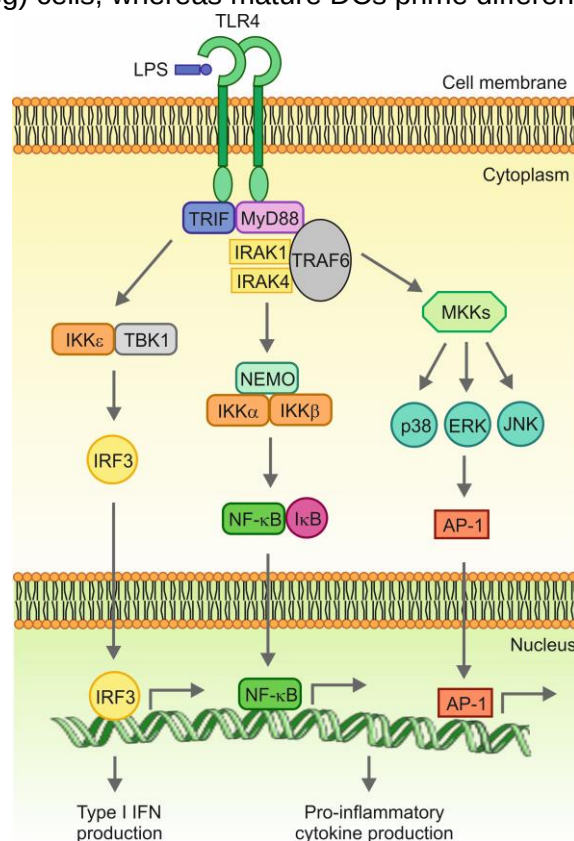


Figure I.3. TLR4 signalling pathways. TLR4 activation triggers the activation of NF-κB, MAPKs and IRF3 signalling pathways, which induce the expression of inflammatory genes and type I interferons.

nuclear factor κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs) (22, 23). This activation triggers the production of cytokines and anti-microbial peptides. Several distinct classes of cellular PRRs, which differ in their structure, localization and specificity, have been described:

- **Toll-like receptors (TLRs).** The TLRs recognize a wide variety of pathogens. They are type I integral membrane glycoproteins that contains leucine-rich repeats in their extracellular domains, transmembrane regions and cytoplasmic domains, which are essential for signalling (24, 25). So far, 10 different functional TLRs have been identified in human. TLRs 1, 2, 4, 5, 6 and 10 are expressed on the plasma membrane, where they recognize PAMPs in the extracellular environment (lipopolysaccharide, flagelin, lipopeptides, etc.). In contrast, TLRs 3, 7, 8 and 9 are mainly expressed on endoplasmic reticulum and endosomal membranes, where they detect nucleic acids. Ligand binding induces TLR dimerization and the recruitment of adaptor proteins, which facilitate the recruitment and activation of protein kinases, leading to the activation of different transcription factors (24, 25). The major transcription factors activated by TLR signalling pathways are NF- κ B and activation protein 1 (AP-1), which induce the expression of inflammatory genes, and interferon response factor 3 (IRF3), which promotes the production of type I interferons (**Figure I.3**) (23, 25).
- **NOD-like receptors (NLRs).** NLRs are cytosolic proteins that contain 3 different domains: a leucine-rich repeated domain that senses the ligands; a NATCH domain, which allows NLRs to form oligomers; and an effector domain, which recruits other proteins to form signalling complexes (26). NLRs recognize ligands from pathogens (peptidoglycan, flagelin, viral RNA), cell products (cholesterol, uric acid) and signals from extracellular environment (alum, UV radiation). Ligand recognition by NLRs induces the activation of NF- κ B and MAPK signalling pathways (26, 27). In addition, some members of NLR family form signalling complexes called inflammasomes, which generate active forms of the inflammatory cytokines IL-1 β and IL-18 (**Figure I.4**) (28).
- **C-type lectin receptors.** They recognize carbohydrates on the surface of pathogens and facilitate the phagocytosis of the pathogens. Furthermore, C-type lectin receptors can recognize self-antigens and favour tolerance induction. The recognition occurs through their extracellular structurally conserved C-type lectin-like domain and is tightly regulated by amino acid motifs and calcium ions. They can signal directly, through integral motifs in their cytoplasmic tails or indirectly through association with Fc gamma receptor (Fc γ R). Some C-type lectin

receptors are mannose receptor, dectin-1, dectin-2 and DC-SIGN (**Figure I.4**) (29-31).

- **RIG-like receptors (RLRs).** RLRs are cytosolic receptors that recognize double-stranded and single-stranded RNA from virus. On binding RNA, the RLRs trigger IRF3 and IRF7 activation that induce the production of type I interferons. The two best-characterized RLRs are RIG-1 (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated gene) (**Figure I.4**) (32, 33).

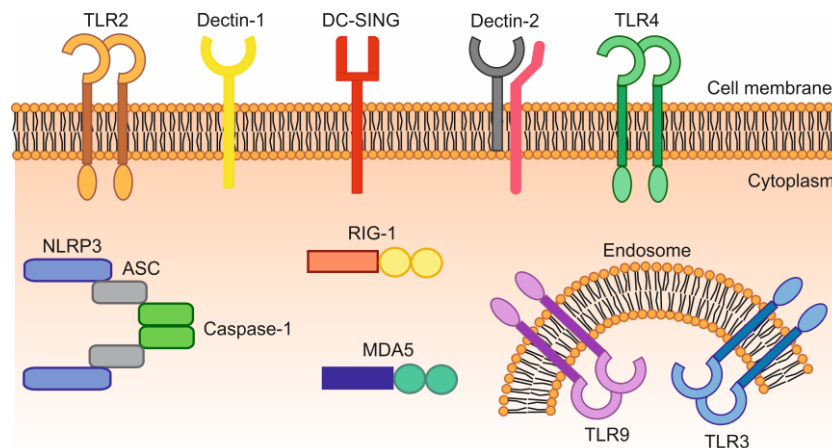


Figure I.4. Some pattern recognition receptors (PRRs) including TLRs, NLRs, C-type lectin receptors and RLRs.

The ontogeny and development of DCs have been mainly studied in mouse (34, 35). Key precursors have been described, including MDPs (monocyte and DC precursor), pre-cDCs (conventional DCs precursor) and pre-pDCs (plasmacytoid DC precursor). Pre-cDCs are precursors of the conventional DCs (cDCs), which comprise two main subsets, the CD11b⁻ DCs (cDC1) or CD11b⁺ DCs (cDC2). cDC1 are involved in CD8⁺ T cell priming and are particularly efficient at cross-presentation. Furthermore, CD11b⁻ DCs can be divided into CD8α⁺ resident DCs and CD103⁺ migratory DCs. cDC2 are specialized in MHCII presentation and CD4⁺ T cell priming. On the other hand, the pre-pDCs are precursors of plasmacytoid DCs (pDCs), characterized by the low expression of CD11c and high expression of CD45RA and involved in the control of viral infections (**Table I.1**) (15, 34-36).

Table I.1. Phenotype and function of mouse DC subsets

	cDC1	cDC2	pDCs	moDCs
Main surface markers	CD11c ⁺ , MHCII ⁺ , CD8α ⁺ (resident), CD103 ⁺ (migratory), CD11b ⁻	CD11c ⁺ , MHCII ⁺ , CD11b ⁺ , CD1c ⁺	CD11c ^{low} , MHCII ^{low} , B220 ⁺ , Ly6C ⁺	CD11c ⁺ , MHCII ⁺ , CD11b ⁺ , Ly6C ⁺ , CD64 ⁺
PRRs	TLR1-6 and 9	TLR1-6 and 9	TLR7, 9 and Ly6C	Not well defined
Main function	CD8 ⁺ T cell priming; Cross presentation	CD4 ⁺ T cell priming	Antiviral immunity	Inflammation
Cytokines	Pro-inflammatory	Pro-inflammatory and anti-inflammatory	Antiviral	Pro-inflammatory and anti-inflammatory

In human, DCs are derived from CD34⁺ hematopoietic precursors generated in the bone marrow. DC precursors migrate to the blood and tissues where they generate the main subsets of DCs: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) (12). Interestingly, it has been described that FMS-like tyrosine kinase ligand (FLT3L) are required for the generation and maintenance of DC subsets (37). Traditionally, DC classification has been based on the expression of surface markers. However, other properties such as the morphology, the migratory capacity and the capacity to stimulate naïve T cells, can help to identify particular DC subsets (15, 35, 38). Although DCs are a heterogeneous population, they are characterized by the expression of class II MHC molecules (HLA-DR) and the absence of lineage markers including CD3 (T cells), CD19/20 (B cells) and CD56 (NK cells) (12, 15, 36). In human, blood DCs are broadly classified in mDCs and pDCs (**Table I.2**).

1.2.1. Human myeloid dendritic cells (mDCs)

Despite the heterogeneity of mDCs, they are characterized by the expression of CD11c and class II MHC (HLA-DR). mDCs can be classified in: CD141⁺ mDCs (mDC1) or CD1c⁺ mDCs (mDC2) (12). CD141⁺ mDCs or mDC1 are present at a frequency of approximately one-tenth compared to mDC2 in human peripheral blood mononuclear cells (PBMC). mDC1 are the major producers of the antiviral cytokine IFN λ , but are poor at producing IL-12p70. The capacity of mDC1 to stimulate naïve CD4⁺ T cells is lower than the capacity of mDC2. However, mDC1 display a superior capacity for cross-presentation and induction of cytotoxic T cell immune responses. Transcriptional and functional analyses have revealed a strong homology of human mDC1 with the mouse CD8 α ⁺/CD103⁺ DCs, suggesting that mDC1 are the human equivalent of the mouse cDC1 (**Table I.2**) (15, 39).

CD1c⁺ mDCs or mDC2 represent approximately 1% of the whole PBMC compartment. This subset is also located in lymphoid tissues, skin, lung and gut. mDC2 express several PRRs and secrete pro-inflammatory and anti-inflammatory cytokines in response to stimuli such as LPS, flagelin, carbohydrates and peptidoglycans. In addition, mDC2 are potent stimulators of naïve CD4⁺ T cells. mDC2 are the human equivalent of mouse cDC2 (**Table I.2**) (12, 39).

1.2.2. Human plasmacytoid dendritic cells (pDCs)

pDCs represent 0.2-0.8% of the whole PBMC compartment and are characterized by the expression of CD123, CD303, CD304 and HLA-DR. pDCs play a key role in viral infections producing high levels of type I IFN, such as IFN α . Furthermore, pDCs are able to detect exogenous nucleic acids by TLR7 and TLR9 (12, 40). On the other hand,

several studies have demonstrated that pDCs from thymus and tonsils can induce FOXP3⁺ Treg cells with potent suppressive capacity (**Table I.2**) (41, 42).

1.2.3. Human monocyte-derived dendritic cells (hmoDCs)

Monocytes represent approximately 10% of total PBMC and play an important role in the innate immune response against pathogens. During inflammation, monocytes are recruited into tissues and become the most abundant DC population (43). *In vitro*, monocytes are differentiated into myeloid DCs in the presence of granulocyte-macrophage stimulating factor (GM-CSF) and interleukin (IL)-4. Human monocyte-derived dendritic cells (hmoDCs) display immature phenotype and reduced antigen-presenting capacity. However, the presence of microbial signals increases their capacity of antigen presentation and T cell stimulation (**Table I.2**) (44, 45). HmoDCs can be generated in bulk numbers in *in vitro* cultures, thus representing a very suitable model for functional research in human.

Table I.2. Phenotype and function of human DC subsets

	mDC1	mDC2	pDCs	hmoDCs
Main surface markers	CD11c ⁺ , HLA-DR ⁺ , CD141/BDCA-3 ⁺	CD11c ⁺ , HLA-DR ⁺ , CD1c/BDCA-1 ⁺ , CD11b ⁺	CD11c ⁻ , HLA-DR ^{low} , CD123 ⁺ , CD303 ⁺ , CD304 ⁺	CD1c ⁺ , CD11c ⁺ , HLA-DR ⁺ , CD11b ⁺
PRRs	TLR1, 2, 3, 5, 6, 8 and 10 STING	TLR1-9, RLR, STING	TLR7, 9 RLR, STING	Not well defined
Main function	CD8 ⁺ T cell priming; Cross-presentation	CD4 ⁺ T cell priming	Antiviral immunity	Inflammation
Cytokines	Pro-inflammatory	Pro-inflammatory and anti-inflammatory	Antiviral	Pro-inflammatory and anti-inflammatory

1.3. Mechanisms involved in polarization of naïve CD4⁺ T cells

As it has been previously mentioned, DCs are able to present antigens and induce T cell activation and differentiation. The initial activation and differentiation of naïve T cells into effector cells occurs mainly in secondary lymphoid organs and three signals are required: antigen recognition, costimulation and cytokines produced by DCs (**Figure I.5**). The TCR (T-cell receptor) of naïve CD4⁺ T cells recognizes peptide-MHC complexes displayed by DCs in a restricted manner (signal 1). In addition, naïve T cell activation and differentiation requires signals provided from costimulatory molecules on DCs (signal 2). The best-characterized costimulatory pathway in T cell activation

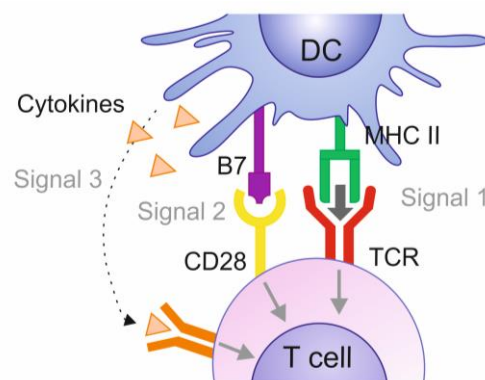


Figure I.5. Signals for T cell activation. The proliferation and activation of T cells require antigen recognition (signal 1), costimulation (signal 2) and cytokines produced by DCs (signal 3). Adapted from *Cellular and Molecular Immunology* (A.K.Abbas).

involves the T cell surface receptor CD28, which binds the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on DCs (46). Moreover, numerous receptors homologous to CD28 have been identified, which are able to activate or inhibit T cells. For example, CD40L is expressed on activated helper T cells, binds CD40 on DCs and activates DCs to make them more potent. On the other hand, cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1) are inhibitory receptors involved in tolerance process (47). To complete T cell activation and polarization, cytokines produced by DCs are needed (signal 3) (16) (**Figure I.5**).

CD4⁺ T cells play a critical role in mediating adaptive immune response against a variety of pathogens. After antigen presentation, CD4⁺ T cells acquire different functions and phenotypes depending on the received signals. Effector CD4⁺ T cells are classified and characterized by their ability to express surface molecules and transcription factors and to secrete cytokines (16, 48). However, CD4⁺ T cells can adapt their functions in response to changing circumstances in a process called plasticity (49). There are three main distinct subsets of CD4⁺ T cells, called Th1, Th2 and Th17, which function against different types of pathogens (48). In addition, regulatory T cells are another population of CD4⁺ T cells that control immune reactions to self and foreign antigens (**Figure I.6**).

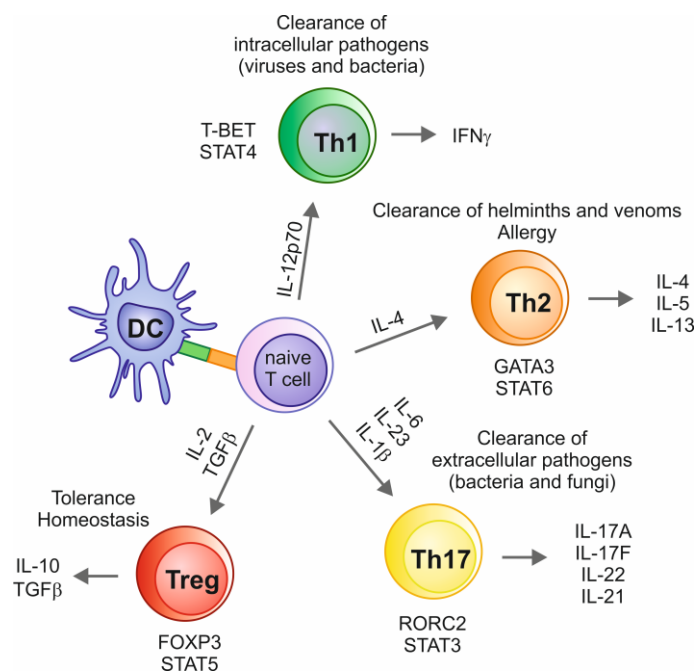


Figure I.6. Differentiation of naïve T cell to functional T cell subsets. Adapted from *Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: Receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2016; 138(4):984-1010.*

1.3.1. T helper 1 (Th1) cells

Th1 differentiation occurs in response to intracellular bacteria, protozoans and virus. The production of IL-12 by DCs and IFNγ by NK cells in response to intracellular pathogens induce Th1 differentiation (**Figure I.6**). The transcription factor T-bet is considered the master regulator of Th1 differentiation and its expression is induced in naïve CD4⁺ T cells in response to antigens, IL-12 and IFNγ (48). Once Th1 cells have developed, T-bet expression promotes IFNγ secretion, which enhances Th1 differentiation and thus

strongly amplifies the reaction. Th1 cells induce cell-mediated inflammatory responses by inducing the activation of macrophages, NK cells, CD8⁺ T cells, which kill infected cells, and B cells that employ humoral response by producing antigen-specific antibodies against targeted pathogens (50). Human data have provided evidence that virus and intracellular bacteria can induce distinct subsets of Th1 cells that can be distinguished based on expression of chemokine receptors and transcription factors. The first type, called classic Th1 cells, express CXCR3 and T-bet, whereas the second type, called nonclassic Th1, express CXCR3 and T-bet as well as CCR6 and RORC2, which are characteristic of Th17 (51).

1.3.2. T helper 2 (Th2) cells

Th2 differentiation occurs in response to helminths, venoms and allergens. Th2 differentiation depends on the presence of IL-4 in the local environment. IL-4 induces the expression of GATA-3, the so-called master regulator of Th2 cell lineage, in naïve T cells. GATA-3 enhances the expression of Th2 cytokines such as IL-4, IL-5 and IL-13 that contribute to eosinophil recruitment and activation, IgE production by B cells, mucus secretion and tissue remodelling (50, 52, 53). Furthermore, GATA-3 might interfere with the development of Th1 and Th17 cells (**Figure I.6**) (48).

1.3.3. T helper 17 (Th17) cells

Th17 cells protect against extracellular bacteria and fungi, especially in mucosal surfaces. IL-1 β and IL-6 produced by DCs and TGF β activate the transcription factor RORC2, which stimulates the differentiation of naïve CD4⁺ T cells to the Th17 subset (54, 55). IL-23, which is also produced by DCs, is needed for the proliferation and expansion of Th17 cells. After differentiation, Th17 cells produce IL-17, IL-21 and IL-22 that are involved in inflammation, neutrophil recruitment and barrier function (**Figure I.6**) (50, 56).

1.3.4. T helper 9 (Th9), 22 (Th22) and follicular (Tfh) cells

Other types of T helper cells are Th9, Th22 and Tfh cells. Th9 cells are characterized by the expression of the transcription factor PU.1 and IL-9 production and play an important role in inflammatory diseases (57, 58). Th22 cell differentiation depends on the aryl hydrocarbon receptor (AhR) and produce IL-22 (59). Tfh are found in the B cell follicles of secondary lymphoid organs and collaborate with B cells to promote and regulate humoral responses (60, 61).

1.3.5. Regulatory T (Treg) cells

Treg cells are a subset of T cells with suppressive capacity, essential for immunological tolerance and homeostasis. The naturally occurring FOXP3⁺ Treg (nTreg) cells are

generated in the thymus. In addition, three different subsets of inducible Treg (iTreg) cells can be generated in the periphery: FOXP3⁺ Treg cells, FOXP3⁻ IL-10 producing Tr1 cells and TGFβ-expressing Th3 cells (**Figure I.7**) (62, 63). Several studies suggest that thymic differentiation is due to self-antigen recognition, whereas peripheral differentiation seems to be required for environmental antigens. Treg cells are characterized by the expression of high levels of the α-subunit of the IL-2 receptor (CD25), the lack of expression of the IL-7 receptor α-subunit (CD127) and the expression of the transcription factor

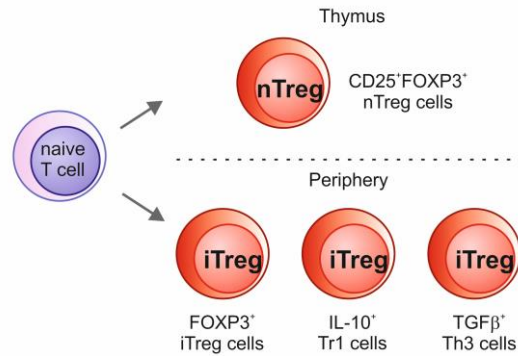


Figure I.7. Different subsets of Treg cells. The naturally occurring CD4⁺CD25⁺FOXP3⁺ Treg (nTreg) cells are generated in the thymus, whereas three different subsets of inducible Treg (iTreg) cells can be generated in the periphery: FOXP3⁺Treg cells, IL-10-producing Tr1 cells and TGFβ-expressing Th3 cells. Adapted from *Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGFβ*. *Genes Immun.* 2014; 15(8):511-520.

FOXP3 (forkhead box P3), which is specifically expressed by Treg cells and it is essential for their development and function (64, 65). Increase evidence has indicated that epigenetic modifications occurring in the regulatory regions of FOXP3 locus are critical for the maintenance of FOXP3 expression and the stabilization of Treg lineage (66-68). DCs play a critical role in the induction of Treg cells. Immature or mature DCs conditioned by specific probiotic or pathogen-derived molecules, by FOXP3⁺ Treg cells and by exogenous signals (for example, vitamin D3 metabolites, adenosine, retinoic acid or mannan) promote the generation of functional Treg cells (69, 70). For that, DCs produce a large number of soluble molecules (IL-10, TGFβ or IL-35), specific enzymes (indoleamine 2,3-dioxygenase; IDO) and metabolites (retinoic acid). In addition, DCs express costimulatory molecules such as CD80, CD86, PD-L1 (programmed death-ligand 1) or ICOSL (inducible costimulatory molecule ligand) (21, 71). Treg cells directly or indirectly are able to suppress T helper cells, B cells, neutrophils, basophils, eosinophils, mast cells and specific inflamed tissue cells as well as inhibit inflammatory DCs, favouring the generation of tolerogenic DCs (**Figure I.8**) (70, 72). Four main mechanism of Treg cell suppression have been described (62, 65):

- Secretion of suppressive cytokines: IL-10, TGFβ and IL-35.
- Metabolic disruption mechanisms: CD25, cAMP, histamine receptor 2, CD39 and CD73.
- Mechanisms using surface molecules: CTLA-4, PD-1.
- Cytolysis: granzymes A and B.

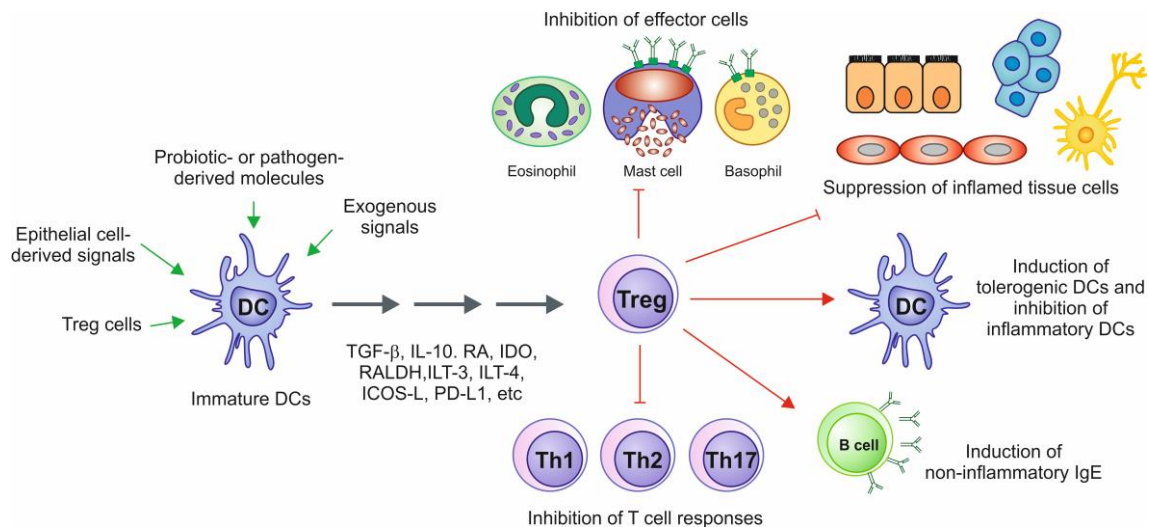


Figure I.8. Treg cells act on different cell and tissues. Immature or mature DCs conditioned by different stimuli promote the generation of Treg cells able to control inflammation by acting on different cells and tissues. Adapted from *Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF β* . *Genes Immun.* 2014; 15(8):511-520.

2. Immunometabolism

2.1. An overview of the main metabolic pathways in immune system cells

The metabolic state of immune cells and the metabolic changes during homeostasis and inflammation have become the focus of intense investigation. Thus, “immunometabolism” comprises the idea that metabolic changes regulate immune cell responses by controlling transcriptional and posttranscriptional events that are key in cell activation. It is clear that different immune cell functions are associated with distinct metabolic status. However, the metabolic configuration of immune cells can undergo reprogramming in response to danger signals, cytokines or receptor activation, which subsequently can lead changes in their function (73). The glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway (PPP), fatty acid oxidation, fatty acid synthesis and amino acid pathways are the main cellular metabolic pathways involved in immunometabolism (**Figure I.9**):

- **Glycolytic pathway.** Glycolysis begins with glucose uptake from the extracellular environment. Then, intracellular processing of glucose in the cytosol produces pyruvate, which can be converted into lactate (lactic acid fermentation) or acetyl coenzyme A (acetyl-CoA), substrate of TCA after transportation into the mitochondria. Glycolytic metabolism is a relatively inefficient pathway for ATP generation: two molecules of ATP per unit of glucose. In addition, glycolysis allows the reduction of NAD⁺ to NADH, which is used by enzymes as a cofactor and as substrate to biosynthetic growth pathways. Many pro-growth signalling pathways, including the PI3K and MAPK pathways, promote cellular glycolytic metabolism, which play an essential role in proliferating cells (73).

- **TCA cycle and oxidative phosphorylation.** The TCA cycle, also called the citric acid cycle or Krebs cycle, takes place in the matrix of the mitochondria. TCA cycle and oxidative phosphorylation are a highly efficient route of ATP generation used by cells whose requirements are energy and longevity. Multiple substrates can feed into TCA cycle. Glucose-derived pyruvate or fatty acids are converted into acetyl-CoA and fuel the TCA cycle, which together the glutamine constitute the main fuel for TCA cycle. The two major products of the TCA cycle are NADH and FADH₂, which can transfer electrons to the electron transport chain to support oxidative phosphorylation and highly efficient ATP generation. This process requires the presence of oxygen (73).

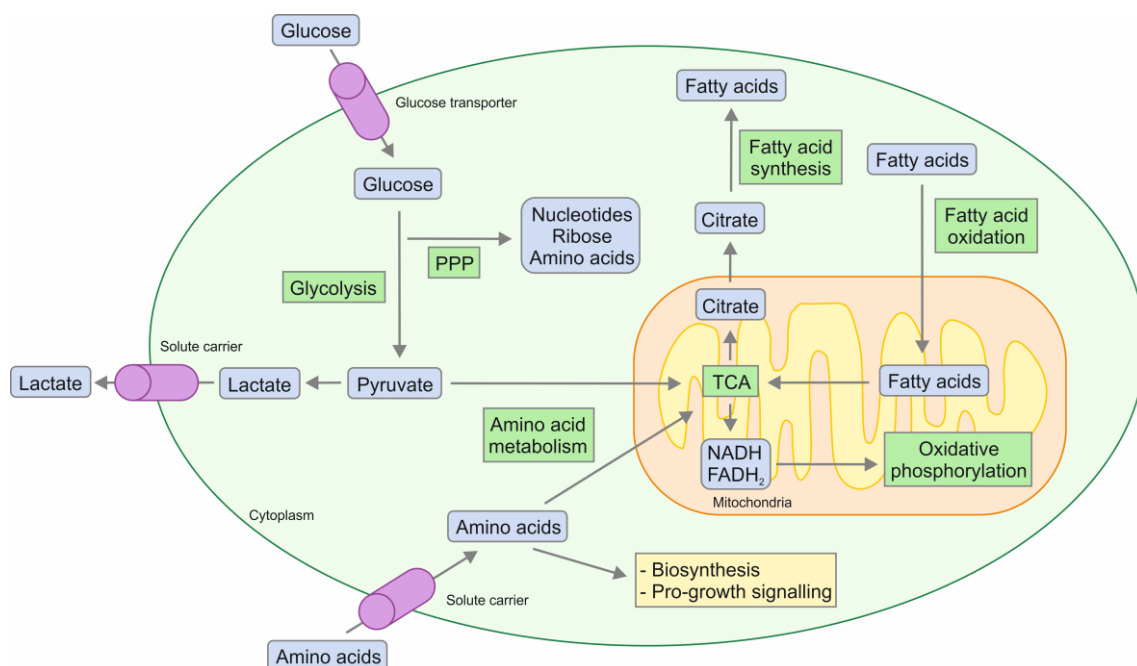


Figure I.9. Six major metabolic pathways involved in immunometabolism: glycolysis, tricarboxylic acid cycle (TCA) followed by oxidative phosphorylation, pentose phosphate pathway (PPP), fatty acid oxidation, fatty acid synthesis and amino acid metabolism. Adapted from *A guide to immunometabolism for immunologists*. *Nat Rev Immunol.* 2016; 16(9):553-565.

- **The pentose phosphate pathway (PPP).** PPP occurs in the cytosol and serves to support cell proliferation and survival. PPP allows the diversion of glycolytic intermediates towards the production of nucleotide and amino acid precursors. PPP generates reducing equivalents of NADPH, which play a key role in the maintenance of suitable cellular redox environment (73).
- **Fatty acid oxidation.** The fatty acid oxidation allows the mitochondrial conversion of fatty acids into acetyl-CoA, NADH and FADH₂ that can be used for energy generation. Fatty acid oxidation can produce huge amounts of ATP with the complete β -oxidation of a single palmitate molecule (a major fatty acid in mammalian cells) (73).

- **Fatty acid synthesis.** Fatty acid synthesis pathway allows the generation of lipids that are necessary for cellular growth and proliferation from precursors derived from other metabolic pathways, such as glycolysis, TCA cycle and pentose phosphate pathway (73).
- **Amino acid metabolic pathways.** Amino acids, as a consequence of their use in protein synthesis, are linked to anabolic cellular pathways. Individual amino acids may play specific roles. For example, glutamine is required for de novo purine and pyrimidine synthesis. In addition, glutamine can be used for ATP production through TCA cycle, or as a source of citrate for fatty acid synthesis (73).

2.2. mTOR and AMPK as key regulators of metabolic routes

The mammalian target of rapamycin (mTOR) is a central regulator of cell growth and proliferation. mTOR is a serine/threonine protein kinase that constitute the catalytic subunit of two distinct protein complexes, mTOR Complex 1 (mTORC1) and 2 (mTORC2). mTORC1 is defined by its components: mTOR, Raptor (regulatory protein associated with mTOR), Deptor and mLST8 (mammalian lethal with Sec13 protein 8). mTORC2 contains mTOR, Rictor (rapamycin insensitive companion of mTOR), mLST8, Protor and Deptor (74, 75). mTORC1 is regulated by multiple

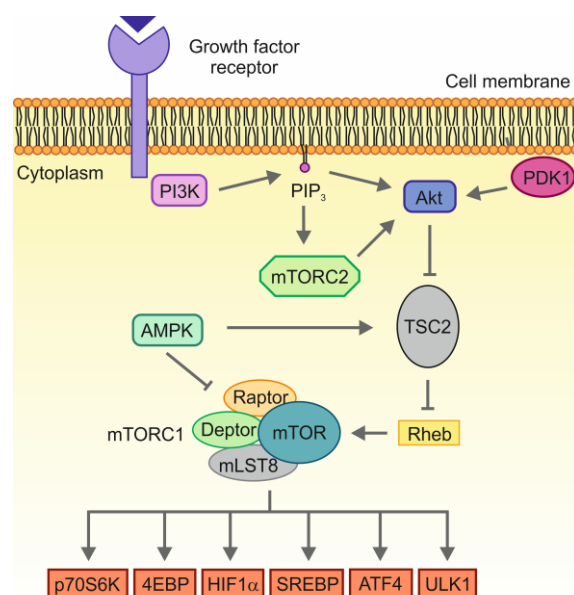


Figure I.10. The mTOR signalling network. The major signalling pathways upstream and downstream of mTOR are shown.

signals, including growth factors, amino acids and cellular energy. Numerous growth factors activate mTORC1 via PI3K and Akt (protein kinase B) signalling. mTORC1 is involved in the regulation of essential cellular processes such as protein biosynthesis through the activation of p70S6 kinase (p70S6K) and eIF4E binding protein (4EBP) and energy expenditure by the activation of hypoxia-inducible factor 1 α (HIF1 α), sterol regulatory element-binding protein (SREBP) and activating transcription factor 4 (ATF4) that promote glucose metabolism and lipid and nucleotide synthesis, respectively. Furthermore, mTORC1 suppresses protein catabolism, mainly autophagy. In contrast, mTORC2 serves as a positive regulator of mTORC1 (**Figure I.10**) (74, 76). Activation of mTORC1 promotes anabolic processes that use energy and nutrients for the biosynthesis of macromolecules required for cell growth and proliferation. Thus,

mTORC1 activates glycolysis, PPP, and lipid synthesis. In addition, mTOR suppresses autophagy, a catabolic process by which the cell breaks down its own organelles and cytosolic components for using them as nutrients (**Figure I.11**) (77).

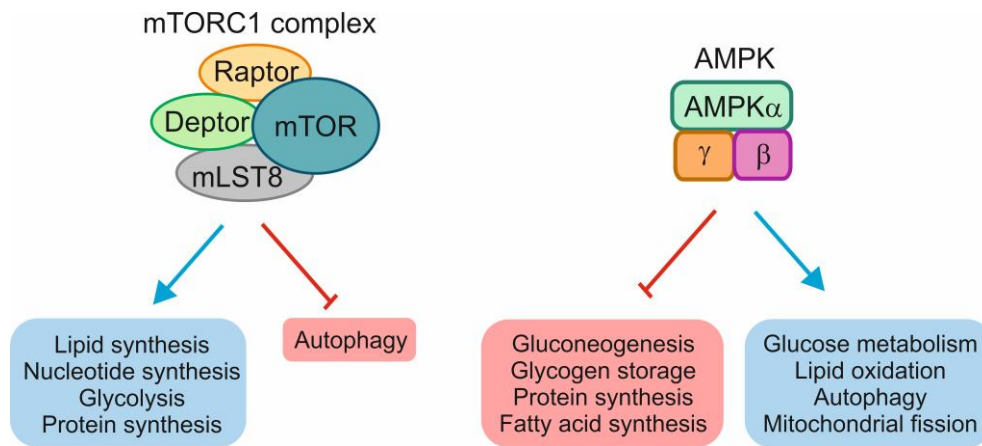


Figure I.11. Metabolic consequences of mTOR and AMPK activation. The activation of mTOR promotes anabolic processes needed for cell growth and proliferation. In contrast, AMPK activation inhibits anabolic processes and induces catabolic processes that regenerate ATP levels.

AMP-activated protein kinase (AMPK) is a major regulator of cellular energy homeostasis by controlling multiple metabolic pathways. AMPK is a heterotrimeric protein consisting of a catalytic α and regulatory β and γ subunits. AMPK is activated in response to an increase in the ratio AMP to ATP in the cell, which could be due to nutrient starvation, ischemia, exercise and hypoxia. Increased intracellular calcium levels also lead to the activation of AMPK (78-80). Activated AMPK phosphorylates key factors involved in energy control. The effect of AMPK on metabolism consists on the inhibition of anabolism to minimize ATP consumption and the stimulation of catabolism to induce ATP production. AMPK inhibits *de novo* lipid synthesis and gluconeogenesis. AMPK inhibits cell growth and protein synthesis, which are the major consumers of ATP in cells, through the modulation of mTOR. AMPK activation inhibits mTORC1 by two mechanisms: activation of the negative mTORC1 regulator TSC2 (tuberous sclerosis complex 2) and the inhibition of the Raptor subunit (**Figure I.10**) (81, 82). On the other hand, AMPK promotes the breakup of macromolecules to produce ATP. AMPK induces the use of glucose and lipid store through glycolysis and β -oxidation, respectively. AMPK promotes mitochondrial biogenesis and transcriptional regulation of genes involved in oxidative phosphorylation to produce more ATP (78, 82). In addition, AMPK induces the activation of autophagy, providing nutrients for ATP production (**Figure I.11**) (83). It is particularly interesting that AMPK can be regulated not only by energy balance, but also by extracellular signals through surface receptors (82).

2.3. Autophagy

Autophagy is a highly conserved catabolic process. It occurs in response to different physiological conditions such as nutrient deprivation, growth factor depletion, infection or hypoxia in order to provide nutrients for cellular functions. In addition, autophagy is involved in the selectively elimination of harmful cytosolic material such as mitochondria or protein aggregates (84, 85).

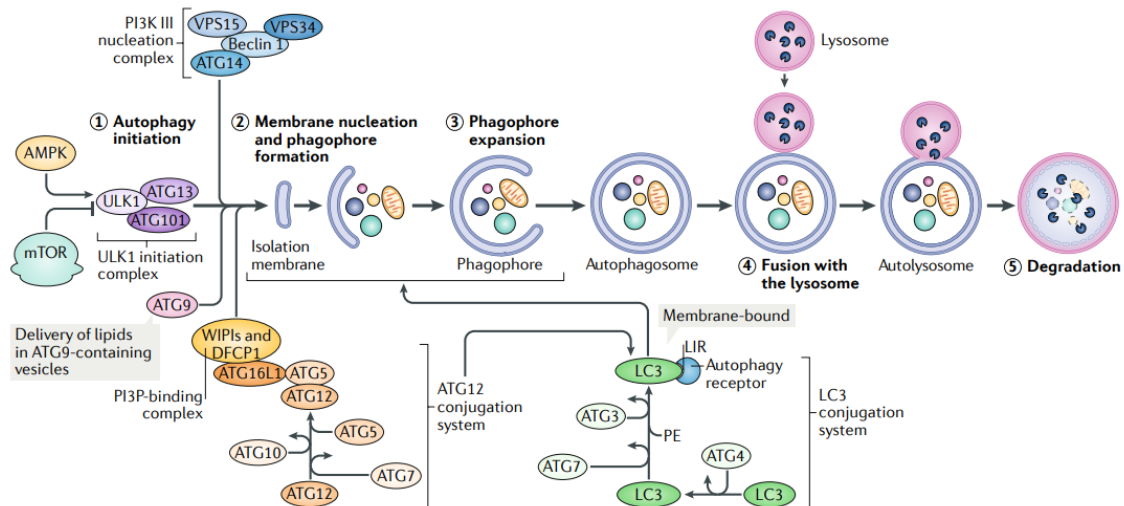


Figure I.12. Overview of the autophagy process. The activation of ULK1 complex results in the generation of phagophore and the recruitment of ATG proteins. Then, the phagophore is closed, forming the autophagosome, which is fused with the lysosome. Adapted from *Mechanisms and medical implications of mammalian autophagy*. *Nat Rev Mol Cell Biol.* 2018; 19(6):349-364.

Autophagy induction can occur after mTOR inhibition, the master cell growth regulator. In high-nutrient conditions, Unc-51 like kinase (ULK1), the protein kinase that initiates autophagy, is phosphorylated by mTORC1 and remains inactive. Upon starvation, ULK1 is dephosphorylated on mTORC1 sites and autophosphorylated, starting the autophagy process. Autophagy can also be induced when the cellular energy levels are low. AMPK senses low ATP levels and inhibits mTORC1 through the previously described mechanisms, promoting autophagy induction. Furthermore, AMPK can directly phosphorylate and activate ULK1 (86, 87).

After ULK1 activation, the ULK complex translocates to a specific localization called phagophore assembly site, where occurs the formation of the isolating membrane known as the phagophore. Here, ULK complex activates class III PI3K complex, whose main components are autophagy-related protein 14 (ATG14), Beclin 1 and the PI3K catalytic subunit and mediates the nucleation step of phagophore formation. Then, others ATG proteins such as ATG12, ATG5, ATG16 and LC3 (microtubule-associated protein 1A/1B-light chain 3) are recruited and participate in the elongation of phagophore, which contains a small portion of cytoplasm containing the material for degradation. Next, the

phagophore is closed forming the autophagosome, which is fused with the lysosome leading to the formation of the digestive compartment termed autolysosome. Finally, lysosomal enzymes degrade the content of the vesicle, providing nutrients for anabolic processes (**Figure I.12**) (84, 85).

2.4. Metabolic control of dendritic cell function

Changes in the cellular metabolism of DCs are essential for the regulation of the activation and function of DCs. The balance of anabolic versus catabolic metabolism controls immunogenicity versus tolerogenicity in DCs (88). In resting DCs, the anabolic demands are low, and the catabolism of proteins and triacylglycerols provides substrates for ATP production by TCA cycle and oxidative phosphorylation (89-91). Immunogenic DC activation induces a metabolic switch from oxidative phosphorylation to glycolysis, in a phenomenon similar to the Warburg effect. Warburg effect is a process by which proliferating or activated cells dramatically increase the rate of glucose uptake and lactate is produced after glycolysis even in the presence of oxygen and fully functioning mitochondria (90, 92). Activated DCs display an increase in glucose uptake and glycolytic pathway to rapidly generate ATP. PPP is increased for purine and pyrimidine production. Although TCA cycle is decreased, citrate is produced and used for fatty acid synthesis, which is critical for size increment of key organelles involved in protein biosynthesis. Thus, immunogenic activation requires anabolic metabolism for the generation of substrates used in biosynthesis and cell growth and can be regulated by Akt/mTORC1 pathway (88, 90, 91, 93). In addition, activated DCs are characterized by the loss or inactivation of AMPK, and subsequently the catabolic processes such as autophagy are inhibited (**Figure I.13**) (91).

On the other hand, tolerogenic DCs induced by resveratrol, vitamin D-3 and dexamethasone exhibit

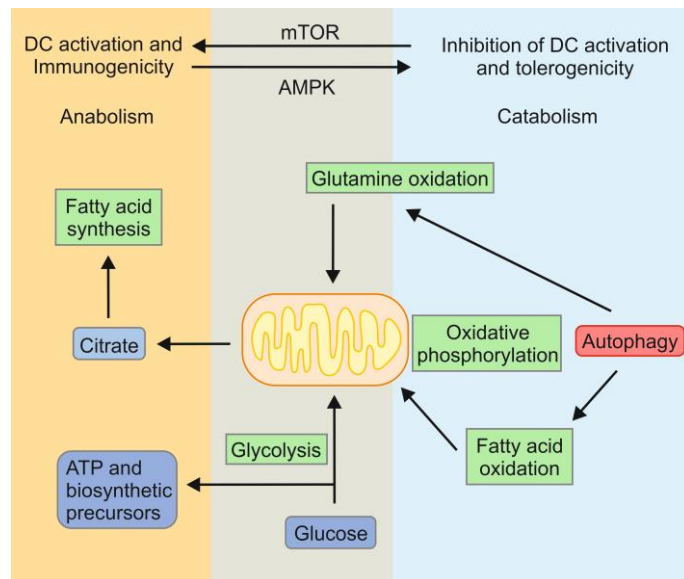


Figure I.13. DC immunogenicity versus tolerogenicity and anabolic versus catabolic metabolism. Cytokines, PRR agonists, nutrients and oxygen levels can influence metabolism. mTOR and AMPK are key regulators in the metabolic balance. Citrate from TCA supports biosynthesis needed for DC activation. In contrast, autophagy and the oxidation of fatty acids and glutamine promote a tolerogenic state in DCs. Adapted from *Immunometabolism governs dendritic cell and macrophage function. J Exp Med. 2016; 213(1):15-23.*

enhanced catabolism characterized by the increased expression of genes related to oxidative phosphorylation and glucose metabolism as well as increased fatty acid oxidation (69, 91, 94, 95). These tolerogenic DCs show enhanced AMPK activity and signalling, which favour catabolic processes. Additionally, AMPK activation through autophagy induction can reduce the ability of DCs to present peptide-MHC complexes to T cells, becoming tolerogenic rather than immunogenic DCs (95, 96). In addition, autophagy can control DC migration and cytokine production (**Figure I.13**) (97, 98).

DCs express a large number of G-protein-coupled receptors (GPCRs) involved in sensing extracellular metabolites such as succinate, butyrate, adenosine/ATP or lactic acid, which can connect metabolic reprogramming with DC function (88). The knowledge of the mechanisms by which GPCR ligands regulate metabolic and functional features of DCs can provide novel targets for immunomodulation.

3. The endocannabinoid system

The cannabis plant *Cannabis sativa* L., *marijuana*, has been used for both therapeutic and recreational effects for many centuries. However, the purification and the chemical characterization of its unique active components, the so called cannabinoids, were not carried out until the 1960s (99). In the early 1990s, specific cannabinoid receptors (CBRs) were cloned and their endogenous ligands were characterized, uncovering the mechanism of action of cannabinoids (100, 101). These findings boosted basic and clinical research to better understand the molecular pathways involved in the way of action of cannabinoids and to explore potential novel therapeutic applications.

The human endocannabinoid system (ECS) is a complex signalling pathway involved in many physiological processes. It acts as a broad-spectrum modulator, controlling several essential processes such as neuronal development, brain plasticity, learning and memory, regulation of appetite, stress and emotions, proliferation, differentiation, cell survival, metabolism and immunity. Therefore, the ECS constitutes a key target for potential therapeutic strategies (102, 103). The main components of the ECS include the endocannabinoid ligands, the cannabinoid receptors (CBRs) and the enzymes related to the ligand synthesis or degradation.

3.1. Endocannabinoids, phytocannabinoids and synthetic cannabinoids

Endocannabinoid ligands are lipid-derived signalling molecules that are endogenously synthesized and can bind and activate CBRs. Anandamide (arachidonylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) are the most widely investigated (**Figure I.14**). Other biochemically and structurally related endocannabinoids such as N-palmitoylethanolamine (PEA), 2-arachidonoylglycerol ether (noladin ether, 2-AGE), O-arachidonylethanolamine (virodhamine) or N-arachidonoyldopamine (NADA) have

been documented (104). AEA is a high affinity partial agonist for GPCRs CB1 (CB1) and a low affinity ligand of the GPCRs CB2, whereas 2-AG is a full agonist with moderate affinity for both CB1 and CB2 (104, 105). In addition, AEA and 2-AG can also interact with other receptors or ion channels, including orphan GPCRs, peroxisome proliferator-activated receptors (PPARs) or transient receptor potential channels (TRPVs) (106).

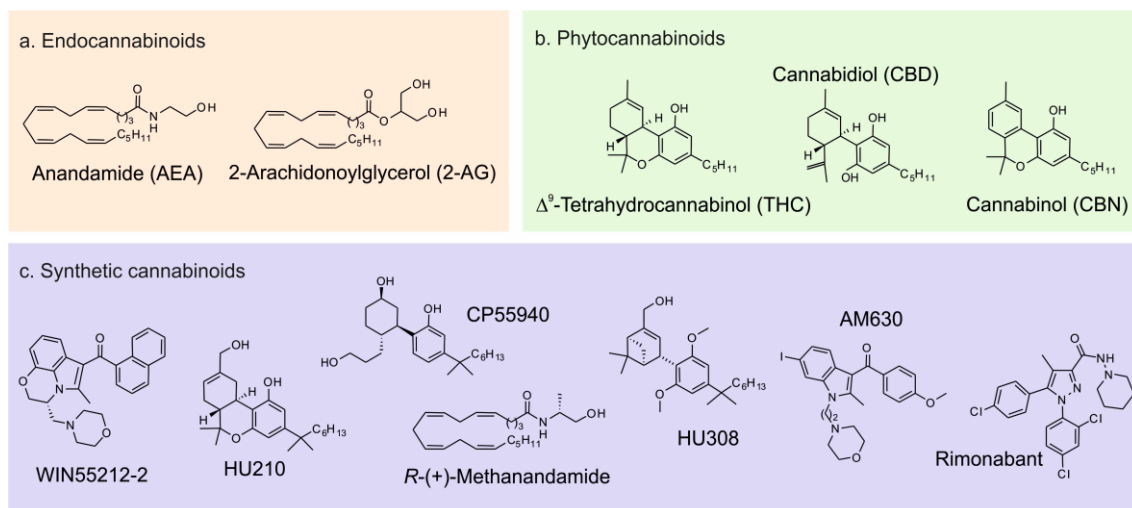


Figure I.14. Cannabinoid ligands. Endocannabinoids (a), plant-derived cannabinoids or phytocannabinoids (b) and synthetic cannabinoids including non-selective agonists, CB1- and CB2-selective agonists and CB1- and CB2-selective antagonists (c) are shown. Adapted from *The role of cannabinoids in allergic diseases*. *Int Arch Allergy Immunol.* 2020; 181(8):565-584.

Other cannabinoid compounds are the phytocannabinoids derived from the plant *Cannabis sativa* L. (*marijuana*) and the synthetic cannabinoids (**Figure I.14**). The most abundant and well-described phytocannabinoid is Δ^9 -tetrahydrocannabinol (THC), which was isolated and structurally characterized in the 1960s (99). THC is the major psychoactive component of cannabis plant and exerts a wide variety of biological effects by activation of CB1 and CB2. The therapeutic effect of THC has been studied in several diseases, including cancer, multiple sclerosis, epilepsy, Alzheimer disease, Parkinson disease and pain treatment (107-109). Currently, THC and its synthetic analogue nabilone (Marinol and Cesamet, respectively) are approved for inhibiting chemotherapy-induced nausea and vomiting (110). Cannabidiol (CBD) and cannabinol (CBN) are other plant-derived cannabinoids produced in significant amounts, but they show lower affinity for CBRs compared to THC. CBD is the main non-psychoactive phytocannabinoid and its therapeutic interest is due to its analgesic, antipsychotic, anti-oxidant and anti-inflammatory properties (108, 109, 111). A mixture of equal doses of THC and CBD, known as Sativex, is marketed for the treatment of spasticity in patients with multiple sclerosis (110, 111).

Since the identification of the ECS, a large number of synthetic cannabinoids have been developed in order to mimic some of the beneficial properties of THC, while

avoiding its negative effects. They include agonists with similar affinity for CB1 and CB2 but greater potency than THC, such as WIN55212-2 (from aminoalkylindol family), HU210 (synthesized at the Hebrew University; HU series) and CP55940. Compounds with specific selectivity have been also described: R-(+)-methanandamide (CB1-selective agonist) and HU308 (CB2-selective agonist). In addition, selective antagonists have been generated such as Rimonabant and AM630, CB1- and CB2-selective antagonist, respectively (**Figure I.14**) (112-115). Numerous cannabinoids, including phytocannabinoids and synthetic cannabinoids have been included in clinical trials (102).

3.2. Synthesis and inactivation of endocannabinoids

Endocannabinoids are synthesized and released on demand from membrane precursors. However, the discovery of storage organelles suggests that endocannabinoids could be accumulated in the cell (116, 117). Although both AEA and 2-AG contain arachidonic acid, their routes of synthesis and degradation *in vivo* are almost completely distinct and are mediated by different enzymes. Synthesis of AEA starts with the hydrolysis of N-acyl-phosphatidylethanolamine (NAPE) by NAPE-hydrolysing phospholipase D (NAPE-PLD). Alternative pathways involving other NAPE phospholipases and enzymes are also described (117, 118). The synthesis of 2-AG consists of the sequential hydrolysis of arachidonoyl-containing phosphatidyl inositol bisphosphate by phospholipase C (PLC) followed by hydrolysis of the resulting diacylglycerol by diacylglycerol lipase (DAGL). The main elements involved in endocannabinoid synthesis are shown in **Figure I.15**. (118).

Endocannabinoids are rapidly inactivated by a two-step process: cellular uptake and intracellular hydrolysis. Cellular uptake of endocannabinoids from extracellular space is mediated by putative endocannabinoid membrane transporters (EMT). To date, EMT have not been cloned and their existence is only based on indirect evidence (119). Degradation of AEA is mediated by the fatty acid amide hydrolase (FAAH), which degrades multiple fatty acid amides, whereas the degradation of 2-AG is primarily due to monoacylglycerol lipase (MAGL). The resulting products of AEA and 2-AG degradation are arachidonic acid in both cases and ethanolamine and glycerol, respectively (**Figure I.15**) (118). Additionally, AEA and 2-AG can be oxidized by cyclooxygenase 2 (COX-2) (118). FAAH and MAGL represent promising therapeutic targets for the treatment of different disorders, and pharmacological inhibitors have been developed. Pharmacological or genetic inhibition of FAAH and MAGL increases AEA and 2-AG levels, prolonging their anti-inflammatory and analgesic effects via action of CBRs. In addition, FAAH and MAGL inhibition induces a reduction of arachidonic acid, a key precursor of pro-inflammatory prostaglandins and thromboxanes, resulting in the

reduction of inflammation. Based on these effects, FAAH and MAGL inhibitors may have a large number of therapeutic applications, including pain, nausea, neurodegenerative pathologies, inflammation, metabolic disorders and cancer (120, 121).

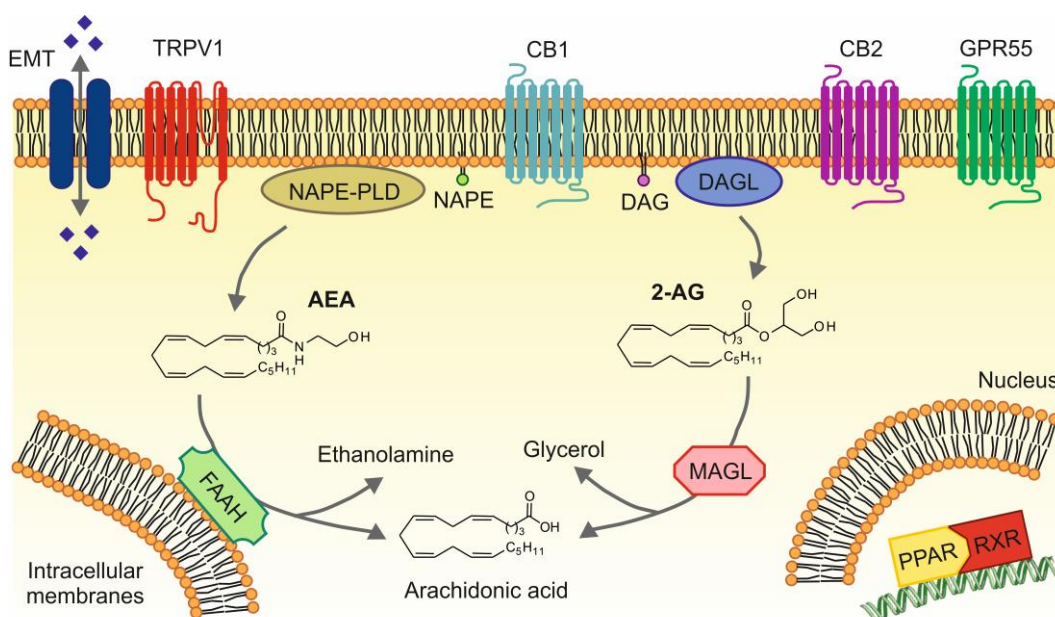


Figure I.15. Main components of the endocannabinoid system (ECS). Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthesized on demand from membrane lipids by N-acyl-phosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively. AEA and 2-AG move across the cell membrane through a purported endocannabinoid membrane transporter (EMT). Cannabinoid receptor 1 (CB1), cannabinoid receptor 2 (CB2), transient receptor potential vanilloid 1 (TRPV1), G protein-coupled receptor 55 (GPR55), and PPARs are the main receptor targets of AEA and 2-AG. AEA and 2-AG are hydrolysed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively, releasing arachidonic acid. Adapted from *The role of cannabinoids in allergic diseases. Int Arch Allergy Immunol.* 2020; 181(8):565-584.

3.3. Cannabinoid receptors (CBRs)

The effects of endocannabinoids are mainly mediated by CB1 and CB2, belonging to the GPCR superfamily. CB1 and CB2 share 44 % sequence identity (101). Both receptors consist of a single polypeptide chain with 7 transmembrane α -helices inserted in the cell membrane, an extracellular N-terminus and intracellular C-terminus domain. Their high-resolution crystal structure in humans has been recently reported (122-125). CB1 and CB2 are generally coupled to inhibitory $G_{i/o}$ proteins thus inhibiting adenylate cyclase and the conversion of ATP to cyclic AMP and protein kinase A (PKA), a positive regulatory signalling pathway of immune response. However, CB1 can also activate adenylate cyclase through G_{α_s} protein stimulation (126). $G\beta\gamma$ subunits coupled to CBRs activate MAPKs and Akt, involved in cellular homeostasis. Other CBR-mediated signalling pathways include modulation of ion channels, ceramide biosynthesis and activation of PLC β (Figure I.16) (127-129).

CB1 is highly expressed in the central nervous system (CNS) where it regulates diverse neuronal functions and behaviours. Far from being restricted to the CNS, CB1 expression is also observed in peripheral tissues including immune cells, liver, pancreas, skeletal muscle and peripheral nervous system, where it has been implicated in other physiologic processes such as control of immunity, metabolism, etc. (110). For a long time it was thought that CB2 was only expressed in peripheral immune system cells, but it has been

shown that CB2 is also expressed in different cell subsets in the CNS, muscle, pancreas, intestine and testis (130). Interestingly, it has been reported that CB1 and CB2 expression can be increased in pathologic or injury conditions (131-133). For example, CB1 gene expression is increased in immune system cells of patients with allergic diseases (131). The wide distribution of CB1 and CB2 in tissues and their ability to modulate several physiological processes turn CB1 and CB2 into promising therapeutic targets for the treatment of different diseases.

3.4. Non-canonical CBRs

Besides CB1 and CB2, other receptors including orphans GPCRs (GPR55, GPR19), transient receptor potential vanilloid 1 (TRPV1) and PPARs are also involved in the endocannabinoid signalling. GPR55 was initially described as a putative “CB3” receptor due to its high affinity to cannabinoid ligands. However, the low sequence similarity with conventional CBRs does not fully endorse this concept (134). GPR55 is widely expressed in the immune system, CNS and peripheral tissues (134). It is coupled to $G\alpha_{12/13}$, signalling through Ras homolog gene family member A (RhoA) and controlling several physiological processes (135). Unlike CB1 and CB2 that mainly trigger inhibitory effects, GPR55 mostly exerts excitatory and stimulatory effects (135). Other orphan

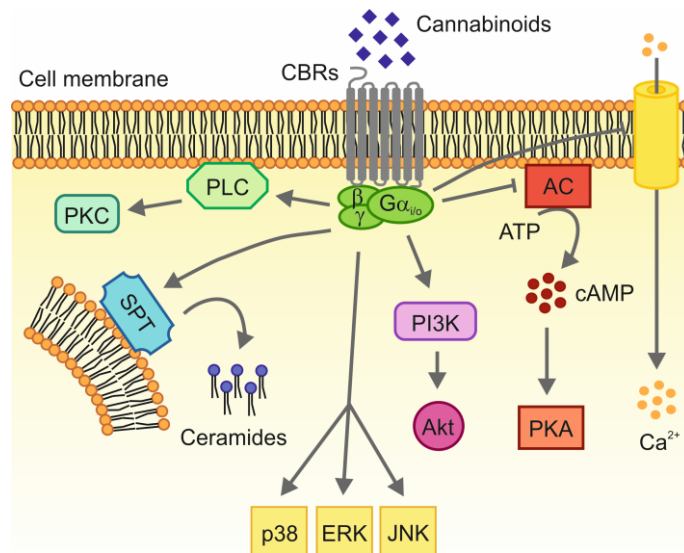


Figure I.16. Cannabinoid receptor (CBR)-induced signalling. After cannabinoid binding, CBRs signal several cellular pathways including inhibition of protein kinase A (PKA) pathway, activation of mitogen-activated protein kinase cascades (p38, JNK, and ERK), activation of protein kinase B (Akt) pathway, inhibition of calcium channels, activation of protein kinase C (PKC), and generation of ceramides. Adapted from *The role of cannabinoids in allergic diseases. Int Arch Allergy Immunol.* 2020; 181(8):565-584.

GPCRs such as GPR18, GPR19 or GPR110 have been also described as potential cannabinoid targets (102).

Transient receptor potential (TRP) channels are a family of trans-membrane channels activated by an excess of chemical and physical stimuli. TRPV1, also known as capsaicin receptor, is a nonselective channel expressed in the CNS and peripheral tissues such as liver, skin or intestine and immune system cells. Upon activation, the pore allows the flux of ions across the membrane. TRPV1 can be activated by heat, capsaicin arachidonic acid derivatives, protons and cannabinoids (136, 137).

Several studies demonstrated the activation of PPAR receptors by some cannabinoid compounds (138, 139). PPARs are nuclear hormone receptors with 3 isoforms (α , β and γ) highly expressed on metabolically active tissues. Upon ligand binding, PPARs heterodimerize with retinoic acid receptor (RXR) and bind to DNA sequences called PPAR response elements, regulating the transcription of genes involved in essential biological processes including metabolism, cell differentiation and inflammation (140). Even though the mechanism of cannabinoid-PPAR interaction is not clear, activation of PPARs by cannabinoids exerts anti-inflammatory and neuroprotective effects in several disease models (139).

3.5. Regulation of immune system by cannabinoids

The ECS plays an important role in the regulation of both innate and adaptive immune responses. Immune cells are not only influenced by cannabinoids, but also produce and secrete endocannabinoids themselves, which in turn modulates the functional features of immune cells (141). CB2 is the most expressed CBR in immune cells and its activation usually mediates immunosuppressive responses, but other CBRs such as CB1, GPR55, and PPARs are also involved in immune cell regulation. In a broad perspective, the anti-inflammatory effects of cannabinoids are a consequence of some specific effects on immune cells such as their capacity to modulate cytokine production, cell migration, T cell responses, cell proliferation, and apoptosis. A detailed summary of the main effects of different cannabinoids on the different immune cells is presented in **Table I.3** (103, 141, 142).

The molecular mechanisms by which cannabinoids exerts their anti-inflammatory effects on immune cells need to be better understood. As described above, TLR activation leads to NF- κ B and MAPK signalling pathway activation, inducing the expression of pro-inflammatory genes (25). Cannabinoids impair pro-inflammatory cytokine and NO production by LPS-stimulated monocytes, macrophages and microglia due to NF- κ B signalling pathway inhibition (143-145). On the other hand, TLR activation

enhances CBR expression and endocannabinoid production, demonstrating the important role of the ECS in the modulation of immune responses (145).

Table I.3. Main effects of cannabinoids in immune cells

Cell type	CBR expression	Role of cannabinoid ligands
Monocytes/ Macrophages	CB1 and CB2	AEA, 2-AG, THC and CBD inhibit cytokine production AEA and PEA stimulate phagocytosis 2-AG, THC and WIN55212-2 modulate ROS production CBD induces apoptosis
Dendritic cells (DCs)	CB1 and CB2	AEA, THC, JWH-015 and JWH-133 inhibit inflammatory cytokine production AEA and THC inhibit the capacity to induce Th1 and Th17 responses THC induces apoptosis THC impairs human monocyte-derived DC differentiation
Neutrophils	CB1 and CB2	AEA, CBD and CB2 signalling reduce cell migration AEA and 2-AG induce cell activation and the release of antimicrobial effectors
NK cells	CB1 and CB2	2-AG and THC inhibit cytolytic activity CB2 signalling reduces cell migration O-1602 induces high cytolytic activity and cytokine production
Eosinophils	CB1 and CB2	2-AG increases cell recruitment WIN55212-2 reduces cell recruitment
Mast cells	CB1 and CB2	AEA and AEA-derived compounds inhibit cell maturation and degranulation AM251 induces cell maturation and degranulation
Innate lymphoid cells (ILCs)	CB2	CBD promotes ILC2 induction CB2 signalling induce high numbers of ILC2
T lymphocytes	CB1 and CB2	AEA, THC and JWH-133 inhibit T cell proliferation AEA and THC suppress T cell responses CBD and JTE907 induce functional Treg generation HU210 and HU308 inhibit cytokine production
B cells	CB1 and CB2	THC and WIN55212-2 increase B cell proliferation CP55940 induces IgE class switching CB2 signalling promotes B-cell retention in bone-marrow or splenic marginal zones

Adapted from *The role of cannabinoids in allergic diseases. Int Arch Allergy Immunol.* 2020; 181(8):565-584.

Novel findings indicate that cannabinoids might also mediate their anti-inflammatory effects by rewiring the metabolic pathways in immune cells (146). Cannabinoids induce AMPK activation inhibiting energetic metabolism. In addition, cannabinoids promote autophagy induction, which can control inflammatory cytokine secretion and inflammation, antigen presentation and lymphocyte development (146, 147).

Controversial results about the mechanisms by which cannabinoids regulate DC function have been described. Cannabinoid treatment induced apoptosis in mice DCs (148). However, *in vitro* models of bacterial infection showed that cannabinoids inhibited cytokine production and costimulatory molecules without apoptosis induction (149-151). Similarly, THC and CB2 agonists inhibited CpG-induced responses in pDCs by inhibition of IRF7 and NF- κ B signalling pathways (152, 153). Moreover, cannabinoids can inhibit

T cell responses in different disease models (154, 155). Human DCs express CBRs and the other components of the ECS, but data of human DCs are still scarce.

The role of cannabinoids in the regulation of immune system cells provided novel insights regarding targets for the therapeutic treatment of immune-related diseases. Therefore, the therapeutic effect of cannabinoids in several immune diseases have been tested. Cannabinoid-based treatment displays anti-inflammatory and beneficial effects in brain injury, inflammatory bowel diseases, vascular inflammation, sepsis, rheumatic disease, multiple sclerosis, airway inflammation and allergy but the actual molecular mechanisms underlying such effects remain to be fully elucidated (142, 156).

OBJECTIVES

Objectives

Several studies have shown that cannabinoid compounds display anti-inflammatory properties in different immune-mediated diseases. However, their potential capacity to regulate the orchestration of immune responses is not well understood, specifically in humans. Human DCs express all the components of the ECS, including classical and alternative CBRs. The ability of cannabinoids to immunomodulate the phenotype and function of DCs, the underlying molecular mechanism and the potential clinical implications in immune-mediated diseases remain elusive.

We hypothesized that synthetic cannabinoids could control immune responses in human DCs, which might well open a new avenue for the development of novel preventive and therapeutic strategies targeting DCs for inflammatory diseases.

Considering all these aspects, the main objective of this thesis is to investigate the role of synthetic cannabinoids in the control of human DC function, the underlying molecular mechanisms and their potential implications in preclinical *in vitro* and *in vivo* models of immune-mediated diseases as potential novel strategies for the prevention and treatment of inflammatory disorders. To achieve our goal we pursue the following aims:

- 1. Development and validation of a fluorescent small molecule (HU210-Alexa488) probe to visualize and quantify CB1 in native systems.**
 - 1.1. Validation of HU210-Alexa488 as a novel probe to identify and quantify the expression of CB1 at the protein level in immune cells.
 - 1.2. Visualization and quantification of CB1-expressing immune cells in human peripheral blood and tonsils with HU210-Alexa488 probe by flow cytometry and confocal microscopy.
 - 1.3. Functional characterization of the identified CB1-expressing T cells in human tonsils.
- 2. Study of the capacity of the synthetic cannabinoid WIN55212-2 to immunomodulate human DCs.**
 - 2.1. Analysis of the CBR expression in human DCs and determination of the anti-inflammatory properties of different synthetic cannabinoids.
 - 2.2. Study of the capacity of WIN55212-2 to regulate the phenotype and function of human DCs.
 - 2.3. Study of the capacity of WIN55212-2-treated human DCs to polarize T cell responses.

3. Elucidation of the molecular mechanism by which the synthetic cannabinoid WIN55212-2 exerts its effects in human DCs.

- 3.1. Study of the main immune signalling pathways involved in the immunomodulatory effects of WIN55212-2 in human DCs.
- 3.2. Study of the main metabolic changes promoted by WIN55212-2 in human DCs.
- 3.3. Study of the contribution of different CBRs and PPARs to the immunomodulatory properties exerted by WIN55212-2 in LPS-stimulated human DCs.

4. Assessment of the immunomodulatory effects promoted by the synthetic cannabinoid WIN55212-2 in human and mice preclinical models.

- 4.1. Study of the immunomodulatory properties of WIN55212-2 in an *in vivo* LPS-sepsis mouse model.
- 4.2. Study of the capacity of WIN55212-2 to immunomodulate *in vitro* peanut-stimulated human DCs.
- 4.3. Study of the immunomodulatory properties of WIN55212-2 in an *in vivo* peanut-induced anaphylaxis mouse model of food allergy.

5. Evaluation of the capacity of the synthetic cannabinoid WIN55212-2 to regulate bronchial epithelial barrier integrity.

- 5.1. Study of the capacity of WIN55212-2 to regulate the integrity of human bronchial epithelial cells during rhinovirus infection in air-liquid interface cultures.
- 5.2. Study of the capacity of WIN55212-2 to modulate T cell responses in human tonsils.

MATERIALS AND METHODS

Materials and methods

1. Media and reagents

RPMI 1640 medium supplemented with 5 % heat inactivated FCS (Amimed), penicillin, streptomycin, kanamycin (Life Technologies), amphotericine B (Bristol-Myers Squibb), ciprofloxacin (Fluka), MEM vitamins, L-glutamine, nonessential amino acids and sodium pyruvate (Life Technologies) was used for isolation and culture of tonsil mononuclear cells (TMC).

We used RPMI 1640 medium (Lonza) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco), normocin (InvivoGen), penicillin, streptomycin, nonessential amino acids, MEM vitamins and sodium pyruvate (all from Life Technologies) for dendritic cell and T cell cultures.

Bronchial epithelial basal medium (Lonza) supplemented with the BEGM SingleQuots (Lonza) was used for the culture of primary human bronchial epithelial cells (HBEC) as monolayers. Bronchial epithelial basal medium supplemented with the BEGM SingleQuots except for retinoic acid and triiodothyronine, and mixed 1:1 with Dulbecco's modified Eagle medium (Gibco) was used for the culture of HBEC in air-liquid interphase (ALI).

The combination of anti-CD2 (clone 4B2 and 6G4), anti-CD3 (clone OKT3) and anti-CD28 (clone B7G5; all from Sanquin) monoclonal antibodies (mAbs) was used to induce T cell proliferation. Lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 and O155:B5 (Sigma-Aldrich) were used for cell cultures and animal models, respectively. Pam3CSK4 (InvivoGen) as TLR2 ligand and a combination of TNF α and IL-1 β (both from PeproTech) as maturing factors were used. CBR agonists WIN55212-2 (Sigma Aldrich) and HU210, selective CB2 agonist HU308, selective antagonists for CB1 (Rimonabant), CB2 (AM630), PPAR α (GW6471), PPAR γ (GW9662) (all from Tocris), autophagy inhibitor 3-methyladenine (3-MA; InvivoGen) and human rhinovirus 16 (HRV-16; Virapur) were used. Peanut extract (Stallergenes Greer) was used for cell cultures and animal models.

2. THP1-XBlue™ cell line

THP1-XBlue™ cell line (InvivoGen) is a human monocytic cell line with stable integration of a NF- κ B/AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct. As a result, THP1-XBlue cells allow the monitoring of NF- κ B/AP-1 activation by determining the activity of SEAP using a SEAP detection reagent called QUANTI-Blue™.

For the optimization of test conditions, THP1-XBlue™ were cultured in 96-well plate at a density of 10^6 cell/mL for 16-20 hours at 37 °C in a humidified 5 % CO₂ incubator with different doses of the synthetic cannabinoids WIN55212-2, HU210 and HU308 in the absence or presence of the TLR2 ligand (TLR2L) Pam3CSK (25 ng/mL). To determine NF-κB/AP-1 activation, 20 µL of the cell supernatant were added to 180 µL of QUANTI-Blue™. The mixture was incubated at 37 °C and the optical density was read with an ELISA EZ Read 400 Microplate Reader (Biochrom). Cell viability was analysed in all the cases with trypan blue (Gibco) exclusion by light microscope.

3. Isolation of different cell populations

3.1. Isolation of tonsil mononuclear cells

Single-cell suspensions of tonsil mononuclear cells (TMC) were isolated by mechanical disruption (source: Swiss Institute of Allergy and Asthma Research (SIAF) Biobank). The tissues were chopped and grounded with medium through a 40 µm mesh. After soft-lysis and several washing steps with medium, the cells were purified by centrifugation on a density gradient (Bicoll; Biochrom) (42, 157).

3.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors (source: Transfusion Centre of Madrid) by Ficoll-Paque Plus (GE-Healthcare) density gradient centrifugation (800 g, 20 minutes) as previously described (158-160).

3.3. Purification of monocytes and hmoDCs generation

Human blood monocytes were obtained from total PBMC using anti-CD14 microbeads (Miltenyi Biotec) following the manufacturer's protocol and using the autoMACS Pro (Miltenyi Biotec). For the differentiation of monocytes to immature human monocyte-derived DCs (hmoDCs), monocytes were cultured in 24-well plate at a density of 2×10^6 cell per well in the presence of 100 ng/mL of IL-4 and GM-CSF (PeproTech) for 6 days at 37 °C in a humidified 5 % CO₂ incubator. The purity and phenotype of monocytes and generated immature hmoDCs were analysed by flow cytometry with lineage-specific markers: HLA-DR, CD86, CD14 and CD83 (158, 160, 161).

3.4. Purification of naïve CD4⁺ T cells

Naïve CD4⁺ T cells were purified from PBMC using the "Naïve CD4⁺ T Cell Isolation Kit" (Miltenyi Biotec) following the manufacturer's protocol and using the autoMACS Pro (Miltenyi Biotec). The purity and phenotype of naïve CD4⁺ T cells were evaluated by flow cytometry with the following markers: CD3, CD4 and CD45RA (160, 161).

3.5. Purification of total blood DCs

Total blood DC fraction was obtained from PBMC using “Blood Dendritic Cell Isolation Kit II” (Miltenyi Biotec) following the manufacturer’s protocol and using the autoMACS Pro (Miltenyi Biotec). We obtained an enriched fraction containing mDCs and pDCs. The purity was analysed using the following markers: HLA-DR, CD1c, CD303 and CD19 (70, 159, 160).

Cell viability was analysed in all the cases with trypan blue (Gibco) exclusion by light microscope.

4. Cell cultures procedures

4.1. Culture of TMC

TMC were seeded at a density of 10^6 cells/mL and stimulated with the following combination of mAbs to induce T cell proliferation: anti-CD2 (0.5 μ g/mL), anti-CD3 (0.5 μ g/mL) and anti-CD28 mAb (0.5 μ g/mL) in the absence or presence of different doses of WIN55212-2, HU210 and HU308 (1 or 10 μ M) for 5 days. After 5 days, cell-free supernatants were collected for cytokine determination by Luminex magnetic bead immunoassays (BioRad) and cell pellets were used for gene expression analysis by quantitative PCR. Proliferative responses were measured by adding 1 μ Ci [3 H]-thymidine during the last 8 hours of culture (157).

4.2. Culture of human DCs

For titration experiments, immature hmoDCs from healthy donors (10^6 cells/mL) were treated with LPS plus different doses of WIN55212-2, HU210 or HU308 (1, 5, 10 or 20 μ M) or with TLR2L (25 ng/mL) or MFs (25 ng/mL IL-1 β and 50 ng/mL TNF α) plus different doses of WIN55212-2 (5, 10 or 20 μ M) for 18 hours at 37 °C in a humidified 5 % CO₂ incubator.

Immature hmoDCs or human total blood DCs from healthy donors (10^6 cells/mL) were stimulated with medium (unstimulated), WIN55212-2 (10 μ M), LPS (0.1 μ g/mL), LPS plus WIN55212-2, CPE (100 μ g/mL) or CPE plus WIN55212-2 for 18 hours at 37 °C in a humidified 5 % CO₂ incubator. For inhibition experiments, hmoDCs were preincubated for 1 hour with Rimonabant (20 μ M), AM630 (20 μ M), GW6471 (25 μ M), GW9662 (10 μ M) or 3-MA (25 μ M) or corresponding vehicle controls prior to activation. Then, the cells were stimulated with LPS plus WIN55212-2 for 18 hours in the presence of the corresponding inhibitors. Cells were used to analyse their phenotype by flow cytometry using the following markers: HLA-DR, CD86 and CD83 and by qPCR for the analysis of the mRNA levels of IL-8, IL-1 β , IL-6, TNF α and IL-10. Cell-free supernatants were used to quantify IL-8, IL-1 β , IL-6, TNF α and IL-10 by sandwich enzyme-linked immunosorbent assay (ELISA). Cell viability was analysed in all the cases with trypan

blue (Gibco) exclusion by light microscope and/or with propidium iodide (InvitroGen) flow cytometry analysis.

4.3. Coculture experiments of DCs and naïve CD4⁺ T cells

HmoDCs or human total blood DC fraction treated with medium (unstimulated), WIN55212-2 (10 µM), LPS (0.1 µg/mL), the combination of WIN55212-2 and LPS, CPE (100 µg/mL) or CPE plus WIN55212-2 for 18 hours were cocultured with purified allogeneic naïve CD4⁺ T cells (DC:T cell ratio of 1:5) for 5 days (**Figure M.17**). IL-17A, IFN γ , IL-13, IL-5 and IL-10 were quantified in cell-free supernatants by ELISA or Luminex magnetic bead immunoassays. Cells were used to analyse T cell phenotype by flow cytometry using the following markers: CD3, CD4, CD127, CD25, FOXP3 and IL-10.

For intracellular IL-10 production, the primed CD4⁺ T cells were washed and restimulated with PMA (25 ng/mL) plus ionomycin (1 mg/mL) (Sigma-Aldrich) for 6 hours, stimulating intracellular cytokine production. Brefeldin A (10 µg/mL) (BD Biosciences) was added during the last 4 hours, inhibiting cytokine secretion to extracellular medium.

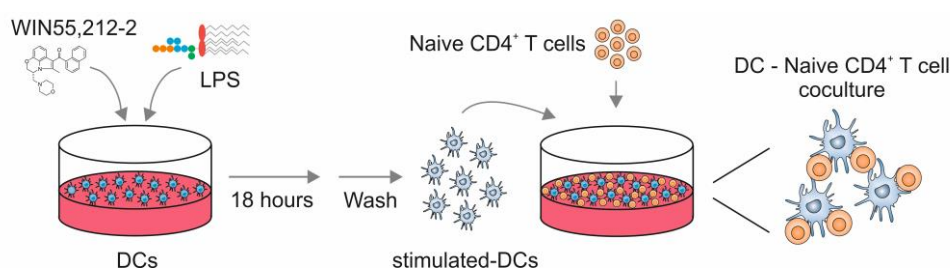


Figure M.17. Scheme of coculture experiments of DCs and naïve CD4⁺ T cells.

4.4. Treg suppression assay

CD4⁺CD25⁺CD127⁻FOXP3⁺ Treg cells induced by allogeneic WIN55212-2 plus LPS-stimulated hmoDCs were purified by cell sorting of CD4⁺CD25⁺CD127⁻ population. Purified Treg cells were mixed with CFSE-labelled autologous PBMC (responder cells) at different ratios and stimulated with the following combination of mAbs to induce T cell proliferation: anti-CD2 (0.5 µg/mL), anti-CD3 (0.5 µg/mL) and anti-CD28 mAb (0.5 µg/mL) for 5 days. For control purposes, CFSE-labelled PBMCs were cultured alone with or without stimulation. Proliferation of CD4⁺ T cells was determined by using CFSE dilution with flow cytometry (70, 159).

4.5. Air – liquid interface (ALI) cultures

Primary HBECs of healthy donors were purchased from Epithelix. Cells were cultured as monolayers placed in a 75 cm² T-flask at 37 °C in 5 % CO₂. HBEC in passage 2 or 3 were seeded in air-liquid interface (ALI) cultures onto 6.5 mm diameter polyester membrane transwells with a pore size of 0.4 µm (Costar) at a density of 150000 cells/well. Medium was changed every two days. Cells were grown submerged for 4 days

to complete confluence. Then, medium in the apical compartment was removed to allow the cell differentiation. Epithelial cell cultures were used on day 21 at the ALI (162, 163). ALI cultures were infected apically with human rhinovirus-16 (HRV-16) with a multiplicity of infection (MOI) of 50 (50 viral particle/cell) in the presence of basal 2.5 μ M WIN55212-2 or vehicle (DMSO) for 72 hours. Basal media samples were collected for cytokine production analysis and cells were used for gene expression profiling or immunofluorescence staining.

5. Flow cytometry

5.1. Surface staining

Cells were washed with PBS/EDTA (2 mM) 0.5 % BSA and labelling of cell-surface markers was performed at room temperature for 15 minutes in darkness with fluorescence-labelled antibodies (**Table M.4**) or corresponding isotype controls. Cells were diluted in 0.1 % PBS/PFA for flow cytometry analysis in the FACScalibur cytometer or FACS Aria III (both from Beckton Dickinson) in the Flow Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid.

5.2. Intracellular staining

After surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 minutes in darkness. Then, cells were washed twice with Perm/Wash Buffer (BD Biosciences) and incubated with fluorochrome-labeled monoclonal antibody to IL-10 for 30 minutes at room temperature. Cells were fixed with 1 % PBS/PFA and analysed by flow cytometry in the FACScalibur cytometer (Beckton Dickinson) in the Flow Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid (160, 161).

5.3. Intranuclear staining

After surface staining, cells were fixed and permeabilized with FOXP3 Fix/Perm buffer for 20 minutes in darkness at room temperature. A second permeabilization was carried out with FOXP3 Perm buffer for 15 minutes in darkness at room temperature. Then, cells were stained with Alexa Fluor 488 anti-human FOXP3 for 30 minutes in darkness at room temperature. Cells were diluted in 1 % PBS/PFA for flow cytometry analysis in the FACScalibur cytometer or FACS Aria III (both from Beckton Dickinson) in the Flow Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid (70, 159).

5.4. CB1 staining

Cells were incubated with Alexa Fluor 488 alkyne or HU210-Alexa488 probe at a final concentration of 1 μ M in PBS for 30 minutes at room temperature with shaking. After

that, cells were washed with PBS and fixed with 0.1 % PBS/PFA. For co-staining with mAbs, cells were first stained with the corresponding fluorescence-labelled antibodies for 15 minutes at room temperature, washed with PBS and then stained with Alexa Fluor 488 alkyne or HU210-Alexa488 probe as described above. Flow cytometry analysis was performed using Galios flow cytometer (Beckman Coulter) (157).

Table M.4. Flow cytometry antibodies

Name	Supplier	Clone	Conjugate
Human FcR Blocking	Miltenyi	-	-
Human HLA-DR	Biolegend	L243	FITC, APC
Human CD86	Miltenyi	FM95	PE
Human CD83	Miltenyi	HB15	APC
Human CD127	Biolegend	A019D5	PE
Human FOXP3	Biolegend	259D	Alexa488
Human CD4	Biolegend	OKT4	PerCP, PE/Cy5.5
Human CD25	Biolegend	BC96	APC
Human IL-10	Biolegend	JES3-19F1	PE
Human CB2	Invitrogen	-	-
Human CD123	Biolegend	6H6	FITC, Alexa647
Human CD1c	Biolegend	L161	FITC, Alexa647
Human CD3	BD Pharmingen	OKT3	APC, Alexa647
Human CD19	Biolegend	HIB19	PE/Cy7
Human CD8	Biolegend	53-6.7	Alexa700
Human CD14	Biolegend	RMO52	PE
Human CD303	Miltenyi	AC144	PE
Mouse FOXP3	Biolegend	MF-14	Alexa488
Mouse CD25	Biolegend	PC61	PE
Mouse CD4	Biolegend	GK1.5	PerCP
Mouse CD11c	Biolegend	N418	APC
Mouse MHII	Biolegend	M5/114.15.2	PE/Cy7
Mouse CD11b	Biolegend	M1/70	PE
Mouse CD103	Biolegend	3E7	FITC
Mouse CD86	Biolegend	GL-1	BV605
Mouse CD40	Biolegend	3/23	PE
Mouse CD80	Biolegend	16-10A1	FITC
Mouse OX40L	Biolegend	RM134L	APC
Mouse CCR7	Biolegend	4B12	BV421

Flow cytometry analyses were performed using Weasel or FlowJo software.

6. Confocal microscopy

6.1. Visualization of CB1 in suspension cells

Cells were suspended in PBS, centrifuged onto glass slides (cytospin, 200 rpm, 2 minutes) and fixed in 4 % PFA for 7 minutes. Then, cells were washed twice with PBS and stained following the CB1 staining protocol as described above. Then, samples were

rinsed twice with PBS and treated with 1 % PBS/PFA and mounted with ProLong Gold antifade reagent with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) for nuclei staining. All assay and control samples were imaged under the same microscope conditions. Specimens were examined under a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy GmbH) (157).

6.2. Immunohistochemistry of tonsil sections

Palatine tonsil tissues (source: Swiss Institute of Allergy and Asthma Research (SIAF) Biobank) were embedded in Tissue-Tek (Sakura Finetek), frozen in liquid nitrogen-cooled 2-methylbutane (Fluka), and stored at -80 °C until cryosections were cut using a HM 500 OM microtome (Mikrotom). Paraformaldehyde-fixed cryosections (7 µm) were sequentially stained with the corresponding antibodies or isotype controls.

The slides were washed with PBS-Tween and PBS and incubated with HU210-Alexa488 probe (50 nM) or Alexa Fluor 488 alkyne (50 nM). After washing with PBS, the sections were treated with 1 % PFA, washed with PBS and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) for nuclei demonstration. Nonspecific fluorescence was determined by colabelling in the presence of 5 µM HU210. Images were acquired and analyzed using the confocal microscope LMS 780 Zeiss (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The general histology of palatine tonsils was demonstrated by staining of the sections with hematoxylin and eosin (H&E) (157).

6.3. Visualization of LC3

Cells were washed with PBS and fixed with 4 % PFA for 10 minutes. Then, cells were washed twice with PBS and permeabilized with 0.03 % Triton X-100 in PBS containing 3 % BSA for 30 minutes. After wash with PBS, cells were incubated with the primary antibody anti-LC3 (1:300) in PBS for 1 hour at room temperature. Then, cells were washed with PBS for 30 minutes, incubated with the secondary antibody (anti-rabbit Alexa 488, 1:1000) in PBS for 1 hour and washed with PBS for 30 minutes (164). Stained cells were subjected to cytospin (600 g for 8 minutes), fixed with ice-cold methanol for 5 minutes, and stained with ProLong Gold-DAPI (Dako) 24 hours before analysis. Images were acquired and analyzed using the confocal microscope LMS 780 Zeiss (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

6.4. Visualization of tight junctions

ALI cultures were fixed with 4 % PFA 72 hours after HRV-16 exposure in the presence or absence of WIN55212-2. ALI samples were blocked with goat serum in 1 % BSA/PBS and incubated with Alexa Fluor 488 anti-occludin antibody (Invitrogen) and anti-zonula occludens 1 (ZO-1)–unlabeled antibody (Invitrogen) and then detected with Alexa Fluor 546–labeled goat anti-rabbit secondary antibody (Invitrogen). Samples were

counterstained with DAPI for nuclear staining and mounted with ProLong-Gold containing antifade reagent (Invitrogen) (162). All assay and control samples were imaged under the same microscope conditions. Specimens were examined under a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy GmbH).

7. Cytokine quantification

7.1. ELISA

Concentrations of human or mouse IL-8, IL-1 β , IL-6, TNF α , IL-10, IFN- γ , IL-13 and IL-5 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences, San Jose, CA). IL-17A levels were quantified by the IL-17 quantikine Elisa Kit (RD Systems). In all cases, manufacturer's instructions were followed with minor modifications. Briefly, the catching mAbs were coated onto microtiter plates that were blocked with assay diluent (PBS 1X with 5 % FBS) and incubated with the standards or samples. After washing, biotinylated anti-human mAbs and streptavidin-labelled peroxidase were added to detect bound cytokines. After washing, chromogenic substrate (0.63 mg/mL OPD, 0.03 % H₂O₂ in 1 M sodium citrate) was added. The colour reaction was stopped by adding 3 N H₂SO₄ and the OD values were measured at 492 or 450 nm according to each kit.

7.2. Luminex

Concentrations of human IL-8, IL-6, IL-15, IL-5, IL-10, IL-17, IL-13, IFN γ GM-CSF, G-CSF, VEGF, FGF, RANTES, IP-10 and MCP-1 in cell-free supernatants were quantified by luminex magnetic bead immunoassays following the manufacturer's instruction (BioRad).

8. RNA extraction, cDNA synthesis, and quantitative real-time PCR

RNA was isolated from harvested cells using the RNeasy Mini Kit or RNeasy Plus Micro Kit (both from Qiagen) according to the manufacturer's instructions. cDNA was generated using a PrimeScript RT reagent Kit (Takara). Real-time quantitative PCR was performed on cDNA using FastStart Universal SYBR Green Master (Roche). The sequences of the employed pair primers are shown in **Table M.5**. Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems). Data were normalized to EF1 α and displayed as fold change ($2^{-\Delta\Delta CT}$) relative to unstimulated cells. ΔCT is defined as the difference between the cycle threshold value for the gene of interest and EF1 α .

Table M.5. Primers for real-time qPCR.

Gene	Forward	Reverse
EF1 α	CTGAACCATCCAGGCCAAAT	GCCGTGTGCAATCCAAT
CB1	AAGGTGACATGGCATCCAAAT	AGGACGAGAGAGACTTGTGTAA
CB2	CACTGATCCCCAATGACTAC	CCACTCCGTAGAGCATAGAT
FAAH	GGCCACACCTTCCTACAGAA	GTTTTGCGGTACACCTCGAT
MAGL	CTGAACCATCCAGGCCAAAT	GCCGTGTGGCAATCCAAT
PPAR α	CATTACGGAGTCCACGCGT	ACCAGCTTGAGTCGAATCGTT
PPAR γ	GAACAGATCCAGTGGTTGCAG	CAGGCTCCACTTTGATTGCAC
IL-1 β	TTTTTGCTGTGAGTCCCGGAG	TTCGACACATGGGATAACGAGG
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL-8	GCAGCTCTGTGTGAAGGTGCAGTT	TTCTGTGTTGGCGCAGTGTGGTC
IL-10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT
ATG5	GCAGATGGACAGTTGCACACAC	GAGGTGTTTCCAACATTGGCTCA
ATG12	TAGAGCGAACACGAACCATCC	CACTGCCAAAACACTCATAGAGA
ATG14	GAGGAAGTAAAGACGGGTGTG	CCATTGTTAGGGAGGCTAATCC
PIK3C3	GGGATTAGTGCTGAGGTCATG	AGTCTATGTGGAAGAGTTGCC
ULK1	GCAAGGACTCTTCCTGTGACAC	CCACTGCACATCAGGCTGTCTG
RUBCN	AGGCCCCAGGAATATCACCA	TCTTCGGAACATGCCTTCCC
BECN1	CCATGCAGGTGAGCTTCGT	GAATCTGCGAGAGACACCATC
ATG16L	TGCAGTTCAGTCCAGGTTT	GCTAAGAGGTAAGATCCAGCAC
Occludin	GATGAGCAGCCCCCAAT	GGTGAAGGCACGTCTGTGT
ZO-1	ACAGTCCCTAAAGCTATTCCTGTGA	TCGGGAATGGCTCCTTGA
Claudin-1	GAGTCAATGCCAGGTACGAATTT	AAGTAGGGCACCTCCCAG
Claudin-4	TGTACCAACTGCCTGGAGGAT	GACACCGGGACTATCACCATA
Claudin-7	GGGCATGAAGTGCACGCGCT	CGGCAAGACCTGCCACGATG
IFN γ	TCTCGGAAACGATGAAATATACAAGTTAT	GTAACAGCCAAGAGAACCCAAAA
IL-5	GACCTTGGCACTGCTTTCTACTC	TGTACAGGAACAGGAATCCTCAGA
IL-13	GCCCTGGAATCCCTGATCA	GCTCAGCATCCTCTGGGTCTT
IL-17	CCATCCCCAGTTGATTGGAA	CTCAGCAGCAGTAGCAGTGACA
GLUT1	GGCTTCTCCAACCTGGACCTC	CCGGAAGCGATCTCATCGAA
HK2	TTCGCACTGAGTTTGACCAG	TCACCAGGATAAGCCTCACC
PFKFB3	AGCCCGGATTACAAAGACTGC	GGTAGCTGGCTTCATAGCAAC
LDHA	ATGGCAACTCTAAAGGATCAGC	CCAACCCCAACAACCTGTAATCT
HIF1A	TCGCATCTTGATAAGGCCTCT	ACAAAACCATCCAAGGCTTTCA
PDHA1	ATGGAATGGGAACGTCTGTTG	CCTCTCGGACGCACAGGATA
IDH3A	AGGTTTTACTGGTGGTGTTC	CCTCCCACTGAATAGGTGCTT
SDHA	CGAACGTCTTCAGGTGCTTT	AAGAACATCGGAACCTGCGAC
ATP5A1	AAGACACGCCAGTTTCTTC	TTTGGGTTTCATCTTTCATTGC
PINK1	CCCAAGCAACTAGCCCCTC	CCCAAGCAACTAGCCCCTC
ACADM	GGAAAGCTACTTGTAGAGCACCA	ACGACCAGAATCAACCTCCC
CPT1A	GACTCTGGAAACGGCCAACT	ATCTTGCCGTGCTCAGTGAA
GLS	GCAGTCTGGAGGAAAGGTTGC	CACACCCACAAATCGGGAC
SLC1A3	CTGGGGACCTCTCAAGTTCTG	GGGAGCACGAATCTGGTGAC
mIL-6	GAAACCGCTATGAAGTCTCTCTG	GTATCCTCTGTGAAGTCTCCTCTCC
mIL-10	GGCCCAGAAATCAAGGAGCA	AGGGGAGAAATCGATGACAGC
mATG5	GCAGATGGACAGTTGCACACAC	GAGGTGTTTCCAACATTGGCTCA

mATG12	TAGAGCGAACACGAACCATCC	CACTGCCAAAACACTCATAGAGA
mATG14	AACCCAAGGACCTGACATGGA	ACACAATGTTGACGAGCTGC
mLC3A	CGCTACAAGGGTGAGAAGCA	GCGGCGCCGGATGAT
mGAPDH	TCCTGCACCACCAACTGCTTA	GCCATCACGCCACAGCTTTC

9. Western blot

Freshly isolated hmoDCs were treated with medium (unstimulated), WIN55212-2 (10 μ M), LPS (0.1 μ g/mL) or the combination of WIN55212-2 and LPS for 15, 30 minutes or 18 hours at 37°C. For inhibition experiments, cells were preincubated during 1 hour with Rimonabant (20 μ M) or GW6471 (25 μ M). After the corresponding time, cells were harvested and lysed with RIPA buffer (Thermo Fisher Scientific) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling) and 1 mM PMSF (Sigma) for 30 minutes at 4°C. Lysates were centrifuged for 15 minutes at 10000 g and 4 °C, and supernatants were collected for protein quantification with Micro BCA Kit (Pierce, Rockford, Ill), following the manufacturer's instructions. Ten micrograms of total protein from cell lysates were separated by means of SDS-PAGE and transferred onto a nitrocellulose or PVDF membrane (Bio-Rad Laboratories). The membrane was blocked with 5 % BSA, 0.1 % Tween-20 in tris buffered saline for 1 hour and incubated with primary antibodies (**Table M.6**). Then, the membrane was washed and incubated with goat anti-rabbit (1:3000; Bio-Rad Laboratories) or goat anti-mouse (1:2500, Pierce) conjugated with horseradish peroxidase as a secondary antibody. Reactive bands were visualized with the Clarity Western ECL Substrate (Bio-Rad Laboratories) in a Fujifilm LAS-3000 developer. The OD of the reactive bands was quantified with Fujifilm Multi Gauge software, and values were expressed relative to the β -actin loading control.

Table M.6. Western blot antibodies

Name	Dilution	Supplier
PPAR α	1:1000	Invitrogen
PPAR γ	1:1000	Cell Signaling
Phospho-IKK α / β (Ser176/177)	1:1000	Cell Signaling
Phospho-IK β (Ser32/35)	1:1000	Cell Signaling
Phospho-p38 MAPK (Thr180/182)	1:1000	Cell Signaling
Phospho-ERK (Thr202/Tyr204)	1:1000	Cell Signaling
Phospho-SAPK/JNK (Thr183/Tyr185)	1:1000	Cell Signaling
Phospho-p70 S6 kinase (Thr389)	1:500	Cell Signaling
Phospho-Akt (Ser473)	1:1000	Cell Signaling
Phospho-AMPK (Thr172)	1:1000	Cell Signaling
LC3-I/II	1:2000	Sigma-Aldrich
B-Actin	1:15000	Sigma-Aldrich

10. Metabolic studies

The Warburg effect in stimulated hmoDC cultures was determined photometrically 18 hours after stimulation by quantifying the OD at 570 nm and calculating the Warburg effect as $1/OD_{570nm}$ normalized to the unstimulated condition. Glucose concentrations in culture supernatants were determined 18 hours after stimulation by using the Glucose (GO) Assay Kit (Sigma-Aldrich). The metabolic rate was derived mathematically in percentage of medium without DCs (glucose concentration in RPMI 1640 = 2 mg/mL). Lactate concentrations in culture supernatants were determined 18 hours after stimulation by using the colorimetric L-Lactate Assay kit (Abcam), according to the manufacturer's recommendations. To measure mitochondrial membrane potential, MitoTracker Red CMXRos was used (250 nM, Thermo Fisher Scientific). NAD⁺ and NADH concentrations in lysates of DCs were determined by using NAD/NADH Assay Kit (Abcam), according to the manufacturer's recommendations. Reactive oxygen species (ROS) were measured by DCFDA Cellular ROS Detection Assay Kit (Abcam, Cambridge, United Kingdom) following manufacturer's instructions. Briefly, total ROS levels were measured using the cell permeant reagent 2',7' -dichlorofluorescein diacetate (DCFDA). Cells were stained with DCFDA for 30 minutes at 37 °C after 18 hours of treatment with the corresponding stimuli. Fluorescence was measured in a FLUOstar Omega microplate reader (Ex/Em = 485/535 nm). ATP concentrations in lysates of DCs were determined by using ATP Determination Kit (Invitrogen), according to the manufacturer's recommendations.

11. Transepithelial electrical resistance (TER) and paracellular flux measurement

TER measurements were done at 0, 4, 24, 48 and 72 hours after HRV-16 infection in the presence or absence of WIN55212-2 using Millicell-ERS Volt Ohm Meter (Millipore). Samples from each ALI culture donor were measured in triplicates (162).

Paracellular flux measurements were performed in ALI cultures after 72 hours of HRV-16 infection in the presence or absence of WIN55212-2. Paracellular permeability was evaluated 24 hours after apical addition of 2 mg/mL fluorescein isothiocyanate (FITC)-dextran based on the intensity of fluorescence in the basolateral medium, as determined with an ELISA reader (Mithra LB; Berthold Technologies) at 480 nm.

12. Animal models

12.1. LPS-induced sepsis mouse model

BALB/c mice (female, six-week-old, Charles River) were randomly divided into body weight-matched groups and intraperitoneally injected with vehicle, WIN55212-2 (5

mg/kg), LPS (10 or 20 mg/kg), LPS plus WIN55212-2 or LPS with WIN55212-2 in the presence of Rimonabant (5 mg/kg), GW6471 (5 mg/kg) or 3-MA (15 mg/kg). The survival rate 72 hours after LPS (20 mg/kg) administration was monitored. The weight loss was evaluated 24 hours after LPS (20 mg/kg) administration. Plasma samples and spleens were collected from mice at 16 hours after LPS administration. For mechanistic studies, assessment score were monitored and spleens were collected 72 hours after LPS (10 mg/kg) administration.

12.2. Plasma collection

Mice were anaesthetized with isoflurane, and peripheral blood was collected via retro-orbital bleeding using lime grass Pasteur pipettes and microtainer tubes (Beckton Dickinson). Samples were centrifuged at 10000 rpm for 10 minutes at room temperature, plasma was collected and stored at -80 °C for further analysis. Concentrations of IL-6 in plasma were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences).

12.3. Spleen processing and DC isolation

Spleens were minced and filtered through 40 µm nylon strainers to obtain a single-cell suspension. Then, red blood cells were lysed with ACK lysis buffer before being resuspended in cRPMI and assessing splenocyte viability using Trypan Blue exclusion by light microscope. Mice DCs from spleen were isolated using “Pan Dendritic Cell Isolation Kit, mouse” (Miltenyi). The purity and phenotype of DCs were analysed by flow cytometry with lineage-specific markers: class II MHC, CD11c, CD11b and CD86. Autophagy and cytokine induction in purified spleen DCs was evaluated by analysing mRNA levels of the genes IL-6, IL-10, ATG5, ATG12, ATG14 and LC3 by real time quantitative PCR. Spleen mice FOXP3⁺ Treg cells were analysed as described above.

12.4. Assesment score

The physical appearance of LPS-induced septic mice were scored according to the appearance of coat, activity and posture. The point of coat: 1, smooth; 2, mild ruffling; 3, significant ruffling. The point of activity: 1, normal; 2, moves slowly; 3, minimal movement with stimulation. The point of posture: 1, moving or resting normally; 2, resting together; 3, huddled. The assessment score is the total sum of the points from the above categories.

12.5. Peanut-epicutaneous sensitization

100 µg of crude peanut extract (CPE, 10 mg/mL) (Greer Laboratories) in the absence or presence of 10 µg WIN55212-2 was directly applied onto shaved and tape-stripped skin for 3 consecutive days. One day later, inguinal lymph nodes (ILN) were collected.

12.6. Inguinal lymph node processing

Inguinal lymph nodes (ILN) were triturated between frosted slides in RPMI medium, washed, and filtered (40 μ M). Phenotype of DCs in ILN was analysed by flow cytometry as described above.

12.7. Peanut-induced anaphylaxis mouse model

Epicutaneous sensitization

100 μ g of crude peanut extract (CPE, 10 mg/mL) (Greer Laboratories) in the absence or presence of 10 μ g WIN55212-2 was directly applied onto shaved and tape-stripped skin for 10 consecutive days.

Challenge

2.5 g of CPE alone or CPE plus 20 μ g of WIN55212-2 in 500 μ L of PBS was injected intraperitoneally 2 weeks after the last sensitization treatment. Mice were monitored for 40 minutes after challenge to assess anaphylactic response:

- 1) Clinical score. Anaphylactic symptom score were obtained by evaluating the following symptoms: hind leg scratching in ear canal, reduced movement, puffy eye and convulsion. The presence of each symptom was pointed with 1 point and the total score was the sum of the different points.
- 2) Core body temperature. Rectal temperature readings were performed every 10 minutes with a rectal probe digital thermometer (VWR).
- 3) Hemoconcentration. Peripheral blood were collected by retro-orbital bleeding into microcapillaries. The tubes were centrifuged for 5 minutes by using a microhematocrit centrifuge (QuercusLab). The hematocrit value was expressed as a percentage of cell volume.

12.8. Plasma peanut-specific immunoglobulins

Peanut-specific IgE and IgG1 were measured by ELISA. High binding 96-well plates (Corning) were coated with CPE (20 μ g/mL) in coating buffer at 4 °C overnight. Coated plates were blocked with FBS (10 %) in PBS for 2 hours at room temperature. Plates were washed and incubated with serum samples overnight at 4 °C. After washing, detection antibody (rat anti-mouse IgE-HRP (Southern Biotech) or goat anti-mouse IgG1-HRP (Thermo Fisher Scientific)) was added and incubated for 2 hours at room temperature. OPD was used to develop the assay and H₂SO₄ (3 N) was added to stop the reaction for absorbance reading at 492 nm.

12.9. Splenocyte cell culture

Spleens were processed and triplicates of 0.8×10^6 splenocytes were cultured in medium alone or with CPE (250 μ g/mL) in flat-bottom 96-well plates (Corning). After 5 days of

culture at 37 °C and 5 % CO₂, the triplicates were pooled, cells were collected for FOXP3 staining, and cell-free supernatants were used to quantify IFN γ , IL-5 and IL-10 by ELISA.

12.10. Peritoneal lavage

Peritoneal lavage fluid was collected 72 hours after challenge and eosinophils were identified by flow cytometry as described above.

13. Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6.0. All the data were expressed as mean $\pm$ SEM of the corresponding parameter. Statistical analysis was calculated using “One-way Anova”, “Paired t test” or “Spearman test”. Differences were considered statistically significant when * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS AND DISCUSSION

Objective 1: Development and validation of a fluorescent small molecule (HU210-Alexa488) probe to visualize and quantify CB1 in native systems

The human endocannabinoid system (ECS) is an essential signalling pathway involved in many physiological processes such as neuronal development, memory, proliferation, metabolism and immunity. Currently, the lack of appropriate tools has hampered the understanding of ECS signalling at the molecular level. Specifically, antibodies for CB1 have shown important limitations in terms of sensitivity and specificity. In addition, the batch-to-batch variations inherent in the way in which antibodies are produced has been recognized as an important problem of accuracy and reliability (165, 166). Therefore, there is a need for the development of tools that enable the direct visualization and quantification of CB1 in immune cells. The development and validation of tools that allows the study of CB1 expression might well contribute to understanding of the role of CB1 in different diseases.

A small molecule fluorescent probe to visualize and quantify CB1 should contain three main components: (i) a high affinity CB1 ligand, (ii) an appropriate fluorophore, and (iii) a suitable spacer to avoid potential steric interferences. The Medicinal Chemistry Lab at Complutense University developed a CB1-specific small molecule fluorescent probe (**Figure R.18**), which consist of the synthetic cannabinoid agonist HU210 (167), a CB1 and CB2 agonist, bound to the fluorophore Alexa Fluor 488. This probe is termed HU210-Alexa488 and keeps its high affinity for CB1, but not for CB2 (157).

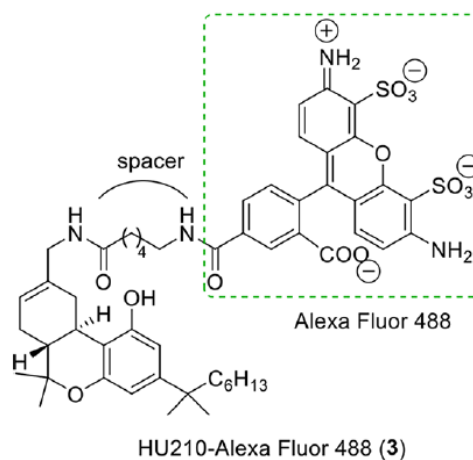


Figure R.18. Structure of HU210-Alexa488 probe.

1.1. Validation of HU210-Alexa488 as a novel probe to identify and quantify the expression of CB1 at the protein level in immune cells.

The capacity of the HU210-Alexa488 probe to identify and quantify peripheral blood CB1-expressing cells was assessed by flow cytometry and confocal microscopy. PBMC from healthy donors were stained with HU210-Alexa488 (1 μ M) or with Alexa Fluor 488 alkyne

(1 μ M) as control. We analysed the expression of CB1 in lymphocytes and monocytes by gating according to cell size and granularity (**Figure R.19**).

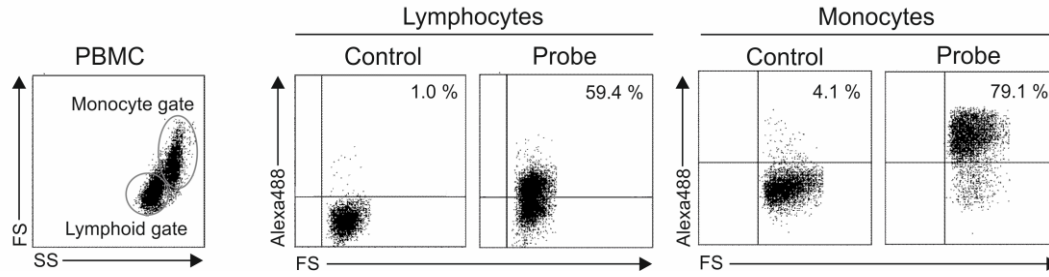


Figure R.19. Validation of HU210-Alexa488 probe in PBMC. Representative flow cytometry dot plots after staining with HU210-Alexa488 probe or Alexa 488 alkyne as control.

Around 60 % of lymphocytes and 80 % of monocytes stained positive with HU210-Alexa488 probe, whereas only around 1 % and 4 %, respectively, did so with control Alexa Fluor 488 alkyne (**Figure R.19**). CB1-expressing cells were visualized by confocal microscopy only in PBMC stained with HU210-Alexa488 probe, supporting the flow cytometry results (**Figure R.20**).

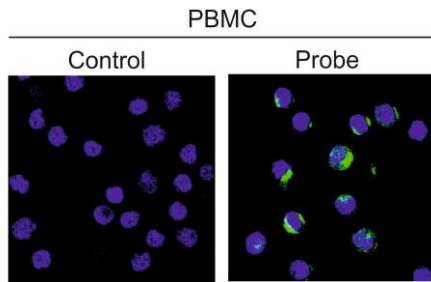


Figure R.20. Representative confocal microscopy images of PBMC after staining with HU210-Alexa488 probe or Alexa488 (control).

As negative control, we also used the HEK293T cell line, which does not constitutively express CBRs. HEK cells did not stain positive with HU210-Alexa488 probe beyond the basal background detected with the control, supporting that HU210-Alexa488 probe did not show no specificity binding (**Figure R.21**), which was also corroborated by confocal microscopy analysis (**Figure R.21**).

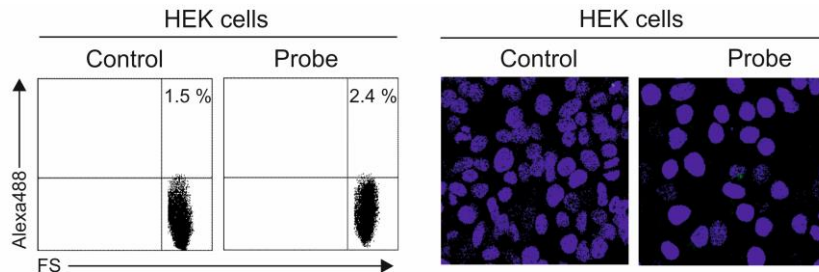


Figure R.21. Validation of the specificity of HU210-Alexa488 probe in HEK293T cell line. Representative flow cytometry dot plots after staining with HU210-Alexa488 probe or Alexa 488 alkyne as control. Representative confocal microscopy images of PBMC after staining with probe or control.

To confirm the specificity of HU210-Alexa488 for CB1, an excess of unlabelled HU210 (50 μ M) or CB2 selective agonist HU308 (167) (50 μ M) was added during the staining with HU210-Alexa488 probe. An excess of HU210, but not of HU308 significantly shifted the positive stained detected with HU210-Alexa488 probe in both lymphocytes and monocytes (**Figure R.22**), thus confirming the specificity of this probe to detect CB1-expressing cells by flow cytometry.

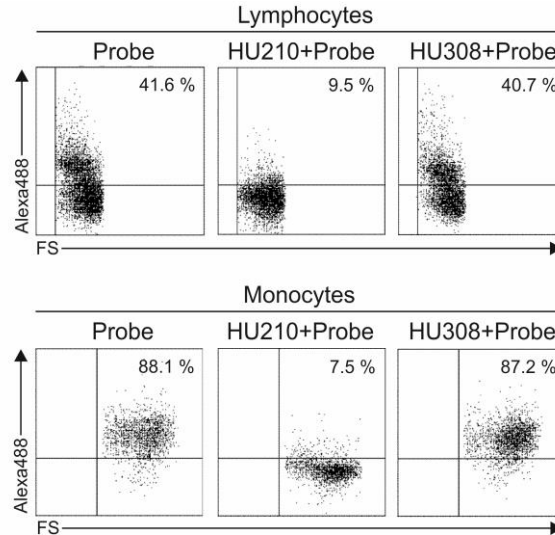


Figure R.22. Representative flow cytometry dot plots after staining with HU210-Alexa488 probe (1 μ M) alone or in the presence of HU210 (50 μ M) or HU308 (50 μ M) after gating in the lymphoid and monocyte areas.

Our cumulative data reveal that the percentage of CB1-expressing cells within monocytes is significantly higher than within lymphocytes. Moreover, mean fluorescence intensity (MFI) analysis for HU210-Alexa488 probe indicated that the density of CB1 was also significantly higher in monocytes than lymphocytes (**Figure R.23a**). Supporting these data, the CB1 mRNA levels were also significantly higher in purified monocytes than lymphocytes as determined by quantitative real-time PCR (**Figure R.23b**).

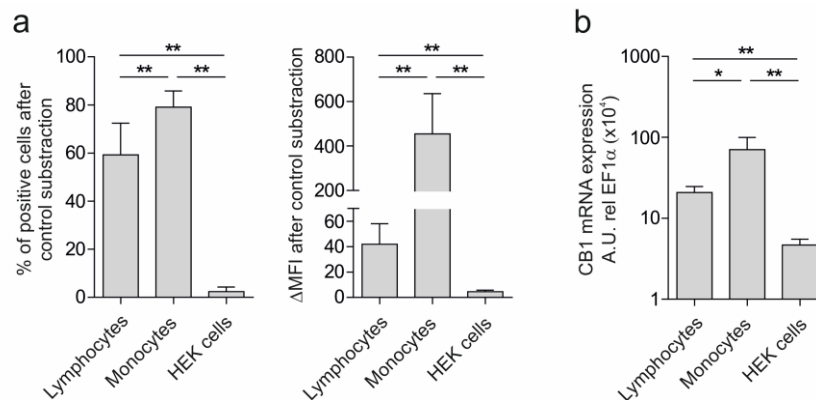


Figure R.23. **a** Percentage and mean fluorescence intensity (MFI) after control subtraction of lymphocytes and monocytes in PBMCs ($n = 6$) and HEK cells ($n = 4$) stained positive with HU210-Alexa488 probe. **b** mRNA expression levels of CB1 in purified lymphocytes, monocytes and HEK cells as determined by quantitative real-time PCR ($n = 4$). Values are mean $\pm$ SEM.

To further confirm the CB1 specificity of HU210-Alexa488 probe, we stained PBMC with the probe and retrieved the obtained positive and negative fractions after cell sorting by flow cytometry to quantify the mRNA expression of CB1. Our data demonstrated that the cells contained in the fraction that stain positive for the probe display significantly higher levels of CB1-specific mRNA than those contained in the negative fraction, supporting the correlation between HU210-Alexa488 probe fluorescence and CB1 expression (**Figure R.24**).

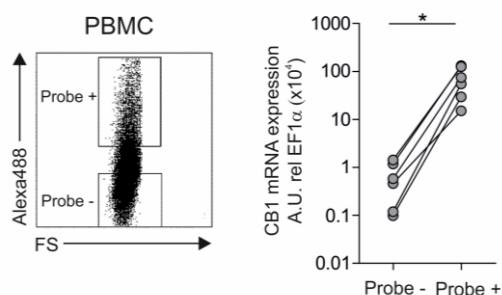


Figure R.24. Representative dot plot of cells stained negative and positive with HU210-Alexa488 probe in PBMC and employed gates for subsequent flow cytometry sorting. mRNA expression levels of CB1 in sorted cells stained negative and positive with the probe ($n = 6$). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$.

Collectively, all these data demonstrated that HU210-Alexa488 probe represent a fluorescent chemical probe suitable to identify and quantify peripheral blood CB1-expressing cells by flow cytometry.

Previous studies demonstrated that plasma membrane CB1 decreases following long agonist exposure as a consequence of finely regulated mechanisms that shuttle the receptor to lysosomes for degradation, contributing to the control of downstream signalling during sustained stimulation (168-170). To determine whether the probe could be used to monitor changes in plasma membrane CB1, we cultured PBMC in medium or stimulated with the synthetic cannabinoid agonists WIN55212-2 or HU210 for 18 hours and we tracked CB1 expression with the probe by flow cytometry.

The percentage of blood lymphocytes expressing CB1 as well as the expression levels of the receptor were significantly downregulated after prolonged stimulation with both synthetic cannabinoids, demonstrating that HU210-Alexa488 probe is able to detect up- and downregulation of plasma membrane CB1 in blood immune cells (**Figure 25a**). Next, we isolated tonsil mononuclear cells (TMC) from the tissue following our previously described protocols and assessed the capacity of HU210-Alexa488 probe to identify tonsil CB1-expressing immune cells by flow cytometry. Tonsils are easily accessible secondary lymph organs located at the main gateway of the respiratory and digestive tracts, representing the first contact point of the immune system with all the pathogens and allergens that enter the body via these ways (42, 171). We previously showed that human tonsils are organs where the induction of oral tolerance occurs through the generation of functional allergen-specific Treg cells (42, 71). Tonsils are organs where immune regulation to viral and bacterial infections, immunometabolism, and allergy takes

place (172-174). Interestingly, the expression of CB1 at the mRNA level is upregulated in TMC from allergic patients compared to healthy donors (131). The availability of chemical fluorescent probes to track up- and downregulation of tonsil CB1-expressing cells is of utmost importance to monitor changes in the expression of this receptor at the protein level. Our data revealed that HU210-Alexa488 probe also allows the detection of CB1-expressing cells in TMC. The percentage of CB1-expressing cells was also significantly downregulated in TMC stimulated with the synthetic cannabinoids WIN55212-2 or HU210 for 18 h compared to the unstimulated condition (**Figure R.25b**).

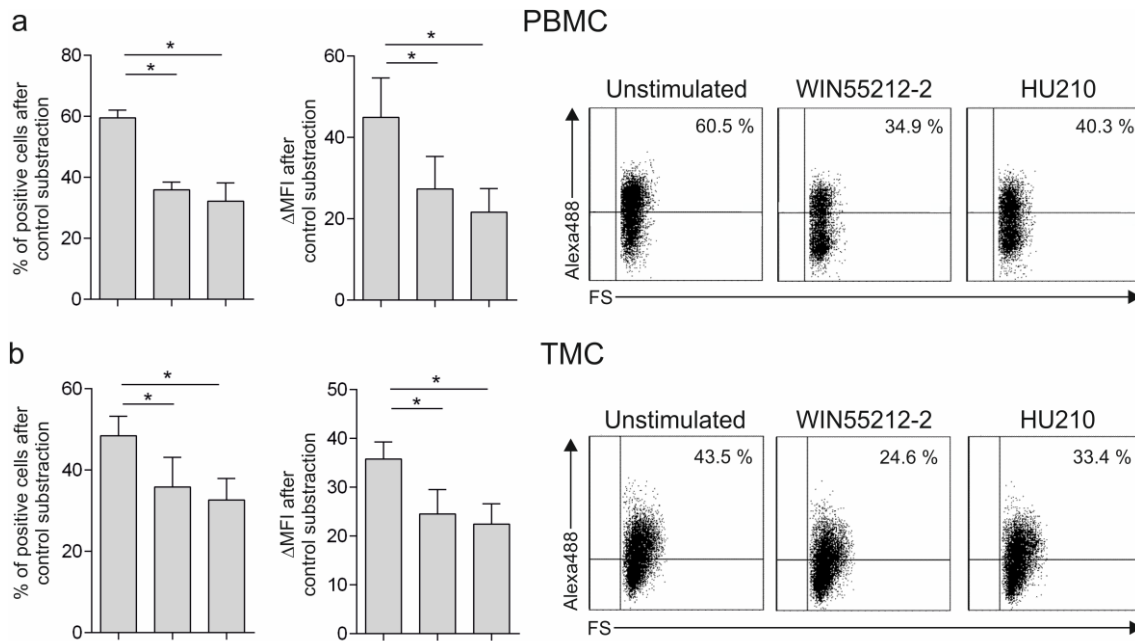


Figure R.25. Percentage and mean fluorescence intensity (MFI) of **a** PBMC and **b** TMC cultured in medium (unstimulated) or with cannabinoid agonists WIN55212-2 or HU210 for 18 hours and stained with HU210-Alexa488 probe ($n = 5$). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$.

Collectively, all these data demonstrated that HU210-Alexa488 probe is a suitable tool to monitor physiological changes in the expression pattern of plasma membrane CB1 in blood and tonsil immune cells by flow cytometry.

1.2. Visualization and quantification of CB1-expressing immune cells in human peripheral blood and tonsils with HU210-Alexa488 probe by flow cytometry and confocal microscopy.

1.2.1. Multiplexed flow cytometry analysis using HU210-Alexa488 probe

The availability of rapid and reliable assays to identify potential novel immune biomarkers remains a challenge (71, 175). In this regard, multiplexed flow cytometry combining lineage-specific markers for different immune cells in a single-step represent a very useful for easy and reproducible routine assays.

We showed that HU210-Alexa488 probe can be employed in combination with specific anti-human antibodies to simultaneously identify and quantify by flow cytometry the percentage of CB1-expressing cells among different immune cell subsets, including B cells (CD19⁺), T helper cells (CD3⁺CD4⁺) and cytotoxic T cells (CD3⁺CD8⁺).

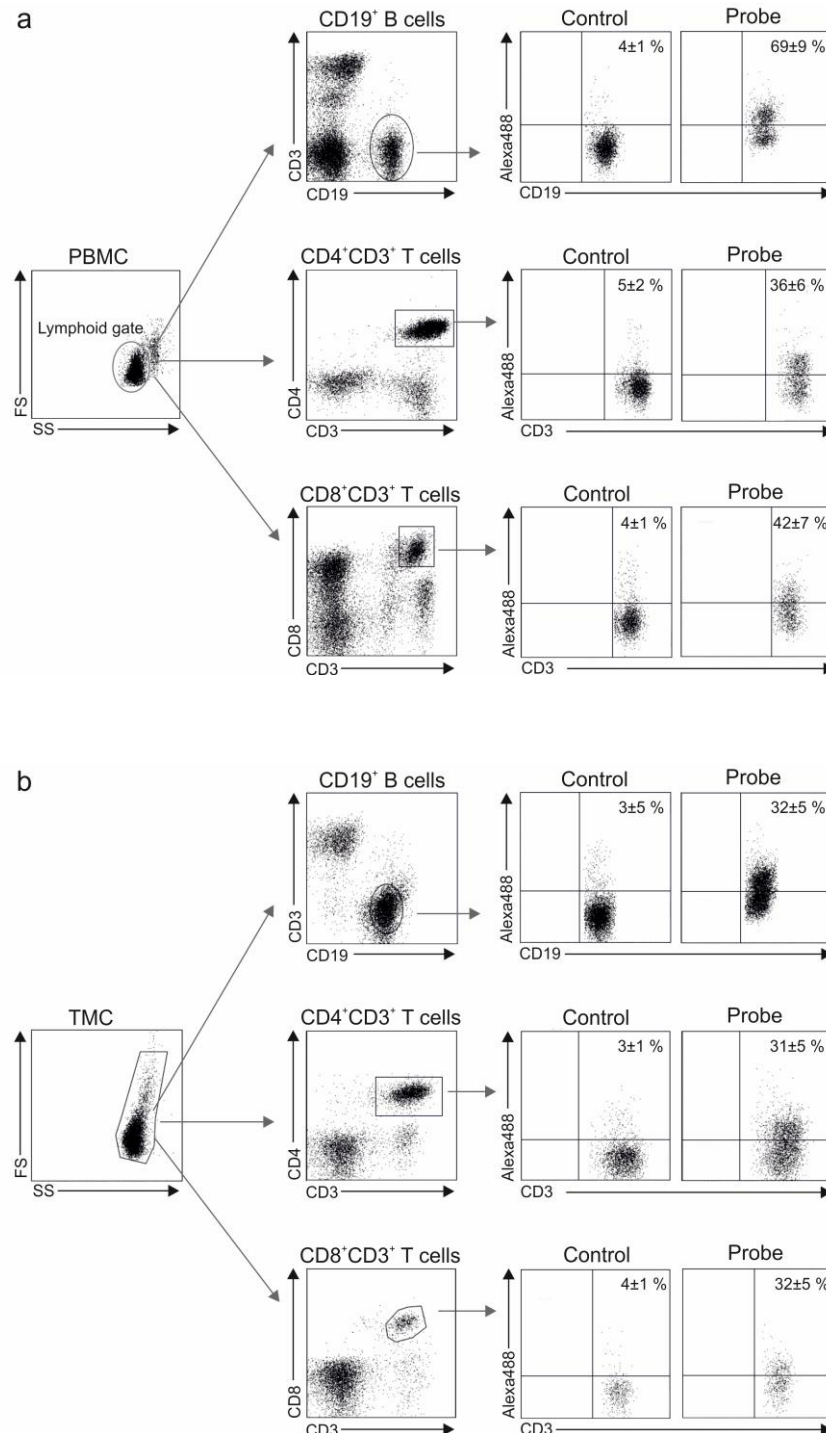


Figure R.26. Multiplexed flow cytometry in PBMC (**a**) and TMC (**b**) using HU210-Alexa488 probe in combination with anti-human lineage specific markers for B cells and T cell subsets. Representative dot plots and gating strategy employed to identify CB1-expressing cells are shown. The percentage of cells stained positive for control or probe are displayed inside the plots and represent means with SEM (n=7).

Our results revealed that HU210-Alexa488 probe allows the quantification of CB1-expressing cells within the B cells (70 %), T helper cells (36 %) and cytotoxic T cells (42 %) in PBMC by flow cytometry (**Figure R.26a**). We perform the same experiments using TMC. Around the 32 % of CD19⁺ B cells, 31 % of CD3⁺CD4⁺ T helper and 32 % of CD3⁺CD8⁺ cytotoxic T cells from TMC express CB1 as detected by flow cytometry with HU210-Alexa488 probe (**Figure R.26b**). Interestingly, the percentage of CB1-expressing B cells in PBMC was significantly higher than in TMC without significant differences at the T cell level (**Figure R.27**). In TMC, B cell represent around the 65 % of the total composition likely due to the high density of tonsil lymphoid follicles and germinal centers, which might well justify why the percentage of CB1-expressing B cells is lower in TMC than PBMC (42, 71).

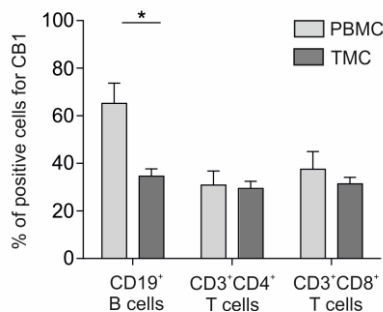


Figure R.27. Comparison of the percentage of CB1-expressing CD19⁺ B cells, CD3⁺CD4⁺ T helper cells and CD3⁺CD8⁺ cytotoxic T cells detected with HU210-Alexa488 probe in PBMC and TMC. Values are mean $\pm$ SEM. Statistical significance was determined using Paired *t* test. * $P < 0.05$.

Collectively, all these data demonstrated that HU210-Alexa488 probe can be used in multiplexed flow cytometry analysis to simultaneously quantify the percentage of CB1-expressing cells within B cells, T helper, and T cytotoxic T cell compartments in PBMC and TMC in an easy, quick and reproducible one-step staining.

1.2.2. Confocal microscopy analysis using HU210-Alexa488 probe.

To assess whether HU210-Alexa488 probe could be also used to visualize CB1-expressing cells subsets directly in tissues by confocal microscopy, we employed human tonsils. Tonsils are fully organized secondary lymphoid organs containing lympho-epithelial areas, B cells follicles with germinal centers, and T cell areas, in which proper immune responses to encounter antigens and pathogens are orchestrated (**Figure R.28**) (42, 171, 173).

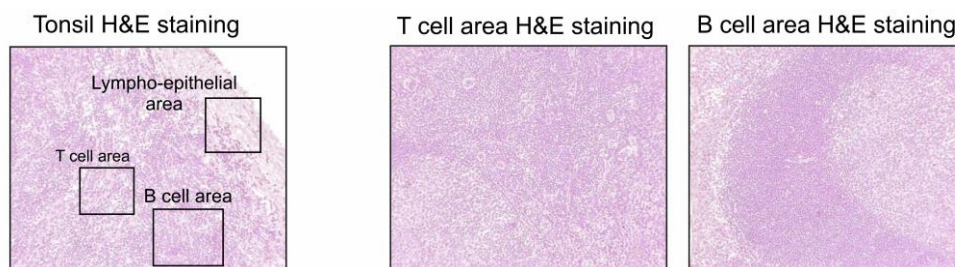


Figure R.28. Human tonsil sections stained with hematoxylin and eosin (H&E) to visualize the localization of lympho-epithelial, T and B cell areas within the tissue.

In a four colour-staining approach, we stained human tonsil with HU210-Alexa488 probe in combination with anti-human CD3, DAPI, and anti-human CD4 or CD8 to identify CD4⁺ T helper cells and CD8⁺ cytotoxic T cells, respectively. Our data showed that HU210-Alexa488 probe is a suitable tool to identify both CD4⁺ and CD8⁺ T cells expressing CB1 *ex vivo* in tonsil T cell areas by confocal microscopy (**Figure R.29a**). Similarly, the probe allowed the identification of CB1-expressing CD20⁺ B cells within the B cell areas by confocal microscopy (**Figure R.29b**).

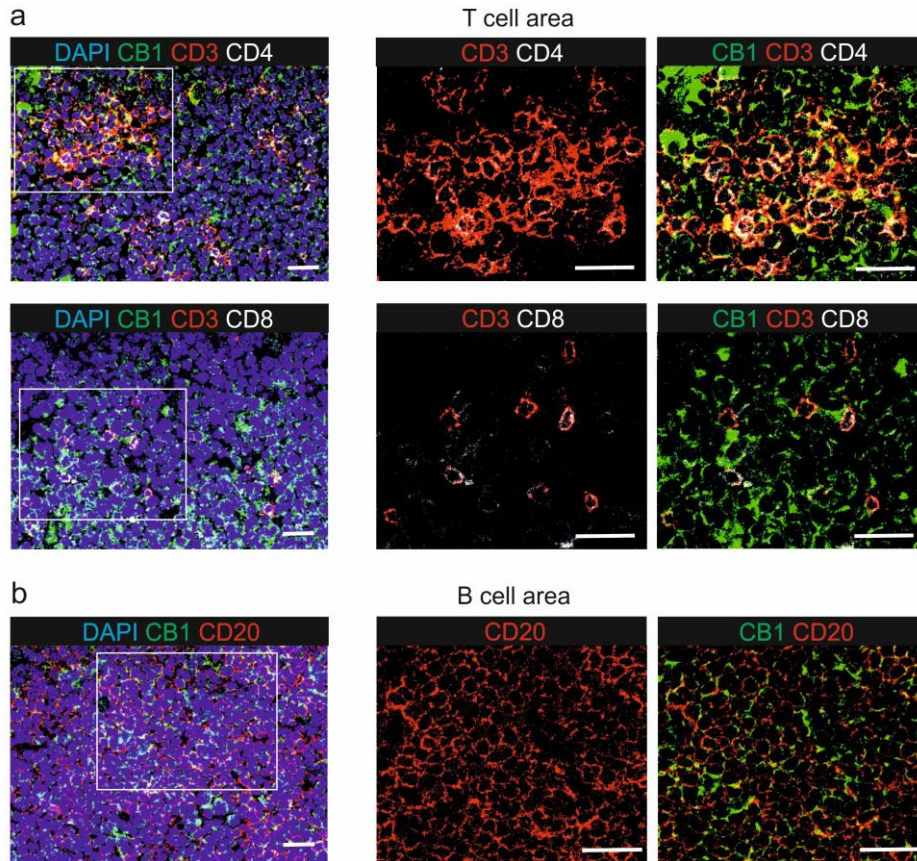


Figure R.29. Visualization of CB1-expressing T and B cells *ex vivo* in human tonsils with HU210-Alexa488 probe by confocal microscopy. **a** Histological sections were stained for CB1 (green) with probe, for CD3 (red), CD4 or CD8 (white) with specific antibodies and with DAPI (blue, nuclei). **b** Histological sections were stained for CB1 (green) with probe, for CD20 (red) with a specific antibody and with DAPI (blue, nuclei). The white square in the left side indicated the T or B cell area where the CB1-expressing cells are visualized. White bars, 10 μ m.

An excess of unlabelled HU210 completely blocked the positive staining detected with HU210-Alexa488 probe in both T and B cell areas, confirming the specificity of this fluorescent probe to visualize *ex vivo* tonsil CB1-expressing T and B cells by confocal microscopy (**Figure R.30**).

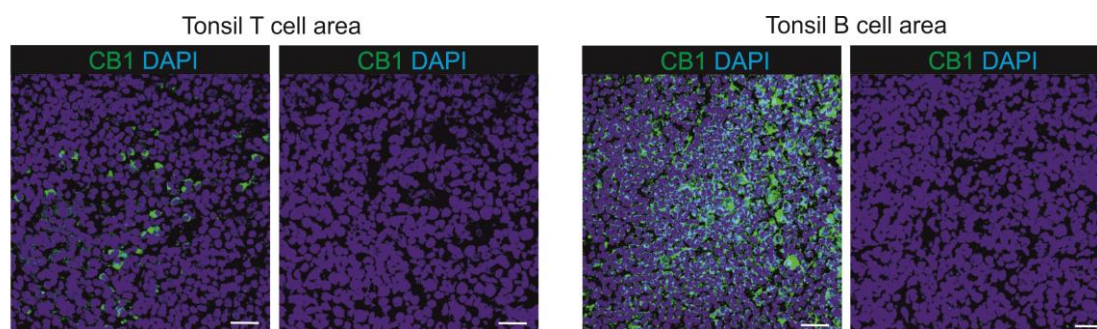


Figure R.30. Tonsil sections stained with HU210-Alexa488 probe alone or in the presence of an excess of unlabelled HU210. T and B areas are displayed. White bars, 10 μ m.

Collectively, our data demonstrated that HU210-Alexa488 probe allows *ex vivo* visualization of tonsil CB1-expressing T and B cells directly in human biopsies by confocal microscopy.

1.3. Functional characterization of CB1-expressing T cells in human tonsils.

Cannabinoids impair inflammation and display immune modulatory effects by mechanisms involving different peripheral blood CB1-expressing immune cells such as monocytes, T cells and to a lesser extent B cells (176, 177). The functional role of CB1 in human tonsils remains largely unknown. We stimulated TMC with anti-CD3/CD2/CD28 antibodies for 5 days to induce T cell proliferation in the presence of different doses of HU210 (CB1/CB2 agonist) or HU308 (CB2-selective agonist) and assessed T cells proliferation by measuring 3H-thymidine incorporation into DNA in proliferating cells. Our results showed that HU210 but not HU308 significantly increased the proliferation of tonsil T cells in a dose dependent manner (**Figure R.31**).

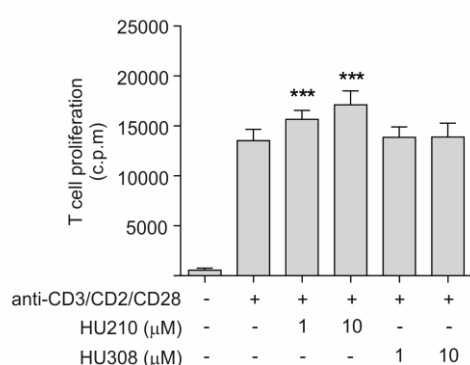


Figure R.31. T cell proliferation measured by using titrated thymidine incorporation as counts per minute (c.p.m.) (n = 6). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. *** P < 0.001.

After 5 days of culture, we measured T cell cytokines at the mRNA and protein level. Both HU210 and HU308 significantly impaired the production of IFN γ and IL-17 in activated TMC. The same tendency was observed at the mRNA level with statistical significance only observed for IFN γ (**Figure R.32**). Interestingly, HU210 but not HU308 significantly reduced the production of the Th2 cytokines IL-5 and IL-13 as well as IL-10

by activated TMC. The same tendency was observed at the mRNA level with statistical significance only observed for IL-10 (**Figure R.32**).

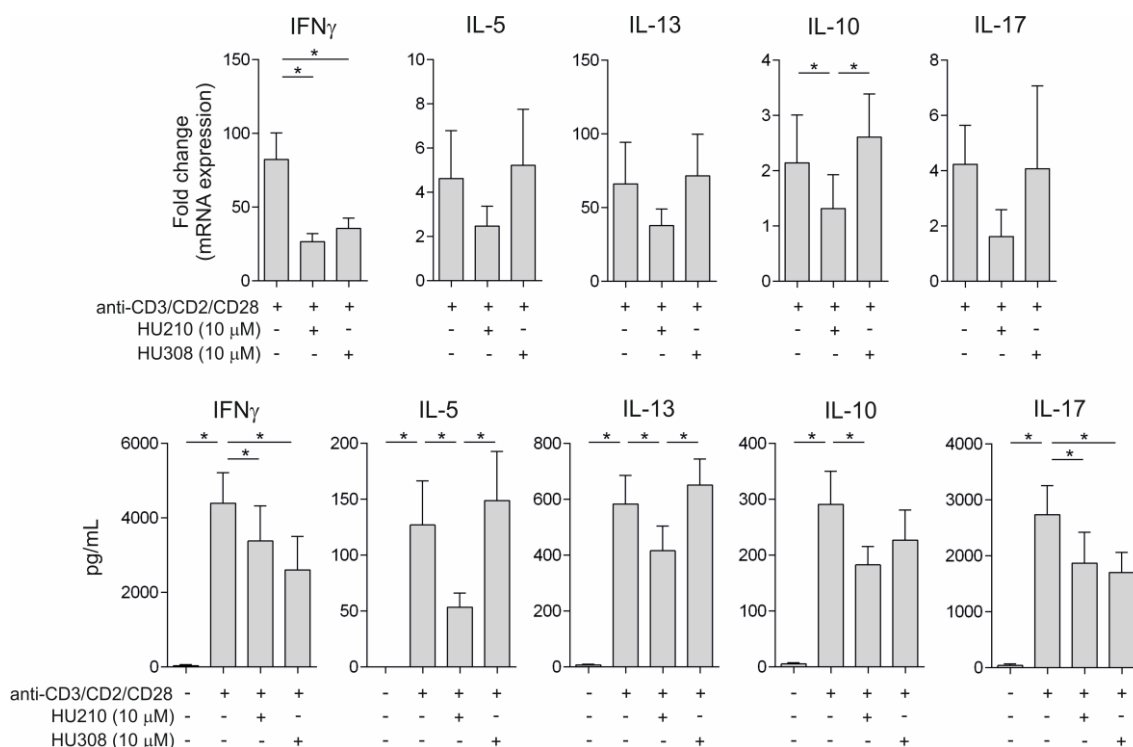


Figure R.32. mRNA and protein expression levels in cell-free supernatants of the indicated T cell cytokines in TMC stimulated for 5 days under the indicated conditions ($n = 6$). Fold changes with respect to the unstimulated conditions are shown. Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova * $P < 0.05$.

These data suggest that CB1 might be the main CBR involved in the increased tonsil T cell proliferation and in the impairment of Th2 and IL-10 cytokines. The fact that CB1 enhanced tonsil T cell proliferation while inhibiting proinflammatory cytokines suggest that CB1 might contribute to the expansion or the novo generation of tonsil Treg cells. Although tonsils contain high numbers of Treg cells and are organs where Treg generation occur, the potential contribution of CB1 to these mechanisms needs to be further investigated. Collectively, these data demonstrated that the tonsil CB1-expressing cells detected with HU210-Alexa488 probe harbour functional plasma membrane CB1 with immunomodulatory capacity that might play a role in the expansion or generation of tonsil Treg cells.

This work shows the development and validation of HU210-Alexa488 probe as the first fluorescent small molecule probe to directly visualize and quantify CB1-expressing immune cells in peripheral blood and tonsils by flow cytometry and confocal microscopy. The probe is compatible with conventional antihuman antibodies used to identify immune cell subsets and can be simultaneously combined with them for the rapid, easy, and reproducible identification of blood and tonsil immune cells expressing

CB1 in multiplexed flow cytometry assays. Human tonsils are the single easily accessible secondary lymphoid organs where immune regulation takes place. Therefore, the possibility of monitoring *ex vivo* CB1-expressing immune cells directly in the tissue opens a new avenue toward the better understanding of the *in vivo* functional role of CB1 in humans, the identification of this receptor as a new potential biomarker in different conditions as well as for the future development of innovative tonsil-targeted therapeutic interventions for many ECS-related diseases.

Objective 2: Study of the capacity of the synthetic cannabinoid WIN55212-2 to immunomodulate human DCs

DCs are professional antigen presenting cells that play a key role in the orchestration of immune responses by linking innate and adaptive immune responses. The main function of DCs is to capture antigens in the periphery and transport them to the closer lymph nodes. There, they present the processed antigens to naïve T cells, inducing T cell differentiation depending on the antigen, costimulatory signals and cytokines produced (14, 16). DCs are potential targets for cannabinoid-mediated modulation (178), but the detailed molecular mechanisms by which cannabinoids might regulate the function of DCs remain poorly understood. In this chapter, we investigated the mechanisms involved in the immunomodulation of DCs by synthetic cannabinoids.

2.1. Analysis of the CBR expression in human DCs and determination of the anti-inflammatory properties of different synthetic cannabinoids.

Currently, there are few studies investigating the expression and function of CBRs in human DCs (179). We wanted to confirm the expression of CB1 and CB2 in different populations of human DCs. For that, human monocyte-derived DCs (hmoDCs), purified human blood plasmacytoid (pDCs) and CD1c⁺ myeloid DCs (mDCs) were stained with HU210-Alexa488 probe, the validated probe for CB1, or with an anti-CB2 polyclonal antibody, and the expression of CB1 and CB2, respectively, was monitored by flow cytometry. HmoDCs and mDCs expressed both CB1 and CB2 at the protein level, whereas pDCs expressed CB1 but not CB2 (**Figure R.33**). Alexa Fluor 488 or proper matched isotype controls for the employed antibodies were negative in all the assayed conditions (**Figure R.33**).

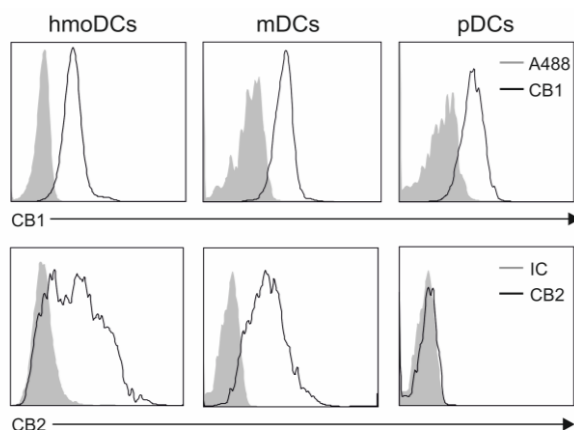


Figure R.33. Human DCs express CBRs at protein level. Flow cytometry histograms of CB1 and CB2 expression in hmoDCs, purified human blood mDCs and pDCs after the staining with HU210-Alexa488 probe or anti-CB2 antibody (black line) and with Alexa Fluor 488 or isotype control (grey). One representative example of three independent experiments is shown.

The expression of CB1 and CB2 at the protein level in different subsets of human peripheral blood DCs was confirmed by confocal microscopy. HmoDCs, mDCs, and pDCs freshly isolated from peripheral blood were stained with HU210-Alexa488 probe in combination with specific anti-human antibodies for HLA-DR, CD1c and CD123 and DAPI. Slides were prepared by cytopspin technique. CB1 was expressed by hmoDCs, mDCs and pDCs, but CB2 was only expressed by hmoDCs and mDCs, confirming the obtained results by flow cytometry (**Figure R.34ab**).

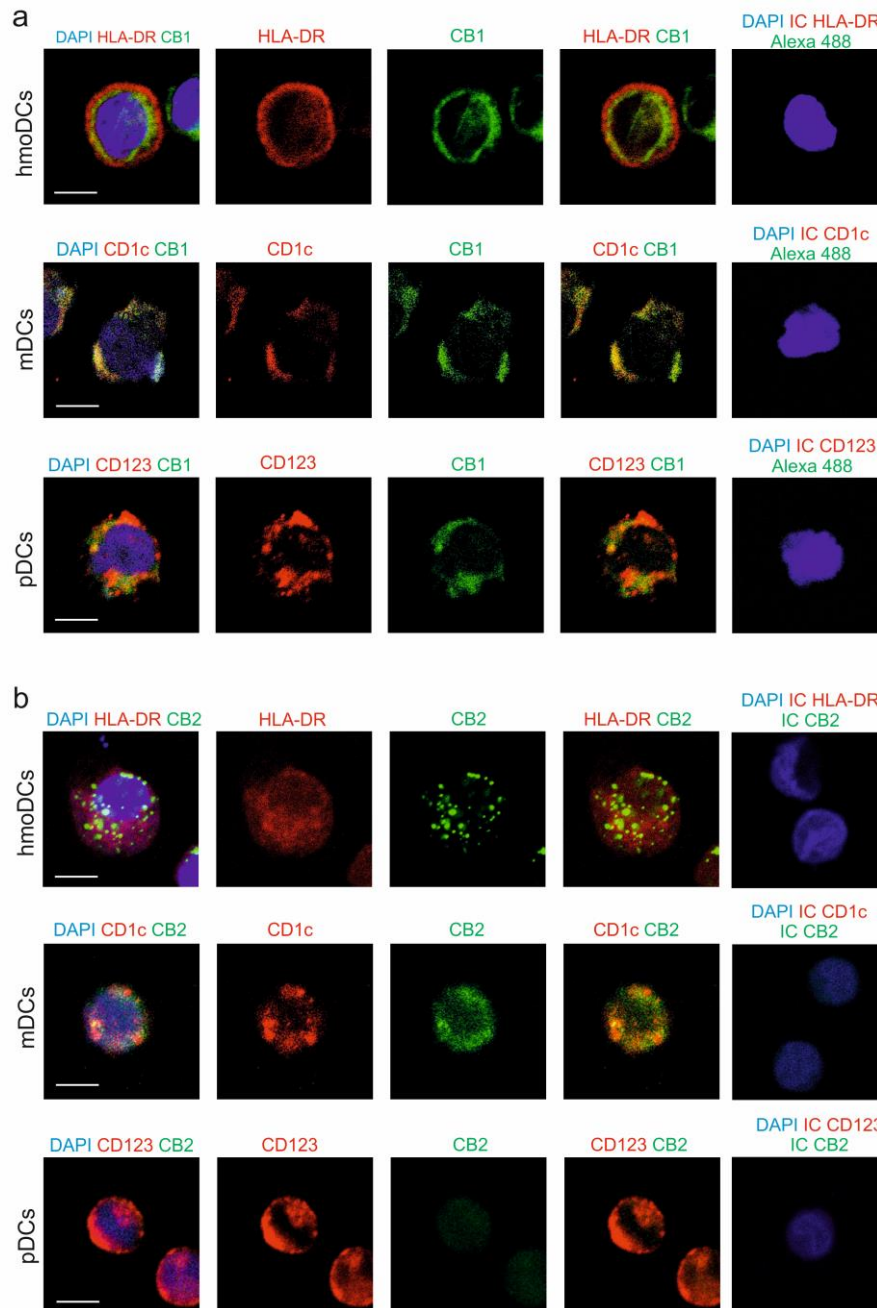


Figure R.34. Human DCs express CBRs at protein level. Visualization of **a** CB1 and **b** CB2 expression in hmoDCs, purified human blood mDCs and pDCs by confocal microscopy. Cells were stained for CB1 or CB2 (green), for HLA-DR, CD1c or CD123 (red) and with DAPI (blue, nuclei) and analysed by confocal microscopy. White bars, 5 μm. One representative example for two independent experiments.

To further explore the expression of CBRs in human DCs in tissues, we analysed *ex vivo* the expression of CB1 and CB2 in mDCs and pDCs from human tonsils, as organs where DCs induce immune responses (42). Human tonsil sections were stained with HU210-Alexa488 probe or anti-CB2 antibody in combination with DAPI and with anti-human CD1c or CD123 antibody to identify mDCs and pDCs. pDCs are the major DC subset in human tonsil, representing around 65 % of total tonsil DCs (42). As is shown in **Figure R.35ab**, CD1c⁺ mDCs and pDCs located at the T cell area of human tonsils expressed CB1. As we previously showed for peripheral blood, CD1c⁺ mDCs, but not pDCs from human tonsil expressed CB2 at the protein level. These data demonstrated that *in vitro* generated hmoDCs, human blood DCs as well as tonsil DCs express CB1 and CB2, which might well contribute to the regulation of DCs.

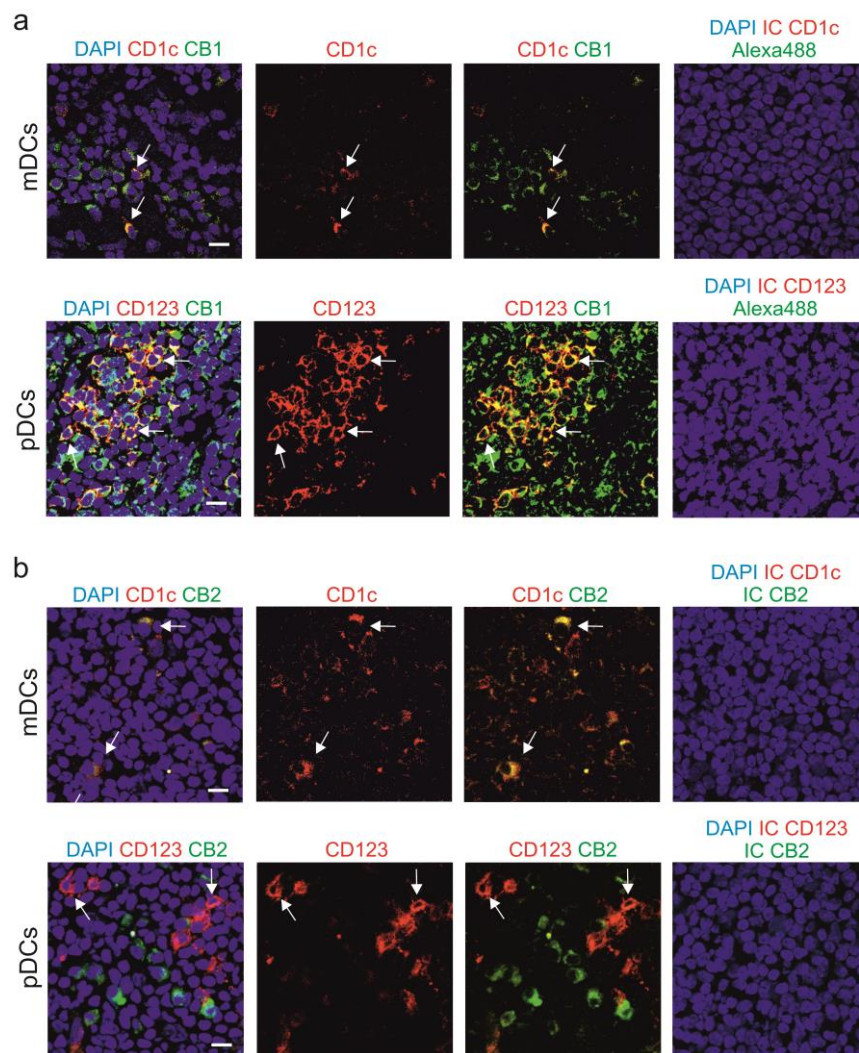


Figure R.35. Visualization of **a** CB1 and **b** CB2 expression in mDCs and pDCs from human tonsils. Human tonsil sections were stained for CB1 or CB2 (green), for CD1c or CD123 (red) and with DAPI (blue, nuclei) and analysed by confocal microscopy. White bars, 10 μ m. White arrows point at mDCs or pDCs in the T cell area of the tonsil tissue. One representative example of three independent experiments.

Next, we wanted to study whether the different populations of DCs expressed the main components of the ECS. For that, we analysed the mRNA expression levels of different genes of the ECS in hmoDCs, purified human blood mDCs and pDCs by quantitative real-time PCR. As shown in **Figure R.36**, hmoDCs, mDCs and pDCs expressed at mRNA level both CB1 and CB2 as well as FAAH and MAGL, which are enzymes involved in the hydrolysis and inactivation of the endocannabinoids anandamide and 2-AG, respectively. Although we did not observe CB2 at protein level, the CB2 mRNA levels were high in human blood pDCs. It could be due to CB2 internalization (180), or to potential different regulation mechanisms between mRNA expression and protein synthesis in human pDCs for this CBR. Further research at this regard is warranted.

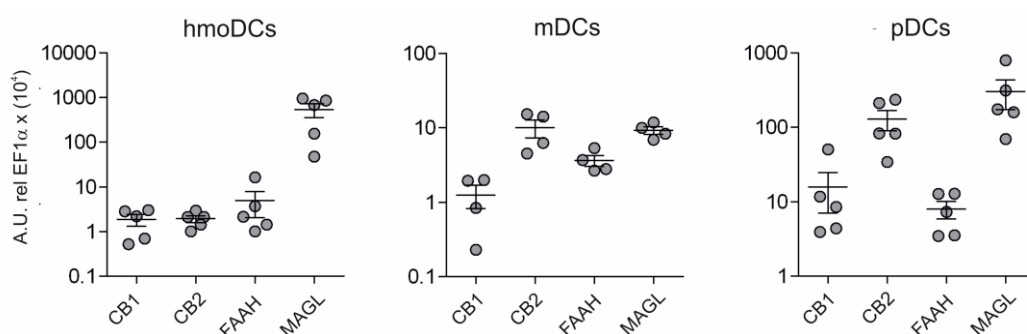


Figure R.36. Human DCs express the main components of the ECS at mRNA level. mRNA expression levels of CB1, CB2, FAAH and MAGL in *in vitro* generated hmoDCs and purified blood mDCs and pDCs ($n = 4/5$). Arbitrary units (A.U.) are $2^{-(\Delta Ct)}$ values multiplied by 10^4 , with ΔCt defined as the difference between the cycle threshold value for each gene and elongation factor 1 α (EF1 α) as housekeeping gene.

Collectively, these data confirmed the expression of different components of the ECS in human DCs. Our data and a previous study in the literature showing the expression of CB1 and CB2 in hmoDCs suggest that the ECS may contribute to the regulation of the function of human DCs (179). Few studies have demonstrated the role of CBRs in the control of human DC function. Roth et al demonstrated that the exposure of human monocytes to THC altered their differentiation into DCs, which displayed reduced capacity of activation (181). However, the detailed mechanism underlying the immune responses triggered by cannabinoids in human DCs remain largely unknown.

After showing that human DCs express CBRs, we wanted to study the capacity of different synthetic cannabinoids to regulate immune responses in human DCs. For that, we initially used the THP1-XBlue™ cell line, a monocytic cell line that expresses many different receptors, including TLRs, whose activation induce the activation of the transcription factors NF- κ B and AP-1, essential transcription factors in the control of pro-inflammatory cytokines. As described in Materials and Methods, THP1-XBlue™ cells

allow the monitoring of NF- κ B/AP-1 activation by using an easy and fast colorimetric assay.

Firstly, we confirmed the expression of CBRs in THP1-XBlueTM cells (182). Western blot analysis showed that CB1 and CB2 were expressed in THP1-XBlueTM cells under unstimulated conditions. The stimulation of THP1-XBlueTM cells with the TLR2 ligand Pam3CSK4 (TLR2L) did not significantly alter the expression of either CB1 or CB2 (**Figure R.37a**). These data were confirmed by flow cytometry experiments, indicating that THP1-XBlueTM cell line is a suitable cell line for the study of the capacity of synthetic cannabinoids to regulate immune responses (**Figure R.37b**).

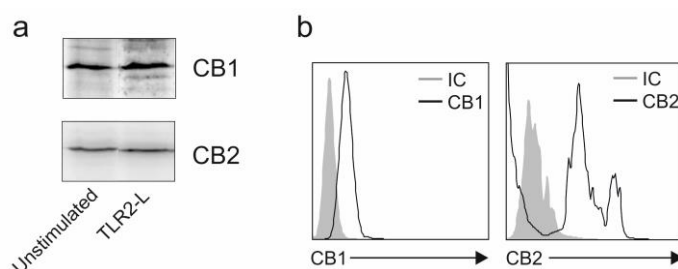


Figure R.37. Expression of CBRs in THP1-XBlueTM cell line. **a** Western blot of CB1 and CB2 expression in unstimulated and TLR2L-activated THP1-XBlueTM cells. **b** Histograms of CB1 and CB2 expression in THP1-XBlueTM cells after the staining with HU210-Alexa488 probe or anti-CB2 antibody (black line) and with Alexa Fluor 488 or isotype control (grey). One representative example of three independent experiments.

Then, THP1-XBlueTM cells were stimulated with TLR2L in the presence of different doses of the selected synthetic cannabinoid agonists WIN55212-2 (CB1/CB2 agonist), HU210 (CB1/CB2 agonist) and HU308 (CB2 selective agonist) for 18 hours. TLR2L promoted the activation of NF- κ B/AP-1 transcription factors as determined by the colorimetric assay. WIN55212-2 significantly reduced TLR2L-induced NF- κ B/AP-1 activation in a dose-dependent manner. Although WIN55212-2 10 μ M and 20 μ M significantly inhibited TLR2L-induced NF- κ B/AP-1 activation, only WIN55212-2 10 μ M did not affect cell viability (**Figure R.38ab**). HU210 also reduced TLR2L-induced NF- κ B/AP-1 activation at 20 μ M without affecting cell viability. In contrast, HU308, the CB2 selective agonist, did not modify TLR2L-induced NF- κ B/AP-1 activation at any of the assayed doses and did not show cytotoxicity (**Figure R.38ab**).

Our data demonstrate that the CB1/CB2 agonists WIN55212-2 and HU210 reduced TLR2L-induced NF- κ B/AP-1 activation but the selective CB2 agonist HU308 did not, suggesting that the observed effects of WIN55212-2 and HU210 are possibly mediated by CB1 and/or other alternative CBRs. Although both WIN55212-2 and HU210 inhibited TLR2L-induced NF- κ B/AP-1 activation, WIN55212-2 induced a higher inhibition at lower doses compare to HU210.

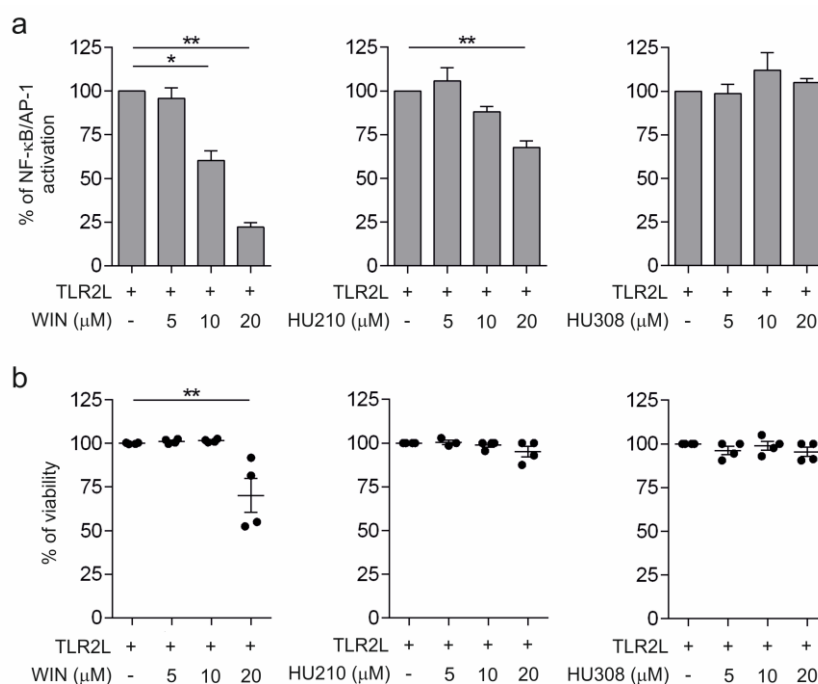


Figure R.38. Synthetic cannabinoids modulate NF-κB and AP-1 signal transduction pathway in THP1-XBlue™ cells. **a** Modulation of TLR2L-induced NFκB/AP-1 activation in THP1-XBlue™ cells by different doses of WIN55212-2 (WIN), HU2120 and HU308 (n = 4). **b** Percentage of cell viability after cannabinoid stimulation (n = 4 independent experiments). Values are mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01.

We also evaluated IL-8 production by THP1-XBlue™ cells after the stimulation with TLR2L in the presence of the different synthetic cannabinoids. WIN55212-2 significantly inhibited TLR2L-induced IL-8 production in a dose-dependent manner. In contrast, HU210 and HU308 did not inhibit TLR2L-induced IL-8 production (**Figure R.39**). Our results demonstrated that WIN55212-2 displays a potent anti-inflammatory capacity in THP1-XBlue™ cells by inhibiting NF-κB/AP-1 activation and IL-8 production. Despite HU210 inhibited NF-κB/AP-1 activation, it did not inhibit IL-8 production, indicating that HU210-mediated inhibition of NF-κB/AP-1 was not enough to block IL-8 production. HU308 did not induce significant changes in TLR2L-induced NF-κB/AP-1 activation and IL-8 production. Collectively, our data indicate that WIN55212-2 displays a potent anti-inflammatory capacity in THP1-XBlue™ cells, thus representing a suitable synthetic cannabinoid for deeper studies at the cellular and molecular level.

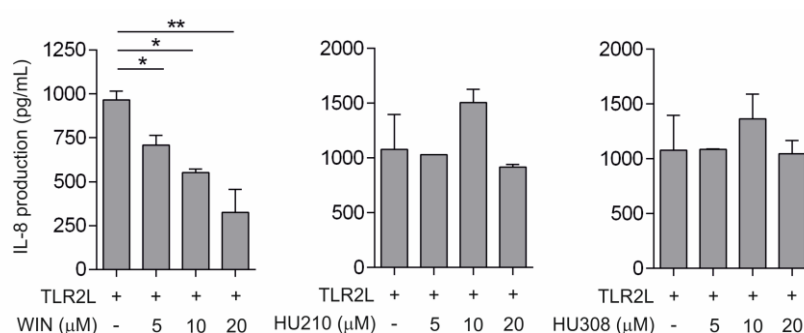


Figure R.39. Production of IL-8 by THP1-XBlue™ cells after the stimulation with TLR2L in the present of WIN55212-2 (WIN), HU210 and HU308 at different doses for 18 hours (n = 4 independent experiments). Values are mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01.

Next, we extended these analyses to investigate the capacity of WIN55212-2, HU210 and HU308 to modulate functional properties of human DCs. We initially employed hmoDCs as *in vitro* model due to the scarcity of primary DCs. The *in vitro* generation of hmoDCs provides high number of cells of a homogeneous population suitable for the study of human DCs (44). HmoDCs were stimulated with the TLR4 ligand LPS in the presence of different doses of WIN55212-2, HU210 and HU308 for 18 hours and the production of different pro-inflammatory cytokines in cell-free supernatants were analysed by ELISA. As previously reported, LPS stimulation induced the production of high levels of TNFα and IL-6 by hmoDCs (183). WIN55212-2 significantly inhibited the production of TNFα and IL-6 by LPS-treated hmoDCs in a dose dependent manner without affecting cell viability. Although HU210 20 μM inhibited IL-6 production by LPS-treated hmoDCs without affecting cell viability, no changes were observed in TNFα production. On the other hand, HU308 did not modify cytokine production or cell viability in LPS-stimulated hmoDCs at the assayed conditions (**Figure R.40ab**).

These results demonstrated and confirmed that WIN55212-2 displays a more potent anti-inflammatory capacity in hmoDCs compared to HU210 and HU308, and that 10 μM is the optimal effective and non-toxic dose. Some studies have described the anti-inflammatory effects of WIN55212-2 in autoimmune models of multiple sclerosis, amyloid β-induced neuroinflammation, or allergen-induced airway inflammation (184-186). However, the effect of WIN55212-2 in the regulation of DCs remains to be fully elucidated, especially in humans.

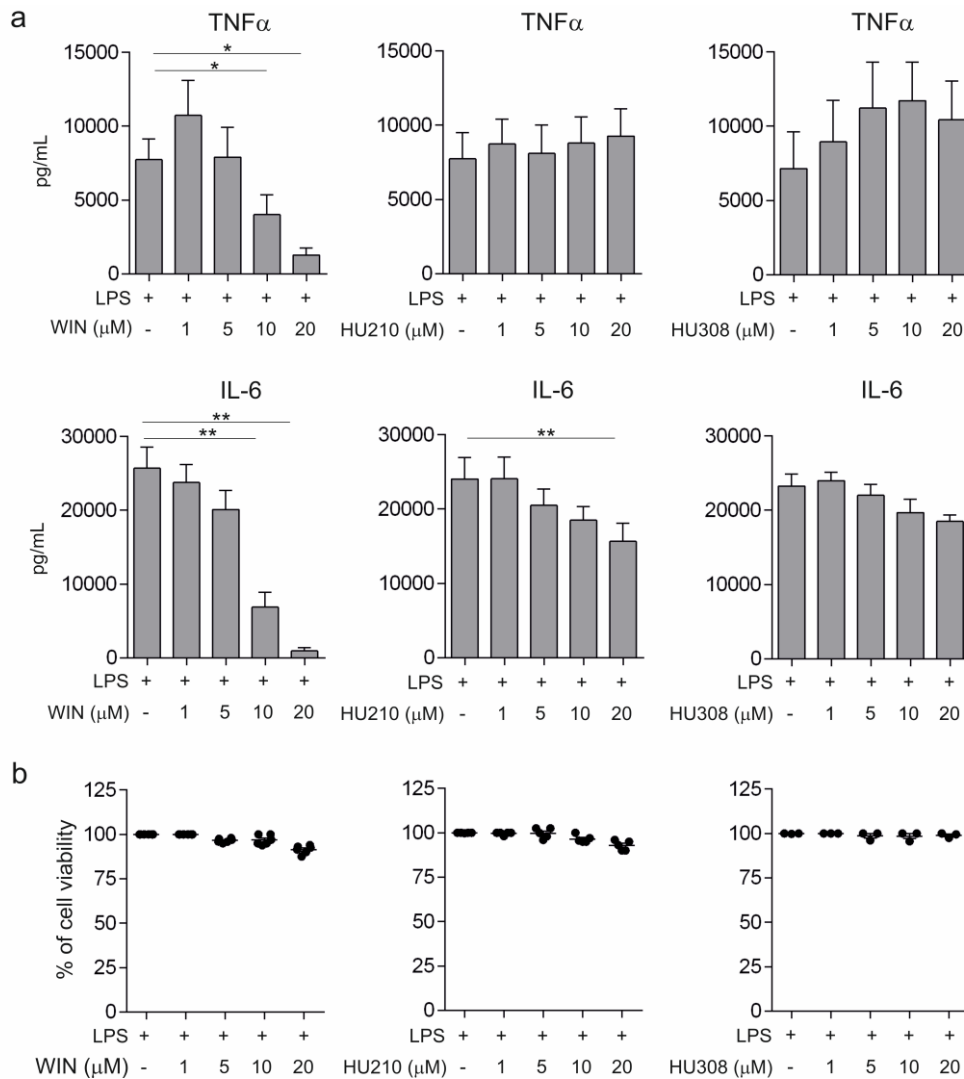


Figure R.40. Effect of synthetic cannabinoids in hmoDCs. **a** Cytokine production by hmoDCs after the stimulation with LPS or LPS plus different doses of WIN55212-2 (WIN), HU210 and HU308 for 18 hours ($n = 4$). **b** Percentage of cell viability after the stimulation of hmoDCs with the indicated conditions ($n = 4$ independent experiments). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$.

2.2. Study of the capacity of WIN55212-2 to regulate the phenotype and function of human DCs.

WIN55212-2 is a potent synthetic cannabinoid ligand belonging to the aminoalkylindole family. WIN55212-2 has been described as potent CB1 and CB2 agonist (187, 188). However, WIN55212-2 also exerts its effects through PPAR α , PPAR γ , TRPV1 and TRPA1 (189-191). WIN55212-2 displays analgesic and anti-inflammatory properties. Its anti-inflammatory properties have been described in several disease models such as autoimmune model of sclerosis multiple, airway inflammation, neuroinflammation, colitis and atherosclerosis (184, 185, 190, 192, 193). However, the effect of WIN55212-2 in the

regulation of human DCs remains unknown. Therefore, we studied the capacity of WIN55212-2 to regulate the phenotype and function of human DCs.

To analyse the impact of WIN55212-2 on the phenotype and function of human DCs, hmoDCs were generated as previously described in Materials and Methods and stimulated with WIN55212-2, LPS and LPS plus WIN55212-2 for 18 hours.

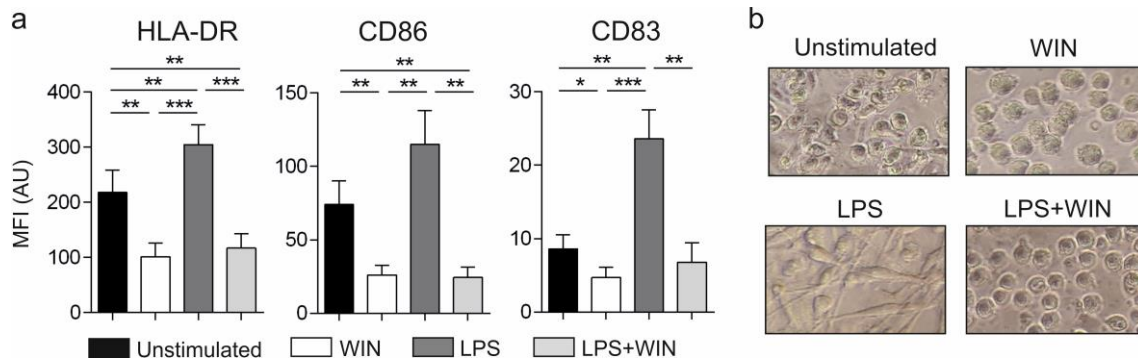


Figure R.41. WIN55212-2 (WIN) modifies the phenotype of LPS-activated hmoDCs. **a** Mean fluorescence intensity (MFI) of the surface markers HLA-DR, CD86 and CD83 in hmoDCs after the stimulation with WIN55212-2, LPS and LPS plus WIN55212-2 for 18 hours ($n = 8$). **b** Representative images of hmoDC morphology after treatment with the indicated stimuli for 18 hours. Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Firstly, the expression levels of the activation surface markers HLA-DR, CD86 and CD83 were analysed by flow cytometry. HLA-DR is the class II MHC molecule involved in antigen presentation to T cells (16). CD86 and CD83 are surface proteins expressed in DCs and other APCs to provide costimulatory signals needed for T cell activation (194). As shown in **Figure R.41a**, WIN55212-2 significantly reduced the expression of HLA-DR and the surface markers CD86 and CD83 in hmoDCs. LPS stimulation increased the expression of HLA-DR, CD86 and CD83 in hmoDCs. In contrast, WIN55212-2 significantly inhibited the expression of HLA-DR, CD86 and CD83 in LPS-activated hmoDCs (**Figure R.41a**). These data demonstrate that WIN55212-2 inhibited the LPS-induced maturation and activation of hmoDCs, reducing the expression of the surface markers HLA-DR, CD86 and CD83 and thus favouring an immature phenotype. Supporting these data, optical microscope images revealed that WIN55212-2 modified the morphology of hmoDCs. WIN55212-2-treated hmoDCs displayed similar morphology than unstimulated hmoDCs. LPS activation induced a mature morphology characterized by the development of long dendrites. WIN55212-2 stimulation inhibited the generation of such classical dendrites and promoted an immature phenotype, which is similar to that observed under unstimulated conditions (**Figure R.41b**).

Next, the cytokine signature of hmoDCs stimulated with WIN55212-2, LPS and LPS plus WIN55212-2 for 18 hours was analysed by ELISA. WIN55212-2 did not induce

any of the cytokines assayed in hmoDCs. However, WIN55212-2 significantly inhibited the production of the pro-inflammatory cytokines TNF α , IL-8, IL-6 and IL-1 β and the anti-inflammatory cytokine IL-10 in LPS-activated DCs (**Figure R.42**).

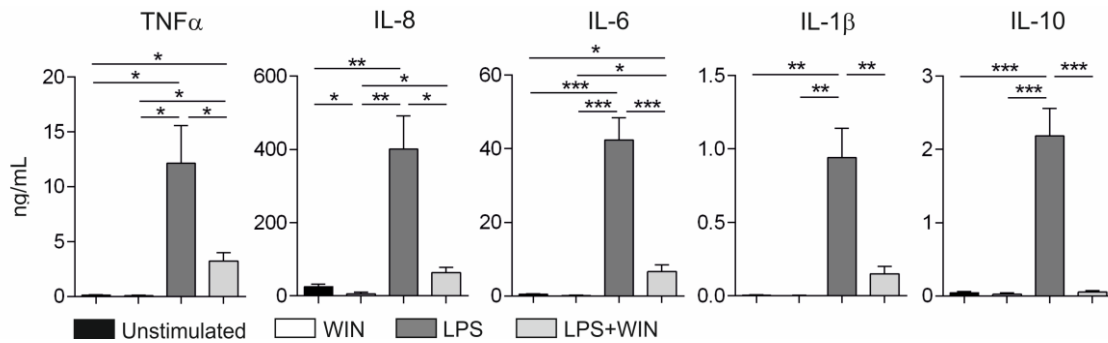


Figure R.42. WIN55212-2 (WIN) inhibits cytokine production by LPS-activated hmoDCs. Cytokine levels in cell-free supernatants after stimulation of hmoDCs with WIN55212-2, LPS and LPS plus WIN55212-2 for 18 hours ($n = 8$). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Our data in THP1-XBlueTM cells demonstrated that WIN55212-2 inhibited TLR2L-induced NF- κ B/AP-1 activation. NF- κ B and AP-1 are essential transcription factors in the control of the mRNA transcription of pro-inflammatory cytokines (25). Therefore, we also wanted to study whether WIN55212-2 could regulate mRNA levels of pro-inflammatory cytokines and the anti-inflammatory cytokine IL-10. As shown in the heatmap of **Figure R.43**, WIN55212-2 reduced the mRNA levels of TNF α , IL-8, IL-6, IL-1 β and IL-10 in LPS-activated DCs. Collectively, these data demonstrated that WIN55212-2 impairs the maturation and activation of LPS-stimulated hmoDCs by inhibiting surface marker expression and cytokine production.

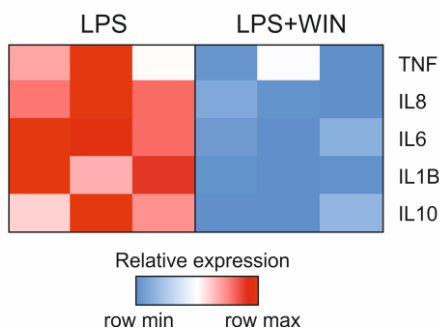


Figure R.43. Heatmap of cytokine gene expression after stimulation of hmoDCs with LPS or LPS plus WIN55212-2 (WIN) for 4 hours ($n = 3$).

To ascertain if the anti-inflammatory effects of WIN55212-2 in hmoDCs were not restricted to LPS-stimulation, we studied the capacity of WIN55212-2 to control the maturation and activation induced by a lipopeptide ligand of TLR2/TLR1 called Pam3CSK4 (TLR2L), and a combination of cytokines TNF α /IL-1 β in hmoDCs. TLR2L and cytokines TNF α /IL-1 β induced the maturation and activation of hmoDCs by increasing the expression of surface markers HLA-DR, CD86 and CD83 and the production of the pro-inflammatory cytokines IL-8 and IL-6. As shown in **Figure R.44**,

WIN55212-2 also inhibited the expression of the assayed surface markers and pro-inflammatory cytokines in a dose-dependent manner in hmoDCs stimulated with TLR2L or the TNF α /IL-1 β inflammatory cocktail in a very similar manner to that observed for LPS. These data demonstrated that WIN55212-2 is able to restore an immature phenotype in hmoDCs upon activation with different pro-inflammatory stimuli.

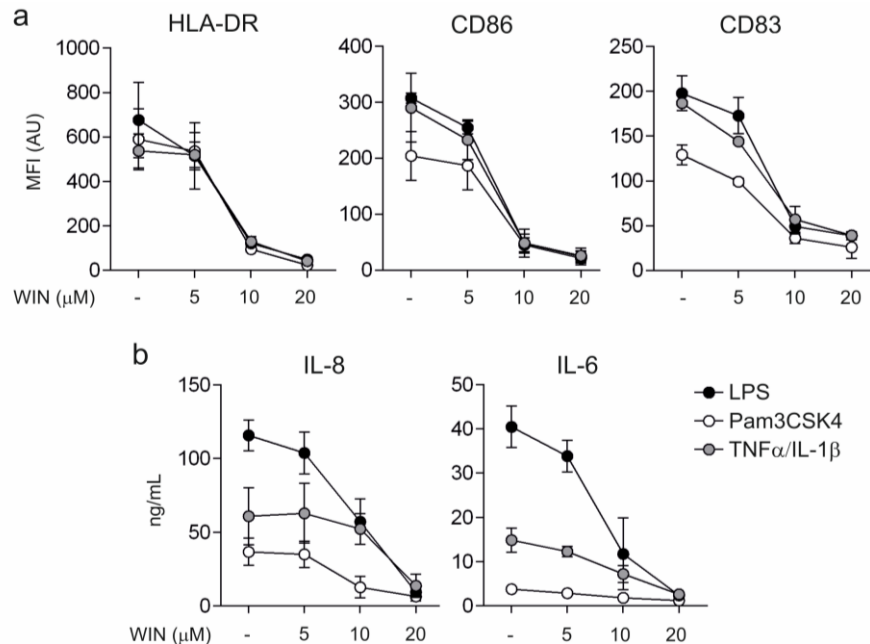


Figure R.44. WIN55212-2 inhibits the mature phenotype induced by different stimuli. **a** Mean fluorescence intensity (MFI) of surface markers and **b** cytokine production after stimulation of hmoDCs with LPS, Pam3CSK4 (TLR2L) and the combination of TNF α /IL-1 β in the presence of different doses of WIN for 18 hours (n = 3).

To confirm the observed anti-inflammatory capacity of WIN55212-2 in physiologically more relevant subsets of human DCs, total DCs from human peripheral blood of healthy donors were purified. As shown in **Figure R.45**, human blood DCs can be broadly classified in mDCs and pDCs. Although both subsets of DCs express HLA-DR, mDCs are characterized by the expression of CD1c and their capacity to secrete pro-inflammatory and anti-inflammatory cytokines (12, 15). However, pDCs are characterized by the expression of CD303 and CD123 and they play a protective key role during viral infections (12, 40).

Total DCs were cultured in the presence of LPS or LPS plus WIN55212-2 for 18 hours and the cytokine production was analysed by ELISA. After LPS stimulation, total DCs produced high levels of the pro-inflammatory cytokines TNF α , IL-8, IL-6 and IL-1 β and the anti-inflammatory cytokine IL-10. WIN55212-2 significantly reduced the production of all the assayed cytokines in LPS-activated total DCs without affecting cell viability (**Figure R.45**).

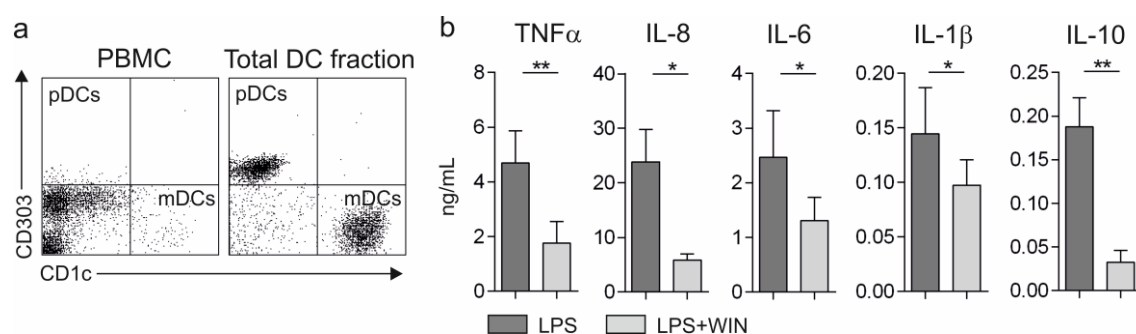


Figure R.45. WIN55212-2 (WIN) inhibits cytokine production in LPS-activated total DCs **a** Representative dot plots of flow cytometry for CD303⁺ pDCs and CD1c⁺ mDCs in PBMC and purified total DC fraction. **b** Cytokine levels in cell-free supernatants after stimulation of purified total DCs with LPS and LPS plus WIN55212-2 for 18 hours (n = 6). Values are mean ± SEM. Statistical significance was determined using Paired *t* test. * *P* < 0.05, ** *P* < 0.01.

Cytokines produced by innate immune cells are involved in the regulation of the immune response both in the activation and in the inhibition of cellular functions. Several studies have demonstrated that TNFα is necessary for Th1 polarization, whereas IL-1β and IL-6 are necessary for Th17 induction (48, 50). IL-8 plays a key role in the recruitment of immune cells, such as neutrophils to inflammation sites (50). In contrast, IL-10 is an anti-inflammatory cytokine that maintains the balance of the immune response (50). Our results showing that WIN55212-2 inhibits LPS-induced cytokine production not only in hmoDCs but also in total blood DCs, thus impairing DC activation and maintaining an immature phenotype.

Consistent with our data, the anti-inflammatory and immunosuppressive effects of cannabinoids in DCs have been described in different inflammatory models (184). The main component of marijuana, THC, suppresses the function of mouse DCs stimulated with *Legionella pneumophila* by inhibiting IL-12 production and the expression of costimulatory molecules CD86 and CD40 (150, 151). THC also suppresses the secretion of IFNα and TNFα by CpG-treated pDCs or by pDCs from HIV-infected donors (152, 153). Remarkably, it has been shown that the synthetic cannabinoid WIN55212-2 prevents microglia cell activation during LPS-induced chronic neuroinflammation in rats (186).

Our data revealed the potent anti-inflammatory and immunosuppressive capacity of WIN55212-2 in hmoDCs and total blood DCs, but the specific role in mDCs and pDCs need to be investigated.

2.3. Study of the capacity of WIN55212-2-treated DCs to polarize T cells responses.

The main function of DCs is to process peripheral antigens and migrate to lymph nodes to present them to naïve T cells, promoting T cell differentiation and activation (16). We

wanted to study the capacity of WIN55212-2 to regulate the function of human DCs, specifically their capacity to prime T cell responses. For that, WIN55212-2-treated hmoDCs were coculture with allogeneic naïve CD4⁺ T cells. After five days of coculture, T cell cytokines were analysed in cell-free supernatants by ELISA.

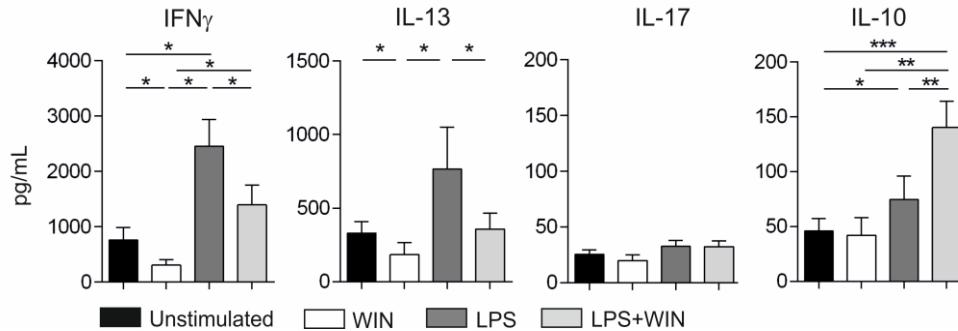


Figure R.46. WIN55212-2 (WIN) modifies the capacity of LPS-activated hmoDCs to polarize CD4⁺ T cell responses. Cytokines produced by allogeneic naïve CD4⁺ T cells primed by unstimulated, WIN55212-2, LPS or LPS plus WIN55212-2-treated hmoDCs after 5 days (n = 8). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01, *** P < 0.001.

As shown in **Figure R.46**, T cells generated by hmoDCs stimulated with WIN55212-2 alone produced lower levels of IFN γ and IL-13 than those generated by unstimulated hmoDCs. T cells generated by LPS-activated hmoDCs produced higher levels of IFN γ , IL-13 and IL-10 than those generated by unstimulated hmoDCs. Remarkably, hmoDCs activated with LPS plus WIN55212-2 generated T cells producing significantly lower levels of IFN γ and IL-13 than hmoDCs stimulated with LPS alone. In contrast, LPS-activated hmoDCs in the presence of WIN55212-2 generated T cells producing significantly more IL-10 than LPS-activated hmoDCs. We did not detect changes in IL-17 production. In addition, the IL-10/IFN γ , IL-10/IL-13 and IL-10/IL-17 ratios were significantly higher when T cells were primed by hmoDCs activated with LPS plus WIN55212-2 than with LPS alone (**Figure R. 47**).

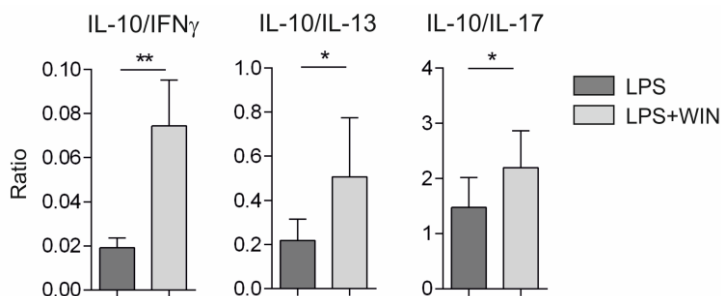


Figure R.47. Cytokine ratios of allogeneic naïve CD4⁺ T cells primed by LPS or LPS plus WIN55212-2 (WIN)-treated hmoDCs after 5 days (n = 8/6). Values are the mean $\pm$ SEM. Paired t test. * P < 0.05, ** P < 0.01.

To confirm the increment of IL-10 production by T cells after the coculture with LPS plus WIN55212-2-treated hmoDCs, intracellular staining experiments at the single cell level were performed. The percentage of IL-10-producing CD4⁺ T cells generated by WIN55212-2-treated hmoDCs was significantly higher than those generated by

unstimulated hmoDCs. LPS plus WIN55212-2-stimulated hmoDCs induced higher percentage of IL-10-producing T cells than hmoDCs activated with LPS alone (**Figure R.48**).

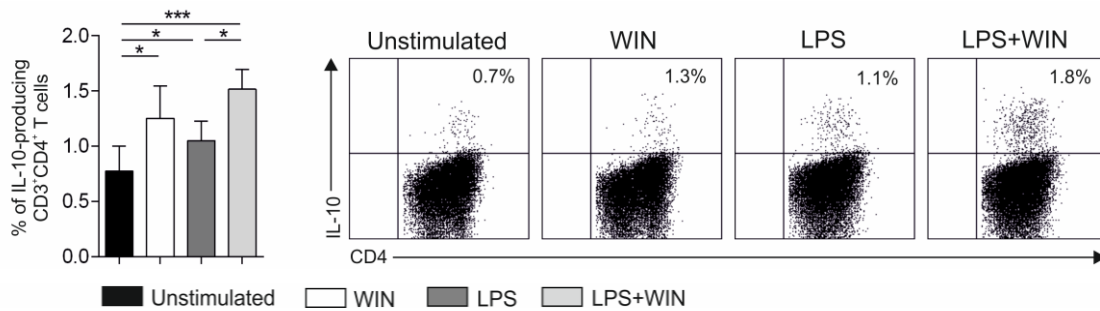


Figure R.48. Percentage of IL-10-producing CD3⁺CD4⁺ T cells after intracellular staining and flow cytometry analysis (n = 5). Representative dot plots of intracellular IL-10 staining are shown. Values are mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05, *** P < 0.001,

These data demonstrated that WIN55212-2 impaired the polarization of effector Th1 and Th2 cells induced by LPS-activated hmoDCs. In addition, WIN55212-treated hmoDCs promoted the generation of IL-10-producing CD4⁺ T cells, suggesting that WIN55212-2-treatment induces tolerogenic hmoDCs, which in turn promote the generation of regulatory responses at the T cell level. To investigate the capacity of WIN55212-2-treated hmoDCs to induce Treg cells, we performed the same allogeneic coculture experiments and intranuclear FOXP3 staining.

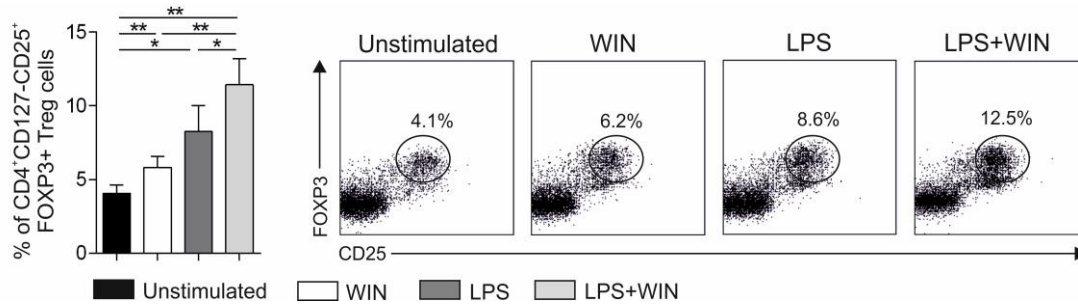


Figure R.49. Percentage of induced CD4⁺CD127⁻CD25⁺FOXP3⁺ Tregs cells by allogeneic WIN55212-2 (WIN), LPS or LPS plus WIN55212-2-stimulated hmoDCs after 5 days (n = 8). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01,

WIN55212-2-stimulated hmoDCs induced significantly higher numbers of CD4⁺CD127⁻CD25⁺FOXP3⁺ Tregs cells compared to unstimulated hmoDCs. Similarly, hmoDCs activated with LPS in the presence of WIN55212-2 induced significantly higher number of FOXP3⁺ Treg cells than hmoDCs treated with LPS alone (**Figure R.49**). These data demonstrated that WIN55212-2 promotes tolerogenic DCs with the capacity of generating FOXP3⁺ Treg cells.

To study the inhibitory capacity of the Treg cells induced by LPS plus WIN55212-2-stimulated hmoDCs, functional experiments were performed. The generated

CD4⁺CD127⁻CD25⁺ Treg cells by LPS plus WIN55212-2-stimulated hmoDCs were sorted by fluorescence-activated cell sorting (FACS) and cocultured with anti-CD3/CD2/CD28-stimulated autologous PBMC. The proliferation of PBMC was analysed by CFSE-dilution experiments by flow cytometry. As shown in **Figure R.50**, purified Treg cells suppressed the proliferation of autologous PBMC in a dose-dependent manner, demonstrating that WIN55212-2-stimulated hmoDCs promote the generation of functional Treg cells.

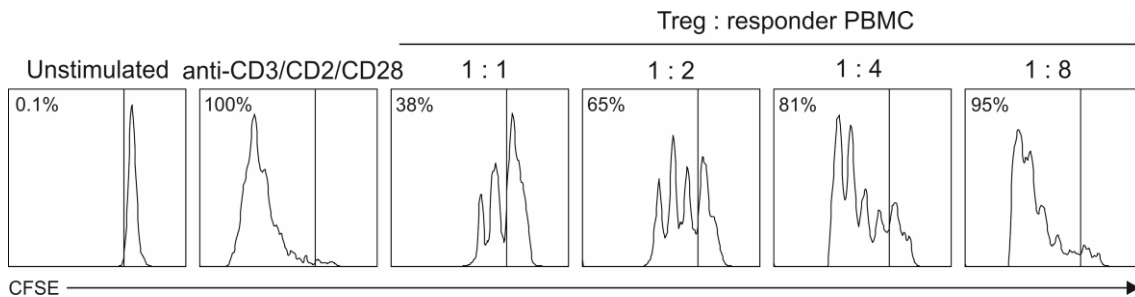


Figure R.50. Proliferation of CFSE-labelled PBMC gated on CD4⁺ T cells after 5 days of culture with autologous purified FOXP3⁺ Treg cells generated by allogeneic LPS plus WIN55212-2-stimulated hmoDCs. Percentage of one-representative example of proliferating responder PBMC is shown.

Finally, coculture experiments using total blood DCs demonstrated that total blood DCs activated with LPS in the presence of WIN55212-2 induced significantly higher numbers of CD4⁺CD127⁻CD25⁺FOXP3⁺ Treg cells than total DCs treated with LPS alone, thus confirming the results obtained with hmoDCs (**Figure R.51**).

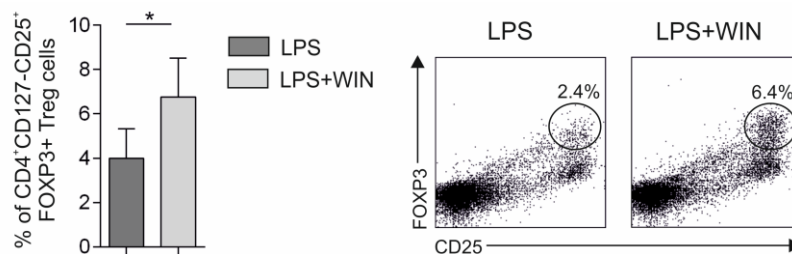


Figure R.51. Percentage of induced CD4⁺CD127⁻CD25⁺FOXP3⁺ Treg cells by allogeneic LPS or LPS plus WIN55212-2 (WIN)-stimulated total DCs after 5 days (n = 6). Representative dot plots of FOXP3 staining is shown. Values are the mean $\pm$ SEM. Paired *t* test. * *P* < 0.05.

Our results demonstrated that the synthetic cannabinoid WIN55212-2 induces tolerogenic DCs with the capacity to block Th1 and Th2 cell responses and to promote the generation of functional FOXP3⁺ Treg cells. In line with our data, it has been reported that THC reduced the capacity of mouse DCs to promote effector CD8⁺ and CD4⁺ T cells in response to different pro-inflammatory stimuli (154, 155). However, to our knowledge, this is the first time that it is reported that WIN55212-2 promoted the generation of functional Treg cells.

Objective 3: Elucidation of the molecular mechanisms by which WIN55212-2 exerts its effects in human DCs

The molecular mechanisms by which cannabinoids can control the function of DCs remain poorly understood. It has been shown that cannabinoid treatment induced apoptosis in mice DCs (148). In contrast, *in vitro* models of bacterial infection showed that cannabinoids inhibited cytokine production and costimulatory molecules without apoptosis induction (150, 151). Similarly, THC inhibited CpG-induced responses in human pDCs by inhibition of NF- κ B signalling (152, 153). Our previous results demonstrated the capacity of WIN55212-2 to imprint tolerogenic features in human DCs. However, the underlying molecular mechanisms needs to be fully elucidated.

To gain further molecular insights in how WIN55212-2 regulates DC function, we investigated the role played by main immune signalling pathways and metabolic routes in the induction of tolerogenic DCs by WIN55212-2.

3.1. Study of the main immune signalling pathways involved in the immunomodulatory effects of WIN55212-2 in human DCs

To elucidate the mechanisms that underline the immunomodulatory effects of WIN55212-2, we initially studied different immune signalling pathways that might be involved in the cross-talk between LPS-induced TLR4 and WIN55212-2-induced CBR signalling in human DCs. HmoDCs were stimulated with WIN55212-2, LPS and LPS plus WIN55212-2 for 15 or 30 minutes and the activation of the different key molecules involved in relevant immune signalling pathways was determined by quantifying their phosphorylation stage by western blot.

It has been described that LPS induces the rapid activation of NF- κ B pathway in human DCs (195). In addition, our previous results demonstrated that WIN55212-2 inhibited TLR2L-induced NF- κ B/AP-1 activation in THP1-XBlue™ cells. Therefore, we evaluated the activation of the inhibitor of NF- κ B (I κ B) and the I κ B kinase complex (IKK α / β). As shown in **Figure R.52**, LPS induced the phosphorylation of IKK α / β (Ser176/177) and I κ B (Ser32/35), activating the NF- κ B signalling pathway. WIN55212-2 inhibited the phosphorylation of IKK α / β and I κ B in LPS-activated hmoDCs, inhibiting the activation of NF- κ B signalling pathway. In addition, we evaluated the activation of the MAPK signalling pathways, including p38 kinase (p38), Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK), which are involved in the activation of the transcription factor AP-1 (25). LPS induced the phosphorylation of p38 (Thr180/182),

JNK (Thr183/Tyr185) and ERK1/2 (Thr202/Tyr204) in hmoDCs. WIN55212-2 inhibited the phosphorylation of p38, JNK and ERK1/2 in LPS-activated hmoDCs. These data demonstrated that WIN55212-2 inhibits NF- κ B and MAPK signalling pathways induced by LPS in hmoDCs, which are involved in the induction of pro-inflammatory genes, such as pro-inflammatory cytokines, supporting our previous data in THP1-XBlue™ cells.

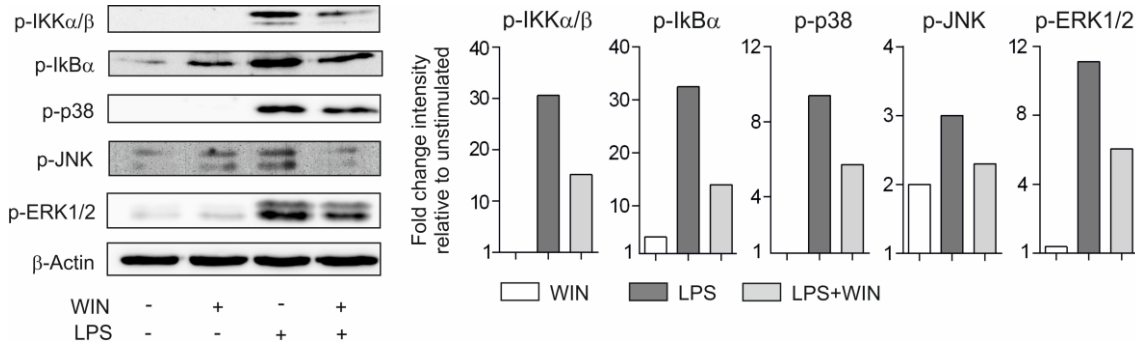


Figure R.52. Western blot analysis of protein extracts from hmoDCs stimulated with WIN55212-2 (WIN), LPS and LPS plus WIN55212-2 for 15 minutes. Quantification of the reactive phosphorylated bands by scanning densitometry is shown. One representative example of two independent experiments.

Other key regulators of inflammation in human DCs are the mammalian target of rapamycin (mTOR) and its negative regulator AMP-activated protein kinase (AMPK) (81). Thus, we studied the phosphorylation stage of the mTOR upstream activator protein kinase B (Akt), the mTOR downstream substrate Ribosomal protein S6 kinase beta-1 (p70S6K) and the mTOR repressor AMPK, which is associated to anti-inflammatory responses (196). LPS induced the phosphorylation of Akt (Ser473) and p70S6K (Thr389), thus indicating the activation of the mTORC1 signalling pathway as previously demonstrated (75). Interestingly, WIN55212-2 inhibited the LPS-induced phosphorylation of Akt and p70S6K in hmoDCs. Moreover, WIN55212-2 induced the phosphorylation of AMPK in LPS-activated hmoDCs (**Figure R.53**). Collectively, these data demonstrated that WIN55212-2 inhibits LPS-activated NF- κ B-, MAPK- and mTOR-mediated signalling pathways, which are involved in the orchestration of inflammatory responses.

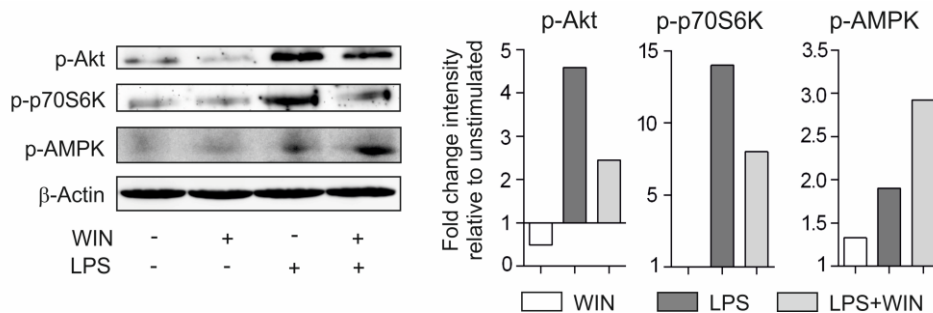


Figure R.53. Western blot analysis of protein extracts from hmoDCs stimulated with WIN55212-2 (WIN), LPS and LPS plus WIN55212-2 for 30 minutes. Quantification of the reactive phosphorylated bands by scanning densitometry is shown. One representative example of two independent experiments.

Besides its capacity to suppress mTORC1, AMPK has been recognized as a cornerstone in autophagy activation (197, 198). Several studies have reported that cannabinoids induce autophagy in different cellular models (199). Salazar et al have demonstrated that THC promotes autophagy-mediated cell death in human and mouse cancer cells. It has been described that THC and synthetic cannabinoids induce AMPK-dependent activation of autophagy in hepatocellular carcinoma and pancreatic cancer cells (147, 200). Therefore, we wanted to investigate whether the synthetic cannabinoid WIN55212-2 might induce autophagy in hmoDCs. For that, we analysed the autophagy protein microtubule-associated protein 1 (LC3) to monitor autophagy induction by western blot. LC3 acts as scaffold for autophagy machinery and as adaptor of selective cargo for recruitment to the autophagosome. LC3 is initially synthesized in its unprocessed form (proLC3). Then, the C terminus of LC3 is cleaved by proteases to form LC3-I. Upon autophagy activation, LC3-I is modified to form the phosphatidylethanolamine (PE)-conjugated form of LC3, named LC3-II. LC3-II is associated to the autophagosome membrane until its degradation in the autophagolysosome. The increase in LC3-II is a hallmark of autophagy. LC3-II has higher electrophoresis mobility than LC3-I and appears as a band of apparent molecular weight lower than LC3-I (164). WIN55212-2-stimulated hmoDCs displayed higher levels of LC3-II than unstimulated hmoDCs. LPS-activated hmoDCs displayed lower levels of LC3-II than unstimulated hmoDCs. In contrast, LPS plus WIN55212-2-stimulated hmoDCs showed higher levels of LC3-II compared to LPS-activated hmoDCs. These data indicating that WIN55212-2 promotes autophagy induction in hmoDCs (**Figure R.54**).

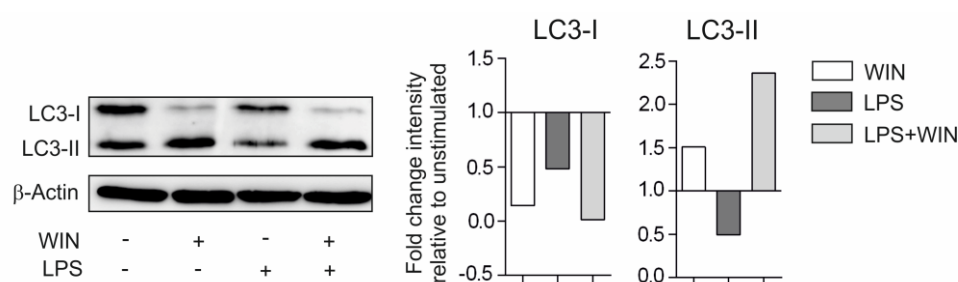


Figure R.54. Western blot analysis of protein extracts from hmoDCs stimulated with WIN55212-2 (WIN), LPS and LPS plus WIN55212-2 for 18 hours. Quantification of the reactive bands by scanning densitometry is shown. One representative example of three independent experiments.

In order to confirm the autophagy induction by WIN55212-2 in hmoDCs, we analysed changes in the subcellular distribution of LC3 by confocal microscopy. We observed more LC3-positive dots, corresponding to LC3-II associated to the autophagosome, in LPS plus WIN55212-2 stimulated hmoDCs than in hmoDCs

stimulated with LPS alone. In addition, the autophagy inhibitor 3-methyladenine (3-MA) impaired the WIN55212-2-induced autophagy in LPS plus WIN55212-2-activated hmoDCs (**Figure R.55**).

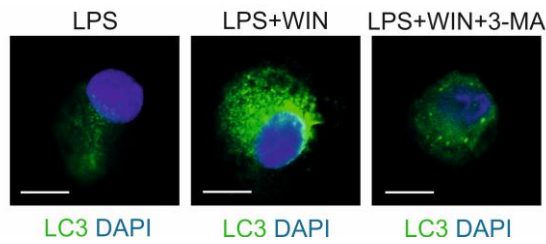


Figure R.55. Representative confocal images of LC3 staining of hmoDCs stimulated with LPS, LPS plus WIN55212-2 (WIN) and LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA for 18 hours. White bars, 5 μ m

Next, we wanted to further explore the capacity of WIN55212-2 to induce autophagy in hmoDCs. For that, we evaluated the mRNA expression levels by quantitative real-time PCR of autophagy related genes involved in the first stages of the autophagy process (85). As shown in the heatmap of **Figure R.56**, LPS plus WIN55212-2 stimulated hmoDCs displayed higher mRNA levels of Unc-51 like kinase (ULK1), autophagy related 14 (ATG14), and PI3K catalytic subunit Type 3 (PIK3C3) than LPS-activated hmoDCs. ULK1 is involved in the initiation of autophagy, and ATG14 and PIK3C3 in the phagophore formation. Autophagy starts with ULK1 complex activation that translocates to the site of autophagosome formation and activates the class III PI3K complex (197, 201), whose main components are ATG14 and PIK3C3. The class III PI3K complex mediates the nucleation step of phagophore formation (197, 201). WIN55212-2 also increased the mRNA levels of ATG12, ATG16L and ATG5 in LPS-activated hmoDCs. ATG12, ATG16L and ATG5 participate in the elongation and autophagophore formation (85). In contrast, WIN55212-2 decreased the gene expression of Rubicon (RUBCN), an inhibitor of canonical autophagy and exclusive protein of LC3-associated phagocytosis (LAP) (202, 203), in LPS-activated hmoDCs (**Figure R.56**). Altogether, our results strongly support that WIN55212-2 is able to promote the induction of canonical autophagy, not LAP in hmoDCs.

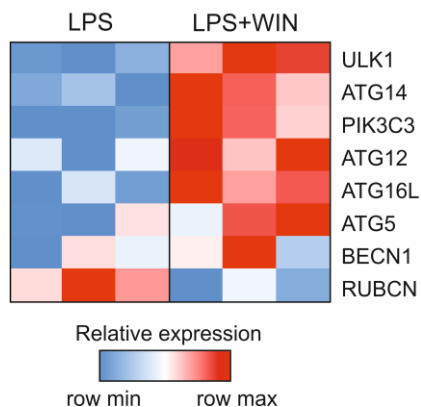


Figure R.56. Heatmap of autophagy-related gene expression after stimulation of hmoDCs with LPS or LPS plus WIN55212-2 (WIN) for 4 hours (n = 3)

mTORC1 is a key regulator of cell growth and activation in DCs and can be activated by a variety of immunological stimuli, including TLR agonists. mTORC1

activation promotes the anabolic metabolism for the biosynthesis of proteins and fatty acids needed for cell activation, growth or division (74, 93). In contrast, AMPK stimulation inhibits mTORC1 activation and enhances autophagy flux, promoting tolerogenic DC differentiation (78, 81). In line with these previously reported observations, our data showed that WIN55212-2 inhibits the pro-inflammatory NF- κ B, MAPK and mTORC1 signalling pathways in LPS-activated hmoDCs and induces AMPK activation and autophagy, which underlies its capacity to generate tolerogenic DCs.

3.2. Study of the main metabolic changes promoted by WIN55212-2 in human DCs.

mTOR and AMPK are key metabolic regulators that act as antagonists controlling catabolism and anabolism (78, 81, 93). Changes in cellular metabolism of DCs are essential to the activation and functionality of DCs (88). DC activation is characterized by an acute increase in glucose metabolism that is required to fuel the high anabolic rates associated with DC activation. Glycolysis inhibition attenuated most aspects of DC activation including antigen presentation or inflammatory cytokines (204, 205). Therefore considering our previous results, we hypothesized that WIN55212-2 could rewire the metabolic profile imprinted by LPS in human DCs.

In order to confirm our hypothesis, we initially evaluated the capacity of WIN55212-2 to rewire glucose metabolism imprinted by LPS in hmoDCs. For that, hmoDCs were stimulated with WIN55212-2, LPS and LPS plus WIN55212-2 for 18 hours and Warburg effect, lactate production and glucose consumption were analysed in cell-free supernatants. Warburg effect is a process by which proliferating or activated cells increase the rate of glucose uptake dramatically and lactate is produced after glycolysis even in the presence of oxygen and fully functioning mitochondria (206).

HmoDCs activated with LPS displayed higher Warburg effect than unstimulated hmoDCs, which was significantly abolished when hmoDCs were stimulated with LPS plus WIN55212-2 (**Figure R.57a**). In order to confirm Warburg effect data, we analysed lactate content in cell-free supernatants. As expected hmoDCs activated with LPS produced higher levels of lactate than unstimulated hmoDCs. WIN55212-2 significantly inhibited lactate production by LPS-activated hmoDCs (**Figure R.57b**). In addition, the activation of hmoDCs with LPS significantly increased the consumption of glucose from the culture medium, which was expressed as metabolic rate. In contrast, hmoDCs stimulated with LPS in the presence of WIN55212-2 showed lower glucose consumption than LPS-stimulated hmoDCs, suggesting that LPS plus WIN55212-2-stimulated hmoDCs needed less glucose for energy production or that other substrates are used as fuel for energy demand (**Figure R.57c**).

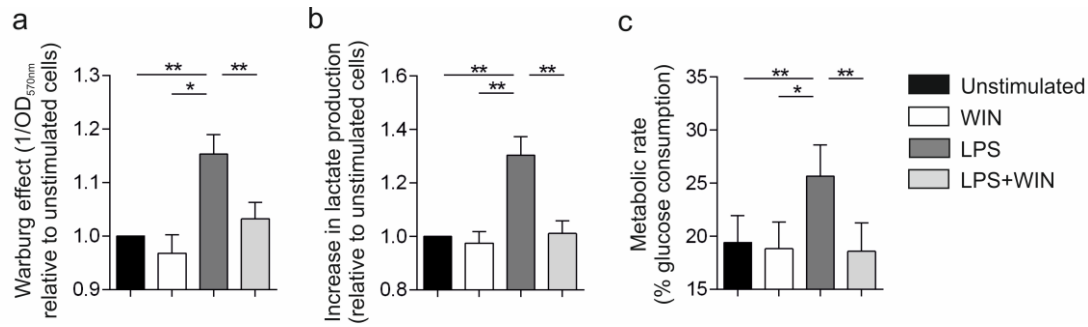


Figure R.57. **a** Quantification of the induced Warburg effect relative to unstimulated condition (n = 8). **b** Lactate content in cell-free supernatants from WIN55212-2 (WIN), LPS or LPS plus WIN55212-2-stimulated hmoDCs for 18 hours relative to unstimulated condition (n = 8). **c** Glucose consumption determined as metabolic rate for the indicated conditions (n = 8). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01.

Intracellular processing of glucose by glycolysis produce pyruvate, which can be converted into lactate (Warburg effect) or acetyl coenzyme A (acetyl-CoA) substrate of TCA cycle and oxidative phosphorylation in the mitochondria (73). Our results showed that WIN55212-2 inhibits Warburg effect in LPS-activated hmoDCs. Therefore, we wanted to study whether WIN55212-2 affects the mitochondrial metabolism in hmoDCs. For that, we monitored changes in mitochondrial membrane potential with Mito Tracker Red CMXRos. The mitochondrial membrane potential was significantly higher in WIN55212-2-stimulated hmoDCs than in unstimulated hmoDCs. Similarly, LPS plus WIN55212-2-treated hmoDCs displayed higher mitochondrial membrane potential than hmoDCs stimulated with LPS alone (**Figure R.58a**). These data suggest that WIN55212-2 promoted high mitochondrial activity. Because mitochondrial activity, specifically oxidative phosphorylation, is an important source of ROS, we analysed ROS content using CM-H2DCFDA. HmoDCs treated with LPS in the presence of WIN55212-2 produced greater levels of ROS compared with LPS-stimulated hmoDCs (**Figure R.58b**). These data showing high mitochondrial activity induced by WIN55212-2 in hmoDCs suggested that WIN55212-2 could be favouring TCA cycle and oxidative phosphorylation.

Nicotinamide adenine dinucleotide in its oxidized form (NAD⁺) and reduced form (NADH) is essential for the activity of enzymes that participate in multiple metabolic processes and the NAD⁺/NADH ratio play a crucial role in regulating the intracellular redox state. Anabolic processes are usually responsible of NAD⁺ generation whereas catabolic metabolism is associated to the generation of NADH (207). Analysis of the redox status showed that the NAD⁺/NADH ratio was slightly lower in hmoDCs stimulated with WIN55212-2 than unstimulated hmoDCs. Similarly, NAD⁺/NADH ratio was significantly lower in hmoDCs stimulated with LPS plus WIN55212-2 than with LPS alone, suggesting high catabolic metabolism (**Figure R.58c**). Catabolic processes are

involved in the synthesis of ATP, which is demanded during the anabolic processes. LPS-activated hmoDCs showed similar levels of intracellular ATP than unstimulated hmoDCs, suggesting that ATP produced by LPS-activated glycolytic pathway was consumed for anabolic processes. In contrast, hmoDCs stimulated with LPS in the presence of WIN55212-2 displayed higher intracellular ATP levels than hmoDCs stimulated with LPS alone (**Figure R.58d**). These results indicate that WIN55212-2 rewires the glucose metabolism of LPS-activated hmoDCs towards decreased glycolysis and anabolism and increased catabolism.

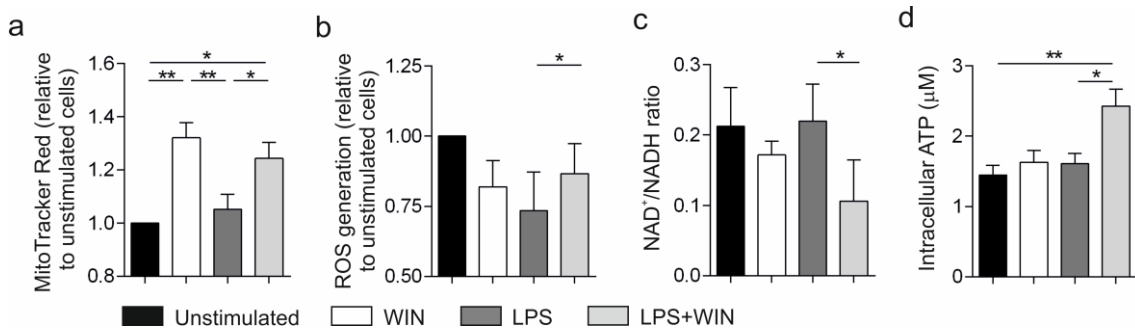


Figure R.58. **a** Fluorescence intensity of stimulated hmoDCs stained with Mito Tracker Red and relative to unstimulated condition ($n = 8$). **b** Intracellular ROS quantification in hmoDCs after 18 hours of stimulation with the indicated conditions ($n = 6$). **c** Cellular redox status analysed based on the determination of NAD^+/NADH ratio ($n = 4$). **d** Intracellular ATP levels of hmoDCs stimulated for 18 hours with the indicated conditions ($n = 6$). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$.

To further investigate the capacity of WIN55212-2 to induced metabolic reprogramming in LPS-activated hmoDCs, we assessed the capacity of WIN55212-2 to regulate the mRNA expression levels of different key metabolism-related genes (**Figure R.59**).

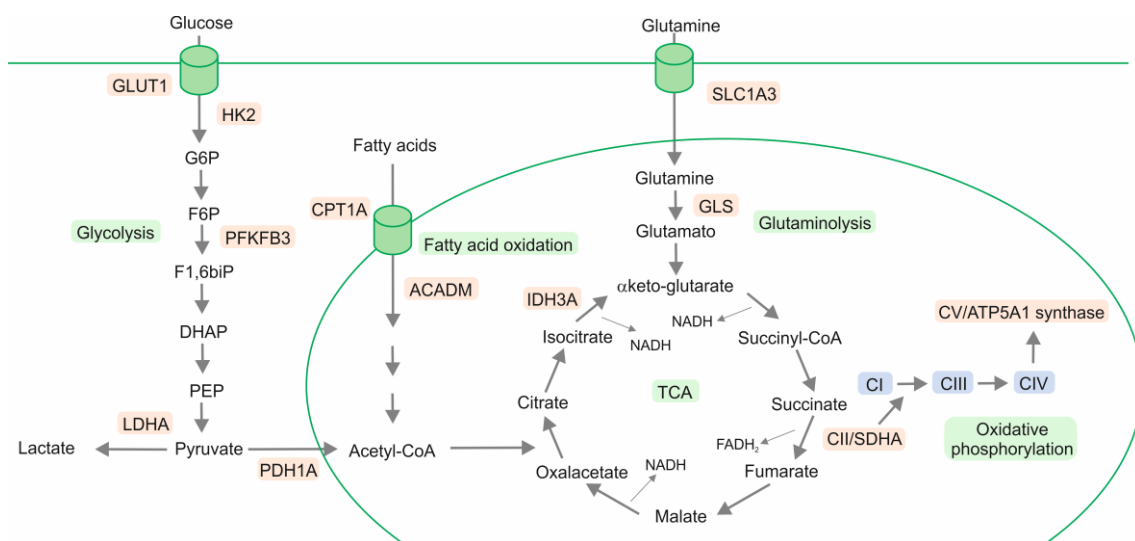


Figure R.59. Scheme of the main metabolic pathways studied: glycolysis, tricarboxylic acid (TCA) cycle, fatty acid oxidation, glutaminolysis and oxidative phosphorylation are shown.

As shown in the heatmap of **Figure R.60**, the mRNA levels of the glucose transporter 1 (GLUT1) and glycolytic enzymes such as hexokinase 2 (HK2) or phosphofructokinase (PFKFB3) were lower in LPS plus WIN55212-2-stimulated hmoDCs than in LPS-activated hmoDCs. HK2 and PFKFB3 are two rate-limiting enzymes of the glycolytic pathway that catalyse the conversion of glucose to glucose 6-phosphate or fructose 6-phosphate to fructose 1,6-bisphosphate, respectively (208). Similarly, the mRNA levels of the lactate dehydrogenase A (LDHA), involved in the conversion of pyruvate to lactate, and hypoxia-inducible factor 1-alpha (HIF1A) (209), an activator of glycolysis were lower in LPS plus WIN55212-2 stimulated hmoDCs than in LPS-activated hmoDCs. These data support our previous results showing that WIN55212-2 inhibited glucose uptake, Warburg effect and lactate production in LPS-activated hmoDCs.

In contrast, enzymes involved in TCA cycle and oxidative phosphorylation were upregulated in LPS plus WIN55212-2-stimulated hmoDCs compared to LPS-stimulated hmoDCs, supporting our previous data that indicated high mitochondrial activity in the presence of WIN55212-2 (**Figure R.60**). These enzymes are pyruvate dehydrogenase (PDHA1), which catalyses the reaction of pyruvate to acetyl-CoA, isocitrate dehydrogenase (IDH3A), which converts isocitrate to α -ketoglutarate, succinate dehydrogenase (SDHA) that is the bridge enzyme between TCA cycle and oxidative phosphorylation, and ATP synthase subunit α (ATP5A1), which is the mitochondrial enzyme involved in ATP production (210). In addition, the mRNA levels of PTEN-induced kinase 1 (PINK1), a mitochondrial kinase that regulates oxidative phosphorylation via mitochondrial fission, were also upregulated in LPS plus WIN55212-2 stimulated hmoDCs compared to LPS-stimulated hmoDCs (211).

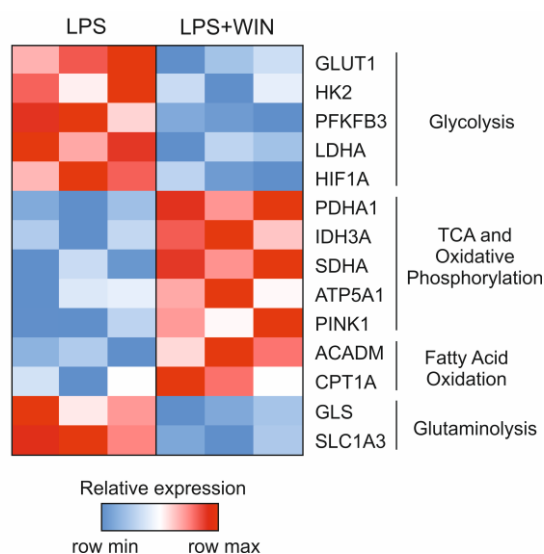


Figure R.60. Heatmap of metabolism-related gene expression after stimulation of hmoDCs with LPS or LPS plus WIN55212-2 (WIN) for 4 hours (n = 3)

Interestingly, the mRNA levels of the proteins involved in fatty acid oxidation, such as acyl-coenzyme A dehydrogenase (ACADM), which catalyse the initial step of the mitochondrial fatty acid oxidation and carnitine O-palmitoyltransferase 1 (CPT1A) that catalyses the essential step for mitochondria uptake of fatty acids also are upregulated in LPS plus WIN55212-2-stimulated hmoDCs compared to LPS-activated hmoDCs, suggesting that WIN55212-2 promotes fatty acid oxidation. In contrast, the mRNA levels of enzymes involved in glutaminolysis, such as glutaminase (GLS) or the amino acid transporter 1 (SLC1A3) are downregulated in LPS plus WIN55212-2-stimulated hmoDCs, suggesting that fatty acids but not glutamine are the main fuel for the activated TCA cycle in LPS plus WIN55212-2-stimulated hmoDCs (**Figure R.60**).

Collectively, our data demonstrated that WIN55212-2 rewires the glucose metabolism of LPS-activated hmoDCs towards decreased glycolysis and enhanced TCA cycle and oxidative phosphorylation. In addition, our data indicated that fatty acids could be used for energy production. Our results are aligned with previous reports showing that tolerogenic DCs display a pronounced catabolic program characterized by glycolysis, oxidative phosphorylation induction and a reduced anabolic metabolism (88). Campbell et al showed that two-plant polyphenols, carnosol and curcumin, imprinted tolerogenic features in LPS-activated DCs through metabolic regulation. DCs treated with these polyphenols displayed reduced rate of glycolysis and increased capacity for oxidative phosphorylation and fatty acid oxidation (212). Malinarich et al demonstrated that tolerogenic DCs showed a high mitochondrial oxidative activity, production of ROS and increased spare respiratory capacity. In addition, fatty acid oxidation activity and the expression of proteins involved in this process were higher in tolerogenic DCs than in mature DCs (94). Vitamin D3 induced regulatory properties in mouse and human DCs, and these DCs exhibit enhanced catabolism, metabolic plasticity, and increased expression of genes involved in oxidative phosphorylation, glycolysis and fatty acid oxidation (69, 91).

Next, we aimed to investigate the role of autophagy in the capacity of WIN55212-2 to induce tolerogenic human DCs. It has been described the importance of autophagy in the modulation of DCs by controlling cytokine production, migration, antigen presentation and T cell differentiation (98, 213). Autophagy is indirectly or directly involved in the immunogenic or tolerogenic properties of DCs depending on the physiological context and micro-environment. Some studies showed the importance of autophagy to induce tolerogenic properties in DCs (98, 214, 215). Autophagy activation in mice DCs contributes to Treg cell stability and homeostasis in inflammatory conditions. In the absence of autophagy, ICOSL is downregulated and DC-Treg cell interaction are

modified, inducing Treg cell instability (215). However, more mechanisms insights in human DCs are required.

To explore the role played by WIN55212-2-induced autophagy in human DCs, we used the autophagy inhibitor 3-MA. As expected by the previous results, WIN55212-2 reduced the mRNA levels of IL-6, TNF α and IL-10 in LPS-activated hmoDCs. The inhibition of autophagy with 3-MA in hmoDCs stimulated with LPS plus WIN55212-2 significantly increased the mRNA expression levels of IL-6, TNF α and IL-10, suggesting that autophagy is involved in the effects induced by WIN55212-2 in hmoDCs (**Figure R.61**).

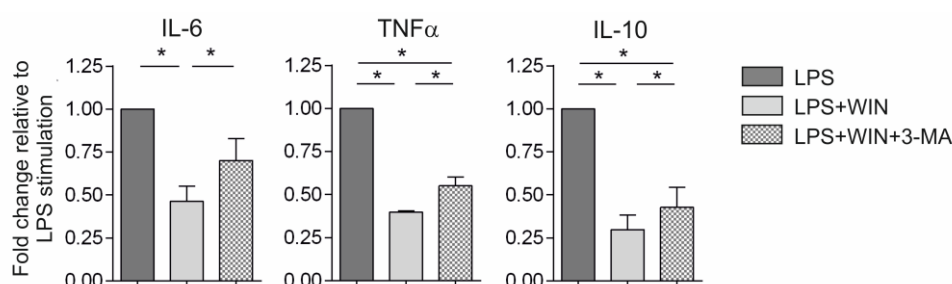


Figure R.61. Influence of autophagy in cytokine mRNA expression in hmoDCs. mRNA expression levels of cytokines in LPS plus WIN55212-2 (WIN) or LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA stimulated hmoDCs relative to LPS-stimulated condition (n = 5). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05.

To assess the relevance of WIN55212-2-induced autophagy in hmoDCs for priming T cell responses, we performed coculture experiments. As expected by the previous results, T cells generated by hmoDCs stimulated with LPS plus WIN55212-2 produced lower levels of IFN γ and higher levels of IL-10 than those generated by LPS-activated hmoDCs. T cells generated by hmoDCs stimulated with LPS plus WIN55212-2 in the presence of 3-MA produced higher levels of IFN γ and lower levels of IL-10 than those generated by LPS plus WIN55212-2-activated hmoDCs, demonstrating the importance of autophagy induction in WIN55212-2-stimulated hmoDCs (**Figure R.62**).

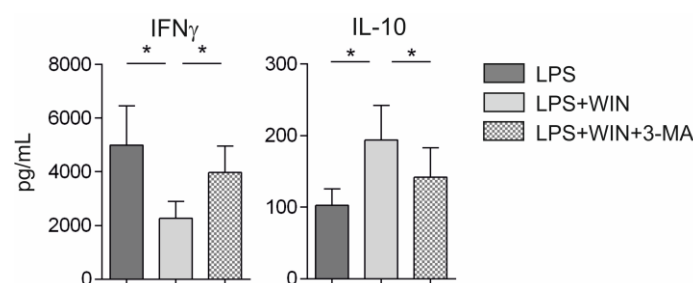


Figure R.62. Influence of autophagy induced in hmoDCs for T cell polarization. Cytokines produced by allogeneic naïve CD4⁺ T cells primed by LPS, LPS plus WIN55212-2 (WIN) or LPS plus WIN in the presence of 3-MA-treated hmoDCs after 5 days (n = 6). Values are mean $\pm$ SEM. Statistical significance determined using one-way Anova * P < 0.05

We analysed the role of autophagy in the capacity of hmoDCs to generate Treg cells. As expected, LPS plus WIN55212-2-stimulated hmoDCs generated higher numbers of CD4⁺CD127⁻CD25⁺FOXP3⁺ Treg cells than LPS-activated hmoDCs, which was significantly impaired by 3-MA, indicating that autophagy induced by WIN55212-2 in hmoDCs is necessary for the acquisition of tolerogenic properties in human DCs and the generation of Treg cells (**Figure R.63**).

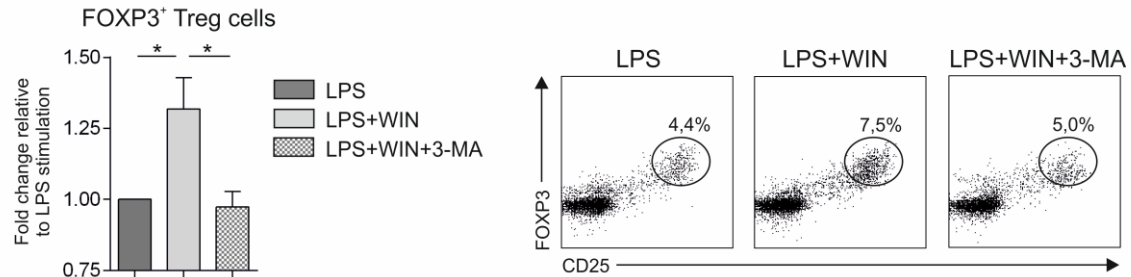


Figure R.63. Influence of autophagy induced in hmoDCs for Treg cell generation. Increment in CD4⁺CD127⁻CD25⁺FOXP3⁺ Treg cell generation relative to LPS-stimulation condition (n = 6). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05.

Next, we investigated whether the metabolic reprogramming associated to WIN55212-2 treatment in LPS-activated hmoDCs was also dependent on autophagy mechanisms. HmoDCs were stimulated with LPS and LPS plus WIN55212-2 in the absence or presence of 3-MA, and the metabolic status was evaluated.

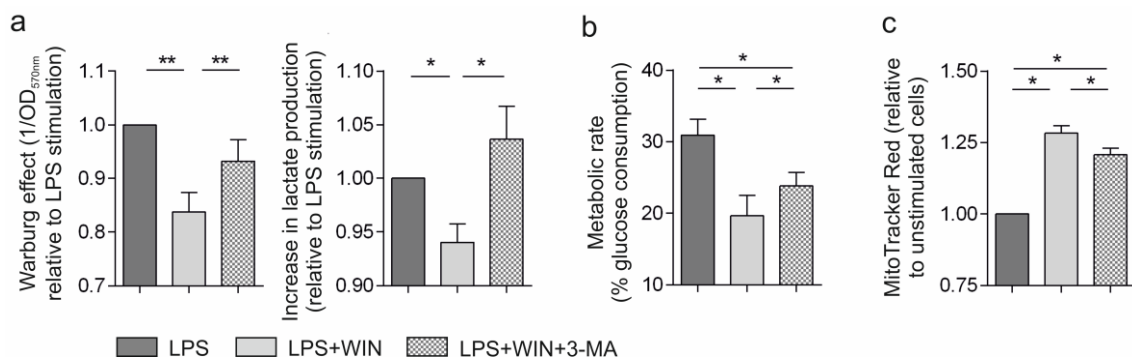


Figure R.64. Role of autophagy in the metabolic reprogramming induced by WIN55212-2 (WIN) in hmoDCs. **a** Quantification of the induced Warburg effect and lactate content in cell-free supernatants relative to LPS stimulation (n = 6). **b** Glucose consumption determined as metabolic rate of the indicated conditions (n = 6). **c** Fluorescence intensity of hmoDCs stained with Mito Tracker Red relative to LPS-stimulated condition (n = 6). Values are mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01.

As we previously described, WIN55212-2 significantly reduced Warburg effect, lactate production and glucose consumption in LPS-activated hmoDCs. Inhibition of autophagy with 3-MA in LPS plus WIN55212-2-stimulated hmoDCs significantly increased Warburg effect, production of lactate and consumption of glucose (metabolic rate) (**Figure R.64ab**). LPS plus WIN55212-2-stimulated hmoDCs displayed higher

mitochondrial membrane potential than hmoDCs activated with LPS alone. The presence of 3-MA reduced the mitochondrial membrane potential in LPS plus WIN55212-2-treated hmoDCs (**Figure R.64c**). These data indicate that WIN55212-2 imprints tolerogenic properties and induce metabolic reprogramming in hmoDCs by mechanisms that depends on the induction of autophagy.

In line with our data, it has been described the role of autophagy in the induction of tolerogenic properties in DCs (98, 213). Autophagy was reported to inhibit IL-1 β production in bone marrow DCs stimulated with LPS and ATP (216). Niven et al showed that autophagy in DCs is required for Treg cell homeostasis and stability during inflammation (215). Similarly, autophagy in bone marrow DCs were required to activate Treg cell for the control of mucosal inflammation caused by a commensal bacteria (214). For the protection of allograft rejection, the induction of tolerogenic DC generation that results in higher induction of FOXP3⁺ Treg cells was associated with enhanced autophagy in the cardiac allograft (217). On the other hand, bone marrow DC infection with respiratory syncytial virus induce the expression of TNF α , IL-6 and IL-12 (218), demonstrating the dual role of autophagy in the control of immune responses.

3.2. Study of the contribution of CBRs and PPARs to the immunomodulatory properties exerted by WIN55212-2 in LPS-stimulated human DCs.

It has been reported that besides the activation of CB1 and CB2, cannabinoids can exert their effects through other receptors, including PPARs (139). WIN55212-2 is a CB1 and CB2 agonist, but its effects can be also mediated through PPAR α and PPAR γ (189, 190). In an experimental model of colitis, WIN55212-2 exerted its anti-inflammatory properties via CB1 and CB2 (192). *In vivo* models showed that WIN55212-2 reduced neuroinflammation and improved memory function via CB1, CB2 and PPAR γ (190). In addition, the protective effects of WIN55212-2 in an autoimmune model of sclerosis multiple was associated to PPAR α activation (184). Therefore, we wanted to investigated the contribution of CB1, CB2, PPAR α and PPAR γ to the observed immunomodulatory properties of WIN55212-2 in hmoDCs.

Firstly, we analysed the expression of PPAR α and PPAR γ at protein and mRNA level in hmoDCs. Western blot analysis using anti-PPAR α and PPAR γ antibodies revealed that hmoDCs express both receptors (**Figure R65a**). Moreover, PPAR α and PPAR γ mRNA levels were detected in hmoDCs (**Figure R.65b**).

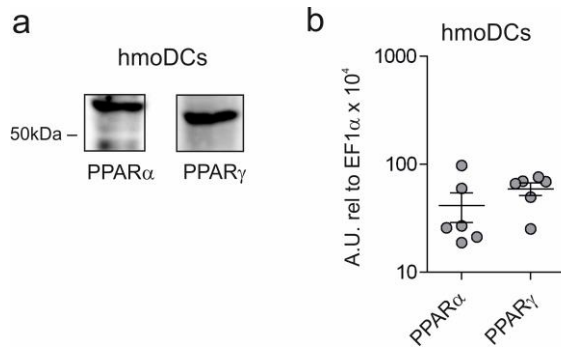


Figure R.65. hmoDCs express PPAR α and PPAR γ at protein and mRNA level. **a** Western blot of PPAR α and PPAR γ expression in hmoDCs. One representative example of three independent experiments. **b** mRNA expression levels of PPAR α and PPAR γ in hmoDCs (n = 6).

Then, to investigate the specific contribution of the classical CB1 and CB2 and other alternative CBRs such as PPAR α and PPAR γ to the immunomodulatory properties imprinted by WIN55212-2 in human DCs, we performed inhibition experiments using pharmacological inhibitors for CB1 (Rimonabant), CB2 (AM630), PPAR α (GW6471) and PPAR γ (GW9662). HmoDCs were pre-incubated with each antagonist for 1 hour and then, stimulated with LPS plus WIN55212-2. CD83 surface expression and cytokine production were analysed by flow cytometry and ELISA, respectively. The blockade of CB1 and PPAR α suppressed the inhibitory effects of WIN55212-2 on CD83 expression in LPS-activated hmoDCs, whereas the blockade of CB2 and PPAR γ did not alter the effects of WIN55212-2. Representative dot plots including the percentage of positive cells for CD83 are shown (**Figure R.66**).

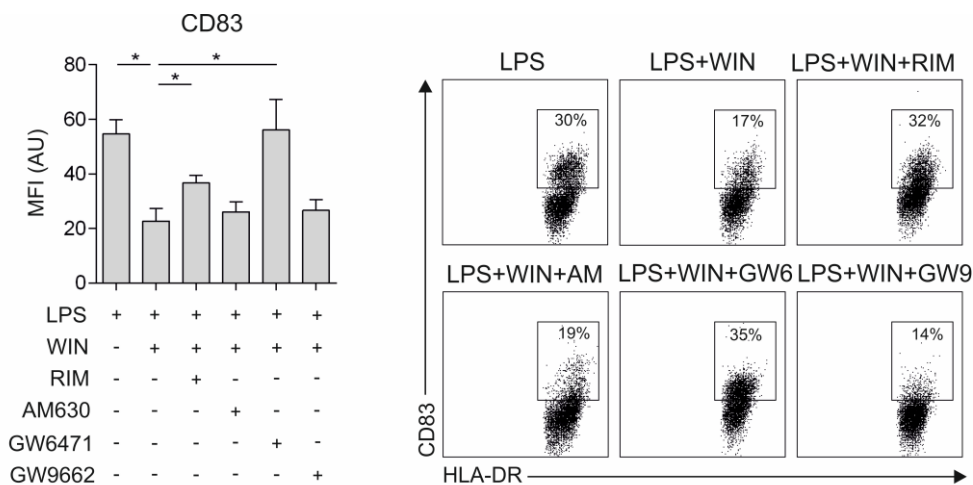


Figure R.66. Contribution of CBRs to the WIN55212-2 (WIN)-induced CD83 inhibition. Mean fluorescence intensity (MFI) of CD83 surface expression in hmoDCs stimulated with LPS or LPS plus WIN55212-2 in the presence or absence of antagonists for CB1 (Rimonabant; RIM), CB2 (AM630), PPAR α (GW6471; GW6) and PPAR γ (GW9662; GW9) for 18 hours (n = 8). Dot plots of CD83 expression and the percentage of CD83⁺ hmoDCs are shown. Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05.

The blockade of CB2 and PPAR γ did not alter the inhibitory effects of WIN55212-2 in cytokine production by hmoDCs. However, TNF α and IL-6 production were significantly higher in hmoDCs stimulated with LPS plus WIN55212-2 in the presence of the CB1 or PPAR α antagonists than in hmoDCs stimulated with LPS plus WIN55212-2,

indicating that CB1 and PPAR α are the main receptors involved in the anti-inflammatory effects of WIN55212-2 in hmoDCs (**Figure R.67a**). We demonstrated that CB1 and PPAR α are the main receptors involved in the features imprinted by WIN55212-2 in hmoDCs. Then, we wanted to study how the inhibition of the different CBRs in hmoDCs affected to the capacity of T cell polarization. For that, the generation of Treg cells was analysed. In the same line that the previous results, the presence of CB1 or PPAR α antagonists in LPS plus WIN55212-2-stimulated hmoDCs impaired their capacity to generate CD4⁺CD127⁺CD25⁺FOXP3⁺ Treg cells, whereas the presence of CB2 and PPAR γ did not (**Figure R.67b**).

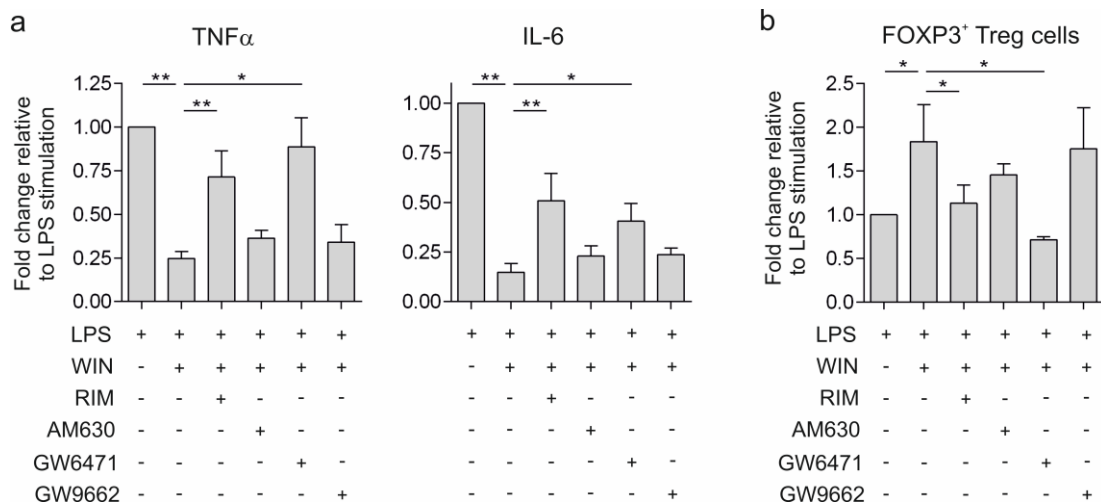


Figure R.67. a Contribution of CBRs to the WIN55212-2 (WIN)-induced cytokine inhibition. Fold change of cytokine production by hmoDCs stimulated with LPS plus WIN55212-2 in the presence or absence of antagonists for CB1 (Rimonabant; RIM), CB2 (AM630), PPAR α (GW6471) and PPAR γ (GW9662) for 18 hours relative to LPS stimulation ($n = 8$). **b** Fold change of FOXP3⁺ Treg induction by hmoDCs stimulated with the indicated conditions relative to LPS stimulation ($n = 8$). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$.

Our data in hmoDCs support previous studies demonstrating that WIN55212-2 can exerts its anti-inflammatory effects not only by CB1 and CB2, but also through PPARs (139). Our results indicate that the blockade of CB1 and PPAR α in hmoDCs before LPS plus WIN55212-2 stimulation inhibits the tolerogenic effects imprinted by WIN55212-2. In line with these results, selective activation of CB1 inhibits K⁺ channels in mice DCs, reducing their capacity to activate T cells (154). PPAR α agonist Wy14643 have been shown to inhibit murine Langerhans cells maturation, migration and capability to prime T cell responses (219). Furthermore, Wy14643 treatment during the differentiation of monocytes to hmoDCs affected the phenotype and the capacity of hmoDCs to respond to LPS stimulation (220).

Next, we assessed the specific contribution of CB1 and PPAR α in WIN55212-2-induced autophagy and the subsequent metabolic reprogramming in hmoDCs. Western

blot analysis revealed that the blockage of CB1 and PPAR α in LPS plus WIN55212-2-stimulated hmoDCs reduced the levels of LC3-II, the lipidated and autophagosome-associated form of LC3, indicating autophagy inhibition (**Figure R.68**).

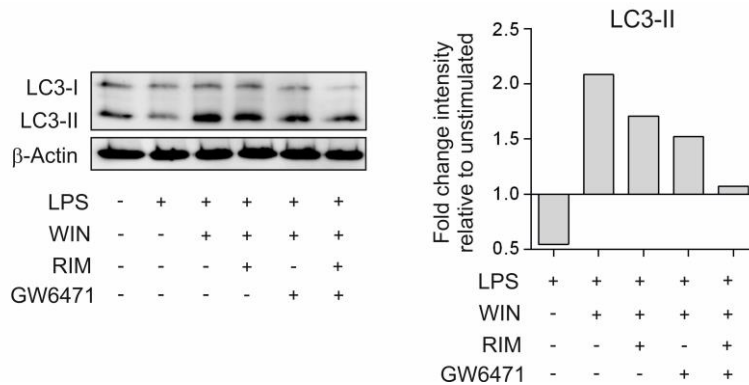


Figure R.68. Contribution of CB1 and PPAR α to autophagy activation by WIN55212-2 (WIN). Western blot analysis of protein extracts from hmoDCs stimulated with LPS and LPS plus WIN55212-2 in the presence or absence of antagonists for CB1 (Rimonabant; RIM) and PPAR α (GW6471) for 18 hours. Quantification of the reactive bands by scanning densitometry is shown. One representative example of two independent experiments

Subsequently, the presence of antagonists for CB1 and PPAR α impaired WIN55212-2-induced metabolic changes in LPS-activated hmoDCs, including the reduction of Warburg effect, the reduction of metabolic rate and the increase of mitochondrial membrane potential (**Figure R.69**).

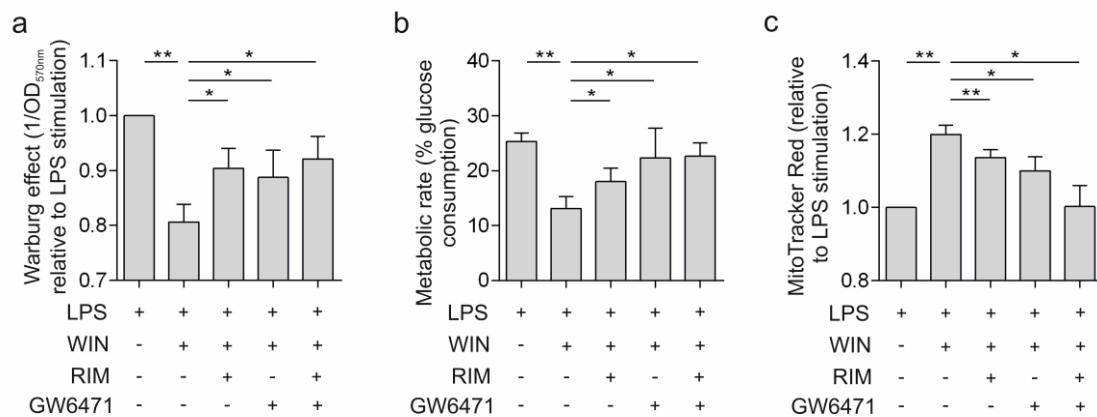


Figure R.69. Contribution of CB1 and PPAR α to the metabolic reprogramming induced by WIN55212-2 (WIN) **a** Quantification of the induced Warburg effect relative to LPS stimulation (n = 6). **b** Glucose consumption determined as metabolic rate for the indicated conditions (n = 6). **c** Fluorescence intensity of hmoDCs stained with Mito Tracker Red relative to LPS-stimulated condition (n = 6). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01.

Collectively, our data showed that CB1 and PPAR α are the main receptors involved in the tolerogenicity imprinted by WIN55212-2 in hmoDCs. Our pharmacological inhibition experiments reported that the blockade of CB1 and PPAR α impaired autophagy activation and the metabolic rewiring induced by WIN55212-2 in LPS-activated hmoDCs. Thus, the blockade of CB1 and PPAR α impaired the generation of FOXP3⁺ Treg cells induced by WIN55212-2-stimulated hmoDCs. Supporting our data, it

has been described that the CB1 agonist arachidonoyl cyclopropamide induces AMPK-dependent autophagy activation in pancreatic cancer cells. In addition, this CB1 agonist inhibits mTOR signalling and the glycolytic pathway (147). Furthermore, PPAR α is well known for its contribution to metabolism. PPAR α plays a key role in metabolic reprogramming of activated-pDCs, favouring oxidative phosphorylation and fatty acid oxidation (221). Similarly, PPAR α agonists induces autophagy and enhances mitochondrial respiration and fatty acid oxidation in macrophages during mycobacterial infection (222). These studies demonstrate the role of CB1 and PPAR α in autophagy induction and metabolic reprogramming in different cell models, including in immune cells.

Objective 4: Assessment of the immunomodulatory effects promoted by the synthetic cannabinoid WIN55212-2 in human and mice preclinical models

4.1. Study of the immunomodulatory properties of WIN55212-2 in an *in vivo* LPS-induced sepsis mouse model.

Sepsis is a lethal disease worldwide characterized by uncontrolled local and systemic inflammatory responses (223). Several animal models to study the molecular mechanism underlying sepsis have been developed. Among them, the administration of LPS, the gram-negative bacterial endotoxin, to mice via different routes has been used as suitable animal model mimicking some of the most relevant pathophysiological mechanisms described in human. Lethal doses of LPS elicit a systemic inflammatory process that result in hepatic and renal failure, myocardial dysfunction, acute lung injury and disseminated intravascular coagulation, which finally lead to lethal multiorgan failure and death (224).

To assess the *in vivo* relevance of our findings, we studied whether WIN55212-2 exerts the previously observed immunomodulatory effects in an *in vivo* preclinical LPS-induced sepsis mouse model. WIN55212-2 (5 mg/kg), LPS (20 mg/kg) alone, LPS in combination with WIN55212-2 and LPS plus WIN55212-2 in the presence of specific pharmacological inhibitor of autophagy (3-MA; 15 mg/kg) or a mixture of CB1 (Rimonabant; 5 mg/kg) and PPAR α (GW6471; 5 mg/kg) specific antagonists were intraperitoneally administered to BALB/c mice and survival monitored for 78 hours (**Figure R.70**).

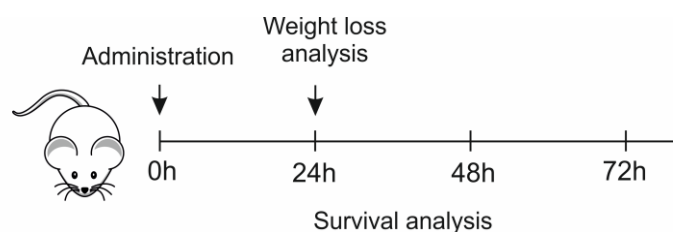


Figure R.70. Scheme of the survival analysis of LPS-induced sepsis model

All mice treated with vehicle and mice treated with WIN55212-2 alone survived for the 78 hours of follow up. As expected, all mice treated with lethal doses of LPS died after 42 hours (**Figure R.71a**). The administration of lethal doses of LPS in combination with WIN55212-2 significantly increased the survival rate (90 %) at 78 hours, demonstrating that WIN55212-2 impaired the LPS-induced death in the used sepsis

model. Remarkably, the administration of the autophagy inhibitor 3-MA or the blockade of CB1 and PPAR α with pharmacological inhibitors in LPS plus WIN55212-2 treated mice significantly reduced the survival rate compared to LPS in combination with WIN55212-2-treated mice (**Figure R71a**). Twenty-four hours after LPS exposure, we monitored weight loss used as a surrogate marker of the clinical severity in this LPS-induced sepsis model. No difference in weight were observed between vehicle- and WIN55212-2-treated mice. In contrast, LPS-treated mice showed a significant weight loss compared to vehicle-treated mice. Interestingly, LPS plus WIN55212-2-treated mice showed less weight loss compared to LPS-treated mice. However, the administration of 3-MA or CB1 and PPAR α antagonists in LPS plus WIN55212-2 treated mice increased the weight loss (**Figure R71b**). These data demonstrated the protective effect of WIN55212-2 in a LPS-induced sepsis model via CB1 and PPAR α through mechanisms that depend on autophagy.

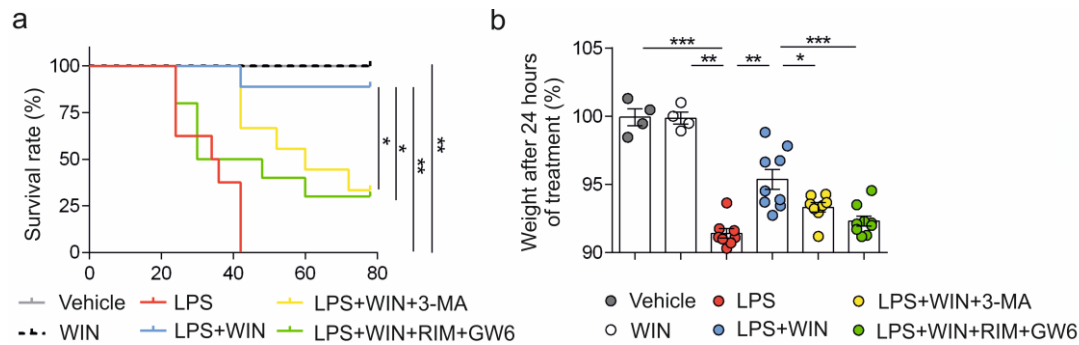


Figure R.71. WIN55212-2 (WIN) improves the survival rate in LPS-induced sepsis. **a** Survival rate of mice treated with WIN55212-2 (5 mg/kg), LPS (20 mg/kg), LPS plus WIN55212-2 and LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA (15 mg/kg) or CB1 (Rimonabant; RIM; 5 mg/kg) and PPAR α (GW6471; GW6; 5mg/kg) antagonists monitored for 78 hours (n = 10 of 3 independent experiments). Survival curves were compared with Log-rank (Mantel-Cox). **b** Percentage of weight after 24 hours of treatment (n = 4/9 of 2 independent experiments). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01. *** P < 0.001.

Then, we wanted to study the effect of WIN55212-2 in the inflammatory response induced by LPS. For that, we monitored the systemic release of the pro-inflammatory cytokine IL-6 as a surrogate marker of systemic inflammation (**Figure R.72**).

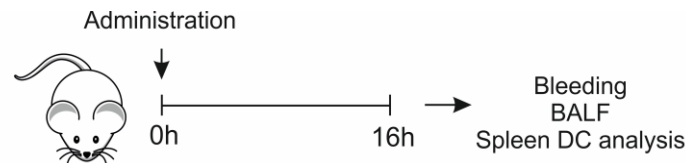


Figure R.72. Scheme of LPS-induced sepsis model

Plasma levels of IL-6 were strongly increased after 16 hours of LPS stimulation and significantly decreased in the presence of WIN55212-2. Autophagy inhibition or blockade of CB1 and PPAR α with the specific pharmacological inhibitors in LPS plus

WIN55212-2-treated mice increased the levels of IL-6 in plasma (**Figure R.73a**). In addition, we analysed the IL-6 levels in the bronchoalveolar fluid (BALF) of lung as example of affected tissue in LPS-induced sepsis. LPS-treated mice showed higher levels of IL-6 in BALF compared to mice treated with vehicle or with WIN55212-2 alone. WIN55212-2 significantly inhibited IL-6 production in BALF from LPS-treated mice. The autophagy inhibition or the blockade of CB1 and PPAR α with pharmacological inhibitors in LPS plus WIN55212-2-treated mice increased the levels of IL-6 in BALF (**Figure R.73b**). Collectively, these data demonstrated that WIN55212-2 exerts *in vivo* anti-inflammatory effects in a LPS-induced sepsis model by mechanisms that rely on autophagy induction via activation of CB1 and PPAR α , thus confirming our previous *in vitro* data in human.

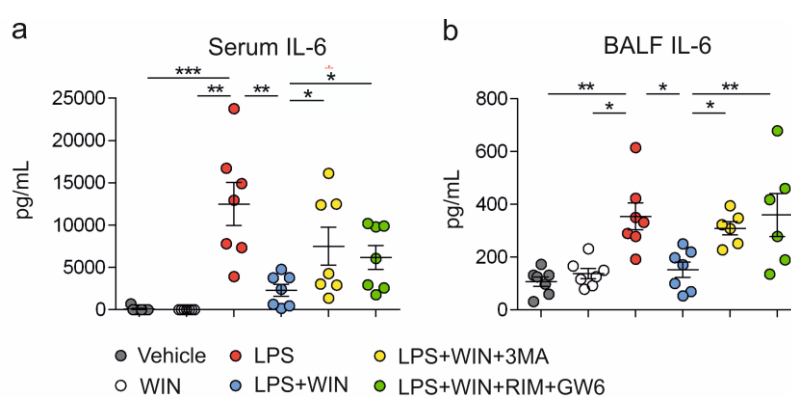


Figure R.73. WIN55212-2 (WIN) inhibits systemic and local inflammation induced in LPS-induced sepsis. **a** Plasma or **b** BALF IL-6 levels after 16 hours of treatment with WIN55212-2 (5mg/kg), LPS (20 mg/kg), LPS plus WIN55212-2 and LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA (15 mg/kg) or CB1 (Rimonabant; RIM; 5 mg/kg) and PPAR α (GW6471; GW6; 5 mg/kg) antagonists ($n = 7$ of 2 independent experiments). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Next, we wanted to study in more detail the potential role of DCs to the observed effects. For that, the percentage of total DCs in spleen was analysed 16 hours after LPS injection (**Figure R.72**). There were no statistically differences in the percentage of total spleen CD11c⁺MHCII⁺ DCs at the studied conditions. However, a slightly increase in the percentage of total spleen DCs was observed in LPS-treated mice compared to mice treated with vehicle. In addition, WIN55212-2 slightly decreased the percentage of total spleen DCs in LPS-treated mice (**Figure R.74a**). The levels of CD86 expression revealed that DCs from LPS-treated mice acquired a more mature phenotype compared to vehicle- or WIN55212-2-treated mice. Interestingly, the presence of WIN55212-2 partially reduced such maturation. Mice treated with LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA showed spleen DCs expressing slightly higher CD86 levels than those DCs from mice treated with LPS in combination with WIN55212-2. Moreover, mice treated with LPS plus WIN55212-2 in the presence of the antagonists

for CB1 and PPAR α showed spleen DCs expressing significantly higher CD86 levels than those DCs from mice treated with LPS in combination with WIN55212-2 (**Figure R.74b**).

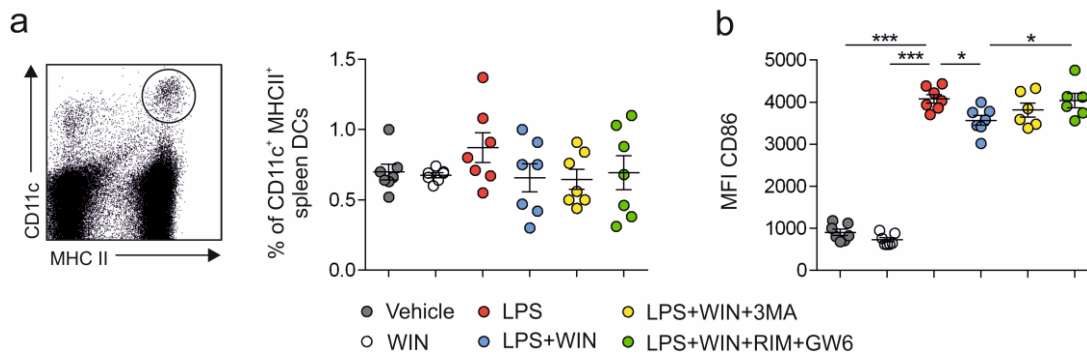


Figure R.74. Spleen DCs in the LPS-induced sepsis mouse model. **a** Percentage of CD11c⁺MHCII⁺ spleen DCs from mice treated with WIN55212-2 (WIN; 5 mg/kg), LPS (20 mg/kg), LPS plus WIN55212-2 and LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA (15 mg/kg) or CB1 (Rimonabant; RIM; 5 mg/kg) and PPAR α (GW6471; GW6; 5 mg/kg) antagonists for 16 hours (n = 7 of 2 independent experiments). Representative dot plot and gating strategy of flow cytometry is shown. **b** Mean fluorescence intensity (MFI) of CD86 expression in spleen DCs. Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05, *** P < 0.001.

Conventional DCs are the main population in secondary lymphoid organs, including spleen. This population can be divided into type 1 and type 2 cDCs. Type 1 cDCs (CD11b⁻ cDC1) are characterized by the expression of CD8 α (resident) or CD103 (migratory) and the lack of CD11b. CD11b⁻ cDC1 are specialized in cross-presentation and are involved in CD8⁺ T cell and Th1 immunity (15, 38). Type 2 cDCs (CD11b⁺ cDC2) are characterized by high expression of CD11b. CD11b⁺ cDC2 are involved in the induction of CD4⁺ T cell immunity in cancer and in Th2 and Th17 immunity (15, 38). Thus, we analysed these two cDC populations in spleen (**Figure R.75a**).

The percentage of spleen CD11b⁻ cDC1 and CD11b⁺ cDC2 did not significantly change in mice treated with WIN55212-2 alone compared to mice treated with vehicle. LPS treated mice showed higher ratio of cDC1/cDC2 than mice treated with vehicle. The presence of WIN55212-2 in LPS-treated mice increased this ratio. Autophagy inhibition with 3-MA did not significantly change this ratio. However, the blockade of CB1 and PPAR α in mice treated with LPS in combination with WIN55212-2 significantly reduced the cDC1/cDC2 ratio (**Figure R.75bcd**). These data suggest that the presence of WIN55212-2 in LPS-treated mice might change the proportions of spleen DCs by increasing the percentage of spleen CD11b⁻ cDC1, which are involved in cross-presentation and predominantly induce Th1 responses as well as Treg cells.

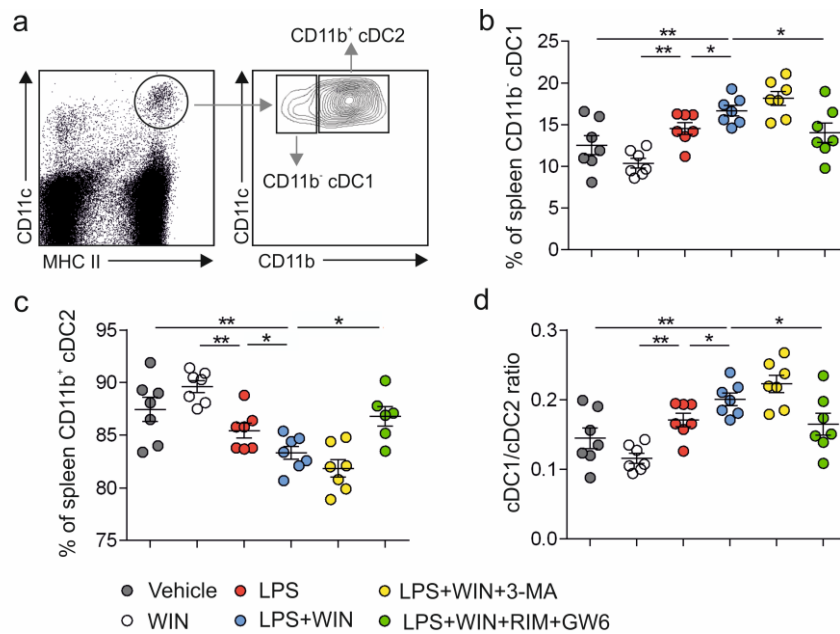


Figure R.75. Changes in the main spleen DC population. **a** Representative dot plot of flow cytometry and gating strategy. **b** Percentage of spleen CD11b⁺ cDC1 or **c** CD11b⁺ cDC2 DCs from mice treated with WIN55212-2 (WIN; 5 mg/kg) alone, LPS (20 mg/mL), LPS plus WIN55212-2 and LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA (15 mg/kg) or CB1 (Rimonabant; RIM; 5 mg/kg) and PPAR α (GW6471; GW6; 5 mg/kg) antagonists for 16 hours ($n = 7$ of 2 independent experiments). **d** cDC1/ cDC2 ratio ($n = 7$ of 2 independent experiments). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$.

DCs play a key role in the induction of T cell responses. Thus, we wanted to study the phenotype and function of MHCII⁺CD11c⁺ total DC fraction from spleen. For that, cytokine mRNA levels in purified total DCs from spleen was analysed. mRNA levels of IL-6 and IL-10 were higher in spleen DCs from LPS-treated mice than vehicle- or WIN55212-2-treated mice. The presence of WIN55212-2 in LPS-treated mice reduced IL-6 mRNA levels, but increased IL-10 mRNA levels in spleen DCs, suggesting the induction of tolerogenic DCs. Autophagy inhibition with 3-MA or blockade of CB1 and PPAR α increased the mRNA levels of IL-6 and reduced the IL-10 mRNA levels in spleen DCs from mice treated with LPS in combination with WIN55212-2, suggesting that WIN55212-2 acts through mechanisms that depend on autophagy induction via CB1 and PPAR α (**Figure R.76**).

To further verify the role of autophagy in the effects induced by WIN55212-2, mRNA levels of autophagy-related genes in spleen total DCs was analysed. mRNA levels of ATG5, ATG12 and LC3A were higher in spleen DCs from WIN55212-2-treated mice than in spleen DCs from untreated mice. Similarly, the presence of WIN55212-2 in LPS-treated mice increased ATG5, ATG12, ATG14 and LC3A mRNA levels in spleen DCs compared to spleen DCs from mice treated with LPS alone. As expected, the administration of 3-MA in LPS plus WIN55212-2 treated mice reduced the mRNA levels of all assayed autophagy-related genes. In addition, the presence of CB1 and PPAR α

antagonists in LPS plus WIN55212-2-treated mice reduced the mRNA expression levels of ATG14 and LC3A, but not the mRNA levels of ATG12 and LC3A (**Figure R.76**).

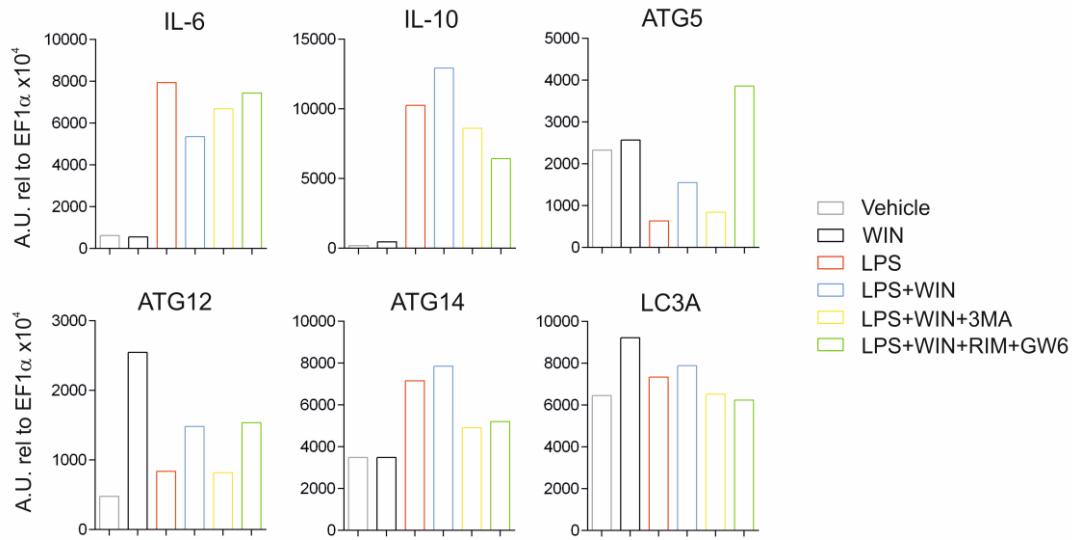


Figure R.76. Role of WIN55212-2 (WIN) in the function of total spleen DCs. mRNA expression levels of the cytokines IL-6 and IL-10 and autophagy-related genes ATG5, ATG12, ATG14 and LC3A in spleen DCs from mice treated with the indicated conditions for 16 hours. Data were obtained from a pull of spleen DCs from four mice treated with the same stimuli. One representative example of two independent experiments is shown. Arbitrary units (A.U.) are $2^{-(\Delta Ct)}$ values multiplied by 10^4 , with ΔCt defined as the difference between the cycle threshold value for each gene and elongation factor 1 α (EF1 α) as housekeeping gene.

Our data indicated that WIN55212-2 induced spleen DCs with tolerogenic phenotype by autophagy induction through CB1 and PPAR α . Our data are aligned with previous studies showing the importance of autophagy in the induction of tolerogenic DCs. Niven et al demonstrated that autophagy is necessary for the induction of tolerogenic DCs that contribute to the stability of Treg cells (215).

Our *in vivo* data showing the protective and anti-inflammatory role of WIN55212-2 in a LPS-induced sepsis model and its capacity to induce tolerogenic DCs encourage us to investigate whether WIN55212-2 could induce Treg cells. For that, we used non-lethal doses of LPS. WIN55212-2 (5 mg/kg), LPS (10 mg/kg) alone, LPS in combination with WIN55212-2 and LPS plus WIN55212-2 in the presence of 3-MA (15 mg/kg) or a mixture of CB1 (Rimonabant; 5 mg/kg) and PPAR α (GW6471; 5 mg/kg) specific antagonists were intraperitoneally administered to BALB/c mice (**Figure R.77**).

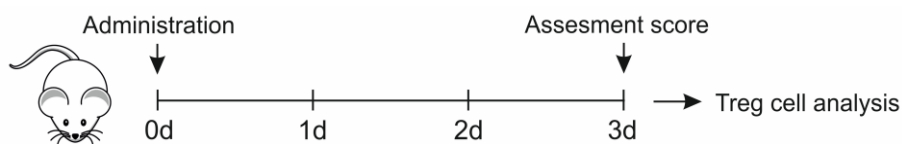


Figure R.77. Scheme of LPS-induced sepsis model

The physical appearance of the experimental animals was evaluated after 3 days of LPS exposure by the assessment score. Animals were scored according to the appearance of coat (1-3), activity (1-3) and posture (1-3), where 1 point correspond to normal conditions and 3 points correspond to the most severe symptoms. The assessment score was the total sum of the points from the above categories (225). Vehicle- and WIN55212-2-treated mice did not develop any clinical symptoms. Mice treated with LPS alone showed significantly higher assessment score than vehicle- or WIN55212-2-treated mice. Interestingly, mice treated with LPS plus WIN55212-2 exhibited significantly lower assessment score compared to mice treated with LPS alone. Mice treated with LPS plus WIN55212-2 in the presence of the autophagy inhibitor 3-MA or the antagonists for CB1 and PPAR α showed significantly higher assessment score than mice treated with LPS in combination with WIN55212-2 (**Figure R.78**).

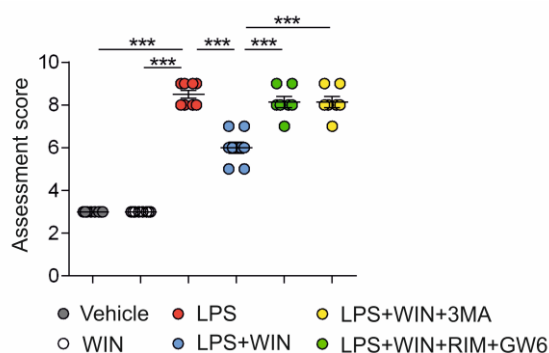


Figure R.78. Assessment score of the physical appearance 3 days after LPS injection (n = 7 of 2 independent experiments). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. *** P < 0.001.

Then, we wanted to evaluate the role of WIN55212-2 in the chronic inflammation induced by LPS. For that, three days after LPS injection, mice were sacrificed and spleens were collected. LPS-treated mice presented significant splenomegaly compared to mice treated with vehicle, indicating chronic systemic inflammation in the mice. In contrast, spleen weight of mice treated with LPS in combination with WIN55212-2 were significantly lower than LPS-treated mice, indicating that WIN55212-2 mitigated the chronic inflammation induced by LPS. The presence of 3-MA or CB1 and PPAR α antagonists in LPS plus WIN55212-2-treated mice significantly increased the spleen weight, suggesting that the anti-inflammatory effect of WIN55212-2 in the LPS-induced chronic inflammation is mediated by autophagy induction via CB1 and PPAR α activation (**Figure R.79**).

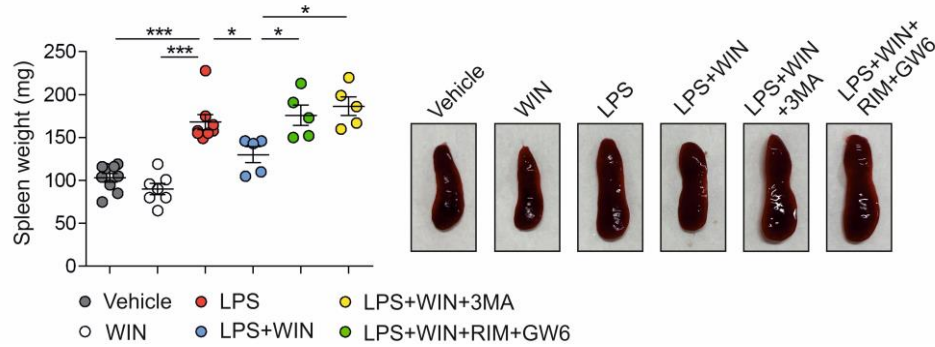


Figure R.79. Analysis of the splenomegaly induced in LPS-treated mice. Spleen weight from mice treated with the indicated conditions for 3 days ($n = 5/7$ of 2 independent experiments). Representative example of spleen size is shown. Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, *** $P < 0.001$.

In order to confirm *in vivo* the capacity of WIN55212-2 to promote the generation of Treg cells, we studied the induction of FOXP3⁺ Treg cells in spleen 3 days after LPS injection. Mice treated with WIN55212-2 showed significantly higher percentage of spleen CD4⁺CD25⁺FOXP3⁺ Treg cells than naïve mice. In addition, the frequency of spleen CD4⁺CD25⁺FOXP3⁺ Treg cells was significantly higher in mice treated with LPS plus WIN55212-2 than mice injected with LPS alone, demonstrating the capacity of WIN55212-2 to induce tolerogenic T cell responses. Interestingly, autophagy inhibition or the blockade of CB1 and PPAR α in LPS plus WIN55212-2-treated mice significantly reduced the percentage of FOXP3⁺ Treg cells, indicating that autophagy induction as well as CB1 and PPAR α activation are essential for the capacity of WIN55212-2 to induce Treg cells (**Figure R.80**).

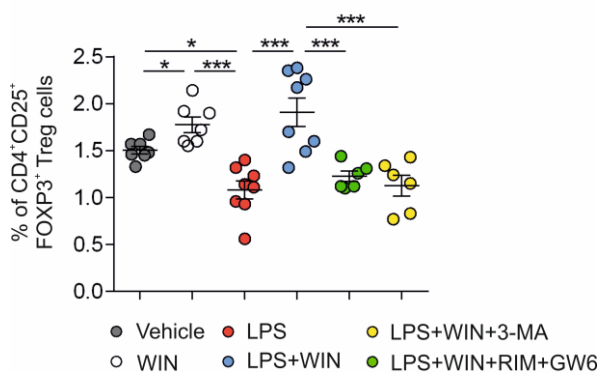


Figure R.80. Analysis of spleen Treg cell generation. Percentage of FOXP3⁺ Treg cells in spleen from mice treated with the indicated conditions for 3 days ($n = 6/8$ of 2 independent experiments). Values are mean $\pm$ SEM. Statistical significance was determined using one-way Anova. * $P < 0.05$, *** $P < 0.001$.

We used a LPS-induced sepsis model, characterized by consistent acute and chronic inflammatory responses, to study the immunomodulatory properties of the synthetic cannabinoid WIN55212-2. Using lethal doses of LPS we demonstrated the capacity of WIN55212-2 to impair lethal acute inflammatory responses by mechanisms depending on autophagy and CB1 and PPAR α activation. Similarly, WIN55212-2 reduced the chronic inflammation in spleen induced by non-lethal doses of LPS and promoted the generation of spleen FOXP3⁺ Treg cells, confirming our *in vitro* human

data. Interestingly, WIN55212-2 promoted Treg cell generation by autophagy induction via CB1 and PPAR α activation. The potential protective role of CB1 in sepsis remains largely unknown. It was demonstrated that cannabidiol inhibits gastrointestinal motility in septic mice by the activation of CB1 but a potential anti-inflammatory role has not been described (226). However, the protective and anti-inflammatory role of CB2 in sepsis has been widely study. Specific CB2R agonists, such as HU308 have been reported to attenuate leukocyte recruitment, oxidative burst, systemic inflammatory mediator release, bacteraemia, and lung tissue damage, while improving survival in different sepsis models (227-229). PPARs have been reported to induce potent anti-inflammatory responses. Specifically, PPAR α activation reduces the LPS-induced acute liver damage by reducing the expression of pro-inflammatory cytokines and leukocyte infiltration by promoting autophagy pathway (230).

The role of Treg cells in the control of immune responses in sepsis is controversial. The deletion of Treg cells results in mortality increase to nonlethal doses of LPS due to an exaggerated immune activation (231-233). Similarly, Ge et al demonstrated that IL-38 enhances the immunosuppressive activity of Treg cells from septic mice. IL-38 administration improves the survival rate of septic mice and the deletion of Treg cells before sepsis induction abolishes IL-28-associated protective response (234). On the other hand, it has been described that Treg cells generating during sepsis can induce long-term immune dysfunction and increase susceptibility to secondary infections (235, 236). Therefore, new therapeutic approaches for restoring the immune function after sepsis have been considered (236).

Taken together, our *in vivo* data indicated that WIN55212-2 impaired lethal acute inflammatory responses and induced spleen Treg cells by mechanisms depending on autophagy induction via CB1 and PPAR α activation. These data together with other previous studies indicate that CBRs might well be potential therapeutic targets for the treatment of the sepsis-induced inflammation.

4.2. Study of the capacity of WIN55212-2 to immunomodulate *in vitro* peanut-stimulated human DCs.

Allergy is a type 2 helper T cell (Th2)-mediated disease of increasing prevalence affecting around 30% of the population worldwide. Allergic diseases constitute a public health problem with a high socio-economic impact (53). The main allergic diseases include allergic rhinitis, allergic asthma, food allergy, atopic dermatitis and anaphylaxis. The immunological mechanisms underlying allergic diseases can be divided into two main phases: (i) sensitization and memory and (ii) effector phase. The sensitization phase occurs during the first contact with the allergen and leads to the generation of

allergen-specific CD4⁺ Th2 cells and allergen-specific IgE antibodies that diffuse and bind to the IgE high-affinity receptor (FcεRI) on the surface of mast cells and basophils, thus leading to the patient sensitization. Upon new allergen encounters, allergen dependent cross-linking of the IgE-FcεRI complexes on sensitized mast cells and basophils triggers the release a plethora of anaphylactogenic mediators, responsible for the immediate clinical symptoms. Late-phase reactions are initiated by the accumulation of mediators and by the activation of allergen-specific memory Th2 via mechanisms depending on IgE-facilitated presentation by DCs and B cells. Th2 cells in cooperation with type 2 innate lymphoid cells (ILC2) activated by epithelial cell-derived alarmins (TSLP, IL-33 or IL-25) produce large amounts of IL-4, IL-5, IL-9 and IL-13 that contribute to maintain allergen-specific IgE levels, eosinophilia, mucus production, inflammatory cell recruitment and tissue inflammation, leading to chronicity and the most severe clinical manifestation of allergy (**Figure R.81**) (53, 71).

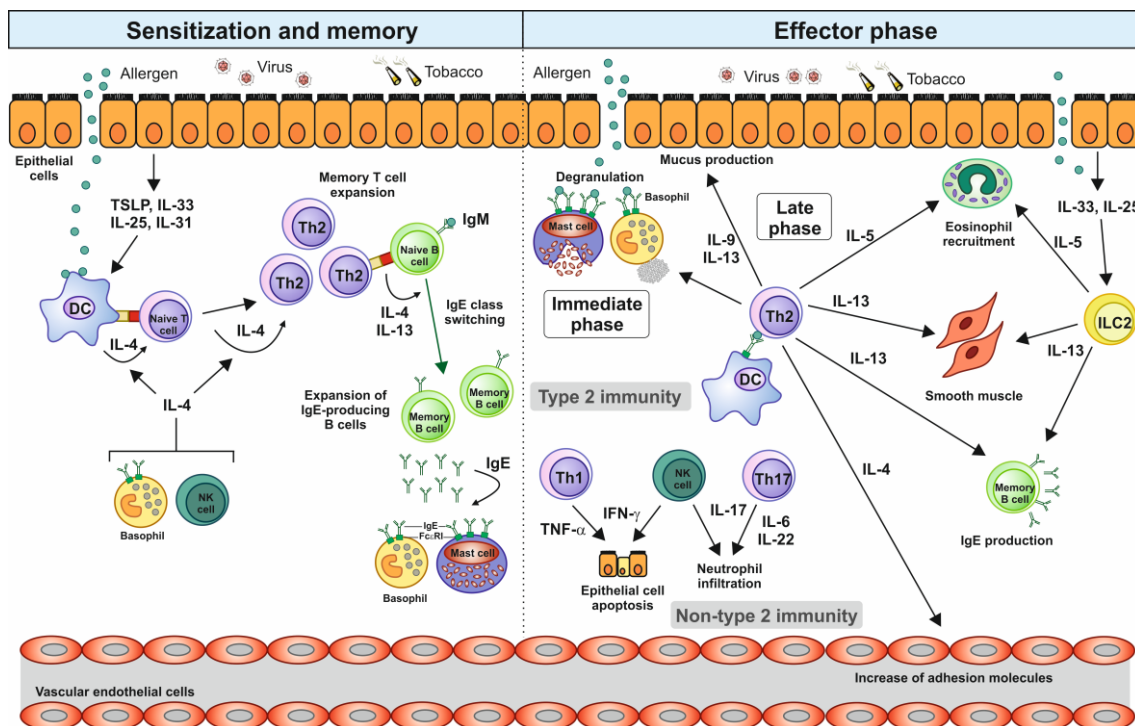


Figure R.81. Mechanisms underlying the different phases of allergy.

Allergen-specific Treg and regulatory B (Breg) cell generation is essential in the induction and maintenance of allergen tolerance in healthy responses and successful allergen-specific immunotherapy (AIT) (21, 71). Although AIT, the single treatment with the capacity to induce long-term modifying effects upon discontinuation, is effective in many cases, it displays several important drawbacks in terms of efficacy, safety and duration. Therefore, the development of novel prophylactic and therapeutic interventions is highly demanded in the field of allergy (70). At this regard, the better understanding of

the role of ECS in the context of allergy might well contribute to open new avenues for the design of novel preventive and curative strategies. To date, the data on the effect of cannabinoids in the context of allergic diseases are still a bit controversial. Some studies reported a potential protective role of ECS in allergen-induced airway inflammation and contact allergy (103, 237, 238). In contrast, other studies in mice showed that CB2 signalling contributes to allergic exacerbation in OVA-asthma models or atopic dermatitis models (103, 239-242).

The prevalence of food allergy is increasing in westernized countries, affecting up to 8% of children and 5% of adults. Even though oral tolerance is the physiological response to ingested antigens, the breakdown of this tolerance triggers the development of allergic sensitization. Such sensitization can occur in the gastrointestinal tract, oral cavity, skin and occasionally in the respiratory tract. Re-exposure to food allergens induces the release of the anaphylactogenic mediators responsible of the clinical symptoms, including anaphylaxis (243). The current standard management and treatment for food allergy is the strict avoidance of the causative food and the use of epinephrine in case of accidental ingestion. Although increasing researches focus on the study of oral (OIT), sublingual (SLIT) and epicutaneous (EPIT) immunotherapy for the treatment of food allergy, only an OIT product for peanut allergy have been recently approved by FDA (244-246). Therefore, the development of novel therapeutic approaches that improve the current strategies of immunotherapy for food allergy are needed. To date, there are no available data associated to the potential role of cannabinoids in food allergy models. The role played by ECS in other allergic diseases suggests that cannabinoids could also modulate food allergic reactions but further research is warranted. Interestingly, we previously reported that the mRNA levels of CB1 are significantly higher in PBMCs from peanut-allergic children than healthy controls, suggesting that CB1 might well also play an immune regulatory role in food allergy (103).

Peanut allergy is the most common cause of fatal food-induced anaphylactic reactions. Although FDA has recently approved an OIT product for peanut allergy (246), the design of novel preventive and curative strategies are needed. Therefore, we wanted to study the potential capacity of cannabinoids to modulate peanut-induced immune responses in hmoDCs. For that, we stimulated hmoDCs from healthy donors with crude peanut extract (CPE) in the presence or absence of the synthetic cannabinoid agonist WIN55212-2. After 18 hours of stimulation, surface marker expression and cytokine production by hmoDCs were analysed by flow cytometry and ELISA, respectively. As shown in **Figure R.82**, CPE stimulation significantly increased the surface expression of HLA-DR and the costimulatory molecules CD86, CD83 and OX40L in hmoDCs. The

presence of WIN55212-2 reduced the expression of HLA-DR, CD86, CD83 as well as OX40L in CPE-stimulated hmoDCs. OX40L is a costimulatory molecule member of the TNF superfamily that is expressed in DCs and it has been involved in the induction of Th2 cell responses (247). These data demonstrated that WIN55212-2 inhibited the maturation and activation induced by CPE in hmoDCs.

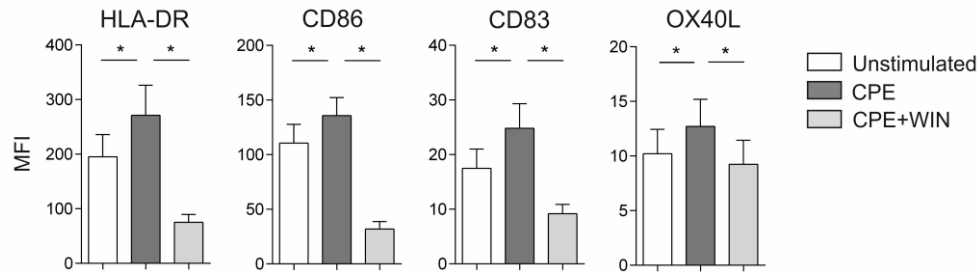


Figure R.82. WIN55212-2 (WIN) modifies the phenotype of CPE-activated hmoDCs. Mean fluorescence intensity (MFI) of the surface markers HLA-DR, CD86 and CD83 in hmoDCs after stimulation with the indicated conditions for 18 hours (n = 8). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05.

CPE stimulation induced the production of the pro-inflammatory cytokines TNF α and IL-6, but not the anti-inflammatory cytokine IL-10. WIN55212-2 inhibited the production of IL-6 but not of TNF α in CPE-stimulated hmoDCs (**Figure R.83**). These data suggested that WIN55212-2 impairs the maturation and activation of CPE-stimulated hmoDCs by inhibiting surface marker expression and IL-6 production.

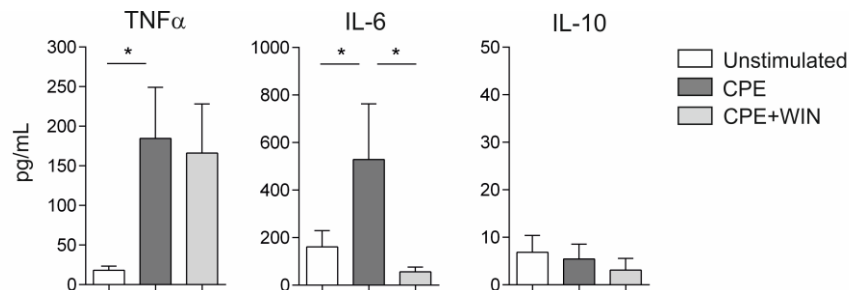


Figure R.83. Effect of WIN55212-2 (WIN) in the cytokine production induced by CPE. Cytokine levels in cell-free supernatants after stimulation of hmoDCs with CPE and CPE plus WIN55212-2 for 18 hours (n = 8). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05.

To evaluate the capacity of WIN55212-2 to modify the polarization of T cells induced by CPE-activated hmoDCs, coculture experiments with allogeneic naïve CD4⁺ T cells were carried out. As expected, CPE-stimulated hmoDCs generated T cells producing higher levels of IL-5 than unstimulated hmoDCs, indicating Th2 polarization that is classically observed in food allergy sensitization. No changes were observed in IFN γ and IL-10 production by T cells. HmoDCs activated with CPE plus WIN55212-2 generated T cells producing lower levels of IFN γ and IL-5 than hmoDCs stimulated with CPE alone. Moreover, CPE-activated hmoDCs in the presence of WIN55212-2

generated T cells producing more IL-10 than CPE-activated hmoDCs (**Figure R.84a**). In addition, the IL-10/IFN γ and IL-10/IL-5 ratios were significantly higher when T cells were primed by hmoDCs activated with CPE plus WIN55212-2 than with CPE alone (**Figure R.84b**). These data demonstrated that WIN55212-2 impaired the Th2 polarization induced by CPE-activated hmoDCs and promoted the generation of IL-10-producing CD4⁺ T cells, suggesting that WIN55212-2-treated hmoDCs promote the generation of tolerogenic responses.

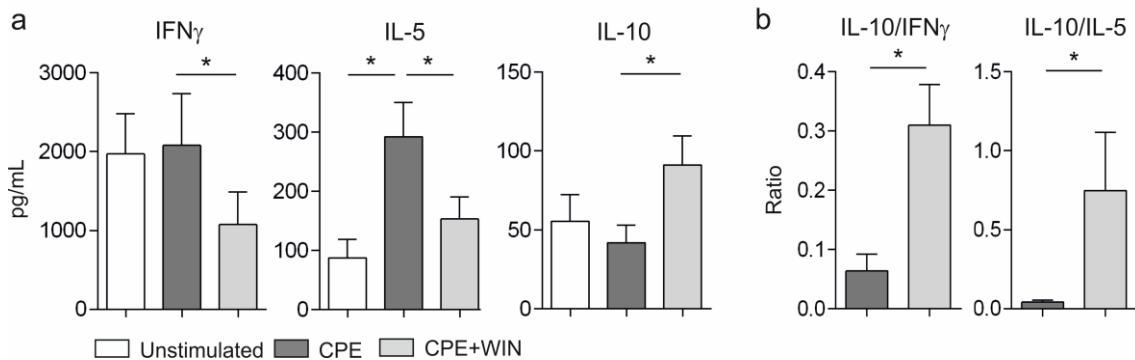


Figure R.84. **a** Cytokines produced and **b** ratios of allogeneic naïve CD4⁺ T cells primed by unstimulated, CPE or CPE plus WIN55212-2 (WIN)-treated hmoDCs after 5 days (n = 8). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05

To investigate the capacity of WIN55212-2-treated hmoDCs to induce Treg cells, we performed the same allogeneic coculture experiments and intranuclear FOXP3 staining. HmoDCs activated with CPE in the presence of WIN55212-2 induced significantly higher number of CD4⁺CD127⁻CD25⁺FOXP3⁺ Treg cells than hmoDCs treated with CPE alone (**Figure R.85**). These data are aligned with our previous data showing that WIN55212-2 promote the generation of Treg cells in our *in vitro* LPS model.

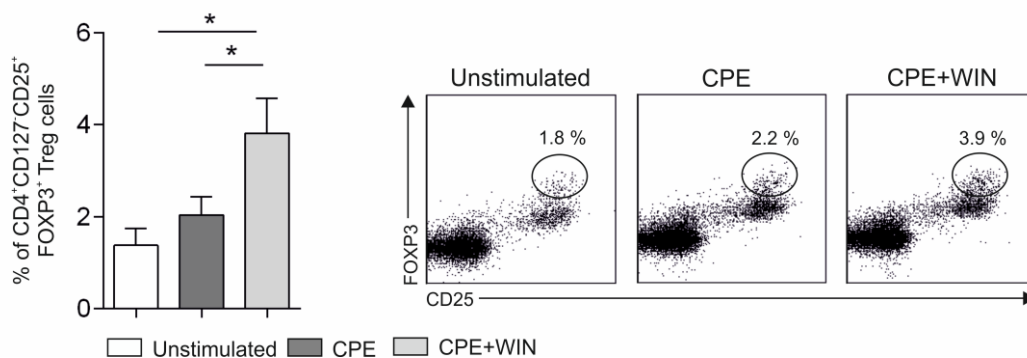


Figure R.85. WIN55212-2 (WIN)-treated hmoDCs promote the generation of Treg cells. Percentage of induced CD4⁺CD127⁻CD25⁺FOXP3⁺ Tregs cells by allogeneic CPE or CPE plus WIN55212-2 (WIN)-stimulated hmoDCs after 5 days (n = 8). Representative dot plots of intranuclear staining are shown. Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05.

Collectively, our data demonstrated that WIN55212-2 induces tolerogenic DCs with capacity to reduce Th2 responses and to promote the generation of FOXP3⁺ Treg

cells. It is well known that strong IL-10/IL-5 ratio and Treg cell generation is associated with healthy immune response to allergens and with successful AIT (70, 159, 243, 244, 248). Thus, the mechanisms by which WIN55212-2 induces tolerogenic responses might well contribute to the design of novel preventive and therapeutic strategies for allergy. Therefore, we wanted to assess the potential therapeutic and prophylactic capacity of WIN55212-2 in peanut allergy mouse model.

4.3. Study of the immunomodulatory properties of WIN55212-2 in an *in vivo* peanut-induced anaphylaxis mouse model of food allergy

Peanut allergy affects 2.2 % of children in USA and its prevalence is increasing. Peanut allergy is the main cause of anaphylaxis, a rapid systemic reaction that can lead to hypotension, respiratory failure and death (249). Recent clinical studies have proposed that the skin is a site for Th2 sensitization to foods (250).

The above-described ability shown by the synthetic cannabinoid WIN55212-2 to induce FOXP3⁺ Treg cells together with previous findings on the role of CB1 and PPAR α in the context of allergic diseases (251, 252), lead us to investigate WIN55212-2 function in a peanut-induced anaphylaxis model, a severe allergic reaction characterized by multiorgan dysfunction and potentially fatal outcome. To our knowledge this has not been previously studied. Firstly, we wanted to study whether WIN55212-2 might inhibit IgE-mediated anaphylaxis and induce allergen-specific Treg cells in the peanut-induced anaphylaxis model. For that, mice were epicutaneously sensitized to peanut using a CPE for 12 days. This sensitization involved the application of allergen onto previously shaved and tape-stripped skin on the lower backs of the mice. (**Figure R.86**).

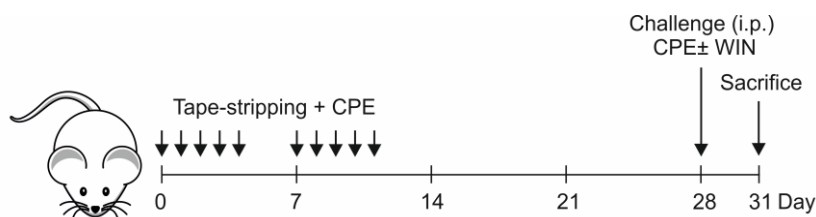


Figure R.86. Scheme of the epicutaneous sensitization protocol

Ten days after the last sensitization, the generation of peanut specific antibodies were analysed in serum after retro-orbital bleeding. CPE-sensitized mice displayed higher levels of peanut-specific IgE and IgG₁ antibodies in plasma than naïve mice, confirming allergic sensitization (**Figure R.87**).

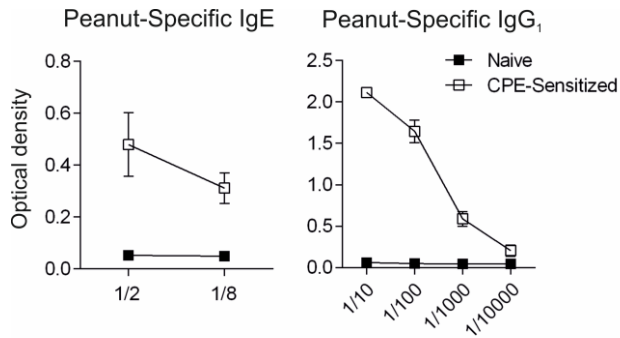


Figure R.87. Confirmation of peanut sensitization. Levels of peanut-specific IgE and IgG₁ in plasma from naïve or epicutaneously CPE-Sensitized mice. (n = 6-10 of 2 independent experiments)

Two weeks after the last sensitization, CPE-sensitized mice were divided into 2 groups for intraperitoneally challenge with CPE (2.5 mg/mouse) or with CPE plus WIN55212-2 (20 µg/mouse) (**Figure R.86**). To assess the development of systemic anaphylaxis after challenge, clinical response was carefully monitored immediately after challenge by the analysis of the clinical symptoms, the induced hypothermia and the hematocrit. The evaluated clinical symptoms were hind leg scratching in ear canal, reduced movement, puffy eyes and convulsion. The presence of each symptom was pointed with 1 point and the total score was the sum of the different points. Mice challenged with CPE in combination with WIN55212-2 exhibited significantly lower clinical symptom scores compared to mice challenge with CPE alone. Naïve mice did not develop any clinical symptoms upon CPE challenge (**Figure R88a**). Because the drop in core body temperature reflects the severity of systemic anaphylaxis, rectal temperature were measured every 10 minutes during the 40 minutes following challenge. Previous studies have demonstrated that the administration of WIN55212-2 can induce hypothermia (253, 254). Therefore, after titration we used 1 mg/kg of WIN55212-2 as the dose that did not induce hypothermia in naïve nor allergic mice. CPE challenge did not induce hypothermia in naïve mice. CPE-sensitized mice challenged with CPE plus WIN55212-2 experienced a significantly lower drop in body temperature than CPE-sensitized mice challenged with CPE alone, indicating that WIN55212-2 prevented the severity of the anaphylaxis. However, the presence of WIN55212-2 during CPE challenge did not completely abolished the hypothermia (**Figure R.88b**). During the immediate effector phase of allergy, anaphylactogenic mediators such as histamine, heparin, prostaglandins or platelet-activating factor are released and induce vascular permeability leading to hematocrit increase, a classical parameter associated with anaphylaxis. WIN55212-2 impaired the hematocrit increase induced by the CPE challenge in CPE sensitized mice, indicating that WIN55212-2 inhibited vascular leakage (**Figure R88c**). Collectively, these data demonstrated that peanut-allergic mice challenged with CPE plus WIN55212-2 showed less anaphylactic reaction, as evidenced

by the development of hypothermia, clinical signs and hemoconcentration, compared to CPE-challenge mice.

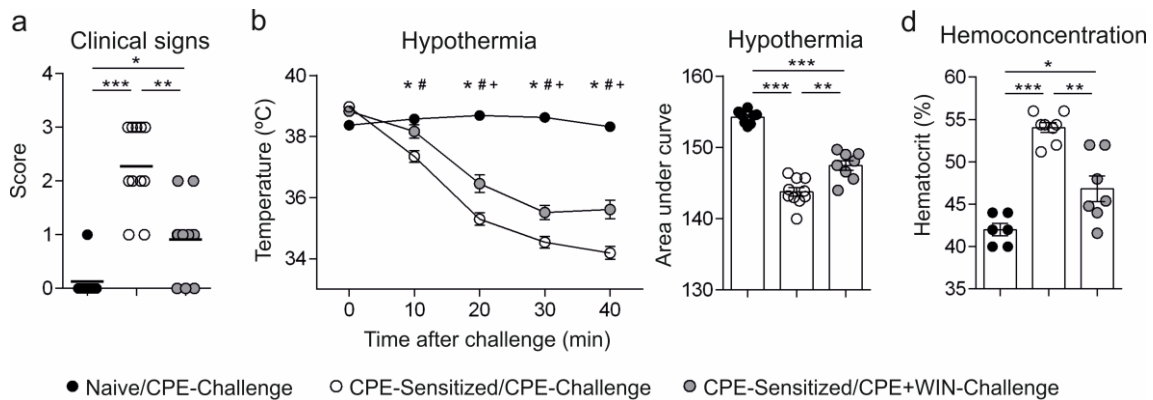


Figure R.88. Analysis of the clinical response during anaphylaxis. **a** Clinical signs observed after challenge with CPE or CPE plus WIN55212-2 (WIN). **b** Changes in body temperature 40 minutes after challenge. * Naïve/CPE Challenge vs CPE-Sensitized/CPE Challenge, # CPE-Sensitized/CPE Challenge vs CPE-Sensitized/CPE+WIN55212-2 Challenge, + Naïve/CPE Challenge vs CPE-Sensitized/CPE+WIN55212-2 Challenge. Area under curve is shown. **c** Hemoconcentration 40 minutes after challenge ($n = 6-10$ of 2 independent experiments). Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Next, we wanted to study the effects of WIN55212-2 in peanut-induced late-phase responses in peanut sensitized mice. For that, seventy-two hours after challenge, mice were sacrificed and spleens were collected. As expected, CPE-challenged mice presented significant splenomegaly compared to naïve mice. Remarkably, spleen weight of mice challenged with CPE plus WIN55212-2 were significantly reduced compared to CPE-challenge mice (**Figure R.89**), suggesting that WIN55212-2 inhibited late-phase responses and chronic inflammation.

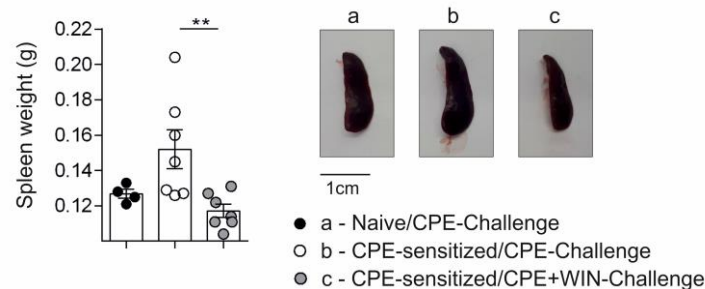


Figure R.89. WIN55212-2 (WIN) reduces splenomegaly induced by CPE challenge. Spleen weight 72 hours after challenge. Representative example of spleen size is shown. ($n = 4-7$) of 2 independent experiments). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova. ** $P < 0.01$.

Next, we assessed how WIN55212-2 reduced the chronic inflammation associated to CPE-challenge. Thus, we studied the generation of FOXP3⁺ Treg cells in the spleen. The percentage of splenic CD4⁺CD25^{high}FOXP3⁺ Treg cells was significantly increased in mice challenged with CPE only under WIN55212-2 presence, suggesting that WIN55212-2 might well contribute to the regulation of late T cell responses after

CPE-induced anaphylaxis in peanut allergic mice by inducing enhanced generation of CPE-specific Treg cells (**Figure R.90**).

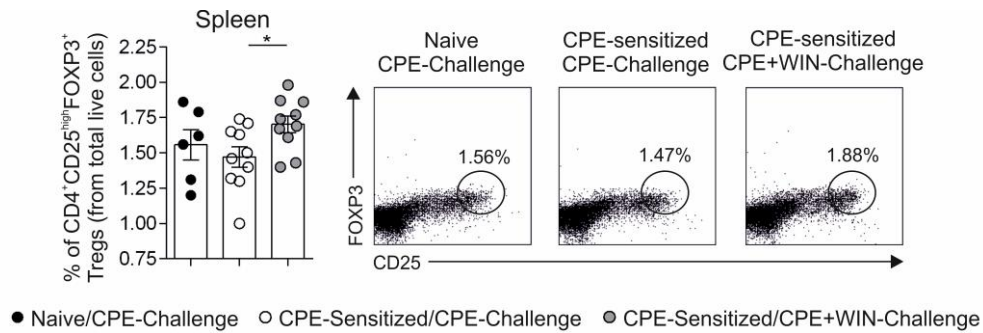


Figure R.90. WIN55212-2 (WIN) induces spleen Treg cells in CPE challenge allergic mice. Percentage of CD4⁺CD25^{high}FOXP3⁺ Treg cells in spleen freshly isolated of naïve CPE-challenge mice, CPE-sensitized and CPE-challenge mice or CPE-sensitized and CPE+WIN55212-2-challenge mice. Representative dot plots of generated CD4⁺CD25^{high}FOXP3⁺ Treg cells in spleen are shown (n = 6-10 of 2 independent experiments). Values are the mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05.

To assess allergen-specific responses, splenocytes from naïve mice, CPE-challenge mice or CPE+WIN55212-2-challenge mice were *in vitro* stimulated with CPE for 4 days. Splenocytes from CPE-challenged allergic mice produced significantly higher levels of IL-5 and IL-10 than naïve mice. In contrast, spleen cells from allergic mice challenged with CPE plus WIN55212-2 produced lower levels of IFN γ and IL-5 than CPE-challenged mice without significant changes detected in IL-10 (**Figure R.91a**). The ratios IL-10/IL-5 and IL-10/IFN γ derived from cytokine production by splenocytes from mice challenged with CPE plus WIN55212-2 were significantly higher than the ratios derived from mice challenged with CPE alone, suggesting the induction of CPE-specific tolerogenic T cell responses (**Figure R.91b**).

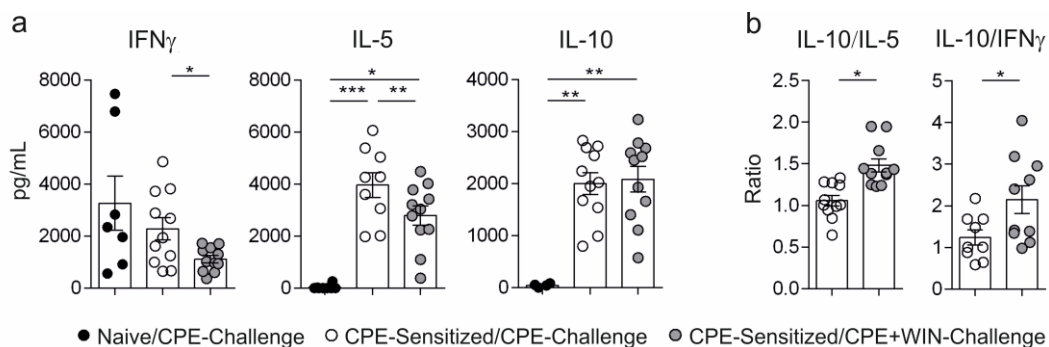


Figure R.91. Allergen-specific T cell response in splenocyte cultures. **a** Cytokine production by splenocytes from naïve mice, CPE-sensitized and CPE-challenge mice or CPE-sensitized and CPE+WIN55212-2 (WIN)-challenge mice after CPE stimulation for 4 days. **b** Cytokine ratios of splenocyte cultures (n = 7-10 of 2 independent experiments). Values are the mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01, *** P < 0.001.

To firmly confirm the generation of CPE-specific Treg cells, we analysed Treg cell induction in the splenocyte cultures by flow cytometry. The increment in FOXP3⁺ Treg cells after CPE stimulation was significantly higher in splenocytes from CPE plus WIN55212-2 challenged mice than CPE-challenge mice (**Figure R92a**). These data

support the capacity of WIN55212-2 to immunomodulate food-induced allergic responses. Interestingly, the Treg cell increment after CPE stimulation was negatively correlated with IL-5 production by splenocytes, highlighting that WIN55212-2-mediated Treg cell induction is associated with lower Th2 responses (**Figure R.92b**).

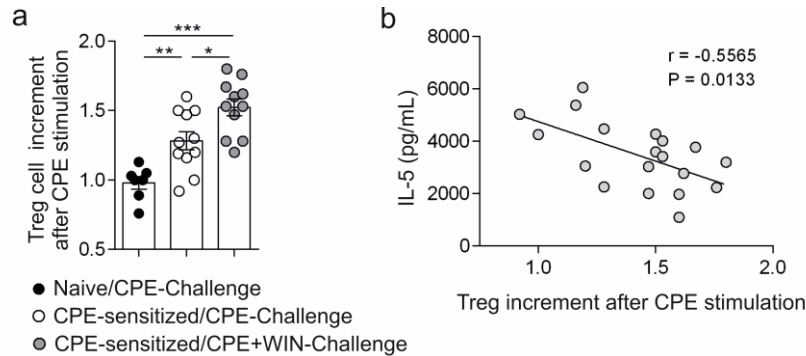


Figure R.92. Induction of CPE-specific Treg cells in splenocyte cultures. **a** Increment of CD4⁺CD25^{high}FOXP3⁺ Treg cells generation in splenocyte cultures after CPE stimulation for 4 days relative to unstimulated condition **b** Correlation of Treg cell increment after CPE stimulation with IL-5 levels produced by CPE-stimulated splenocytes (n = 7-10 of 2 independent experiments). Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova and Spearman test. * P < 0.05, ** P < 0.01, *** P < 0.001.

Collectively, these results show that WIN55212-2 protects peanut-sensitized mice from anaphylactic reactions and favours the generation of Treg cells able to impair peanut-induced late effector phase responses, which indicates immunomodulatory capacity of WIN55212-2 not only in type 1 immune context, but also under type 2 inflammatory conditions. Although more studies are required, these data suggest that WIN55212-2 might well represent a useful future strategy for the treatment of peanut allergy.

Then, we wanted to study whether WIN55212-2 might prevent IgE-mediated allergic sensitization to peanut and the potential role of DCs in peanut sensitization. Thus, we evaluated the ability of WIN55212-2 to prevent DC activation and migration *in vivo*, in a preclinical model of peanut allergy. BALB/c mice were subjected to epicutaneous sensitization to CPE (100 μ g/day) for three consecutive days in the absence or presence of WIN55212-2 (10 μ g/day). One day later, mice were sacrificed and the draining inguinal lymph nodes were collected to analyse DC migration and activation (**Figure R.93**).

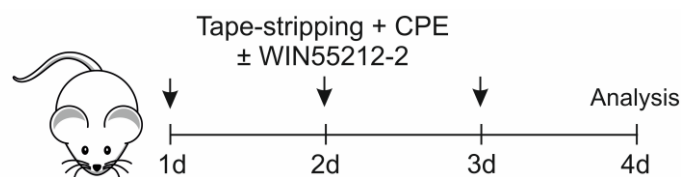


Figure R.93. Scheme of the epicutaneous sensitization protocol

CPE-sensitized mice displayed significantly higher percentage of total MHCII⁺CD11c⁺ DC migrating to the inguinal lymph nodes than naïve mice. Interestingly, the presence of WIN55212-2 during CPE sensitization significantly impaired the migration of DCs to the draining lymph nodes (**Figure R.94**)

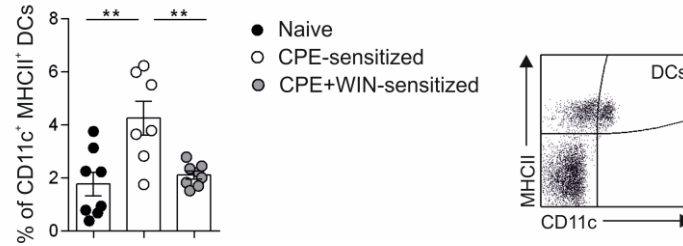


Figure R.94. Analysis of inguinal lymph node DCs. Percentage of CD11c⁺MHCII⁺ DCs from inguinal lymph nodes (n = 7/8 of 2 independent experiments). Gate strategy of the flow cytometry analysis. Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. ** P < 0.01.

We also analysed the expression of the mature and activation markers CD40, CD80, CD86 and OX40L and the migration marker CCR7 in inguinal lymph node DCs. Mice sensitized with CPE increased the number of DCs expressing mature and activation markers in inguinal lymph nodes. Interestingly, mice sensitized in the presence of WIN55212-2 showed a significantly lower number of mature DCs in the draining lymph nodes as compared to CPE-sensitized mice (**Figure R.95**). Collectively, these data indicated that WIN55212-2 could impair DC activation and migration to inguinal lymph nodes in preclinical models of peanut allergy.

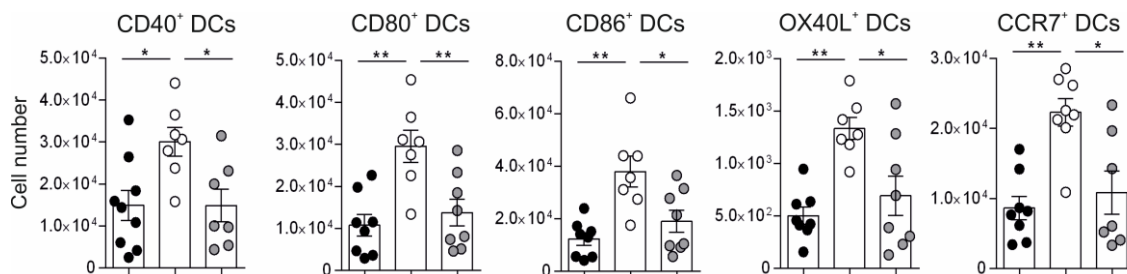


Figure R.95. Analysis of activated inguinal lymph node DCs. Cell number of positive cells for CD40, CD80, CD86, OX40L and CCR7 in DCs from inguinal lymph nodes (n = 7/8 of 2 independent experiments). Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01.

To further explore the capacity of WIN55212-2 to control *in vivo* Th2 priming during CPE sensitization, we employed the same epicutaneous mice model of peanut allergy previously described and analysed the prophylactic capacity of WIN55212-2 to prevent IgE peanut-sensitization. In this model, mice were epicutaneously sensitized by application of allergen (100 μ g/day) in the presence or absence of WIN55212-2 (10 μ g/day) onto previously shaved and tape-stripped skin on the lower backs of the mice

(**Figure R.96**). Ten days later, the generation of peanut specific antibodies in serum were analysed prior to challenge with CPE.

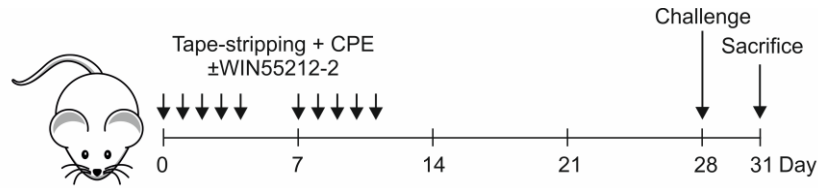


Figure R.96. Scheme of the epicutaneous sensitization protocol

CPE-sensitized mice displayed higher levels of peanut-specific IgE and IgG₁ antibodies in plasma than naïve mice. The presence of WIN55212-2 during peanut sensitization induced a significant reduction in peanut-specific plasma IgE and IgG₁ antibodies, demonstrating that WIN55212-2 is able to modify epicutaneous peanut sensitization (**Figure R.97**). However, the presence of WIN55212-2 during peanut sensitization did not completely abolished IgE and IgG₁ in the assayed conditions.

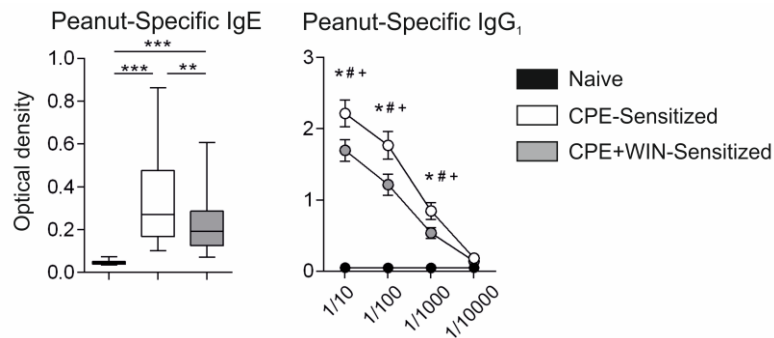


Figure R.97. Levels of peanut-specific IgE and IgG₁ in plasma from naïve, CPE-sensitized mice or CPE+WIN55212-2 (WIN)-sensitized mice (n = 7-10 of 3 independent experiments). Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. ** P < 0.01, *** P < 0.001. * Naïve vs CPE-sensitized; # CPE-sensitized vs CPE+WIN55212-2-sensitized; + naïve vs CPE+WIN55212-2-sensitized.

To assess the effects of prophylactic treatment with WIN55212-2 in immediate and late phase, mice were challenge with CPE (2.5 mg/mice) two weeks after the last sensitization. The clinical response was carefully monitored immediately after challenge by the analysis of the clinical symptoms (hind leg scratching in ear canal, reduced movement, puffy eyes and convulsion), the induced hypothermia and the haematocrit. Mice sensitized with CPE plus WIN55212-2 exhibited significantly lower clinical symptom scores compared to mice sensitized with CPE alone. Naïve mice did not develop any clinical symptoms upon CPE challenge (**Figure R.98a**). Rectal temperature were measured every 10 minutes during the 40 minutes following challenge and then, haematocrit was analysed as a surrogate objective markers of anaphylaxis. Mice sensitized with CPE plus WIN55212-2 displayed only a slightly non-significant increase in body temperature compared to mice sensitized with CPE alone (**Figure R.98b**). In

addition, mice sensitized with CPE plus WIN55212-2 also showed a slightly non-significant lower hematocrit than CPE-sensitized mice (**Figure R.98c**). Overall, although there is a reduction of clinical score, the administration of WIN55212-2 did not prevented CPE-induced anaphylaxis in peanut sensitized mice at the assayed conditions. Despite CPE sensitization in the presence of WIN55212-2 reduced the levels of CPE-specific IgE, this reduction was not enough to abolish CPE-induced anaphylaxis.

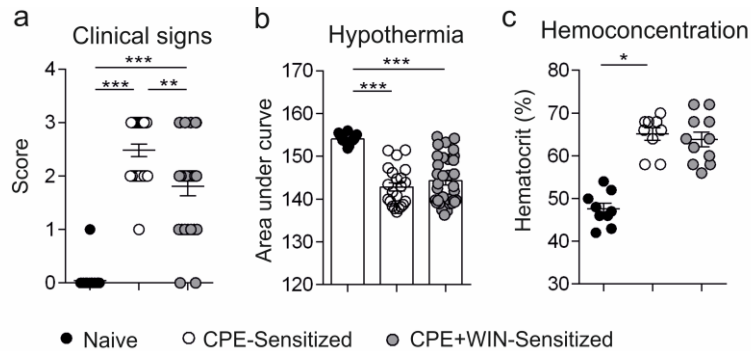


Figure R.98. Analysis of the clinical response during anaphylaxis. **a** Clinical signs observed after challenge in CPE or CPE plus WIN55212-2 (WIN)-sensitized mice. **b** Changes in body temperature 40 minutes after challenge represented as area under curve is shown. **c** Hemoconcentration 40 minutes after challenge ($n = 15-20$ of 3 independent experiments). Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Seventy-two hours after challenge, mice were sacrificed and peritoneal cells, inguinal lymph nodes and spleens were collected. The percentage of eosinophils in the peritoneal cavity was analysed by flow cytometry. CPE-sensitized mice showed higher percentage of eosinophils in the peritoneal cavity than naïve mice, demonstrating the recruitment of these cells after challenge. Interestingly, CPE plus WIN55212-2-sensitized mice displayed significantly lower percentage of eosinophils than CPE-sensitized mice, suggesting that WIN55212-2 could act in DCs reducing IgE-mediated presentation and reducing late Th2 response (**Figure R.99**).

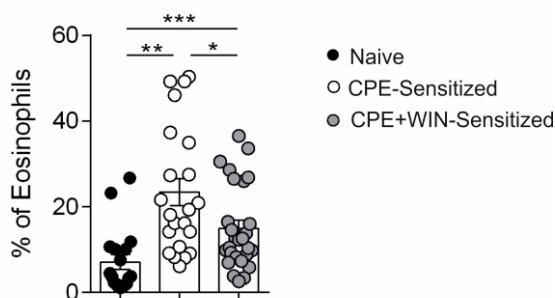


Figure R.99. Percentage of eosinophils in the peritoneal cavity 72 hours after challenge ($n = 15-20$ of 3 independent experiments). Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Next, we investigated the generation of FOXP3⁺ Treg cells in freshly isolated spleen and inguinal lymph nodes. The percentage of CD4⁺CD25^{high}FOXP3⁺ Treg cells in spleen and inguinal lymph nodes was significantly increased in mice sensitized with CPE in the presence of WIN55212-2, suggesting that WIN55212-2 controlled late T cell

responses after CPE-induced anaphylaxis in peanut allergic mice by the induction of CPE-specific Treg cells (**Figure R.100**).

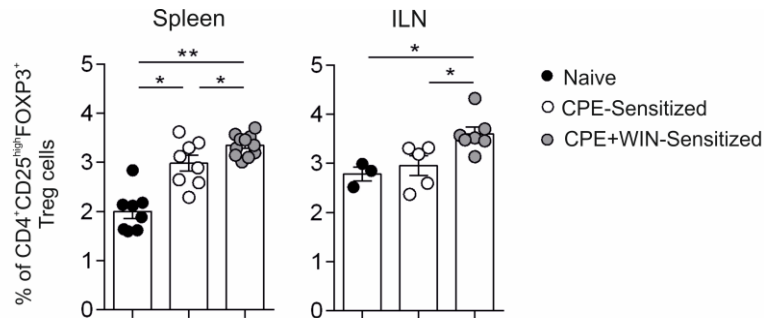


Figure R.100. Treg induction in the late effector phase of peanut allergy. Percentage of CD4⁺CD25^{high}FOXP3⁺ Treg cells in spleen and inguinal lymph nodes (ILN) (n = 5-10 of 3 independent experiments). Values are the mean ± SEM. Statistical significance was determined using One-way Anova * P < 0.05, ** P < 0.01.

To assess allergen-specific responses, splenocytes were *in vitro* stimulated with CPE for 4 days. Splenocytes from CPE-sensitized mice produced significantly higher levels of IL-5 and IL-10 than naïve mice. In contrast, spleen cells from mice sensitized with CPE plus WIN55212-2 produced lower levels of IL-5 and higher levels of IL-10 than CPE-sensitized mice without affect IFN γ production (**Figure R.101a**). Consequently, the ratios IL-10/IL-5 and IFN γ /IL-5 derived from cytokines produced by splenocytes from mice sensitized with CPE plus WIN55212-2 were significantly higher than the ratios derived from mice sensitized with CPE alone (**Figure R.101b**), suggesting a reduction in the peanut-specific Th2 responses and an increase of regulatory T cell response.

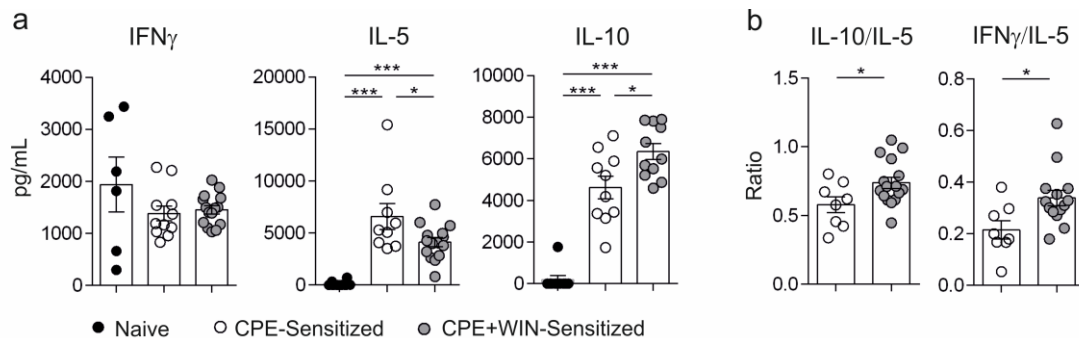


Figure R.101. **a** Cytokine production by splenocytes from naïve, CPE-sensitized and CPE+WIN55212-2 (WIN)-sensitized mice after CPE stimulation for 4 days. **b** Cytokine ratios of splenocyte cultures (n = 6-10 of two independent experiments). Values are the mean ± SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01, *** P < 0.001.

To confirm the generation of CPE-specific Treg cells, we analysed Treg cell induction in the splenocyte cultures after CPE stimulation by flow cytometry. The increment in CPE-specific FOXP3⁺ Treg cell after CPE stimulation was significantly higher in splenocytes from CPE plus WIN55212-2 sensitized mice, than in mice sensitized with CPE (**Figure R.102**). These data indicated that WIN55212-2 induced

Treg cells in the late effector phase, demonstrating the capacity of WIN55212-2 to control type 2 inflammatory conditions.

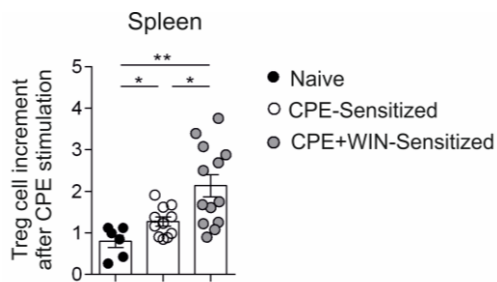


Figure R.102. a Increment of CD4⁺CD25^{high}FOXP3⁺ Treg cells generation in splenocyte cultures after CPE stimulation for 4 days relative to unstimulated condition (n = 7-14 of three independent experiments). Values are the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * P < 0.05, ** P < 0.01.

Collectively, these data demonstrated the capacity of WIN55212-2 to interfere in CPE sensitization by reducing CPE-specific IgE and IgG₁ plasma levels. However, we did not observed an inhibition of anaphylaxis in CPE plus WIN55212-2 sensitized mice. This could be due to several reasons: the reduction of IgE was not enough to abolish anaphylaxis, other IgE-independent specific or unspecific mechanisms are taking place in our model, or the assayed doses of WIN55212-2 were not completely effective. Remarkably, at the late effector-phase, the presence of WIN55212-2 during peanut sensitization reduced eosinophil infiltration and promoted Treg cell generation in spleen, indicating the potential capacity of WIN55212-2 to control Th2 chronic inflammation.

However, more detailed studies to know the mechanisms by which WIN55212-2 can control Th2 responses are needed. The generation of allergen-specific Treg cells is essential to induce tolerance to allergens (21, 71, 248). Therefore, these results might well help to the design of future-novel allergen-specific vaccines for the immunotherapy of allergy.

Objective 5: Evaluation of the capacity of WIN55212-2 to regulate bronchial epithelial barrier integrity

Bronchial epithelial cells constitute the first physical barrier with mucociliary clearance and immunologic defence capacity against environmental inhaled insults. The integrity of the bronchial epithelial barrier depends on a complex network of protein interactions generating tight junctions (TJs), adherent junctions and desmosomes (255, 256). TJs located at the most apicolateral borders involve transmembrane proteins such as occludin or claudins linked to the actin cytoskeleton via zonula occludens (ZO) proteins. TJs build an impermeable wall blocking the free passage of noxious agents into the paracellular space, regulate cell polarity and paracellular ion and small molecule transportation (255, 256). Disruption of TJs and epithelial barrier dysfunction is a hallmark of chronic inflammatory airway diseases such as rhinitis, chronic rhinosinusitis with nasal polyps (CRSwNP) or asthma (53, 163, 257). Many environmental factors have been reported to damage epithelial barrier and a need to restore the leaky epithelium exists as a treatment or prevention modality for mucosal inflammatory diseases (255).

There is a strong link between common cold rhinovirus (RV) infections and asthma development early in life or asthma exacerbations in children and adults (53, 258, 259). Rhinovirus-mediated respiratory tract infections induces disruption of the airway epithelial barrier. RV-infected bronchial epithelial cells produce a large amount of pro-inflammatory mediators. RV also infects and replicates in B cells inducing their proliferation and inhibit the generation of Treg cells, thus promoting both innate and adaptive pro-inflammatory responses (260, 261). Therapeutic strategies that improve and restore human epithelial barrier integrity would be promising candidates for the treatment respiratory diseases.

5.1. Study of the capacity of WIN55212-2 to regulate the integrity of human bronchial epithelial cells during rhinovirus infection in air-liquid interface cultures

Cannabinoids have been demonstrated as potent anti-inflammatory agents in several inflammatory conditions (103). However, very little is known about their potential capacity to regulate the bronchial epithelial barrier integrity upon RV infection. We initially investigated the capacity of the synthetic cannabinoid WIN55212-2 to regulate the integrity of human bronchial epithelial cells (HBEC) during human rhinovirus-16 (HRV-16) infection in air-liquid interface (ALI) cultures. First, we performed dose- and time-

response experiments to assess the capacity of WIN55212-2 to directly disturb the barrier integrity by monitoring changes in transepithelial electric resistance (TER). Basolateral stimulation of ALI cultures with WIN55212-2 drastically decreased TER relative to unstimulated conditions at concentrations of 5 or 10 μM due to cytotoxic effects as determined by the accumulation of LDH into the culture medium. In contrast, at concentrations of 1 or 2.5 μM WIN55212-2 did not significantly affect TER (**Figure R.103**).

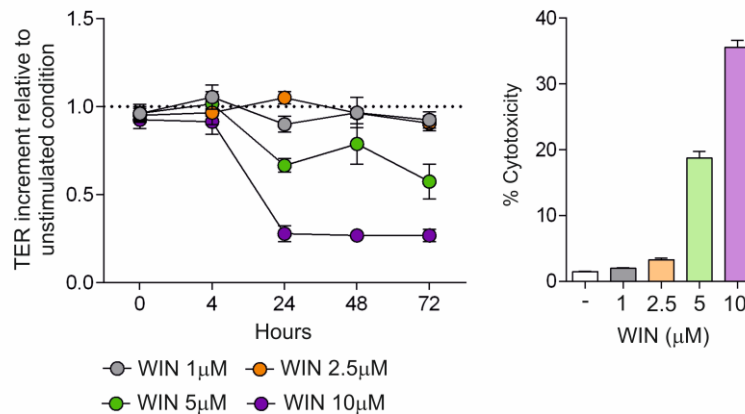


Figure R.103. Fold change in TER with respect to unstimulated condition in ALI-cultured HBEC after basolateral stimulation with the indicated doses of WIN55212-2 (WIN) at different times ($n = 2$ independent experiments). Each determination was performed by triplicate. The graph at the right shows the percentage of primary HBEC cell lysis (cytotoxicity) at the indicated conditions after 24 hours of stimulation quantified by measuring LDH release in the medium ($n=2$).

Different studies previously demonstrated that RV infection abolishes airway epithelial barrier integrity (260, 261). We investigated whether non-toxic concentrations of WIN55212-2 could impact HRV-16-induced epithelial barrier disruption. ALI cultures of HBEC were apically infected with HRV-16 at a multiplicity of infection (MOI) of 50, based on previous titration experiments to ensure barrier disruption, in the absence or presence of basolateral 2.5 μM WIN55212-2 and TER was monitored over 72 hours as previously described. We also included WIN5212-2 alone and ultraviolet (UV)-inactivated HRV-16 as controls.

As shown in **Figure R.104**, TER was slightly reduced after 4 hours of stimulation in all the assayed conditions, likely due to the application procedure. After 24 hours, TER values were restored in the unstimulated, 2.5 μM WIN55212-2 alone and UV-inactivated HRV-16 conditions and remained without significant changes up to 72 hours. HRV-16, but not UV-inactivated HRV-16, significantly decreased TER after 24 hours of infection and these values lasted almost unchanged up to 72 hours, suggesting that HRV-16-induced barrier disruption strongly depend on the infectious capacity of the virus rather

than in a solely antigen response. After 24 hours of HRV-16-infection in the presence of WIN55212-2, TER was also significantly decreased. However, a significant increment of TER in the presence of WIN55212-2 compared to HRV-16-infection alone was observed after 48 hours. Remarkably, in the presence of WIN55212-2, TER values were similar to that observed for unstimulated, WIN55212 alone or UV-inactivated HRV-16 conditions after 72 hours, thus demonstrating that WIN55212-2 is able to restore the barrier integrity of HRV-16-infected ALI-cultured HBEC.

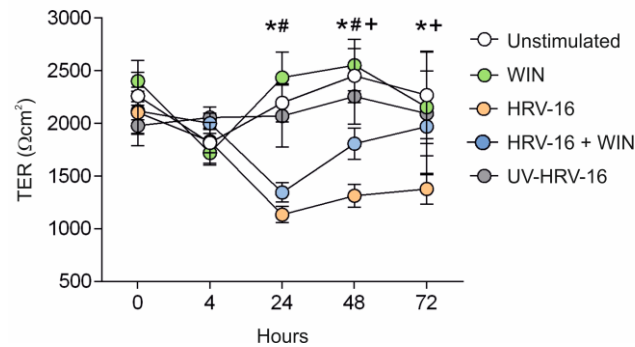


Figure R.104. TER in ALI-cultured HBEC after apical HRV-16 infection with or without basolateral WIN55212-2 (WIN) (2.5 μ M) treatment, WIN55212-2 alone or UV-inactivated HRV-16 (UV-HRV-16) at the indicated times ($n = 4$ independent experiments). Each determination was performed by triplicate. * Unstimulated vs HRV-16, # Unstimulated vs HRV-16+WIN55212-2, + HRV-16 vs HRV-16+WIN55212-2. Data represent the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$.

Supporting these data, the presence of WIN55212-2 significantly inhibited the HRV-16-induced increase of paracellular flux of fluorescein isothiocyanate (FITC)-conjugated 4 kDa dextran, thus fully restoring the impaired paracellular permeability of HRV-16-infected ALI-cultured HBEC to the levels observed for the unstimulated, WIN55212-2 alone or UV-inactivated HRV-16 conditions (**Figure R.105**).

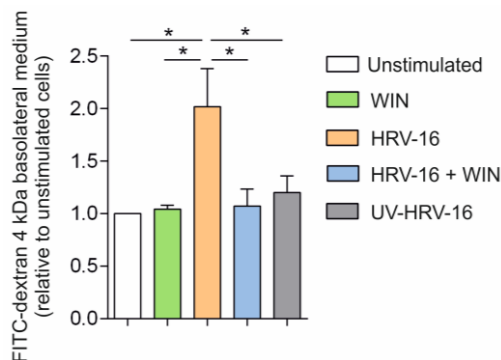


Figure R.105. Paracellular flux of FITC-dextran in ALI-cultured HBEC unstimulated, infected with HRV-16 alone or in the presence of WIN55212-2 (WIN) (2.5 μ M), WIN55212-2 alone or UV-HRV-16 over 72 hours. FITC-dextran was apically added after 72 hours of treatment and quantified in the basolateral compartment after 24 hours ($n = 4$ independent experiments). Data represent the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$.

To gain further molecular insights into the mechanisms by which WIN55212-2 restores barrier integrity upon HRV-16 infection, we assessed the distribution of TJ proteins in ALI-cultured HBEC under the different conditions by immunofluorescence

staining and confocal microscopy. RV infection impairs barrier assembly of airway epithelial cells by disrupting occludin and ZO-1 TJ proteins through mechanisms depending on Nod-like receptor X-1- and NADPH oxidase 1-mediated ROS generation (262, 263). After 72 hours, HRV-16-infected ALI-cultured HBEC showed lower occludin and ZO-1 immunofluorescence intensity at the cell boundaries than unstimulated or cells stimulated with WIN55212-2 alone, indicating the disruption of these TJ proteins and the subsequent impairment of the barrier integrity. Remarkably, HRV-16-infected HBEC in the presence of WIN55212-2 showed higher occludin and ZO-1 immunofluorescence intensity compared to cells infected with HRV-16 alone and similar to that observed under unstimulated or WIN55212-2 conditions (**Figure R.106**).

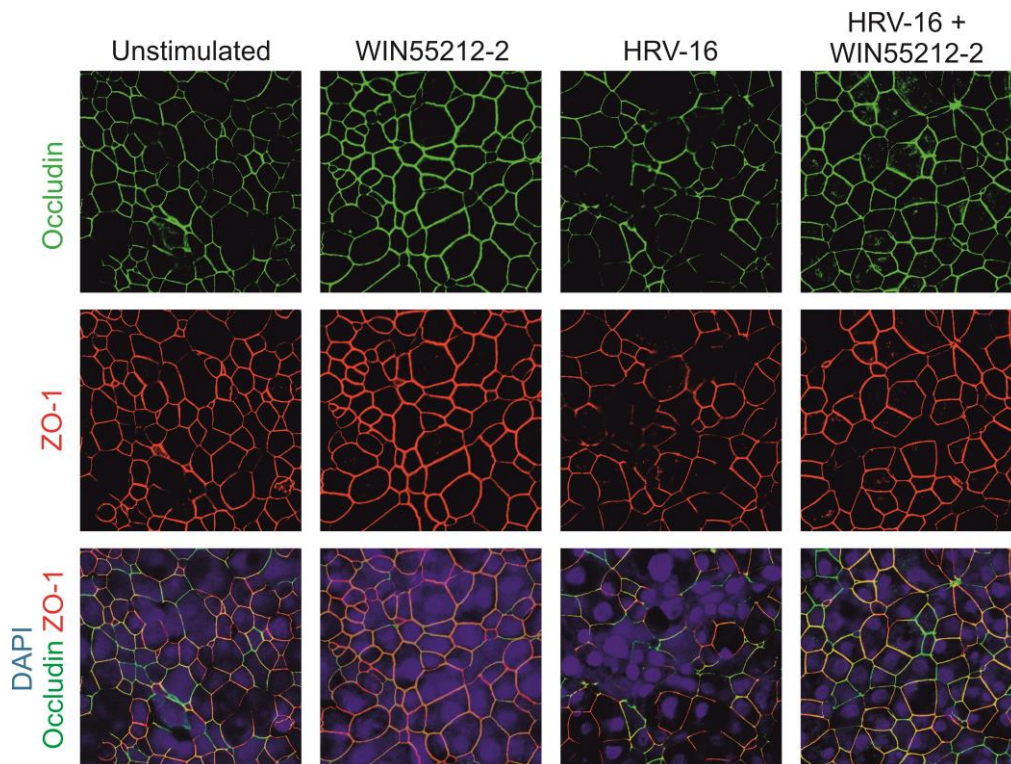


Figure R.106. Representative confocal microscopy of HBEC after apical HRV-16 infection with or without basolateral WIN55212-2 (WIN) (2.5 μ M) treatment, WIN55212-2 alone or UV-inactivated HRV-16 (UV-HRV-16) for 72 hours ($n = 2$ independent experiments). ALI-cultured HBEC were stained for occludin (green) and ZO-1 (red) with specific antibodies and with DAPI (nuclei, blue).

In addition, mRNA levels of TJ were evaluated. WIN55212-2 or UV-inactivated HRV-16 did not significantly alter the mRNA expression of TJ genes. WIN55212-2 significantly increased the expression of occludin and claudin-7 mRNAs with respect to HRV-16-infected cells without significant changes observed for ZO-1, claudin-1 and claudin-4 (**Figure R.107**).

Our results are aligned with a previous report showing that the phytocannabinoid THC impairs TNF α -induced increase in airway epithelial permeability (264). Another study showed that the endocannabinoid anandamide disrupted the bronchial epithelial

integrity, but such effects were driven by the cyclooxygenase and lipoygenase downstream arachidonic acid-derived metabolites resulting from the accumulation of arachidonic acid after the degradation of anandamide and not by the direct action of anandamide itself (265).

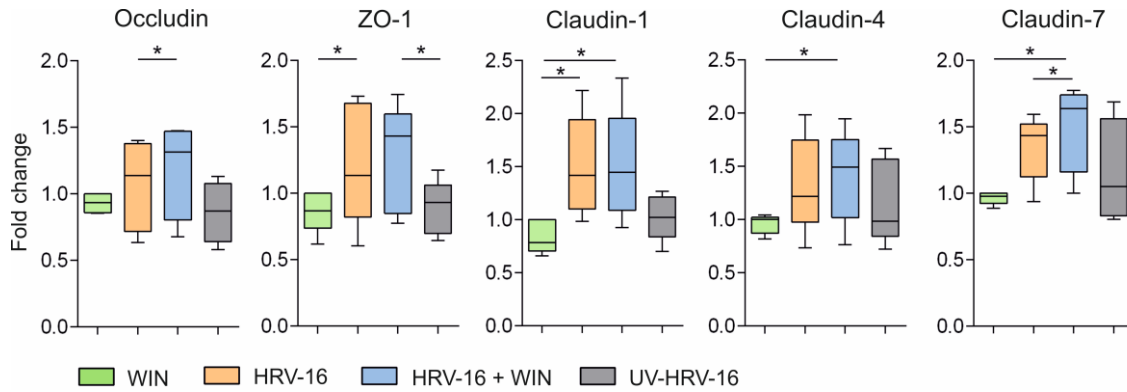


Figure R.107. mRNA expression levels of the indicated TJ genes in ALI-cultured HBEC after 24 hours of stimulation with WIN55212-2 (WIN) (2.5 μ M) alone, HRV-16 infection in the presence or absence of WIN55212-2 as determined by quantitative PCR ($n = 4$ independent experiments). Fold change with respect to the unstimulated condition was calculated as $2^{-\Delta\Delta C_t}$, where ΔC_t correspond to the difference between the cycle threshold value for the gene of interest and EF1 α as housekeeping gene. Data represent the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$.

During viral infections, epithelial cells produce pro-inflammatory cytokines and chemokines that enhance the recruitment of peripheral inflammatory cells into the airway submucosa to orchestrate immune responses (255, 256, 266). WIN55212-2 alone or UV-inactivated HRV-16 did not induce cytokine production in HBEC. In contrast, HRV-16-infected ALI-cultured HBEC produced significantly higher levels of TNF α , IL-8, IP-10, MCP-1 and G-CSF than unstimulated cells after 24 hours, which were not significantly inhibited by WIN55212-2, suggesting that WIN55212-2 does not alter the innate immune responses triggered by HRV-16 infection in HBEC (**Figure R.108**).

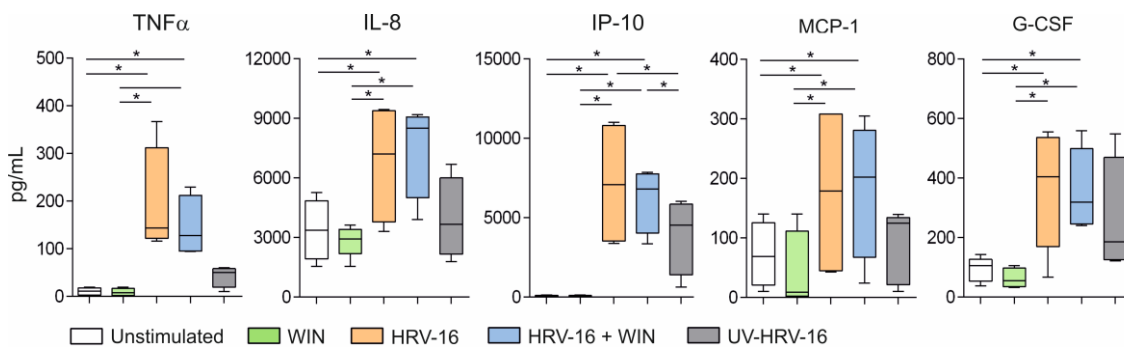


Figure R.108. Levels of the indicated cytokines and chemokines in cell-free supernatants produced by ALI-cultured HBEC unstimulated, stimulated with WIN55212-2 (WIN) (2.5 μ M) alone, UV-HRV-16, infected with HRV-16 or with HRV-16 plus WIN55212-2 for 24 hours as determined by cytometric bead array ($n = 4$ independent experiments). Data represent the mean $\pm$ SEM. Statistical significance was determined using One-way Anova. * $P < 0.05$.

Collectively, the synthetic cannabinoid WIN55212-2 repairs HRV-16-induced airway epithelial damage by restoring the integrity of TJ proteins without affecting epithelial cell-mediated innate immune responses. In conclusion, these data show the potential clinical applications of WIN55212-2 in restoring impaired barrier function during viral infections.

5.2. Study of the capacity of WIN55212-2 to modulate T cell responses in human tonsils

Tonsils are secondary lymphoid organs located in the gateway of the gastrointestinal and respiratory tracts where allergen-specific regulatory T cells are generated (42). Immune responses in tonsils are conditioned by viral infections and allergy and represent a very suitable model for *ex vivo* studies (172).

We stimulated TMC with anti-CD3/CD2/CD28 antibodies for 3 days to induce T cell proliferation and activation in the absence or presence of WIN55212-2 and monitored T cell proliferation by measuring ^3H -thymidine incorporation. WIN55212-2 alone did not induce T cell proliferation. WIN55212-2 significantly reduced the polyclonally-induced proliferation of tonsil T cells in a dose-dependent manner after 3 days of culture without affecting cell viability (**Figure R.109ab**).

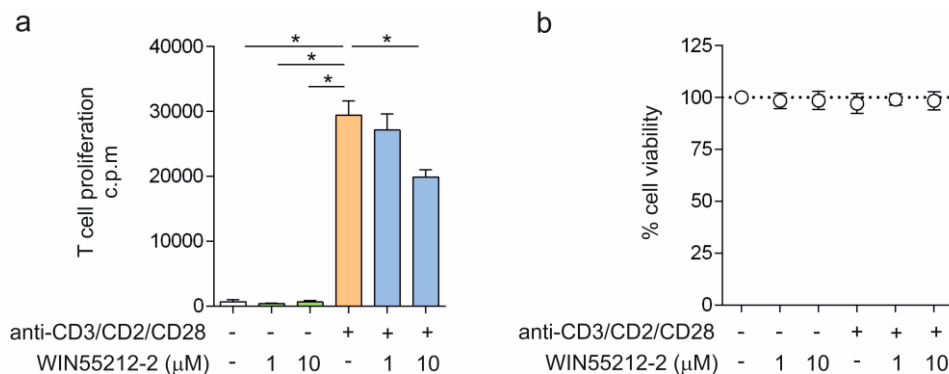


Figure R.109. **a** T cell proliferation measured by using titrated thymidine incorporation as counts per minute (c.p.m.) ($n = 6$). **b** Percentage of cell viability for the indicated conditions as determined by trypan blue exclusion using a light microscope ($n = 3$). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova * $P < 0.05$.

To evaluate the capacity of WIN55212-2 to control T cell responses in TMC, T cell cytokines at mRNA and protein level was analysed. WIN55212-2 (10 μM) significantly reduced IFN γ , IL-5, IL-13 and IL-17, but not IL-10 mRNA levels after 3 days (**Figure R.110a**). At the protein level, WIN55212-2 10 μM significantly inhibited the production of IFN γ , IL-5, IL-13, IL-17 and IL-10 after 3 days of polyclonal stimulation of tonsil T cells (**Figure R.110b**).

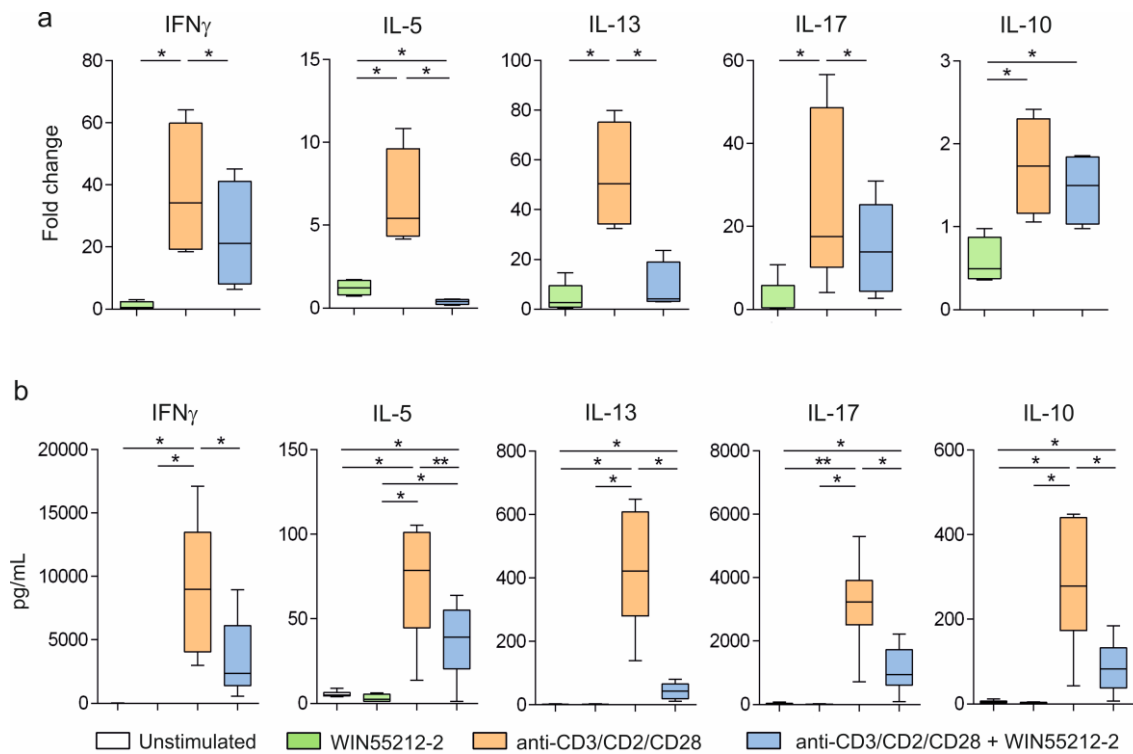


Figure R.110. **a** mRNA expression levels and **b** protein levels in cell-free supernatants of the indicated T cell cytokines in TMC stimulated for 3 days under the indicated conditions ($n = 6$). Fold change with respect to the unstimulated condition was calculated as $2^{-\Delta\Delta C_t}$, where ΔC_t correspond to the difference between the cycle threshold value for the gene of interest and EF1 α as housekeeping gene. Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova * $P < 0.05$, ** $P < 0.01$.

In addition, the activation of T cells with anti-CD3/CD2/CD28 antibodies in human TMC for 3 days induced the production of a plethora of pro-inflammatory cytokines (IL-8, IL-6, IL-15), chemokines (RANTES, IP-10 or MCP-1) and growth factors (GM-CSF, G-CSF, VEGF or FGF), which were also significantly inhibited by WIN55212-2 in all the cases (**Figure R.111**).

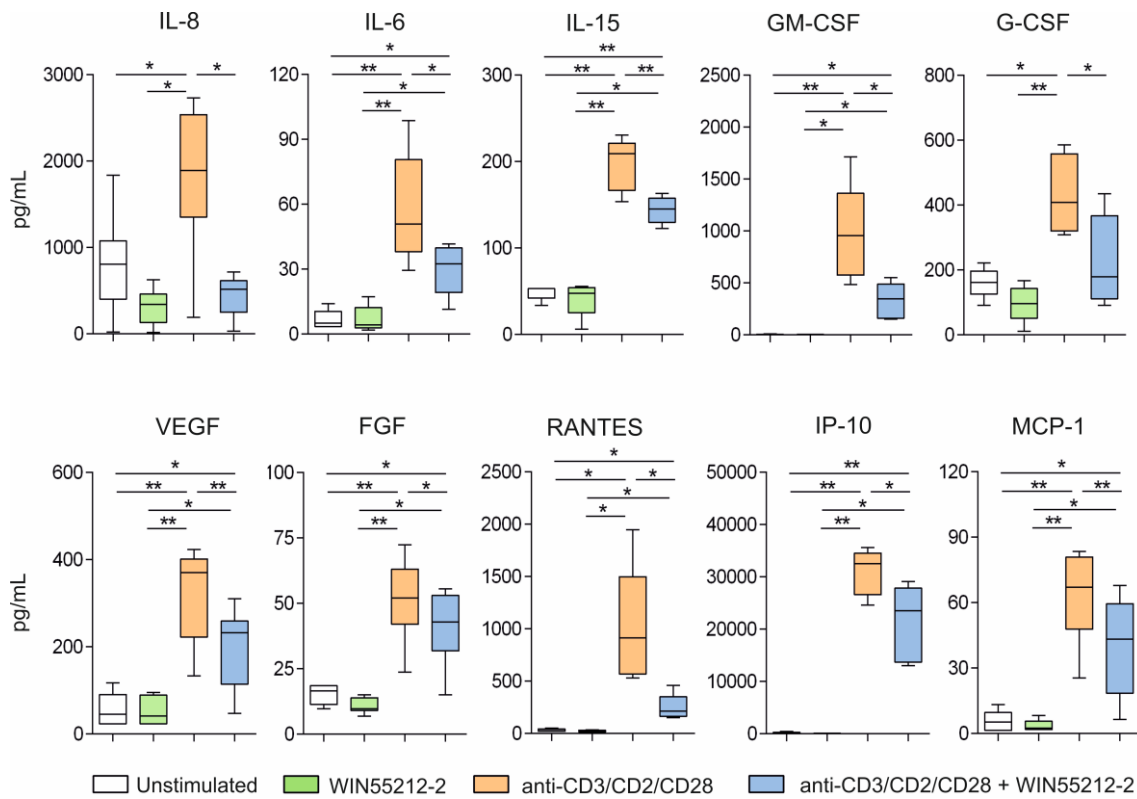


Figure R.111. Protein level in cell-free supernatants of the indicated pro-inflammatory cytokines, chemokines and growth factors in TMC stimulated for 3 days under the indicated conditions (n = 6). Values are mean $\pm$ SEM. Statistical significance was determined using One-way Anova * $P < 0.05$, ** $P < 0.01$.

Collectively, WIN55212-2 displays a potent anti-inflammatory capacity, as it is able to suppress the proliferation and activation of tonsil T cell-mediated immune responses. RV infection induces severe airway epithelial barrier damage and potent inflammatory T cell responses, which play a significant role in the pathophysiology and clinical manifestations of different airway chronic inflammatory diseases, such as rhinitis, CRSwNP or asthma. An impaired bronchial barrier allows higher penetration of allergens, toxins, pollutants and pathogens into submucosal tissues, leading to allergic sensitization, acute and chronic tissue inflammation and asthma exacerbations. Up to date, few drugs have been reported to prevent or restore RV infection-induced respiratory epithelial barrier alterations. Herein, we uncover a previously unknown capacity of the synthetic cannabinoid WIN55212-2 to regulate the integrity of the airway epithelial barrier during RV infection and to suppress tonsil T cell-mediated inflammation, which might well pave the way for the future development of potential novel preventive and therapeutic approaches for different chronic inflammatory airway diseases.

GLOBAL DISCUSSION

Global discussion

The human ECS is a complex signalling network involved in many physiological processes such as neuronal development, memory, metabolism and immunity (102). Currently, the lack of appropriate tools has hampered the understanding of ECS signalling at the molecular level. Specifically, the scarcity of suitable antibodies for CB1 due to drawbacks in specificity and reliability (165, 166) prompted scientists to develop chemical probes that allow the visualization and quantification of CB1 in native systems. We developed a CB1-specific small molecule fluorescent probe termed HU210-Alexa488 that consists of the synthetic cannabinoid agonist HU210, a CB1 and CB2 agonist, bound to the fluorophore Alexa Fluor 488 (157). This fluorescent probe keeps its high affinity for CB1, but not for CB2 (157). In this work, we have shown that HU210-Alexa488 probe represents a fluorescent chemical probe suitable to visualize and quantify CB1-expressing cells in peripheral blood and tonsils by flow cytometry and confocal microscopy. This probe is compatible with anti-human antibodies used to identify immune cell subsets and can be simultaneously combined with them for the rapid and reproducible identification of CB1-expressing cells in human blood and histological tonsil sections by flow cytometry and confocal microscopy. Therefore, the possibility of monitoring *ex vivo* CB1-expressing immune cells opens a new avenue toward the better understanding of the *in vivo* role of CB1 in human, the identification of this receptor as a new potential biomarker and the future development of novel CB1-targeted therapeutic interventions for any ECS-related diseases.

Dendritic cells are essential in the orchestration of immune responses by linking innate and adaptive immunity (14, 16). Due to its indispensable role in the induction of immune responses, DCs are regarded as ideal targets for therapeutic interventions. Although data for human DCs are still scarce, it was shown that they express all the components of the ECS (179). Here, we confirmed the expression of the ECS in hmoDCs, mDCs and pDCs from human peripheral blood and mDCs and pDCs from human tonsils. While some studies investigated the role of cannabinoids in the control of mouse DC function, studies assessing the capacity of cannabinoids to modulate the function of human DCs are comparatively scarce. Roth et al showed that the exposure of human monocytes to THC alters their differentiation into DCs, reducing their capacity of activation (181). It was also reported that THC and CB2 agonists reduce the production of cytokines in human pDCs (152, 153). Here, we performed a comprehensive immunologic study of the capacity of synthetic cannabinoids, specifically WIN55212-2, to modulate human DC function. WIN55212-2 significantly decreases the expression of HLA-DR and the surface mature markers CD86 and CD83 as well as the

production of pro-inflammatory cytokines in hmoDCs stimulated with LPS, TLR2 ligand Pam3CSK4 or with a mixture cytokines TNF α /IL-1 β . At the T-cell level, WIN55212-2 impairs the polarization of effector Th1 and Th2 cells induced by LPS-activated human DCs, whereas increases the generation of functional FOXP3⁺ Treg cells (**Figure D.112**). Although WIN55212-2 is a CB1 and CB2 agonist, it might also exert its anti-inflammatory effects through PPAR α , PPAR γ , TRPV1 and TRPA1 (139, 191). Our data demonstrated that WIN55212-2 imprints tolerogenic features in hmoDCs by mechanisms depending on the activation of CB1 and PPAR α .

The molecular mechanisms by which cannabinoids control the function of DCs remain poorly understood. Previous studies showed the cross-talk between TLR- and CBR-mediated signalling pathways in monocytes, macrophages and microglia (143-145). WIN55212-2 inhibits NF- κ B and MAPK signalling pathways induced not only by LPS in hmoDCs, but also by Pam3CSK4 in THP1-XBlue™ cells. Moreover, WIN55212-2 inhibits the activation of mTOR and induces the activation of AMPK, a key regulator of autophagy (**Figure D.112**). Salazar et al showed that THC promotes autophagy-mediated cell death in human and mice cancer cells (199). Similarly, THC and synthetic cannabinoids induces AMPK-dependent autophagy in hepatocellular carcinoma and pancreatic cancer cells (147, 200). WIN55212-2 promotes canonical autophagy activation in hmoDCs, which is leading the acquisition of the tolerogenic properties imprinted by WIN55212-2 in human DCs. We showed that the inhibition of WIN55212-2-induced autophagy in DCs correlates with a reduction on the generation of FOXP3⁺ Treg cells. Tomic et al found that graphene quantum dots suppresses pro-inflammatory T cell responses through the autophagy-dependent induction of tolerogenic human DCs (267). Supporting this data, mice with DCs lacking autophagy displayed Treg cells with reduced suppressive function and stability (215).

mTOR and AMPK are key metabolic regulators of catabolism and anabolism (81). mTOR promotes anabolic processes for the synthesis of macromolecules needed for cell growth, proliferation and activation (74, 75). In contrast, AMPK inhibits anabolism and activate catabolic processes for ATP production (78, 80). Metabolic reprogramming plays a significant role in the control of DC function by regulating tolerogenicity vs immunogenicity (88, 91). LPS-activated hmoDCs display a high rate glycolysis and lactic acid fermentation (Warburg effect) (94, 205, 268), features that are abolished by WIN55212-2. In addition, WIN55212-2 down-regulates the expression of genes involved in glycolysis in LPS-activated hmoDCs. Consistent with the results presented here, Malinarich et al have reported that hmoDCs matured with LPS are more glycolytic than immature hmoDCs, whereas tolerogenic hmoDCs display less glycolytic capacity than

mature DCs (94). The polyphenols carnosol and curcumin attenuate the increase in glycolysis in LPS-activated human DCs by the activation of AMPK, which is necessary for the immunosuppressive properties of these polyphenols (212). Tolerogenic DCs have been reported to possess a greater capacity for oxidative phosphorylation and fatty acid oxidation (95). For example, tolerogenic hmoDCs generated by treatment with dexamethasone or vitamin D3 exhibit enhanced catabolism and metabolic plasticity and increased expression of genes involved in oxidative phosphorylation and fatty acid oxidation (69, 94). We showed that WIN55212-2 favours mitochondrial activity and increases the expression of genes involved in oxidative phosphorylation and fatty acid oxidation in LPS activated-hmoDCs. We also demonstrated that autophagy activation by WIN55212-2 is required to mediate not only the immunomodulatory features but also the metabolic rewiring induced by WIN55212-2 in LPS-stimulated hmoDCs (**Figure D.112**). WIN55212-2 induces autophagy and subsequent metabolic reprogramming via CB1 and PPAR α . In line with our study, Dando et al have described that the CB1 agonist arachidonoyl cyclopropamide inhibits the glycolytic pathway and induces AMPK-dependent autophagy in pancreatic cells (147). PPAR α agonists also induces autophagy and enhances mitochondrial respiration in macrophages during mycobacterial infection (222).

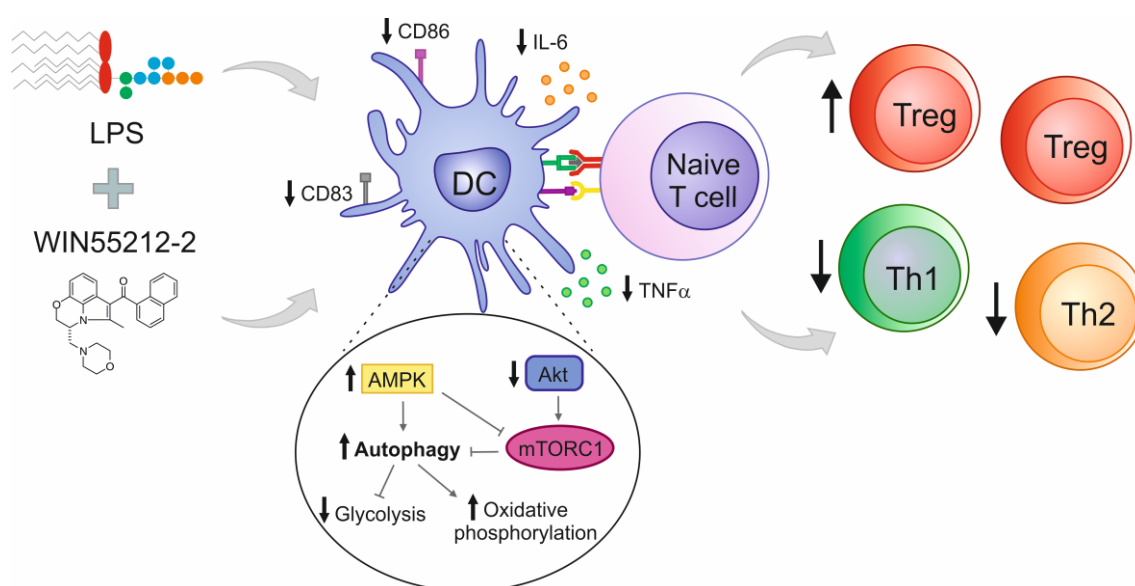


Figure D.112. Graphical abstract of the mechanisms by which the synthetic cannabinoid WIN55212-2 immunomodulates LPS-activated hmoDCs.

We assessed the potential *in vivo* relevance of our findings in a preclinical LPS-induced sepsis mouse model, characterized by a local and systemic inflammatory responses (223). We demonstrated that WIN55212-2 exerts *in vivo* protective and anti-inflammatory effects in LPS-induced sepsis model by mechanisms that rely on autophagy induction via activation of CB1 and PPAR α . In line with our data, Jiao et al

described that PPAR α activation reduces LPS-induced acute liver damage by promoting autophagy (230). Supporting our *in vitro* data, WIN55212-2 imprints tolerogenic properties in spleen DCs and promotes the generation of Treg cells by mechanisms depending on autophagy induction in LPS-induced septic mice. Treg cells together with IL-10 are essential for the survival of septic mice and the control of excessive immune responses, avoid deleterious consequences for the tissues and contribute to pathogen clearance by keeping homeostasis (231, 232). However, if the immunosuppression induced by Treg cells persists, the susceptibility to secondary infections can increase. Therefore, further researches investigating the immunomodulatory capacity of WIN55212-2 in sepsis is needed.

Remarkably, we confirmed the immunoregulatory properties of WIN55212-2 in a peanut allergy model, which is the most common cause of fatal food-induced anaphylactic reactions (245, 246). We demonstrated that WIN55212-2 in the presence of peanut induces human tolerogenic DCs with capacity to reduce Th2 responses and to promote the generation of Treg cells *in vitro*. Allergen-specific Treg cells are essential in the induction and maintenance of allergen tolerance in healthy responses and successful AIT (243, 244). Our *in vivo* data showed that the administration of WIN55212-2 during peanut challenge in allergic mice reduces the immediate anaphylactic reaction and promotes the generation of spleen allergen-specific Treg cells that is associated with lower Th2 responses in the late-phase. On the other hand, we showed that WIN55212-2 interferes in peanut sensitization by reducing the peanut-specific IgE and IgG1 plasma levels, but it does not inhibit the anaphylaxis.

Bronchial epithelial cells constitute the first physical and immunologic barrier against environmental inhaled insults (255, 256). Disruption of barrier integrity is a hallmark of chronic inflammatory airway diseases such as rhinitis or asthma (53, 163, 257). RV-mediated respiratory tract infections induces disruption of the airway epithelial barrier and contribute in the pathophysiology and clinical manifestations of rhinitis and asthma (256, 260, 261). In this study, we demonstrated that the synthetic cannabinoid WIN55212-2 repairs HRV-16-induced airway epithelial damage by restoring the integrity of TJ proteins (**Figure D.113**). Our data are aligned with a previous reports showing that the THC impairs TNF α -induced increase in airway epithelial permeability (264). Another study showed that anandamide disrupts the bronchial epithelial integrity, but such effects are driven by cyclooxygenase and lipoxygenase downstream arachidonic acid-derived metabolites resulting from the accumulation of arachidonic acid after the degradation of anandamide, not by the direct action of anandamide itself (265). During viral infections, epithelial cells produce pro-inflammatory cytokines and chemokines that enhance the

recruitment of peripheral inflammatory cells into airway submucosa to orchestrate immune responses (255, 266). We showed that WIN55212-2 does not alter the innate immune responses triggered by HRV-16 infection in airway epithelial cells. Viral infections condition immune responses in tonsils (172), secondary lymphoid organs located in the gateway of the gastrointestinal and respiratory tracts and that represent a suitable model for *ex vivo* studies. We demonstrated that WIN55212-2 displays a potent anti-inflammatory capacity, as it is able to suppress the proliferation and activation of tonsil T cell-mediated immune responses.

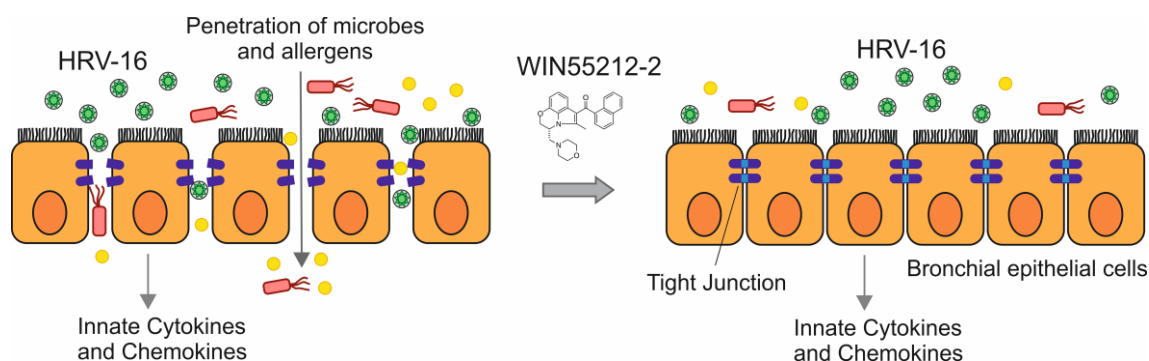


Figure D.113. Graphical abstract of the capacity of the synthetic cannabinoid WIN55212-2 to restore RV-induced airway epithelial barrier disruption.

In summary, we uncover the previously unknown capacity of the synthetic cannabinoid WIN55212-2 to immunomodulate the phenotype and function of human DCs. We show for the first time molecular mechanisms by which WIN55212-2 imprints tolerogenic features in human DCs, which include CB1- and PPAR α -mediated autophagy induction and metabolic reprogramming. In addition, we have demonstrated the potential clinical applications of WIN55212-2 in different *in vitro* and *in vivo* human and mouse models such as sepsis, peanut allergy or RV-induced inflammation. Our results might well contribute to pave the way for the future development of novel cannabinoid-based strategies for the prevention and treatment of immune-mediated diseases.

CONCLUSIONS

Conclusions

The main conclusions of this Thesis are:

1. HU210-Alexa488 probe represents a suitable probe to identify, visualize and quantify CB1-expressing cells in human peripheral blood and tonsils by flow cytometry and confocal microscopy.
2. Tonsil CB1-expressing cells detected with HU210-Alexa488 probe harbour functional plasma membrane CB1 with immunomodulatory capacity.
3. HmoDCs, mDCs and pDCs express all the components of the ECS at mRNA level. HmoDCs, mDCs and pDCs from peripheral blood express CB1 at protein level, whereas only hmoDCs and mDCs express CB2 at protein level.
4. The synthetic cannabinoid WIN55212-2 displays a more potent anti-inflammatory capacity than HU210 and HU308 in THP1-XBlue™ cells and hmoDCs.
5. The synthetic cannabinoid WIN55212-2 inhibits LPS-induced maturation and activation in hmoDCs. In addition, WIN55212-2-treated hmoDCs promote the generation of functional FOXP3⁺ Treg cells and reduce IFN γ - and IL-13-producing T cells. Thus, the synthetic cannabinoid WIN55212-2 imprints tolerogenic features in human DCs.
6. The synthetic cannabinoid WIN55212-2 inhibits LPS-induced activation of NF- κ B, MAPKs and Akt/mTORC1 signalling pathways in hmoDCs. WIN55212-2 activates AMPK signalling and autophagy in hmoDCs and induces subsequent metabolic reprogramming via CB1 and PPAR α activation.
7. The synthetic cannabinoid WIN55212-2 impairs lethal acute inflammatory responses and induces FOXP3⁺ Treg cells in LPS-induced septic mice by mechanisms depending on autophagy induction via CB1 and PPAR α activation.
8. The synthetic cannabinoid WIN55212-2 inhibits the maturation and activation of hmoDCs stimulated with CPE. CPE plus WIN55212-2-treated hmoDCs promotes the generation of FOXP3⁺ Treg cells and reduces the induction of IL-5-producing T cells.
9. The administration of the synthetic cannabinoid WIN55212-2 during peanut challenge protects peanut-allergic mice from the immediate anaphylactic reaction. The administration of the synthetic cannabinoid WIN55212-2 during peanut sensitization reduces the levels of peanut-specific IgE and IgG1

antibodies in mice serum. WIN55212-2 administration promotes the generation of allergen-specific spleen FOXP3⁺ regulatory T cells in the late-effector phase in both mice models.

10. The synthetic cannabinoid WIN55212-2 repairs HRV-16-induced airways epithelial disruption by restoring the integrity of TJ proteins without affecting epithelial cell-mediated innate immune response. WIN55212-2 also suppress the proliferation and activation of T cell-mediated immune responses.

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APPENDIX

The Role of Cannabinoids in Allergic Diseases: Collegium Internationale Allergologicum (CIA) Update 2020



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Keywords

Cannabinoids · Cannabinoid receptors · Allergy · Asthma · Atopic dermatitis · Immunosuppression · Immunomodulation

Abstract

The human endocannabinoid system (ECS) is a complex signalling network involved in many key physiological processes. The ECS includes the cannabinoid receptors, the endocannabinoid ligands, and the enzymes related to their synthesis and degradation. Other cannabinoids encompass the phytocannabinoids from *Cannabis sativa* L. (marijuana) and the synthetic cannabinoids. Alterations in the ECS are associated with different diseases, including inflammatory and immune-mediated disorders such as allergy. Allergy is a global health problem of increasing prevalence with high socioeconomic impact. Different studies have convincingly demonstrated that cannabinoids play a role in allergy, but their actual contribution is still controversial. It has been shown that cannabinoids exert anti-inflammatory properties in the airways and the skin of allergic patients. Other studies reported that cannabinoids might exacerbate asthma and atopic dermatitis mainly depending on CB2-mediated sig-

nalling pathways. A better understanding of the molecular mechanisms involved in the mode of action of specific cannabinoids and cannabinoid receptors on relevant immune cells under different biological contexts might well contribute to the design of novel strategies for the prevention and treatment of allergic diseases. Future research in this promising emerging field in the context of allergy is warranted for the upcoming years.

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Introduction

The cannabis plant *Cannabis sativa* L. (marijuana) has been used for both therapeutic and recreational effects for many centuries. However, the purification and the chemical characterization of its unique active components, the so-called cannabinoids, were not carried out until the 1960s [1]. In the early 1990s, specific cannabinoid receptors (CBRs) were cloned and their endogenous ligands were characterized, uncovering the mechanism of action

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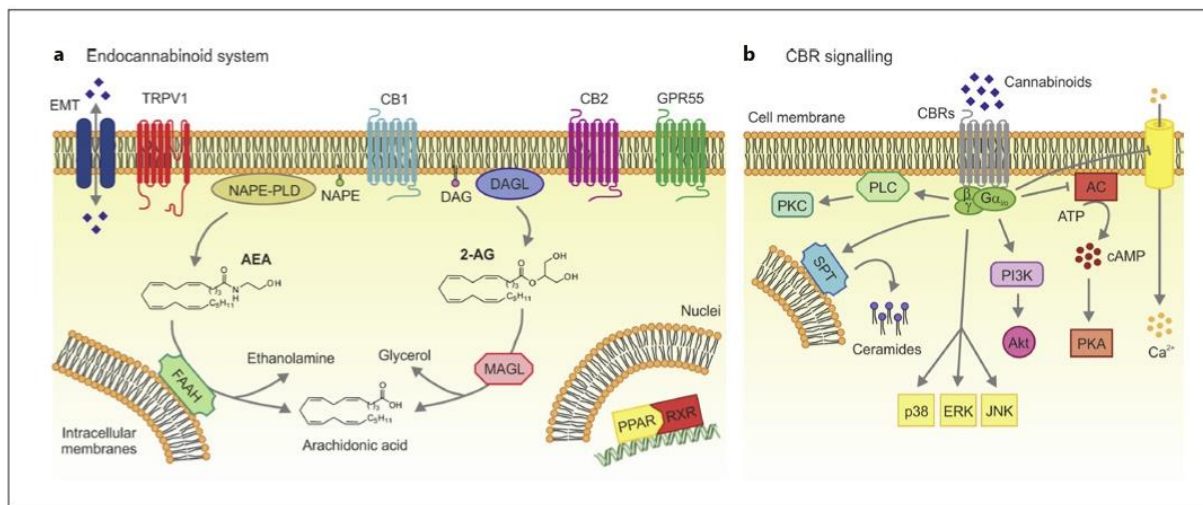


Fig. 1. **a** Main components of the endocannabinoid system (ECS). Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthesized on demand from membrane lipids by *N*-acyl-phosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively. AEA and 2-AG move across the cell membrane through a purported endocannabinoid membrane transporter (EMT). Cannabinoid receptor 1 (CB1), cannabinoid receptor 2 (CB2), transient receptor potential vanilloid 1 (TRPV1), G-protein-coupled receptor 55 (GPR55), and PPARs are the main re-

ceptor targets of AEA and 2-AG. AEA and 2-AG are hydrolysed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively, releasing arachidonic acid. **b** Cannabinoid receptor (CBR)-induced signalling. After cannabinoid binding, CBRs signal several cellular pathways including inhibition of protein kinase A (PKA) pathway, activation of mitogen-activated protein kinase cascades (p38, JNK, and ERK), activation of protein kinase B (Akt) pathway, inhibition of calcium channels, activation of protein kinase C (PKC), and generation of ceramides.

of cannabinoids [2, 3]. These findings boosted basic and clinical research to better understand the molecular pathways involved in the mode of action of cannabinoids and to explore potential novel therapeutic applications. The human endocannabinoid system (ECS) encompasses the CBRs, the endocannabinoid ligands, and the group of enzymes responsible for their synthesis, transport, and degradation [4]. The ECS is involved in a large number of vital physiological processes in the body, such as the control of neuronal activity, energy metabolism, cardiovascular tone, and immunity. Alterations in the ECS have been associated with different diseases, including inflammatory and immune-mediated disorders, which suggests the ECS components as promising novel therapeutic targets [5, 6]. Cannabinoids regulate immune responses by promoting anti-inflammatory properties in brain injury, inflammatory bowel diseases, sepsis, multiple sclerosis, airway inflammation, and allergy [7].

Allergy represents a global health problem of increasing prevalence, affecting almost one billion patients worldwide. The main clinical manifestations of allergy include allergic rhinitis, allergic asthma, atopic dermatitis (AD) and other skin diseases, food allergy, and anaphy-

laxis. Allergic diseases can be generally considered as type 2-mediated diseases characterized by the production of high levels of IgE against innocuous substances called allergens [8, 9]. Allergic disorders significantly affect patients' quality of life, decrease productivity at work, and enhance sanitary costs of health care systems, thus representing a significant health problem with high socio-economic impact [10, 11]. Therapeutic strategies that not only treat allergic symptoms but also might prevent or modify the course of the disease are highly demanded. Currently, allergen-specific immunotherapy (AIT) remains as the only treatment with potential long-term disease-modifying capacity for allergic disorders [12, 13]. AIT consists of the administration of high doses of the causative allergens to induce a state of permanent tolerance upon treatment discontinuation. AIT is a successful treatment strategy for many patients, but it displays some important drawbacks related to efficacy, safety, and duration. Therefore, the design and development of novel therapeutic approaches for the prevention and treatment of allergic diseases is fully demanded.

Several studies have demonstrated the participation of the ECS in the development and maintenance of allergic

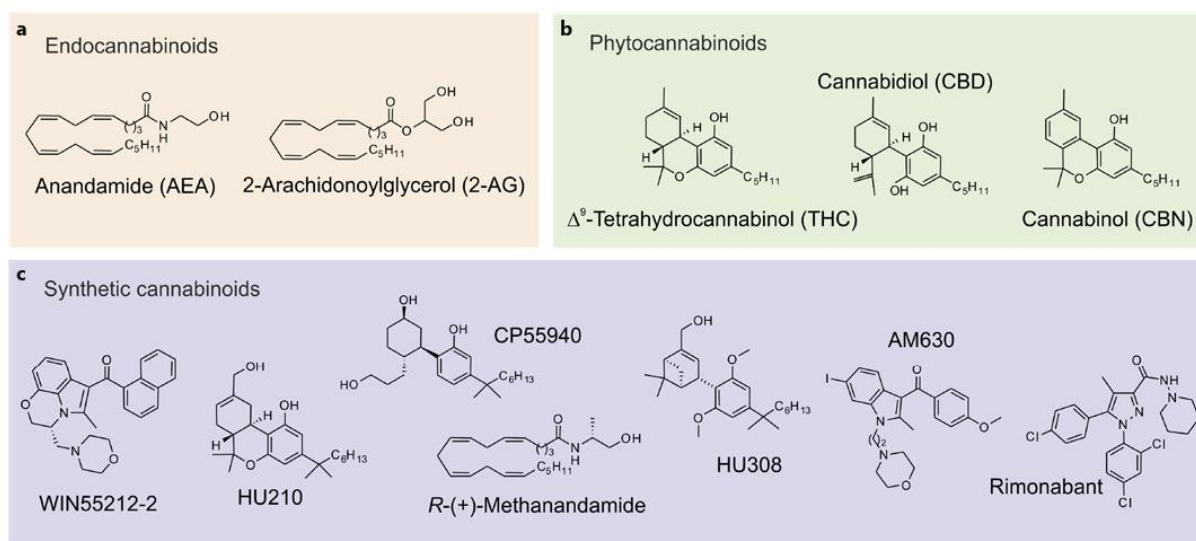


Fig. 2. Cannabinoid ligands. **a** Endocannabinoids. **b** Plant-derived cannabinoids or phytocannabinoids. **c** Synthetic cannabinoid including non-selective agonists, CB1- and CB2-selective agonists, and CB1- and CB2-selective antagonists.

diseases, but the data related to the actual role of cannabinoids in allergy are still controversial. The capacity of the ECS to suppress inflammation in mouse models of allergen-induced airway inflammation, AD, and contact allergy has been well documented [14, 15]. In contrast, other studies have shown that CBR 2 (CB2)-mediated signalling contributes to the exacerbation of asthma and AD [16, 17]. Future basic and clinical research will help to delineate the detailed molecular mechanisms by which cannabinoids might exert their functions on different immune cells in the context of allergy as well as their potential clinical implications. The development of novel chemical probes is enabling a better understanding of these pathways, which will be of outmost value to firmly confirm how cannabinoids can modulate allergic responses. In this article, we will comprehensively review our current knowledge on the role played by the ECS and the potential therapeutic applications of cannabinoid-based drugs in the context of the main allergic diseases.

The Human ECS

The human ECS is a complex and essential signalling pathway involved in many physiological processes. The ECS is involved in the control of many relevant physio-

logical processes such as neuronal development, brain plasticity, learning and memory, regulation of appetite, stress and emotions, proliferation, differentiation, cell survival, metabolism, and immunity. Alterations in the ECS have been associated with a plethora of diseases, and preclinical and clinical studies have indicated cannabinoids as novel potential therapeutic tools in cancer, neurological, inflammatory, and immune-mediated diseases [6, 18, 19]. The main components of the ECS include the endocannabinoid ligands, the enzymes related to their synthesis and degradation, and the CBRs (Fig. 1).

Endocannabinoids are lipid-derived signalling molecules that are endogenously synthesized and can bind and activate CBRs. Anandamide (arachidonylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) are the most widely investigated. However, other biochemically and structurally related endocannabinoids such as *N*-palmitoylethanolamine (PEA), 2-arachidonoylglycerol ether (noladin ether, 2-AGE), *O*-arachidonylethanolamine (virodhamine), and *N*-arachidonoyldopamine (NADA) have also been recognised [20]. AEA is a high-affinity partial agonist of the G-protein-coupled receptors (GPCRs) CBR 1 (CB1) and a low-affinity ligand of the GPCR CB2, whereas 2-AG acts as a full agonist with moderate affinity for both CBRs [20, 21]. AEA and 2-AG can also interact with other receptors or ion channels, including

different GPCRs, peroxisome proliferator-activated receptors (PPARs), and transient receptor potential channels (TRPVs) [22]. Other cannabinoid compounds encompass the phytocannabinoids derived from the plant *Cannabis sativa* L. (marijuana) and the synthetic cannabinoids (Fig. 2). The most abundant and well-characterized phytocannabinoid is Δ^9 -tetrahydrocannabinol (THC), which was isolated and structurally characterized in the 1960s [1]. THC is the major psychoactive component of marijuana and exerts a wide variety of biological effects by activation of CB1 and CB2 [23]. The potential therapeutic effects of THC have been studied in several diseases, including cancer, multiple sclerosis, epilepsy, Alzheimer disease, Parkinson disease, and pain treatment [23]. Currently, THC and its synthetic analogue nabilone (Marinol and Cesamet, respectively) are approved for inhibiting chemotherapy-induced nausea and vomiting [6]. Cannabidiol (CBD) and cannabitol (CBN) are other plant-derived cannabinoids produced in significant amounts. CBD is the main non-psychoactive phytocannabinoid and its therapeutic interest is due to its analgesic, antipsychotic, antioxidant, and anti-inflammatory properties [24]. A mixture of equal doses of THC and CBD, known as Sativex, is marketed for the treatment of spasticity in patients with multiple sclerosis [6, 24]. Since the identification of the ECS, a large number of synthetic cannabinoids have been developed in order to mimic some of the beneficial properties of THC, while avoiding its negative effects. They include agonists with similar affinity for CB1 and CB2 but greater potency than THC, such as WIN55212-2 (from the aminoalkylindole family), HU210 (synthesized at the Hebrew University; HU series), and CP55940. Compounds with specific selectivity have also been described: R(+)-methanandamide (CB1-selective agonist) and HU308 (CB2-selective agonist). In addition, selective antagonists have been generated such as Rimonabant and AM630, CB1- and CB2-selective antagonists, respectively [25, 26] (Fig. 2). Numerous cannabinoids, including phytocannabinoids and synthetic cannabinoids, have been included in clinical trials [5].

Endocannabinoids are synthesized and released on demand from membrane precursors, but it is suggested that they could also be accumulated in storage organelles within the cell [27, 28]. As shown in Figure 1, synthesis of AEA starts with the hydrolysis of *N*-acyl-phosphatidylethanolamine (NAPE) by NAPE-hydrolysing phospholipase D (NAPE-PLD). Alternative pathways involving other NAPE phospholipases and enzymes are also described [4, 28]. The synthesis of 2-AG consists of the sequential hydrolysis of arachidonoyl-containing phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC)

followed by hydrolysis of the resulting diacylglycerol by diacylglycerol lipase (DAGL) [4]. Endocannabinoids are rapidly inactivated by a two-step process: cellular uptake and intracellular hydrolysis. Cellular uptake of endocannabinoids from extracellular space is mediated by putative endocannabinoid membrane transporters, the existence of which is only based on indirect evidence as it has not been cloned yet [29]. Degradation of AEA is mediated by the fatty acid amide hydrolase (FAAH), whereas the degradation of 2-AG is primarily due to monoacylglycerol lipase (MAGL). The resulting products of AEA and 2-AG degradation are arachidonic acid, which is required for prostaglandin synthesis, and ethanolamine and glycerol, respectively [4] (Fig. 1). FAAH and MAGL represent promising therapeutic targets for the treatment of different disorders, and pharmacological inhibitors have been developed. Pharmacological or genetic inhibition of FAAH and MAGL increases AEA and 2-AG levels, prolonging their anti-inflammatory and analgesic effects via CBRs [30]. Remarkably, FAAH and MAGL inhibition also reduces arachidonic acid, a key precursor of pro-inflammatory prostaglandins and thromboxanes. FAAH and MAGL inhibitors may have a large number of therapeutic applications, including pain, nausea, neurodegenerative pathologies, inflammation, metabolic disorders, and cancer [31, 32].

The effects of endocannabinoids are mainly mediated by CB1 and CB2 but other receptors acting as CBRs have been proposed. CB1 and CB2 share 44% sequence identity [3]. Both receptors consist of a single polypeptide chain with 7 transmembrane α -helices inserted in the cell membrane, as well as an extracellular N-terminus and intracellular C-terminus domain. Their high-resolution crystal structure in humans has been recently reported [33–35]. CB1 and CB2 are generally coupled to inhibitory $G_{i/o}$ proteins, thus inhibiting adenylate cyclase and the conversion of ATP to cyclic AMP and protein kinase A activation, a positive regulatory signalling pathway of immune response. CB1 can also activate adenylate cyclase through $G_{q,s}$ protein stimulation [36]. $G_{\beta\gamma}$ subunits coupled to CBRs activate mitogen-activated protein kinases (MAPKs) and protein kinase B (Akt) with important consequences in the maintenance of cellular homeostasis. Other CBR-mediated signalling pathways include modulation of ion channels, ceramide biosynthesis, and activation of PLC β (Fig. 1) [37, 38]. CB1 is highly expressed in the central nervous system (CNS) where it regulates diverse neuronal functions and behaviours. Far from being restricted to the CNS, CB1 expression is also observed in

peripheral tissues including immune cells, liver, pancreas, skeletal muscle, and peripheral nervous system, where it has been implicated in other key physiologic processes such as control of immunity, metabolism, etc. [6]. For a long time it was thought that CB2 was only expressed in peripheral immune system cells, but it has been shown that CB2 is also expressed in different cell subsets in the CNS, muscle, pancreas, intestine, and testis [39, 40]. Interestingly, CB1 and CB2 expression can be increased in pathologic or injury conditions. CB1 is significantly increased in murine models of colitis inflammation or lipopolysaccharide-stimulated macrophages [41, 42]. CB1 gene expression is also increased in human immune system cells from patients with allergic diseases [43]. CB2 expression is upregulated in chronic inflammation of the immune system, as well as in brain injury [44, 45].

Other receptors including orphan GPCRs (GPR55, GPR19), TRPV1, and PPARs are also involved in the cannabinoid-induced signalling pathways. GPR55 was initially described as a putative “CB3,” but the low sequence similarity with conventional CBRs does not fully endorse this concept [46, 47]. GPR55 is widely expressed in the immune system, CNS, and peripheral tissues [47]. It is coupled to Gα12/13, signalling through RhoA and controlling several physiological processes [48]. Unlike CB1 and CB2 that mainly trigger inhibitory effects, GPR55 mostly exerts excitatory and stimulatory effects [49, 50]. AEA, 2-AG, THC, and HU210 have been described as GPR55 ligands [51]. Other orphan GPCRs such as GPR18, GPR19, and GPR110 have also been described as cannabinoid targets [5]. TRPV1, also known as capsaicin receptor, is a non-selective channel expressed in the CNS and periphery tissues, including liver, skin, intestine, and immune system cells. Upon activation, the pore allows the flux of ions across the membrane. TRPV1 can be activated by heat, capsaicin, arachidonic acid derivatives, protons, and cannabinoids [52]. AEA shows a similar affinity for TRPV1 as capsaicin, but less potency [53]. CBD has also been described as TRPV1 activator, whereas THC does not modulate this channel [22, 54]. Some synthetic cannabinoids such as WIN55212-2 or AM1241 are also TRPV1 ligands [22]. Several studies demonstrated the activation of PPARs by some cannabinoids [55, 56]. PPARs are nuclear hormone receptors with 3 isoforms (α, β, and γ) highly expressed on metabolically active tissues. Upon ligand binding, PPARs heterodimerize with retinoic acid receptor and bind to the PPAR response element DNA sequences, regulating the transcription of genes involved in metabolism, cell differentiation, and inflammation [57]. AEA and 2-AG bind and activate both PPARα and

PPARγ, and phytocannabinoids can mainly bind to PPARγ. Although the detailed mechanisms of cannabinoid-PPAR interaction are not clear, activation of PPARs by cannabinoids exerts anti-inflammatory and neuroprotective effects in several disease models [58, 59].

The ECS and the Immune System

The ECS play an important role in the regulation of both innate and adaptive immune responses. Immune cells are not only influenced by cannabinoids, but also produce and secrete endocannabinoids themselves, which in turn modulates the functional features of immune cells [60]. CB2 is the most expressed CBR in immune cells and its activation usually mediates immunosuppressive responses, but other CBRs such as CB1, GPR55, and PPARs are also involved in immune cell regulation. In a broad perspective, the anti-inflammatory effects of cannabinoids are a consequence of some specific effects on immune cells such as their capacity to modulate cytokine production, cell migration, T-cell responses, cell proliferation, and apoptosis [7, 60].

Regulation of Immune Cells by Cannabinoids

AEA, 2-AG, THC, and CBD control macrophage function by inhibiting cytokine production, nitric oxide release, and phagocytosis [61–63]. In human monocyte-derived dendritic cells (DCs), the expression of all the components of the ECS has been described [64] and several inflammatory models showed the capacity of cannabinoids to modulate DC function. AEA and THC inhibit pro-inflammatory cytokine production and the capacity of DCs to polarize Th1 and Th17 responses [65–67]. THC induces apoptosis in murine DCs, providing a potential immunosuppression mechanism of immune cells [68].

The contribution of ECS signalling in the regulation of neutrophils and natural killer (NK) cell function is still controversial. Neutrophils express low levels of CBRs, but the lack of CB2 enhances their migration [69, 70]. In contrast, 2-AG induces neutrophil activation and the release of antimicrobial mediators [71]. NK cells express high levels of CB1, CB2, and GPR55, but controversial results are reported. THC treatment impairs the cytolytic activity of NK cells [72], but GPR55 activation induces the release of cytokines and cytolytic activity [49]. Although there is scarce data about the effect of cannabinoids on eosinophils, AEA inhibits the activation, maturation, and degranulation of mast cells [73]. Mice lacking CB2 display a low number of type 2 innate lymphoid cells (ILC2s),

Table 1. Main effects of cannabinoids in immune cells

Cell type	CBR expression	Role of cannabinoid ligands
Monocytes/macrophages	CB1 and CB2 [184, 185]	AEA, 2-AG, THC, and CBD inhibit cytokine production [61, 81, 186, 187] AEA and PEA stimulate phagocytosis [188, 189] 2-AG, THC, and WIN55212-2 modulate ROS production [187, 190] CBD induces apoptosis [191]
Dendritic cells (DCs)	CB1 and CB2 [43, 64]	AEA, THC, JWH-015, and JWH-133 inhibit inflammatory cytokine production [65, 66] AEA and THC inhibit the capacity to induce Th1 and Th17 responses [65, 67] THC induces apoptosis [68] THC impairs human monocyte-derived DC differentiation [192]
Neutrophils	CB1 and CB2 [70, 193]	AEA, CBD, and CB2 signalling reduce cell migration [69, 70] AEA and 2-AG induce cell activation and the release of antimicrobial effectors [70, 71, 194]
NK cells	CB1 and CB2 [60]	2-AG and THC inhibit cytolytic activity [72, 74] CB2 signalling reduces cell migration [74] O-1602 induces high cytolytic activity and cytokine production [49]
Eosinophils	CB1 and CB2 [195]	2-AG increases cell recruitment [195, 196] WIN55212-2 reduces cell recruitment [197]
Mast cells	CB1 and CB2 [73, 103, 198]	AEA and AEA-derived compounds inhibit cell maturation and degranulation [73, 141, 199] AM251 induces cell maturation and degranulation [103]
Innate lymphoid cells (ILCs)	CB2 [200]	CBD promotes ILC2 induction [200] CB2 signalling induces high numbers of ILC2 [74]
T lymphocytes	CB1 and CB2 [76, 201]	AEA, THC and JWH-133 inhibit T-cell proliferation [201–203] AEA and THC suppress T-cell responses [201] CBD and JTE907 induce functional Treg generation [77, 204] HU210 and HU308 inhibit cytokine production [76]
B cells	CB1 and CB2 [76, 205]	THC and WIN55212-2 increase B-cell proliferation [206] CP55940 induces IgE class switching [207] CB2 signalling promotes B-cell retention in bone marrow or splenic marginal zones [78, 79]

suggesting the role of CB2 signalling in the induction of ILC2s [74].

Cannabinoids mainly suppress adaptive T-cell responses by inhibiting proliferation and cytokine production [75, 76]. CBD has been shown to induce tolerogenic responses by favouring the generation of regulatory T (Treg) cells [77]. B cells express the highest levels of CB2, which is essential for mouse B-cell subset formation and for retention of immature B cells in bone marrow and splenic marginal zones [78, 79]. A more detailed summary of the main effects of cannabinoids on the different immune cells is presented in Table 1.

Anti-Inflammatory Mechanisms of Cannabinoids

Compelling experimental evidence supports that cannabinoids exert powerful anti-inflammatory effects on immune cells, but the mechanisms by which they exert such effects need to be better understood [7]. Toll-like

receptor (TLR) activation leads to NF- κ B and MAPK signalling pathway activation, inducing the expression of pro-inflammatory genes [80]. Cannabinoids impair pro-inflammatory cytokine and nitric oxide production by LPS-stimulated monocyte, macrophages, and microglia due to NF- κ B signalling pathway inhibition [62, 81]. In vivo models of LPS-induced inflammation support the capacity of cannabinoids to interfere in TLR signalling [82, 83]. TLR activation enhances CBR expression and endocannabinoid production, suggesting an important role of the ECS in the modulation of TLR-mediated immune responses [42, 82].

Novel findings indicate that cannabinoids might also mediate their anti-inflammatory effects by rewiring the metabolic pathways in immune cells [84]. Metabolic reprogramming can govern the function of T cells, macrophages, and DCs. Immune activation is mainly linked to a glycolysis-driven upregulation of anabolic processes,

whereas the tolerance state is characterized by increased catabolic processes [85–87]. AMP kinase (AMPK) is a master regulator of catabolism promoting mitochondrial biogenesis, oxidative phosphorylation, and autophagy. Simultaneously, AMPK also downregulates anabolic processes, antagonising immune cell activation [88, 89]. In pancreatic cancer cells, cannabinoid agonists induce AMPK activation depending on ROS-mediated increase of AMP/ATP ratio [90]. Similarly, THC and JWH015 activate AMPK through CB2 and inhibit energetic metabolism [91]. In these studies, AMPK activation leads to autophagy induction, a catabolic process involved in cellular homeostasis [88, 90, 91]. Autophagy has also been involved in immune system control by clearance of intracellular bacteria, control of inflammatory cytokine secretion and inflammation, antigen presentation, and lymphocyte development [92, 93]. CBD and AEA attenuate inflammation in an autophagy-dependent manner [94]. Considering all these aspects, cannabinoid-based treatment demonstrated anti-inflammatory and beneficial effects in brain injury, inflammatory bowel diseases, vascular inflammation, sepsis, rheumatic disease, multiple sclerosis, airway inflammation, and allergy [7, 95].

Cannabinoids in Allergic Diseases

Allergy is a type 2 helper T cell (Th2)-mediated disease of increasing prevalence affecting around 30% of the population worldwide. Allergic diseases constitute a public health problem with a high socio-economic impact. The main allergic diseases include allergic rhinitis, allergic asthma, food allergy, AD, and anaphylaxis [9]. The immunological mechanisms underlying allergic diseases can be divided into two main phases: (i) sensitization and memory and (ii) effector phase. The sensitization phase occurs during the first contact with the allergen and leads to the generation of allergen-specific CD4⁺ Th2 cells and allergen-specific IgE antibodies that diffuse and bind to the IgE high-affinity receptor (FcεRI) on the surface of mast cells and basophils, thus leading to patient sensitization. Upon new allergen encounters, allergen-dependent cross-linking of the IgE-FcεRI complexes on sensitized mast cells and basophils triggers the release of a plethora of anaphylactogenic mediators, responsible for the immediate clinical symptoms. Late-phase reactions are initiated by the accumulation of mediators and by the activation of allergen-specific memory Th2 via mechanisms depending on IgE-facilitated presentation by DCs and B cells. Th2 cells in cooperation with ILC2s activated by ep-

ithelial cell-derived alarmins (TSLP, IL-33, or IL-25) produce large amounts of IL-4, IL-5, IL-9, and IL-13 that contribute to maintain allergen-specific IgE levels, eosinophilia, mucus production, inflammatory cell recruitment, and tissue inflammation, leading to chronicity and the most severe clinical manifestation of allergy [8, 9, 96].

Allergen-specific Treg and regulatory B (Breg) cell generation is essential in the induction and maintenance of allergen tolerance in healthy responses and successful AIT [9, 97]. Although AIT, the single treatment with the capacity to induce long-term modifying effects upon discontinuation, is effective in many cases, it displays several important drawbacks in terms of efficacy, safety, and duration. Therefore, the development of novel prophylactic and therapeutic interventions is highly demanded in the field of allergy. In this regard, a better understanding of the role of ECS in the context of allergy might well contribute to open new avenues for the design of novel preventive and curative strategies. To date, the data on the effect of cannabinoids in the context of allergic diseases are still a bit controversial. Some studies reported a potential protective role of ECS in allergen-induced airway inflammation and contact allergy [14, 15, 98, 99]. In contrast, other studies in mice showed that CB2 signalling contributes to allergic exacerbation in OVA-asthma models or AD models [16, 17, 100]. Our group has previously shown that the gene expression of CB1 is significantly increased in peripheral blood and tonsils of atopic patients, but the functional significance of these findings remains to be fully elucidated [43]. In the next sections, we will comprehensively review our current knowledge on the role of the ECS and cannabinoid-based drugs in the context of different allergic diseases.

Allergic Rhinitis

Allergic rhinitis is a highly prevalent disorder in western countries, especially in children. It is defined by chronic inflammation of the nasal mucosa that promotes the appearance of its main clinical symptoms: sneezing, itch, nasal congestion, and rhinorrhoea [101, 102]. The pathophysiology of allergic rhinitis is mediated by a type 2 immune response where Th2 cells, ILC2s, B cells, mast cells, basophils, and eosinophils together with structural cells from the nasal mucosa interact and release a range of mediators that end up in the development of classical features of rhinitis [8, 9]. Several pathophysiological mechanisms of allergic rhinitis are also present in the lower respiratory tract of asthmatic patients. In fact, both diseases coexist in many patients and the diagnosis of al-

lergic rhinitis is an important risk factor for the future development of asthma [101, 102].

Studies addressing the role of the ECS in allergic rhinitis are scarce. As mentioned above, we previously showed that the expression of CB1 was significantly up-regulated in tonsils from allergic rhinitis patients compared to non-atopic donors [43]. CB1 limits mucosal mast cell activation and maturation from nasal polyps [103], indicating a protective role in allergen-induced airway diseases. Conversely, in patients with allergic rhinitis, nasal stimulation of TRPV1 by capsaicin or olvanil during the pollen season resulted in an increased perception of itch compared to placebo-treated controls [104]. Intriguingly, no effects were found when TRPV1 was activated by AEA, possibly due to a high degradation rate of AEA in the nasal mucosa [104]. These findings suggest that the ECS may contribute to some extent to the pathophysiology of allergic rhinitis, but future research is required.

Allergic Asthma

Asthma is a heterogeneous syndrome characterized by chronic inflammation of the conducting airways affecting up to 358 million people worldwide. It encompasses several phenotypes with different pathophysiological mechanisms that share common clinical symptoms such as bronchial hyperreactivity (BHR), reversible airflow obstruction, and intermittent periods of wheezing, cough, and chest tightness as well as airway remodelling and exacerbations in the most severe manifestations [105–107]. Allergic asthma represents one of the most common and well-studied asthma phenotypes [108]. It is associated with an early age onset, increased levels of total and allergen-specific serum IgE, and type 2 immune responses [8, 108]. Corticosteroids and bronchodilators are the mainstay treatment for asthma and many patients are properly controlled with them. However, in patients with severe asthma, the treatment of the disease is still challenging [106, 107]. Over the last years, biologicals have significantly improved the asthma control and quality of life of many severe asthma patients, but novel safe and cost-effectiveness treatments are still demanded [109–111].

First evidence on the therapeutic potential of cannabinoids in the airways dates back to the 1970s when several studies pointed out the bronchodilatory properties of marijuana smoke and oral administration of THC [112]. However, its therapeutic exploitation was hampered due to concerns of its psychotropic effects at the CNS and the paradoxical bronchoconstrictory responses reported in

some asthmatic patients [113]. Since then, different studies have addressed the potential involvement of the ECS in asthma. Increased levels of AEA have been reported in the bronchoalveolar lavage fluid of allergic asthma patients upon allergen challenge and the mRNA levels of CB1 are increased in asthmatics [43, 114]. The expression of CB2 is enhanced in peripheral blood eosinophils from allergic patients with seasonal respiratory symptoms compared to healthy controls [16]. These data, together with findings in mice showing decreased levels of PEA and upregulation of CB2 and GPR55 receptors after OVA sensitization [115], suggest a potential role of the ECS in the pathophysiology of asthma. However, whether they are a cause or consequence of the ongoing disease needs to be considered carefully.

AEA might play a dual role in the pathogenesis of asthma as it promotes an increase in airway epithelial cell permeability while it also reduces prostaglandin D4-induced bronchospasm in guinea pigs [33, 116]. Besides, AEA controls capsaicin-induced BHR via CB1 in axon terminals of airway nerves, but it also promotes bronchospasm when the vagus nerve constricting tone is removed [117]. This could explain the above-mentioned paradoxical bronchoconstriction in some asthmatic patients treated with cannabinoid compounds [113, 118]. PEA significantly inhibited BHR as well as inflammatory cell recruitment to the airways [115]. In addition to the role of CB1 as a suppressor of mast cell degranulation in the airways, CB1 activation prevented BHR through a modulatory control of nerve-mediated cholinergic contractions in mice and humans, confirming a probable protective role in asthma [119, 120]. In contrast, CB2-mediated signaling strongly potentiates eosinophil chemotaxis and responsiveness, leading to worsening of airway hyperreactivity in mice [16]. Supporting this data, CB2 knockout animals developed a significantly attenuated allergic airway inflammation after house dust mite exposure compared to wild-type mice [74]. This result correlated with increased levels of NK cells and reduced numbers of ILC2s in the lungs of mice lacking CB2, which led to the discovery that NK cells are key negative regulators of ILC2s. However, CB2 activation may also play a protective role in asthma since stimulation of CB2 inhibited antigen-induced plasma extravasation and electrical field-induced contraction of bronchial smooth cells by acting on C-fibres in guinea pig airways [121, 122].

The pleiotropic functions exerted by different ECS components highlight the complexity of the system and its therapeutic potential in the pathogenesis of asthma [123]. In mice, THC attenuated allergic inflammation

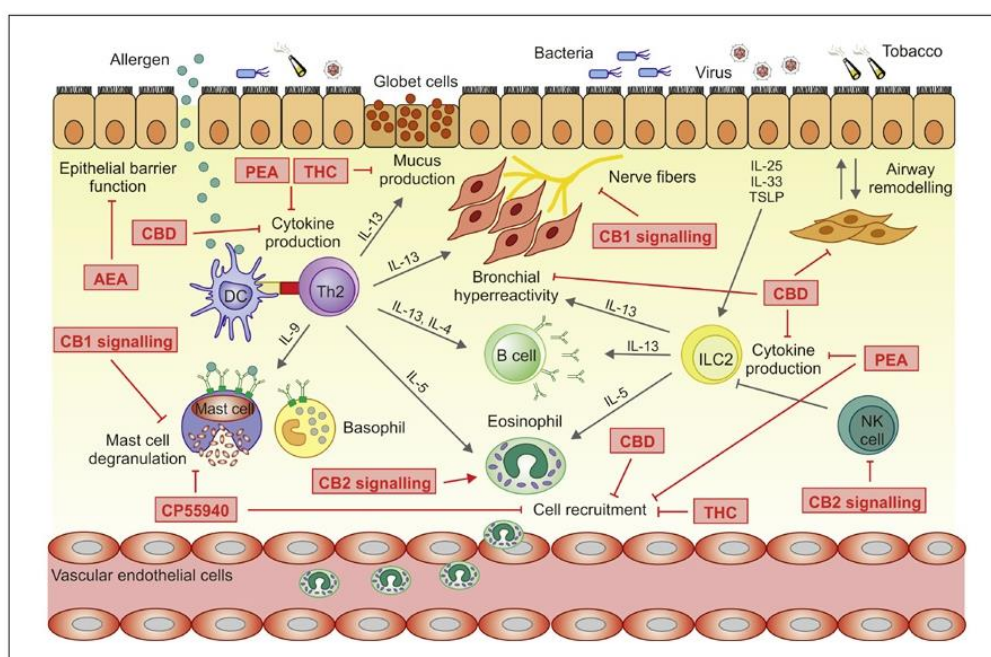


Fig. 3. Modulatory pathways of the ECS in asthma pathophysiology. In allergen-sensitized patients, antigen exposure results in IgE-FcεRI cross-linking in the surface of mast cells and basophils that lead to the release of their anaphylactogenic mediators, causing increased vascular permeability, bronchoconstriction, and/or mucus production. Following this early process, APC-activated Th2 cells and alarmin-activated ILC2s produce large amounts of

Th2 cytokines (IL-4, IL-13, IL-5, and IL-9) that contribute to the activation and recruitment of eosinophils and other inflammatory cells, contraction of smooth muscle, and bronchial hyperreactivity. If the inflammatory environment persists, it may trigger the remodelling of the airways. The contribution of the ECS to the different asthma pathways is highlighted with the corresponding arrow.

in the airways by reducing Th2 cytokine production, total cell infiltration, mucus secretion, and serum IgE levels in a CB1- and CB2-independent manner [14, 124]. In an antigen-induced asthma model in guinea pigs, CP55940 decreased respiratory clinical abnormalities, histological changes in the lung, mast cell degranulation, and airway cell recruitment [125]. Furthermore, the non-psychotropic cannabinoid CBD improved lung function and reduced airway inflammation in a murine model of LPS-induced acute lung injury [126]. Supporting these findings, CBD ameliorated the outcomes of a murine model of experimental allergic asthma by decreasing cytokine production, airway hyperresponsiveness and remodelling, and restoring lung function [99]. Finally, the potential application of manipulating endocannabinoid levels by using inhibitors of the cannabinoid degrading enzymes has also been assessed in the airways. Both MAGL and FAAH inhibitors, after intraperitoneal administration, prevented

BHR and lung inflammation in a murine model of LPS-induced airway inflammation [127].

In summary, the ECS seems to be clearly involved in the pathophysiology of asthma by acting in structural cells and by regulating immune responses (Fig. 3). Different strategies targeting immune cells with different types of immunomodulators have been previously shown as promising therapies for allergic diseases [128–130]. Thus, the rational design of novel immunomodulatory drugs targeting the ECS may be of potential interest for the development of new therapies for allergic asthma treatment.

Allergic Skin Diseases

Despite their low mortality rates, allergic skin disorders such as AD and allergic contact dermatitis (ACD) have a great impact on patients' quality of life. Skin allergies are complex diseases initiated by allergens and multiple environmental factors on genetically susceptible individuals that rely on the communication be-

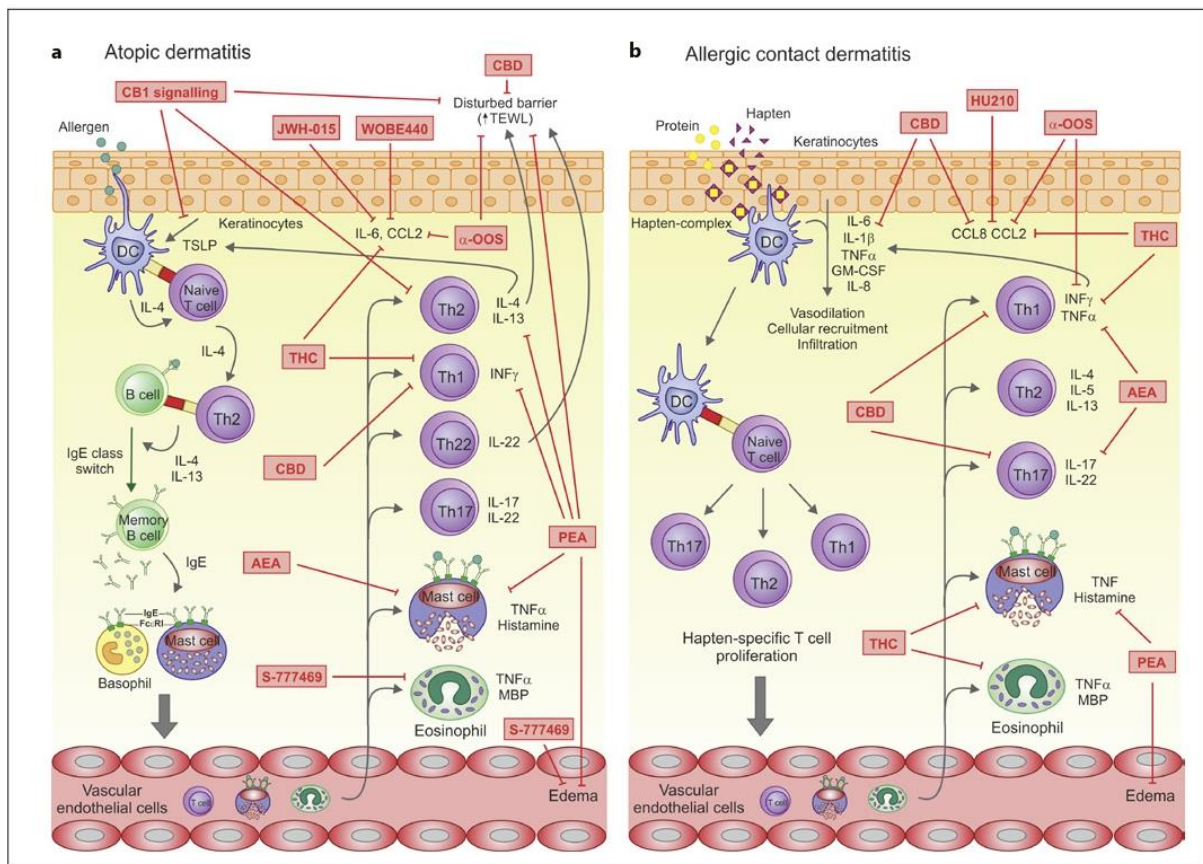


Fig. 4. Modulatory pathways of the ECS in the pathogenesis of atopic dermatitis (AD) and allergic contact dermatitis (ACD). **a** In the early phase of AD, allergen encounter and presentation to naïve T cells lead to a Th2 inflammatory response. In this context, cytokines and chemokines drive the infiltration of inflammatory cells to the skin. In the chronic phase of AD, bacterial colonization of the skin promotes a Th1, Th2, Th17, and Th22 effector response. Th2 and Th22 during the early and late phase of AD contribute to epithelial barrier impairment and transepithelial water loss (TEWL). **b** The sensitization phase of ACD is hallmarked by the

haptens-induced secretion of pro-inflammatory mediators leading to vasodilation, recruitment, and infiltration of immune cells. During the effector phase, hapten-specific T cells are recruited into the skin, activating keratinocytes and endothelial cells that secrete pro-inflammatory mediators that further amplify the immune response. The contribution of the ECS to every process in AD and ACD is highlighted with the corresponding arrow. TNF α , tumour necrosis factor α ; GM-CSF, granulocyte macrophage colony stimulating factor.

tween immune cells and other cell types such as keratinocytes and fibroblasts [8]. Regardless of the triggering factor, allergic skin disorders involve increased interleukins and chemokines, leading to the expansion of different T-helper subsets and activation of effector cells that may cause the chronicity of the disease [131]. Nevertheless, the cells residing in the skin are also able to secrete anti-inflammatory cytokines and chemokines, responsible for the regulation of local immune responses [9, 97, 132].

Different studies demonstrated that the ECS and endocannabinoids are expressed in the skin [15]. Cannabinoids have various effects over different skin cell types, ranging from activation or inhibition of keratinocyte proliferation to anti-inflammatory and anti-pruritic properties [133, 134]. Topical cannabinoids have shown high safety profiles, and their local application as oils, emollients, or creams have not been related to any adverse systemic effects, rendering them as an attractive therapeutic option [135].

AD is a chronic inflammatory skin disease where a number of polymorphisms associated with IL-4 secretion/signalling and structural proteins such as claudin and filaggrin compromise epidermal integrity [136, 137]. Initial disruption of the epidermis caused by scratching, microbial toxins, or allergens triggers the release of inflammatory mediators, including alarmins (TSLP, IL33, or IL-25), GM-CSF, TNF α , or IL-1 β , by epidermal keratinocytes, mast cells, or DCs. These mediators play an important role in the infiltration of inflammatory cells into the skin, being Th2 cells and their secreted cytokines the cornerstones of the early phase of AD inflammation [8]. In the chronic phase, Th2 cells but also Th1, Th17, and Th22 predominate in the skin together with epidermal hyperplasia and bacterial colonization.

CB1 in keratinocytes is essential to preserve repair responses and membrane integrity, and prevent transepithelial water loss as shown in AD-like CB1^{-/-} mouse models [138, 139]. Mice with CB1^{-/-} keratinocytes presented higher skin inflammation, eosinophil infiltration, and expression levels of IL-4, TSLP, and CCL8 when challenged with fluorescein isothiocyanate [138]. Topical administration of AEA and α -oleoyl oleylamine serinol (α -OOS), a synthetic CB1 agonist, accelerated barrier recovery and reduced chemokines in both oxazolone- and tetradecanoylphorbol acetate-induced AD models [140, 141]. CB1 activation also showed anti-inflammatory functions, reduced mast cell recruitment, proliferation, and degranulation, and decreased Th2 cytokines [73, 141, 142]. PEA, another CB1 agonist, reduced the secretion of pro-inflammatory chemokines in human keratinocytes in vitro and showed relieving effects in acute dermatitis and pruritus in clinical studies [143, 144]. Supporting these findings, FAAH and NAAA inhibitors decreased pro-inflammatory cytokine secretion in keratinocytes, reduced oedema in AD mouse models, and also had an impact systemically, normalizing serum IL-4, IL-5, IFN γ , and IgE levels [145, 146]. CBD inhibited Th1, Th2, and Th17 responses and suppressed B cells [147–150]. In clinical studies, CBD ameliorated transepithelial water loss and improved skin barrier by restoring the IFN γ -mediated inhibition of skin ceramide synthesis [151, 152]. Preclinical studies have shown that the synthetic CB2 antagonist S-777469 suppressed swelling, epidermal thickness, and mast cell and eosinophil infiltration in 2,4-dinitrofluorobenzene (DNFB) and house dust mite-induced AD models [153]. The effects of cannabinoids in AD are summarized in Figure 4.

ACD is an inflammatory response of the skin after contact with certain chemicals (haptens) whose charac-

teristics and low molecular weight render them as highly reactive and capable of penetrating the skin barrier. During sensitization, chemicals react with epidermal proteins generating hapten carrier complexes that stimulate innate immune cells via TLRs [154–156]. The secretion of pro-inflammatory mediators (IL-1 β , IL-18, TNF α) by innate cells leads to the activation of DCs, which uptake encountered hapten complexes and migrate to lymph nodes where they prime antigen-specific naïve T cells to differentiate into Th1 and Th17 cells. During the effector phase, repeated hapten complex exposure induces the recruitment of allergen-specific IFN γ - and IL-17-producing T-effector cells into the skin, which activate keratinocytes and endothelial cells to produce pro-inflammatory cytokines and mediators. This promotes vasodilation and the infiltration of macrophages and neutrophils, which in turn further amplify the recruitment of effector cells [157–159].

The protective role of CB1 in ACD has been extensively studied. In a mouse model with CB1^{-/-} keratinocytes, myeloid immune cell skin infiltration and CCL8 expression were increased [98]. Moreover, CB1 agonists alone have proven strong anti-inflammatory effects in vitro and in vivo. AEA pre-treatment of HaCaT keratinocytes prevented the secretion of Th1 and Th17 polarizing cytokines in an IFN γ -induced pro-inflammatory context [63]. AEA levels may be increased by other cannabinoids such as CBD, which suppressed the inflammation in poly-(I:C)-induced ACD in human keratinocyte cells [160]. The systemic and local administration of THC significantly reduced inflammation and myeloid immune cell infiltration in DNFB-induced contact hypersensitivity mouse models [15, 134]. THC not only decreased ear swelling in vivo, but also inhibited the production of IFN γ by T cells and the secretion of the pro-inflammatory mediators CCL2, CCL8, and CXCL10 by keratinocytes in vitro. Other synthetic cannabinoids and endocannabinoids displayed potent CB2-mediated anti-inflammatory effects both in vitro and in vivo. PEA decreased ear swelling, mast cell number, and the angiogenic factor VEGF in a contact dermatitis mouse model [161]. However, there is also conflicting evidence regarding the role of CB2, which might show either an exacerbation or suppression of inflammatory responses depending on the context and assayed conditions [15, 134]. The effects of cannabinoids in ACD are summarized in Figure 4.

The complexity of cannabinoids and the role of ECS in skin homeostasis and pathology are evident. Cannabinoids may play different roles depending on the origin of disease and pre-clinical studies have unveiled some of

their possible mechanisms of action. However, limitations such as the low number of double-blinded clinical trials and the high variation of cannabinoids tested, as well as their delivery routes, hinder the interpretation of clinical data. Further investigation is needed to understand the potential therapeutic role of cannabinoids in allergic skin diseases.

Food Allergy

The prevalence of food allergy is increasing in westernized countries, affecting up to 8% of children and 5% of adults. Even though oral tolerance is the physiological response to ingested antigens, the breakdown of this tolerance triggers the development of allergic sensitization. Such sensitization can occur in the gastrointestinal tract, oral cavity, skin, and occasionally in the respiratory tract. Re-exposure to food allergens induces the release of the anaphylactogenic mediators responsible of the clinical symptoms, including anaphylaxis [162]. The current standard treatment for food allergy is the strict and causative avoidance of the causative food and the use of epinephrine in the case of accidental ingestion. Although increasing research studies focus on the study of oral (OIT), sublingual (SLIT) and epicutaneous (EPIT) immunotherapy for the treatment of food allergy, only an OIT product for peanut allergy has been recently approved by the FDA [163, 164]. Therefore, the development of novel therapeutic approaches that improve the current strategies of immunotherapy for food allergy are needed. To date, there are no available data associated with the potential role of cannabinoids in food allergy models. The role played by ECS in other allergic diseases suggests that cannabinoids could also modulate food allergic reactions, but further research is warranted. Interestingly, we previously reported that the mRNA levels of CB1 are significantly higher in PBMCs from peanut-allergic children than healthy controls, suggesting that CB1 might well also play an immune regulatory role in food allergy [43].

Chemical Probes for ECS Research

The development of multiple cannabinoid agonists and antagonists has marked a milestone in the understanding of the effects of ECS signalling at the molecular level. However, the poor translational outcomes shown in clinical trials and their adverse side effects [165, 166] indicate that further validation and characterization of the receptors triggering the observed effects is necessary. In this context, the lack of suitable antibodies due to draw-

backs in specificity and reliability [167, 168] prompted scientists to develop chemical probes to address the issue. In recent years, various probes have been validated for the assessment of CBR expression (Table 2). Biotinylated probes overcame the drawbacks shown in previous attempts to design high-affinity CBR tools [169]. Their ability to bind different streptavidin tags made them suitable to study and visualize CBRs in native systems [170]. Moreover, several CB2-specific probes based on photoaffinity and fluorescent labelling have also been developed [171–175]. Their capacity to track CB2 in various cell types and settings may be of quite some interest in studies monitoring the expression of CB2 and its interactions in biological systems (Table 2). Regarding CB1, the conjugation of HU210, a dual agonist of CB1 and CB2, with the fluorescent tag Alexa Fluor 488 resulted in a high-affinity CB1-specific probe [76]. The probe was suitable for use in common biochemical and immunological techniques such as confocal microscopy and flow cytometry. It was also validated for the visualization of CB1 in different immune cell subsets [76], suggesting it to be a promising implement for future ECS research in immune-related diseases such as allergy. Besides, novel probes that provide reliable information on the allosteric motifs of CB1 have been designed. These probes could be useful for drug discovery, thus helping to overcome the limited translational potential of the orthosteric ligands [176]. These advances, together with the generation of compounds such as the recently developed THC-based photoaffinity probe allowing the identification of cannabinoid off-targets [173], might well contribute to widen the knowledge on the molecular mechanisms and receptors involved in cannabinoid-induced effects.

Other strategies to overcome the limitations displayed by drugs based on cannabinoid agonists and antagonists have focused on the manipulation of the enzymes involved in endocannabinoid metabolism [5]. Consequently, various chemical probes have been developed and used for the discovery and validation of novel inhibitors of the ECS metabolic enzymes [177] (Table 2). In this way, the generation of a photoaffinity probe that binds to the endocannabinoid membrane transport has been a remarkable finding that may help with the identification of the proteins involved in endocannabinoid membrane trafficking and the investigation of novel modulators of this process [178]. Furthermore, activity-based protein profiling (ABPP) probes have been used to evaluate the activity of hydrolase inhibitors and visualize additional targets in human leukocytes [179]. In fact, the use of tailored ABPP probes has provided important information on the

Table 2. Main small molecules and probes developed and used for ECS research

Type of compound	Targets	Validated uses	Potential use in ECS research	Ref.
Fluorescent probe	CB1	Identification and quantification of CB1 expression in different human immune cell subsets	Monitoring CB1 expression and function in biological systems	[76]
Electrophilic/photoaffinity probes	CB1	Covalent binding to CB1 allosteric site and negative allosteric modulation	Characterization and mapping of CB1 ligand binding sites	[176]
Biotinylated probes	CB1/CB2	Visualization of CBRs in native cell systems	Functional and imaging studies of CBRs	[170]
Photoaffinity probe	CB2	Monitorization of CB2 expression in human immune cells	Monitoring CB2 expression and ligand occupancy	[208]
Fluorescent probe	CB2	Assessment of CB2 expression in mice immune cells	Monitoring CB2 expression and ligand interactions	[171]
Fluorescent probe	CB2	Visualization of CB2 in human cell line	Monitoring CB2 expression	[174]
Fluorescent probe	CB2	High-affinity binding to CB2	Monitoring CB2 expression	[172]
Fluorescent probe	CB2	Imaging of CB2 in human tumour cell line	Monitoring CB2 expression and function	[175]
Photoaffinity probe	–	Identification of additional targets of THC in mouse neural cell line	Elucidation of novel THC off-targets as a tool for new drug discovery	[173]
ABPP probe	DAGL	Evaluation of inhibitor activity in biological systems	Development of novel inhibitors of endocannabinoid biosynthesis	[209]
ABPP probe	DAGL	Development of selective inhibitors of DAGL α	Development of novel inhibitors of endocannabinoid biosynthesis	[210]
Fluorescent probe	MAGL	High-throughput assessment of MAGL activity in vitro	Studying MAGL activity and development of new MAGL inhibitors	[211]
ABPP probes	Serine hydrolases	Tracking the dynamic expression and function of the serine hydrolases family	Monitoring serine hydrolase expression and development of novel inhibitors of endocannabinoid degradation pathways	[212]
ABPP Probe	Serine hydrolases	Visualization of endocannabinoid hydrolases and assessment of compound inhibitor activities on biological systems	Monitoring serine hydrolase expression and development of novel inhibitors of endocannabinoid degradation pathways	[213]
Photoaffinity probe	-	Irreversible blockage of endocannabinoid membrane transport	Identification of membrane proteins involved in endocannabinoid trafficking and generation of pharmacological modulators of EC transport	[178]

endogenous activity of MAGL in macrophages and DCs, leading to the identification of DAGL β as a potential target for chronic inflammation [180, 181]. However, targeting the endocannabinoid metabolism has also shown relevant side effects [5, 182]. Future perspectives in ECS research include multitargeted therapies and a better clinical study of plant-derived cannabinoids and allosteric modulators of CBRs [5]. The use of small molecules and probes has provided reliable information on CBR expression and imaging, the identification of novel off-targets, and the expression and activity assessment of the ECS enzymes in biological systems. Therefore, their thoughtful design may help in achieving future goals in this field of research. Particularly, in the context of allergic diseases, it is of note that despite the huge amount of

preclinical studies confirming a role for the ECS in allergy, to our knowledge, only one drug for AD has reached clinical trials [183]. This information highlights the need of ECS research tools to improve the translational potential of preclinical studies in allergic diseases and underlines their relevance in the future of this area.

Conclusions and Future Perspectives

Our knowledge on the underlying molecular mechanisms by which the ECS and cannabinoids regulate vital physiological processes in diverse biological contexts has significantly improved over the last years. These advances have led to the development of different cannabinoid-

based strategies for therapeutic interventions in several pathologic conditions, such as cancer and neurological disorders. The participation of the ECS and cannabinoids in allergy is still controversial. Different studies have convincingly demonstrated the anti-inflammatory properties exerted by cannabinoids in the airways and the skin in the context of allergic diseases both in mice and humans. On the other hand, other studies reported that cannabinoids might exacerbate asthma and AD via mechanisms depending on CB2-mediated signalling pathways. It seems evident that much more basic and clinical research is needed to better understand the role of cannabinoids in the pathophysiology of allergic diseases. In this regard, the rational design of small molecules and chemical probes targeting specific components of the ECS might well represent outstanding tools for its manipulation. Similarly, a deeper knowledge on how the specific endocannabinoids, phytocannabinoids, and synthetic cannabinoids contribute to immunomodulate the functional features of relevant immune cells involved in the orchestration of innate and adaptive immune responses, as well as on their receptor target profiling, will be of utmost importance to delineate future novel strategies for the prevention and treatment of allergic diseases.

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Author Contributions

O.P. conceived and designed the review. A.A., M.P.-D., J.L.-A., and O.P. wrote the manuscript. All authors approved the final version of the paper.

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A Fluorescent Probe to Unravel Functional Features of Cannabinoid Receptor CB₁ in Human Blood and Tonsil Immune System Cells

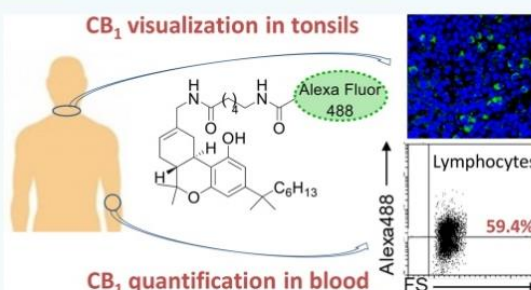
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Supporting Information

ABSTRACT: The human endogenous cannabinoid system (ECS) regulates key physiological processes and alterations in its signaling pathways, and endocannabinoid levels are associated with diseases such as neurological and neuropsychiatric conditions, cancer, pain and inflammation, obesity, and metabolic and different immune related disorders. Immune system cells express the G-protein coupled cannabinoid receptor 1 (CB₁), but its functional role has not been fully understood, likely due to the lack of appropriate tools. The availability of novel tools to investigate the role of CB₁ in immune regulation might contribute to identify CB₁ as a potential novel therapeutic target or biomarker for many diseases. Herein, we report the development and validation of the first fluorescent small molecule probe to directly visualize and quantify CB₁ in blood and tonsil immune cells by flow cytometry and confocal microscopy. We coupled the cannabinoid agonist HU210 to the fluorescent tag Alexa Fluor 488, generating a fluorescent probe with high affinity for CB₁ and selectivity over CB₂. We validate HU210-Alexa488 for the rapid, simultaneous, and reproducible identification of CB₁ in human monocytes, T cells, and B cells by multiplexed flow cytometry. This probe is also suitable for the direct visualization of CB₁ in tonsil tissues, allowing the *in vivo* identification of tonsil CB₁-expressing T and B cells. This study provides the first fluorescent chemical tool to investigate CB₁ expression and function in human blood and tonsil immune cells, which might well pave the way to unravel essential features of CB₁ in different immune and ECS-related diseases.



The human endogenous cannabinoid system (ECS) is a complex signaling network involved in a large number of physiological processes.^{1,2} The ECS comprises the endocannabinoid ligands, the proteins related to their synthesis and degradation, and the cannabinoid receptors (CBRs).² The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) bind to the G-protein coupled cannabinoid receptor 1 (CB₁) and 2 (CB₂). CB₁ is largely expressed in the central nervous system and also in peripheral tissues and immune cells.^{2–4} The high-resolution crystal structure of human CB₁ and agonist-bound complexes have been recently reported.^{5–7} CB₂ is mainly expressed in immune cells and also in other cell types.^{2,3} The activation of CBRs triggers a complex network of signaling pathways leading to the regulation of key physiological processes such as proliferation, differentiation, and cell survival.^{2–4,8} Alterations in the ECS signaling pathways and changes in the endocannabinoid levels have been associated with different diseases such as neurological and neuropsychiatric conditions, cancer, pain, inflammation, obe-

sity, metabolic disorders, septic shock, and different immune system related disorders.^{2,8–11}

The immune system encompasses a complex interactive network of cells and molecules that protect the host against potentially dangerous pathogens, while keeping a state of tolerance to self- and innocuous non-self-antigens.^{12,13} For that, the immune system employs a plethora of tightly regulated mechanisms, and alterations in these processes lead to immune tolerance-related diseases such as autoimmunity, tumor tolerance, rejection of organ transplants, or allergy. For a long time, it was thought that CB₁ and CB₂ modulated neurological and immune functions, respectively. However, several studies demonstrated that immune system cells also express functional CB₁ that could play a role in the immune regulation of T cells, B cells, and innate immune cells such as

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monocytes, dendritic cells (DCs), or macrophages.^{8,14–18} Therefore, there is an increased interest in studying the role of CB₁ in the control of immune responses. A better understanding of the role of CB₁ in immune regulation might well contribute to identifying CB₁ as a potential novel therapeutic target as well as to determine whether it can be a candidate biomarker of disease, prognosis, or treatment response.

In the particular case of allergic diseases, mouse models showed different roles for the ECS.^{19–23} In humans, CB₁ plays a potent inhibitory role on mast cell activation in the airway mucosa and skin.^{24,25} We showed that the mRNA expression levels of CB₁ are upregulated in tonsils and peripheral blood immune cells of allergic patients.¹⁶ Although it is plausible that the ECS may contribute to the regulation of allergic diseases, the underlying molecular mechanisms are not fully understood and human data are still scarce. In the same way, whether CB₁ might represent a potential novel biomarker in the context of allergy or other immune tolerance-related diseases remains completely unknown and requires further investigation. Currently, the lack of appropriate tools has hampered these particular studies. In this regard, antibodies for CB₁ could be employed, but they have important limitations in terms of sensitivity and specificity likely due to the poor immunogenicity reported for CBRs.^{26,27} In addition, the batch-to-batch variations inherent in the way in which antibodies are produced has been recognized as an important problem of accuracy and reliability.^{28,29} Therefore, there is a need for the development of tools that enable the direct visualization and quantification of CB₁ in immune cells. The availability of such tools would allow the quantification of up- or down-regulation of CB₁ as a disease biomarker in a straightforward manner in both peripheral blood and tissue samples, which might well contribute to stratifying patients for personalized medicine.

In this work, we develop and validate the first fluorescent small molecule probe that allows the visualization and quantification of CB₁ in peripheral blood and tonsil immune cells. For this purpose, we coupled the synthetic cannabinoid agonist HU210³⁰ to the fluorescent tag Alexa Fluor 488. The resulting fluorescence probe displays high affinity for CB₁ and selectivity over CB₂. We validate this probe as a chemical tool for the rapid, simultaneous, and reproducible identification of functional CB₁ in human monocytes, T cells, and B cells from peripheral blood and tonsils by flow cytometry in a single multiplexed manner. In addition, we demonstrate that HU210-Alexa488 is suitable for the visualization of CB₁ in tonsil tissues, which allows the *ex vivo* identification of tonsil T and B cell subsets expressing this receptor. This study provides the first fluorescent chemical tool to interrogate CB₁ expression in blood and tonsil immune cells, which might well pave the way to unravel the functional features of CB₁ and to uncover the potential role of this receptor as a novel biomarker not only for allergy but also for other immune related diseases. The applicability of this validated fluorescent probe could be also extended to other ECS-related diseases.

RESULTS AND DISCUSSION

Development of a CB₁-Specific Fluorescence Small Molecule Probe. A small molecule fluorescent probe to visualize and quantify CB₁ should contain three main components: (i) a high affinity CB₁ ligand with a position amenable for derivatization, (ii) an appropriate fluorophore, and (iii) a suitable spacer to avoid potential steric interferences

of the bulky fluorophore that could produce the loss of affinity for the receptor under study (Figure 1A). Regarding the ligand,

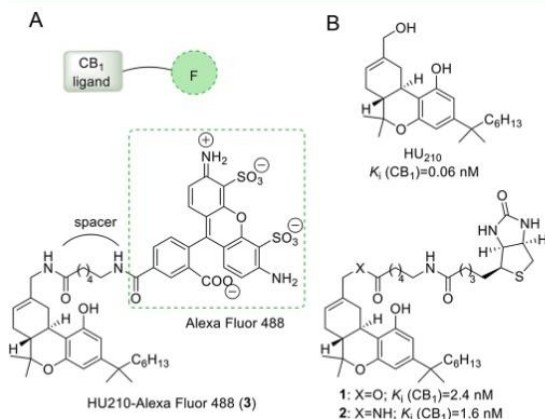
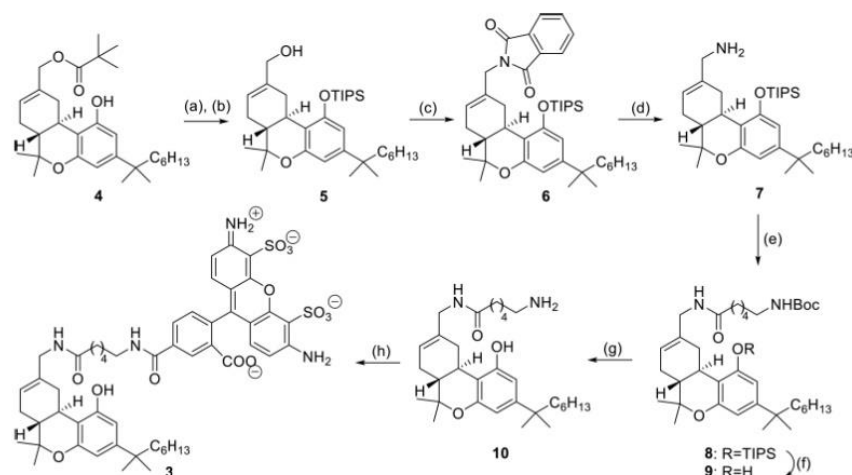


Figure 1. Development of a CB₁ fluorescent probe. (A) Scheme of the main components of a fluorescent probe and structure of the HU210-Alexa Fluor 488 probe 3. (B) Structure of the cannabinoid ligand HU210 and biotin-based probes 1 and 2 previously described.

among the cannabinoids that could be initially considered, the main endocannabinoids (AEA, 2-AG) were ruled out because our previous studies revealed that the attachment of tags to these ligands reduces the affinity of the probes, which restricts their use to the visualization of the CBRs only to transfected cells.³¹ We focused our efforts on the synthetic high-affinity cannabinoid ligand HU210 [K_i (CB₁) = 0.061 nM, K_i (CB₂) = 0.52 nM],³⁰ because we previously demonstrated that this ligand admits the introduction of biotin in the free allylic hydroxyl group without any important loss of affinity (Figure 1B).^{16,32} Biotinylated probes 1 and 2 have been successfully used for the visualization of CB₁ in neurons and in different immune cells, but they require a two-step labeling process and additional steps to block endogenous biotin, which do not make them suitable for flow cytometry clinical routine or tissue staining. To avoid these drawbacks, we selected the bright fluorescent Alexa Fluor 488 dye that is commonly used for flow cytometry and confocal microscopy imaging. The spacer between the ligand and fluorophore consists of a 6-amino-hexanoyl linker that will be attached to the HU210 through amide functionality to avoid the potential lability of the ester group (Figure 1A).

The preparation of the desired fluorescent compound 3 involved the synthesis of the HU210 scaffold functionalized as the corresponding allylic amine, followed by the incorporation of the appropriate spacer and further coupling with the commercially available tetrafluorophenyl (TFP) ester of Alexa Fluor 488 (Scheme 1). Thus, phenol 4³³ was protected as triisopropylsilyl (TIPS) ether under microwave (MW) irradiation, followed by removal of the pivaloyl group with lithium aluminum hydride to afford alcohol 5, which was transformed into the corresponding allylic amine 7 through Mitsunobu reaction with phthalimide and subsequent deprotection, using hydrazine as the cleaving reagent. Condensation of 7 with 6-(N-Boc-amino)hexanoic acid and further removal of TIPS and Boc protecting groups yielded 6-amino-hexanamide 10, which was finally coupled with Alexa Fluor 488 TFP ester to afford fluorescent derivative 3.

Scheme 1. Synthesis of the Fluorescent Probe 3⁴⁴

⁴⁴Reagents and conditions: (a) TIPS-Cl, imidazole, DMF, MW, 200 °C, 95%; (b) LiAlH₄, THF, 0 °C, 72%; (c) phthalimide, PPh₃, DEAD, THF, rt, 93%; (d) (i) N₂H₄·H₂O, EtOH, reflux; (ii) HCl/H₂O 1:1 reflux to rt, 96%; (e) 6-(N-Boc-amino)hexanoic acid, DCC, DCM, rt, 74%; (f) TBAF, THF, 0 °C, 81%; (g) TFA, DCM, rt, 84%; (h) Alexa Fluor 488 TFP ester, DCM, DMF, rt, 66%.

Affinity of synthesized compound 3 for CB₁ and CB₂ was assessed by radioligand competitive binding assays using membranes of HEK-293-EBNA cells transfected with human CB₁ and CB₂, respectively, and [³H]-CP55940 as radioligand. Interestingly, fluorescent probe 3 exhibited affinity values in the nanomolar range toward CB₁, while showing almost no affinity for CB₂ (submicromolar range) [K_i (CB₁) = 27 ± 4 nM; K_i (CB₂) = 0.8 ± 0.2 μ M]. Therefore, the newly identified fluorescent derivative 3 displays an optimized profile in terms of affinity and selectivity (30-fold over CB₂) to carry out its validation as a chemical tool to interrogate CB₁ in immune system cells.

Validation of HU210-Alexa488 Probe 3 to Detect and Quantify CB₁-Expressing Immune Cells by Flow Cytometry. To assess the capacity of the new fluorescent probe 3 to identify and quantify peripheral blood CB₁-expressing immune cells by flow cytometry, we isolated peripheral blood mononuclear cells (PBMC) from healthy donors and stained with probe 3 (1 μ M) or with Alexa Fluor 488 alkyne (1 μ M) as control (Figure 1A). We analyzed the expression of CB₁ in lymphocytes and monocytes by gating according to cell size and granularity (Figure 2A). Around 60% of lymphocytes and 80% of monocytes stained positive with HU210-Alexa488 fluorescent probe 3, whereas only around 1% and 4%, respectively, did so with control Alexa Fluor 488 alkyne (Figure 2A). As negative control, we also used the HEK293T cell line (Figure 2B), which does not constitutively express CBRs. HEK cells did not stain positive with probe 3 beyond the basal background detected with the control supporting that the probe 3 did not show nonspecific binding (Figure 2B). An excess of unlabeled HU210 (50 μ M), but not of the CB₂ selective agonist HU308^{4,30} (50 μ M) significantly shifted the positive staining detected with probe 3 (1 μ M) in both lymphocytes and monocytes (Figure S1), thus confirming the specificity of this fluorescent probe to detect CB₁-expressing cells in peripheral blood by flow cytometry. These data strongly suggest that the fluorescent signal provided by probe 3 is due to CB₁ but not to CB₂ expression. We also analyzed the stained

PBMC and HEK cells after cytospin by confocal microscopy (Figure 2C). CB₁-expressing cells were only visualized in PBMC but not in HEK cells, supporting the flow cytometry results. Our cumulative data revealed that the percentage of CB₁-expressing cells within monocytes is significantly higher than within lymphocytes (Figure 2D). Mean fluorescent intensity (MFI) analysis for probe 3 indicated that the density of CB₁ was also significantly higher in monocytes than lymphocytes (Figure 2D). Supporting these data, the CB₁ mRNA levels were also significantly higher in purified monocytes than lymphocytes as determined by quantitative PCR (Figure 2E). To further confirm the CB₁ specificity, we stained PBMC with probe 3 and retrieved the obtained positive and negative fractions after cell sorting by flow cytometry to quantify the mRNA expression of CB₁ (Figure 2F). Our data demonstrated that the cells contained in the fraction that stain positive for probe 3 display significantly higher levels of CB₁-specific mRNA than those contained in the negative fraction, supporting the correlation between probe 3 fluorescence signal and CB₁ expression (Figure 2F). Collectively, all these data demonstrated that HU210-Alexa488 probe 3 represents the first fluorescent chemical probe suitable to identify and quantify peripheral blood CB₁-expressing immune cells by flow cytometry.

HU210-Alexa488 Probe 3 as a Flow Cytometry Tool to Monitor Changes in CB₁ Expression in Blood and Tonsil Immune Cells. Plasma membrane CB₁ decreases following long agonist exposure as a consequence of finely regulated mechanisms that shuttle the receptor to lysosomes for degradation, contributing to the control of downstream signaling during sustained stimulation.^{34–36} To determine whether the probe 3 could be used to monitor changes in plasma membrane CB₁, we cultured PBMC in medium or stimulated with the cannabinoid agonists WIN552122 or HU210 for 18 h and we tracked CB₁ expression with probe 3 (1 μ M) by flow cytometry (Figure 3A). The percentage of blood lymphocytes expressing CB₁ as well as the expression levels of the receptor were significantly downregulated after

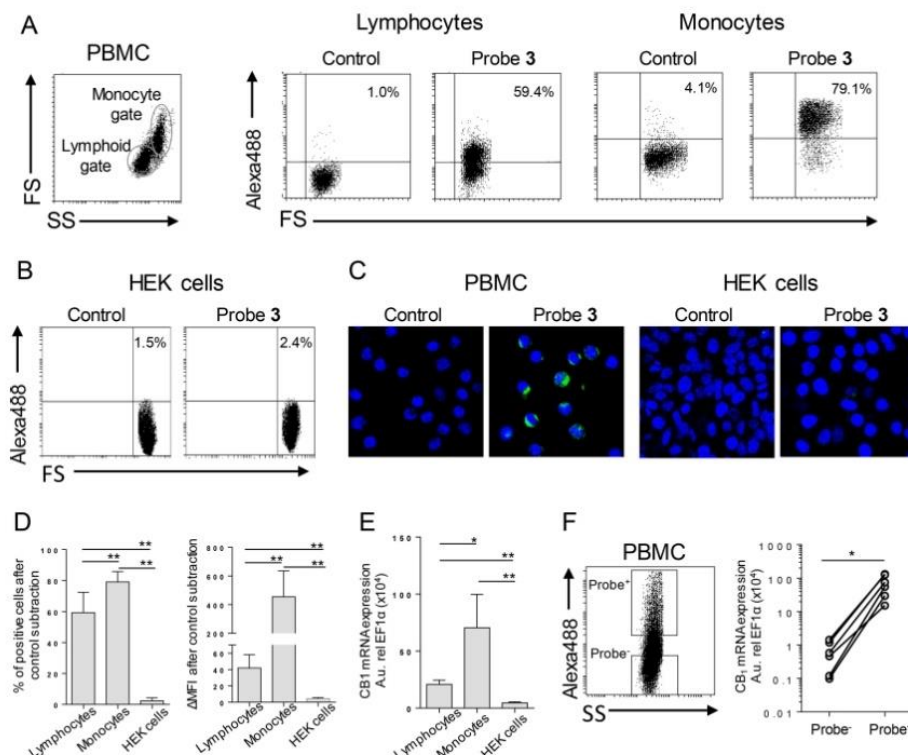


Figure 2. Validation of HU210-Alexa488 probe 3 to detect and quantify CB₁-expressing immune cells by flow cytometry. Representative flow cytometry dot plots after staining with HU210-Alexa488 probe 3 (1 μ M) or Alexa Fluor 488 alkyne [2-(6-amino-3-imino-4,5-disulfonato-3H-xanthen-9-yl)-5-(prop-2-yn-1-ylcarbamoyl)benzoate] (1 μ M) as control of (A) gated lymphocytes and monocytes from whole PBMC or (B) HEK cells. (C) Representative confocal microscopy images of PBMC and HEK cells after staining with probe 3 or control. (D) Percentages and mean fluorescent intensity (MFI) after control subtraction of lymphocytes and monocytes in freshly isolated PBMC ($n = 6$ independent experiments) and HEK cells ($n = 4$ independent experiments) stained positive with probe 3. (E) mRNA expression levels of CB₁ in sorted lymphocytes, monocytes, and HEK cells ($n = 4$ independent experiments) as determined by quantitative real-time RT-PCR. Arbitrary units (a.u.) are $2^{-\Delta\Delta C_t}$ values multiplied by 10^4 , with ΔC_t , defined as the difference between the cycle threshold value for CB₁ gene and elongation factor 1 α (EF1 α) as a housekeeping gene. (F) Representative dot plot of the cells stained negative or positive with probe 3 in whole freshly isolated PBMC and employed gates for subsequent flow cytometry cell sorting. mRNA expression levels of CB₁ in sorted cells stained negative and positive with probe 3 ($n = 6$ independent experiments). * $P < 0.05$ and ** $P < 0.01$. Data represent means with SEMs.

prolonged stimulation with both synthetic cannabinoids (Figure 3A), demonstrating that probe 3 is able to detect up- and downregulation of plasma membrane CB₁ in blood immune cells.

Next, we isolated tonsil mononuclear cells (TMC) from the tissue following our previously described protocols³⁷ and assessed the capacity of probe 3 to identify tonsil CB₁-expressing immune cells by flow cytometry (Figure 3B). Tonsils are easily accessible secondary lymph organs located at the main gateway of the respiratory and digestive tracts, representing the first contact point of the immune system with all the pathogens and allergens that enter the body via these ways.^{12,37,38} We previously showed that human tonsils are organs where the induction of oral tolerance occurs through the generation of functional allergen-specific regulatory T (Treg) cells.^{12,13,37} Tonsils are organs where immune regulation to viral and bacterial infections, immunometabolism, and allergy takes place.^{39–41} Interestingly, the expression of CB₁ at the mRNA level is upregulated in TMC from allergic patients compared to healthy donors.¹⁶ The availability of chemical fluorescent probes to track up- and downregulation of tonsil

CB₁-expressing cells is of utmost importance to monitor changes in the expression of this receptor at the protein level. Our data revealed that probe 3 (1 μ M) also allows the detection of CB₁-expressing cells in TMC (Figure 3B). The percentage of CB₁-expressing cells was also significantly downregulated in TMC stimulated with the synthetic cannabinoids WIN552122 or HU210 for 18 h compared to the unstimulated condition (Figure 3B). Collectively, all these data demonstrated that HU210-Alexa488 probe 3 is a suitable tool to monitor physiological changes in the expression pattern of plasma membrane CB₁ in blood and tonsil immune cells by flow cytometry.

Multiplexed Flow Cytometry Using HU210-Alexa488 Probe 3 Allows the Rapid and Simultaneous Identification of Immune Cell Subsets Expressing CB₁. The availability of rapid and reliable assays to identify potential novel immune biomarkers remains a challenge.^{12,42,43} In this regard, multiplexed flow cytometry combining lineage-specific markers for different immune cells in a single-step represents a very useful alternative for easy and reproducible routine assays. We showed that probe 3 (1 μ M) can be employed in

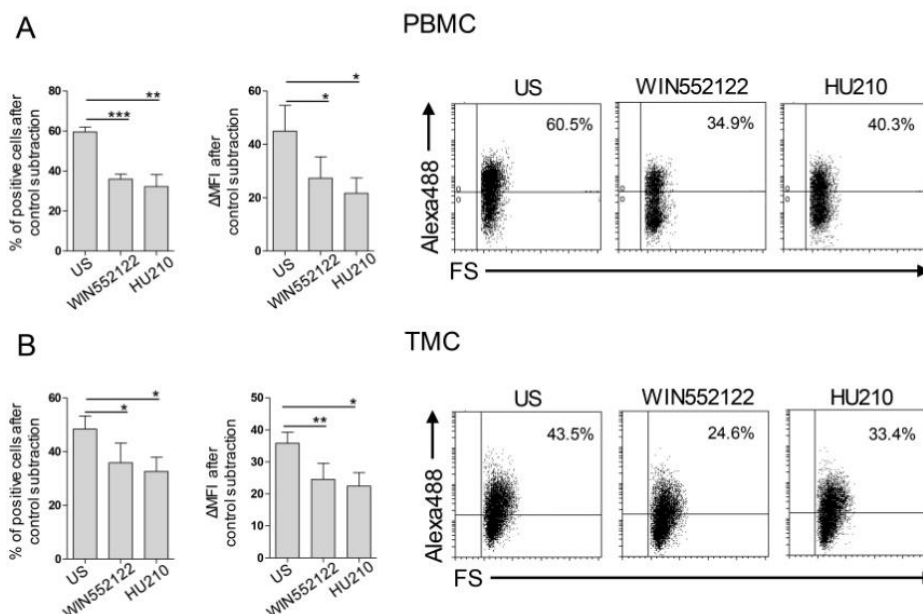


Figure 3. HU210-Alexa488 probe 3 allows the tracking of changes in CB₁ expression in blood and tonsil immune cells by flow cytometry. Percentages and mean fluorescent intensity (MFI) of (A) PBMC and (B) TMC cultured in medium (US) or with cannabinoid agonists WIN552122 or HU210 for 18 h ($n = 5$ independent experiments) stained with probe 3 (1 μ M) after control subtraction. Representative flow cytometry dot plots are also displayed in each case. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Data represent means with SEMs.

combination with specific anti-human antibodies to simultaneously identify and quantify by flow cytometry the percentage of CB₁-expressing cells among different immune cell subsets, including B cells (CD19⁺), T helper cells (CD3⁺CD4⁺), and cytotoxic T cells (CD3⁺CD8⁺) (Figure 4 and Figure S2). Our results revealed that probe 3 allows the quantification of CB₁-

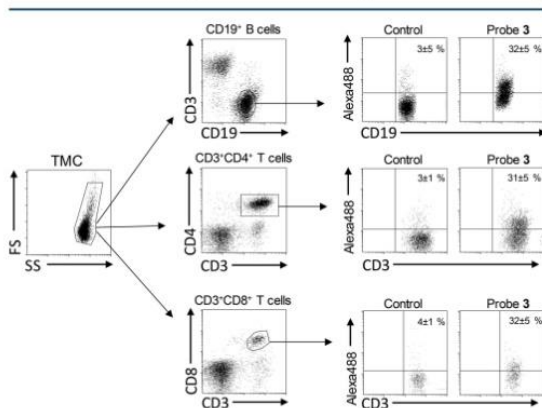


Figure 4. Multiplexed flow cytometry in TMC using HU210-Alexa488 probe 3 in combination with anti-human lineage specific markers for B cells and T cell subsets. Representative flow cytometry dot plots and the gating strategy employed to identify and quantify CB₁-expressing B cells, T helper, and cytotoxic T cell in freshly isolated TMC are shown. The percentages of CD19⁺ B cells, CD3⁺CD4⁺ T helper cells, and CD3⁺CD8⁺ cytotoxic T cells stained positive for control or probe 3 (1 μ M) are displayed inside the plots. Data represent means with SEMs of 7 independent experiments.

expressing cells within the B cells (32%), T helper cells (31%), and cytotoxic T cells (32%) in TMC by flow cytometry (Figure 4). We performed the same type of experiments using PBMC (Figure S2). Around 70% of CD19⁺ B cells, 36% of CD3⁺CD4⁺ T helper cells, and 42% of CD3⁺CD8⁺ cytotoxic T cells from PBMC express CB₁ as detected by flow cytometry with probe 3 (Figure S2). The percentage of CB₁-expressing B cells in PBMC was significantly higher than in tonsils without significant differences at the T cell level (Figure S3). In TMC, B cells represent around 65% of the total composition likely due to the high density of tonsil lymphoid follicles and germinal centers, which might well justify why the percentage of CB₁-expressing B cells is lower in TMC than PBMC.^{12,37} Collectively, all these data demonstrated that HU210-Alexa488 probe 3 can be used in multiplexed flow cytometry analysis to simultaneously quantify the percentage of CB₁-expressing cells within B cells, T helper, and cytotoxic T cell compartments in PBMC and TMC in an easy, rapid, and reproducible one-step staining.

CB₁ Immunomodulates T Cell Responses in Cells from Human Tonsils. Cannabinoids impair inflammation and display immune modulatory effects by mechanisms involving different peripheral blood CB₁-expressing immune cells such as monocytes, T cells, and to a lesser extent B cells.^{15,44} The functional role of CB₁ in human tonsils remains largely unknown. We stimulated TMC with anti-CD3/CD2/CD28 antibodies for 5 days to induce T cell proliferation in the presence of different doses of HU210 (CB₁/CB₂ agonist) or HU308 (CB₂-selective agonist) and assessed T cell proliferation by measuring ³H-thymidine incorporation into DNA in proliferating cells (Figure 5A). Our results showed that HU210 but not HU308 significantly increased the proliferation of tonsil T cells in a dose dependent manner (Figure 5A). After

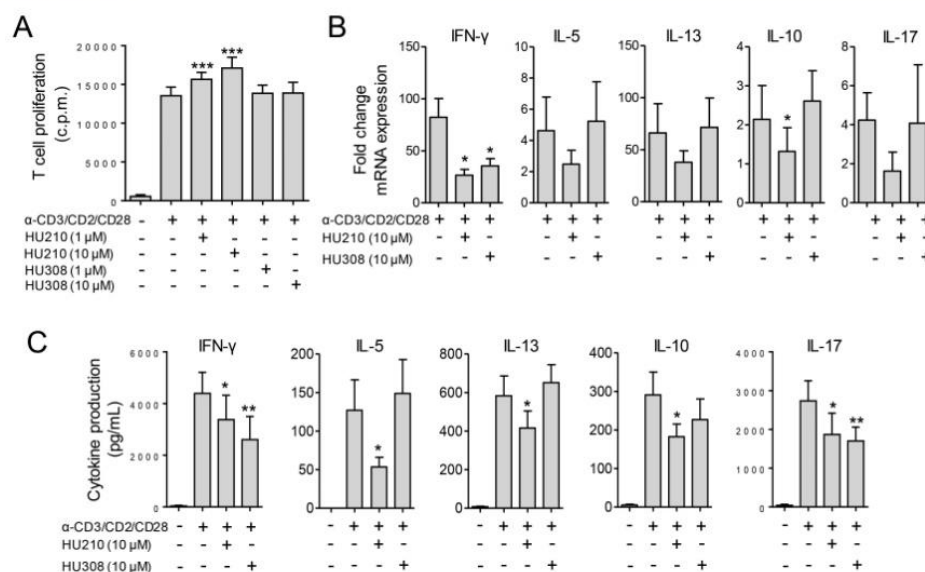


Figure 5. CB₁ activation immunomodulates T cell responses in TMCs. (A) Freshly isolated TMC ($n = 10$ independent experiments) were cultured in medium or in the presence of the indicated stimulus: anti-CD2/CD3/CD28 alone to induce T cell proliferation or in the presence of the indicated doses of the synthetic agonist HU210 or HU308 for 5 days. Proliferative responses were measured by using titrated thymidine incorporation as counts per minute (c.p.m.). (B) mRNA expression levels of the indicated T cells cytokines in TMC stimulated for 5 days under the indicated conditions ($n = 6$ independent experiments) as determined by quantitative real-time RT-PCR. Fold changes with respect to the unstimulated conditions were calculated as $2^{-\Delta\Delta C_t}$, with ΔC_t defined as the difference between the cycle threshold value for the gene of interest and elongation factor 1 α (EF1 α) as a housekeeping gene. (C) Levels of the indicated T cell cytokines in cell-free supernatants ($n = 6$ independent experiments) were also quantified after 5 days by cytometric bead array. * $P < 0.05$. Data represent the mean with SEMs.

5 days of culture, we measured T cell cytokines at the mRNA (Figure 5B) and protein level (Figure 5C). Both HU210 and HU308 significantly impaired the production of IFN- γ and IL-17 in activated TMC (Figure 5C). The same tendency was observed at the mRNA level with statistical significance only observed for IFN- γ (Figure 5B). Interestingly, HU210 but not HU308 significantly reduced the production of the Th2 cytokines IL-5 and IL-13 as well as IL-10 by activated TMC (Figure 5C). The same was observed at the mRNA level with statistical significance only observed for IL-10 (Figure 5B). These data suggest that CB₁ might be the main CBR involved in the increased tonsil T cell proliferation and in the impairment of Th2 and IL-10 cytokines. The fact that CB₁ enhanced tonsil T cell proliferation while inhibiting proinflammatory cytokines suggests that CB₁ might contribute to the expansion or the novo generation of tonsil Treg cells. Although tonsils contain high numbers of Treg cells and are organs where Treg cell generation occur,^{12,37,40} the potential contribution of CB₁ to these mechanisms needs to be further investigated. Collectively, all these data demonstrated that the tonsil CB₁-expressing cells detected with fluorescent probe 3 harbor functional plasma membrane CB₁ with immunomodulatory capacity that might play a role in the expansion or generation of tonsil Treg cells.

HU210-Alexa488 Probe 3 Allows the Visualization of CB₁-Expressing T and B Cells in Human Tonsils. To assess whether probe 3 could be also used to visualize CB₁-expressing cell subsets directly in tissues, we employed human tonsils. Tonsils are fully organized secondary lymphoid organs containing lympho-epithelial areas, B cells follicles with germinal centers, and T cell areas, in which proper immune

responses to encountered antigens and pathogens are orchestrated (Figure 6A).^{37,38,40} In a four color-staining approach, we stained human tonsil sections with probe 3 (50 nM) in combination with anti-human CD3, DAPI (to visualize nuclei), and anti-human CD4 or CD8 to identify CD4⁺ T helper cells and CD8⁺ cytotoxic T cells, respectively. Our data showed that probe 3 is a suitable tool to identify both CD4⁺ and CD8⁺ T cells expressing CB₁ *in vivo* in tonsil T cell areas by confocal microscopy (Figure 6B). Similarly, probe 3 also allowed the identification of CB₁-expressing CD20⁺ B cells within the B cell areas by confocal microscopy (Figure 6C). Alexa Fluor 488 alkyne or proper matched isotype controls for the employed antibodies were negative in all the assayed conditions (Figure S4). An excess of unlabeled HU210 (5 μ M) completely blocked the positive staining detected with probe 3 (50 nM) in both T and B cell areas (Figure S5), confirming the specificity of this fluorescent probe to visualize *in vivo* tonsil CB₁-expressing T and B cells by confocal microscopy. Collectively, our data demonstrated that HU210-Alexa488 probe 3 allows the *in vivo* visualization of tonsil CB₁-expressing T and B cells directly in human biopsies by confocal microscopy.

CONCLUSIONS

This work shows the development and validation of HU210-Alexa488 probe 3 as the first fluorescent small molecule probe to directly visualize and quantify CB₁-expressing immune cells in peripheral blood and tonsils by flow cytometry and confocal microscopy. Probe 3 is compatible with conventional anti-human antibodies used to identify immune cell subsets and can be simultaneously combined with them for the rapid, easy, and

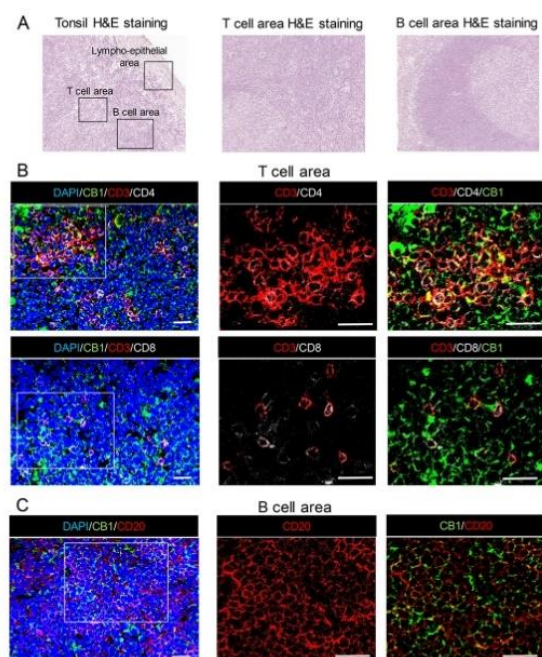


Figure 6. Visualization of CB₁-expressing T and B cells *in vivo* in human tonsils with HU210-Alexa488 probe 3. (A) Human tonsil sections stained with hematoxylin and eosin (H&E) to visualize the localization of lympho-epithelial, T and B cell areas within the tissue (left). Magnification of T cell areas (center) and B cell areas (right) are also displayed. (B) The sections were stained for CB₁ (green) with probe 3 (50 nM), for CD3 (red), CD4, or CD8 (white) with specific antibodies and with DAPI (blue, nuclei) and analyzed by confocal microscopy. The white squares in the images at the left side indicate the T cell areas where the CB₁-expressing T cells (up for CD4⁺ and down for CD8⁺) were visualized. (C) Sections were stained for CB₁ (green) with probe 3 (50 nM), for CD20 (red) with a specific antibody, and with DAPI (blue, nuclei) and analyzed by confocal microscopy. The white square in the image at the left side indicates the B cell area where the CB₁-expressing B cells were visualized. Data are from 1 of at least 3 tissue samples with similar results. White bars, 10 μ m. DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride.

reproducible identification of blood and tonsil immune cells expressing CB₁ in multiplexed flow cytometry assays. Human tonsils are the single easily accessible secondary lymphoid organs where immune regulation takes place. Therefore, the possibility of monitoring *in vivo* CB₁-expressing immune cells directly in the tissue opens a new avenue toward the better understanding of the *in vivo* functional role of CB₁ in humans, the identification of this receptor as a new potential biomarker in different conditions as well as for the future development of innovative tonsil-targeted therapeutic interventions for many ECS-related diseases.

METHODS

Details of experimental procedures are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00680.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB, cannabinoid receptor; DCs, dendritic cells; ECS, endogenous cannabinoid system; EF1 α , elongation factor 1 α ; MFI, mean fluorescent intensity; PBMC, peripheral blood mononuclear cells; TIPS, triisopropylsilyl; TFP, tetrafluorophenyl; TMC, tonsil mononuclear cells

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The cannabinoid WIN55212-2 restores rhinovirus-induced epithelial barrier disruption

To the Editor,

Bronchial epithelial cells constitute the first physical barrier with mucociliary clearance and immunologic defence capacity against environmental inhaled insults.¹ Disruption of tight junctions (TJs) and epithelial barrier dysfunction is a hallmark of chronic inflammatory airway diseases such as rhinitis,

chronic rhinosinusitis with nasal polyposis or asthma.^{2,3} Rhinovirus infections induce the disruption of the airway epithelial barrier and the production of pro-inflammatory cytokines.² Cannabinoids are lipid-derived mediators with anti-inflammatory properties in different disorders.⁴

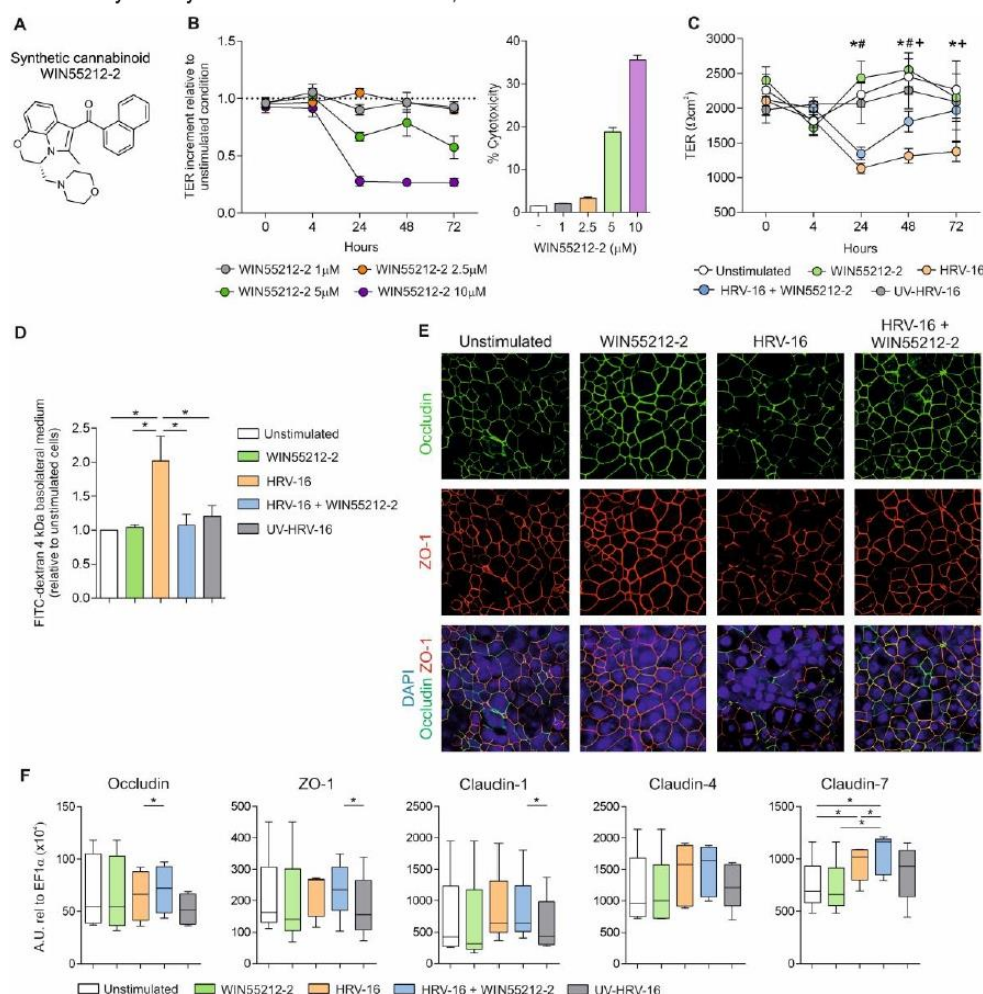


FIGURE 1. **A**, Chemical structure of WIN55212-2. **B**, Fold change in TER with respect to unstimulated condition in ALI-cultured HBEC after basolateral stimulation with the indicated doses of WIN55212-2 ($n = 2$ different donors). The graph at the right shows cytotoxicity after 24 hours of stimulation quantified by LDH release in the medium ($n = 2$ different donors). **C**, TER values in ALI-cultured HBEC after apical HRV-16 infection (MOI of 50) with or without basolateral WIN55212-2 (2.5 μ M), WIN55212-2 alone or UV-inactivated HRV-16 ($n = 4$ different donors). Each TER determination was performed by triplicate. * Unstimulated vs HRV-16, # Unstimulated vs HRV-16+WIN55212-2, * HRV-16 vs HRV-16+WIN55212-2. **D**, Paracellular flux of FITC-dextran in ALI-cultured HBEC in the indicated conditions. FITC-dextran was apically added after 72 h of treatment and quantified in the basolateral compartment after 24 h ($n = 4$ different donors). **E**, Representative immunofluorescence staining and confocal microscopy images of occludin (green) and ZO-1 (red) in ALI-cultured HBEC after 72 h of stimulation with the indicated conditions. DAPI (blue) was included for nucleus visualization. **F**, mRNA expression levels of the indicated TJs genes in ALI-cultured HBEC after 24 h of stimulation with the indicated conditions as determined by qPCR. Arbitrary units (A.U.) are $2^{-(\Delta\text{Ct})}$ values multiplied by 10^4 , with ΔCt defined as the difference between the cycle threshold valued for each gene and elongation factor 1 α (EF1 α) as housekeeping gene ($n = 4$ different donors). Data represent the mean $\pm$ SEM. Statistical significance determined using one-way ANOVA with Tukey's post-hoc comparisons (ANOVA P value < 0.05). * $P < 0.05$.

Cannabinoid receptor 1 (CB1) agonists inhibited the development of arthritis by restoring the intestinal barrier in the in the pre-phase of arthritis.⁵ CB1 expression is significantly increased in atopic patients.⁶ WIN55212-2 is a non-selective synthetic cannabinoid belonging to the aminoalkylindole group (Figure. 1A) with anti-inflammatory properties,⁴ but its capacity to modulate the bronchial epithelial barrier function during viral infections remains elusive.

Basolateral stimulation of human bronchial epithelial cells (HBEC) in air-liquid interface (ALI) cultures with 5 and 10 μ M but not at 1 and 2.5 μ M WIN55212-2 drastically decreased transepithelial electric resistance (TER) due to cytotoxic effects (Figure 1B). We investigated whether non-toxic concentrations of WIN55212-2 could impact human rhinovirus-16 (HRV-16)-induced epithelial barrier disruption. ALI cultures of HBEC were apically infected with HRV-16 in the absence or presence of basolateral 2.5 μ M WIN55212-2. TER was slightly and equally reduced after 4 h of stimulation in all the assayed conditions (Figure 1C), likely due to potential little physical alterations induced during the application of the stimulus. After 24 h, TER values were restored in the unstimulated, 2.5 μ M WIN55212-2 alone and ultraviolet (UV)-inactivated HRV-16 conditions and remained without significant changes up to 72 h. HRV-16 significantly decreased TER after 24 h and these values continued almost unchanged up to 72 h (Figure 1C). After 24 h of HRV-16-infection in the presence of WIN55212-2, TER was also significantly decreased. However, a significant

increment of TER in the presence of WIN55212-2 compared to HRV-16-infection alone was observed after 48 h. Remarkably, in the presence of 2.5 μ M WIN55212-2, TER values were similar to that observed for unstimulated, WIN55212-2 alone or UV-inactivated HRV-16 conditions after 72 h, demonstrating that WIN55212-2 is able to restore the barrier damage caused by HRV-16 infection of ALI-cultured HBEC (Figure 1C). Supporting these data, the presence of WIN55212-2 also significantly restored the paracellular flux (Figure 1D). Interestingly, after 72 h, HRV-16-infected HBEC in the presence of WIN55212-2 showed higher occludin and ZO-1 immunofluorescence intensity compared to cells infected with HRV-16 alone and similar to that observed under unstimulated or WIN55212-2 conditions (Figure 1E). WIN55212-2 significantly increased the expression of occludin and claudin-7 mRNAs with respect to HRV-16-infected cells without significant changes observed for zonula occludens (ZO)-1, claudin-1 and claudin-4 (Figure 1F).

During viral infections, epithelial cells produce pro-inflammatory cytokines and chemokines that enhance the recruitment of peripheral inflammatory cells into the airway submucosa.¹ WIN55212-2 alone or UV-inactivated HRV-16 did not induce cytokine production in HBEC. In contrast, HRV-16-infected ALI-cultured HBEC produced significantly higher levels of TNF α , IL-8, IP-10, MCP-1 and G-CSF than unstimulated cells after 24 h, which were not significantly inhibited by WIN55212-2 (Figure 2), suggesting that WIN55212-2

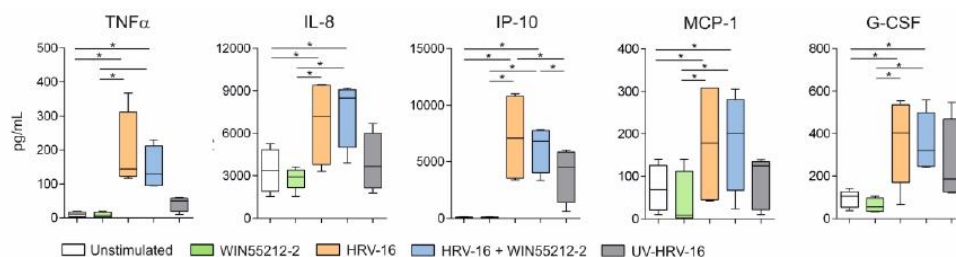


FIGURE 2. Levels of the indicated cytokines and chemokines in cell-free supernatants produced by ALI-cultured HBEC unstimulated, stimulated with WIN55212-2 (2.5 μ M) alone, UV-HRV-16, infected with HRV-16 (MOI of 50) or with HRV-16 + WIN55212-2 (2.5 μ M) for 24 h as determined by cytometric bead array ($n = 4$ different donors). Data represent the mean $\pm$ SEM. Statistical significance determined using one-way ANOVA with Tukey's post-hoc comparisons (ANOVA P value < 0.05). * $P < 0.05$.

does not alter the innate immune responses triggered by HRV-16 infection in HBEC.

Rhinovirus infection induces severe airway epithelial barrier damage, which plays a significant role in the pathophysiology of different airway chronic inflammatory diseases.¹ Up to date, few drugs have been reported to prevent or restore rhinovirus infection-induced respiratory epithelial barrier alterations.¹ Herein, we uncover a previously unknown capacity of the synthetic cannabinoid WIN55212-2 to regulate the integrity of the airway epithelial barrier during rhinovirus infection, which might well pave the way for the future development of potential novel preventive and therapeutic approaches for different chronic inflammatory airway diseases.

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