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The cannabinoid WIN55212-2 suppresses effector T-cell responses and promotes regulatory T cells in human tonsils

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To the Editor,

The human endocannabinoid system (ECS) is a complex molecular network encompassing cannabinoid receptors (CBRs), endocannabinoid ligands and the enzymes involved in their synthesis and degradation.^{1, 2} The role of cannabinoids in allergic diseases is not yet fully understood. Several studies reported that cannabinoids display beneficial anti-inflammatory properties, whereas others suggested potential proinflammatory and harmful effects.² The mRNA expression of functional cannabinoid receptor 1 (CB1) is upregulated in tonsils and peripheral blood of allergic patients.³ Functional CB2 is also highly expressed in immune cells, and it is involved in the control of dendritic cell function.² We showed that the synthetic cannabinoid WIN55212-2 restores rhinovirus-induced epithelial barrier disruption⁴ and promotes the generation of functional FOXP3⁺ Treg cells in peripheral blood.⁵ Blood dendritic cells (DCs) expressing CBRs are potential targets for cannabinoid-mediated modulation via autophagy and metabolic reprogramming.⁵ Whether tonsil DCs preserve the ECS expression and how WIN55212-2 immunomodulates T-cell responses in human tonsils remains unknown. Tonsils are first line secondary lymphoid organs located in the main entrance of the respiratory and gastrointestinal tracts where the generation of allergen-specific FOXP3⁺ Treg cells occurs by mechanisms involving plasmacytoid DCs (pDCs).⁶ Type 2 conventional DCs (cDC2s), which can be also found in the tonsil T-cell area, play an important role in the control of allergic diseases.⁶ In the present study, we sought to investigate the protein expression pattern of CB1 and CB2 in tonsil DCs and whether WIN55212-2 could immunomodulate T-cell responses in human tonsils.

To study whether tonsil DC subsets express the ECS, we purified tonsil pDCs and type 2 conventional DCs (cDC2s) and quantified the *in vivo* mRNA expression levels of main ECS components (Figure [1A](#)). Purified pDCs and cDC2s expressed CB1 and CB2, as well as the enzymes involved in the degradation of the endocannabinoids (anandamide and 2-arachidonoylglycerol), fatty acid amide hydrolase (FAAH), and monacylglycerol lipase (MAGL) at mRNA level, indicating that tonsil DCs express *in vivo* main components of the ECS. To further explore the expression pattern of CBRs in human tonsil DCs, we analyzed CB1 and CB2 protein expression in pDCs and cDC2s by confocal microscopy. Human tonsil sections

were stained with the validated HU210-Alexa488 probe for CB1 identification or with anti-CB2 polyclonal antibody in combination with anti-human CD123 (pDCs) or CD1c (cDC2s) antibodies. pDCs and cDC2s located at the T-cell area of human palatine tonsils expressed CB1 at the protein level (Figure [1B](#)). Although CB2 mRNA levels were detected in pDCs and cDC2s, only cDC2s, but not pDCs expressed CB2 at the protein level in tonsils (Figure [1B](#)), which is in accordance with our previous findings for circulating blood DCs subsets.⁵ Interestingly, the synthetic cannabinoid WIN55212-2 significantly reduced cytokine production induced by TLR stimulation on purified tonsil pDCs and cDC2s, demonstrating the functionality of these receptors (Figure [S1A,B](#)).

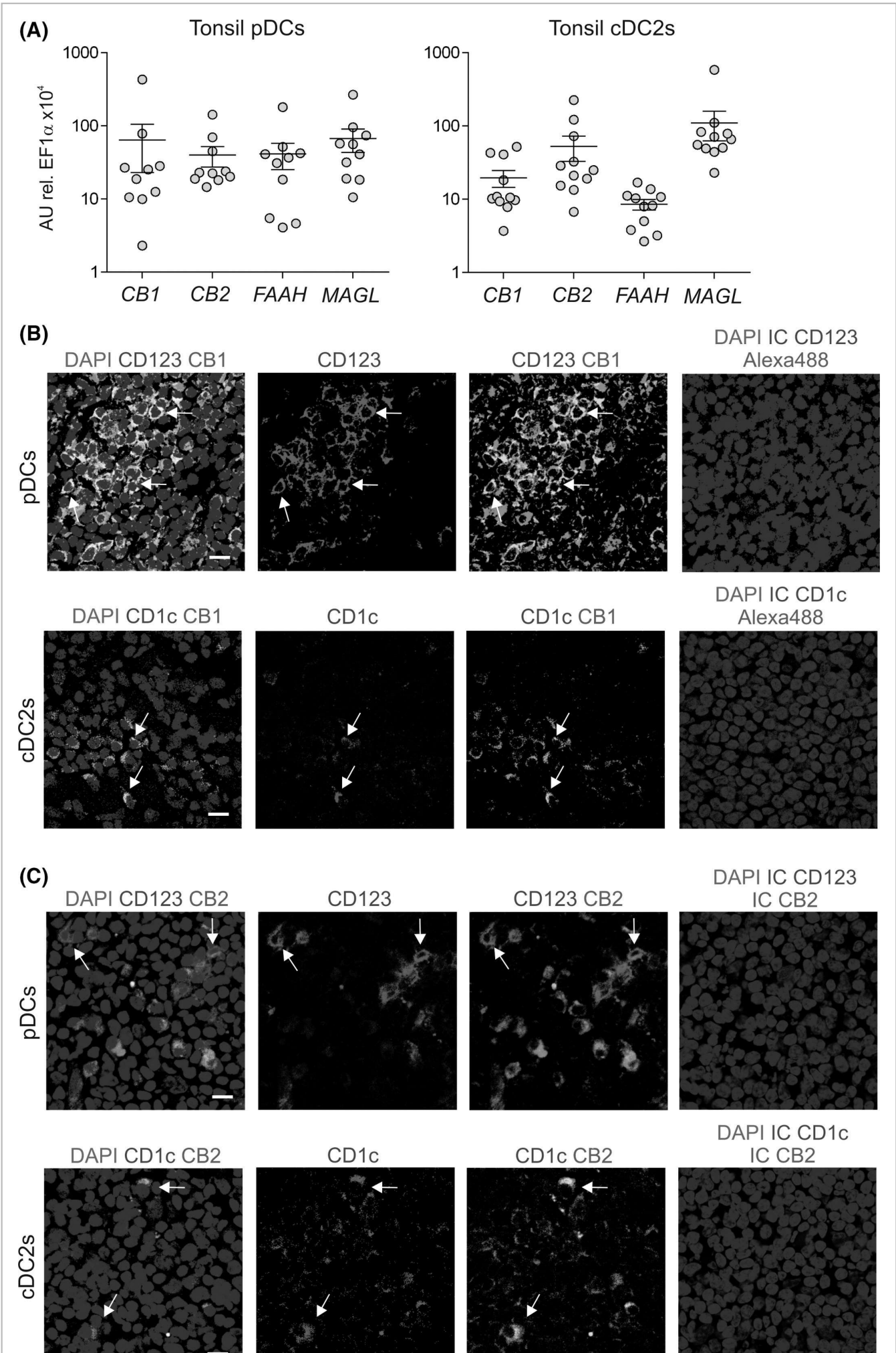


**FIGURE 1**
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Tonsil DCs subsets express the main components of the ECS at mRNA level and CB1/CB2 at the protein level. (A) mRNA expression levels of CB1, CB2, FAAH, and MAGL in purified tonsil pDCs and cDC2. Arbitrary units (A.U.) are $2^{-(\Delta Ct)}$ 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. Visualization of (B) CB1 and CB2 expression in cDC2 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 analyzed by confocal microscopy. White bars, 10 μ m. White arrows point at cDC2 or pDCs in the T-cell area of the tonsil tissue. One representative example of three independent experiments with similar results. DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride

Next, we wanted to investigate how the synthetic cannabinoid WIN55212-2 affects T-cell responses in human tonsils. We stimulated tonsil mononuclear cells (TMC) with anti-CD3/CD2/CD28 antibodies for 3 days to induce T-cell proliferation in the presence of different doses of WIN55212-2 and assessed T-cell proliferation by measuring ^3H -thymidine incorporation. WIN55212-2 decreased the proliferation of tonsil T cells in a dose-dependent manner (Figure [2A](#)) without affecting cell viability (Figure [2B](#)). After 3 days, WIN55212-2 significantly impaired the production of IFN γ , IL-5, IL-13, and IL-17 in activated TMC both at the mRNA and protein levels (Figure [2B,C](#)). Remarkably, WIN55212-2 significantly increased the percentage of CD25 $^+$ CD127 $^-$ FOXP3 $^+$ Treg cells in activated TMC (Figure [2E](#)), without increasing IL-10 levels. These data are indicating that WIN55212-2 is able to suppress the activation of tonsil Th1, Th2, and Th17 effector cells while promoting the expansion or the novo generation of Treg cells.

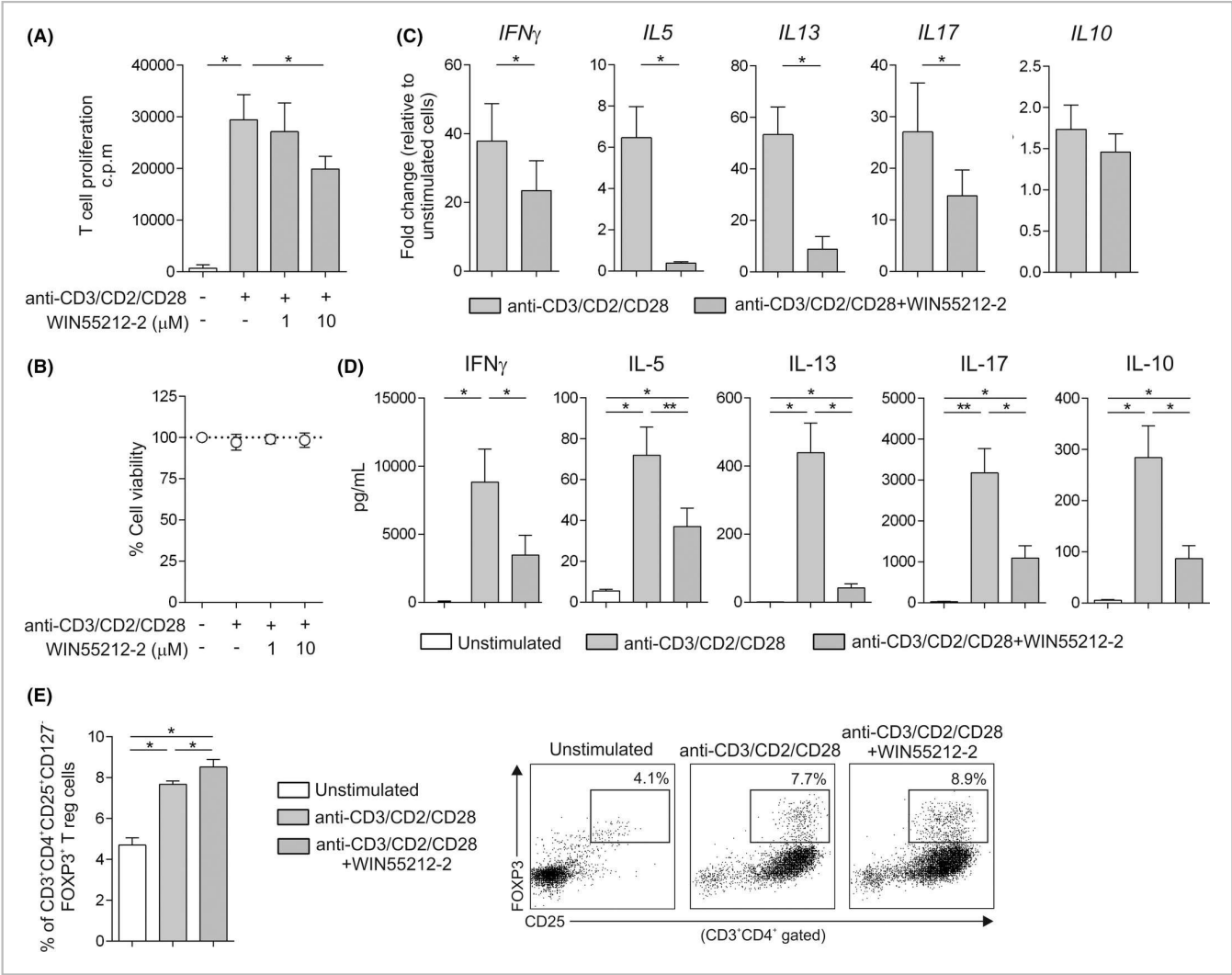


FIGURE 2

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WIN55212-2 suppress effector T-cell responses and promotes Treg cells in human tonsils. (A) Freshly isolated TMC ($n = 5$ 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 cannabinoid WIN55212-2 for 3 days. Proliferative responses were measured by using ^3H -thymidine incorporation as counts per minute (c.p.m.). (B) Cell viability at the indicated conditions. (C) mRNA expression levels of the indicated T cells cytokines in TMC stimulated for 3 days under the indicated conditions ($n = 5$ independent experiments) as determined by quantitative real-time RT-PCR. Fold changes with respect to the unstimulated conditions were calculated as $2^{-(\Delta\Delta\text{Ct})}$, with ΔCt 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. (D) The levels of the indicated T-cell cytokines in cell-free supernatants ($n = 5$ independent experiments) were also quantified after 3 days by cytometric bead array. (E) Percentage of FOXP3⁺ Treg cells in TMC after 3 days of stimulation under the indicated conditions ($n = 4$ independent experiments). Flow cytometry representative dot plots are shown. * $P < .05$; ** $P < .01$. Data represent the mean with SEMs

Tonsils are the only easily accessible secondary lymphoid organs in humans. Our findings showing that tonsil pDCs and cDC2s express functional CBRs and that WIN55212-2 suppresses effector T-cell responses while enhancing FOXP3⁺ Treg cells might well open new

avenues for the development of future novel vaccines targeting DCs in human tonsils for the prevention and treatment of allergy and other immune-mediated diseases.

Funding information

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None.

CONFLICT OF INTEREST

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Supporting Information

Filename	Description
all15160-sup-0001-Supinfo.docx	Word document, 27.9 KB Supplementary Material
all15160-sup-0002-FigureS1.tif	TIFF image, 2.1 MB Figure S1

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TITLE PAGE

The cannabinoid WIN55212-2 suppresses effector T cell responses and promotes regulatory T cells in human tonsils

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CAPSULE SUMMARY: The synthetic cannabinoid WIN55212-2 suppresses the activation of Th1, Th2 and Th17 cells and induces FOXP3⁺ Treg cells in human tonsils. Human tonsil dendritic cells express cannabinoid receptors CB1 and CB2.

KEYWORDS: cannabinoids, tonsils, plasmacytoid dendritic cells, type 2 conventional dendritic cells, effector T cells, regulatory T cells.

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CONFLICT OF INTERESTS: Dr. Cezmi Akdis reports grants from Allergopharma, grants from Idorsia, Swiss National Science Foundation, Christine Kühne-Center for Allergy Research and Education, European Commission's Horizon's 2020 Framework Programme, Cure, Novartis Research Institutes, Astra Zeneca, Scibase, advisory role in Sanofi/Regeneron, grants from Glaxo Smith-Kline, advisory role in Scibase. Dr. Oscar Palomares received research grants from Inmunotek S.L., Novartis, MINECO and Ministerio de Ciencia e Innovación and fees for giving scientific lectures or participation in Advisory Boards from: Allergy Therapeutics, Amgen, AstraZeneca, Diater, GlaxoSmithKline, S.A, Inmunotek S.L, Novartis, Sanofi-Genzyme and Stallergenes. The rest of authors declare no conflict of interests.

Online Supplementary Methods

Media and reagents

RPMI 1640 supplemented with 5% heat inactivated FCS (Amimed), antibiotics (penicillin, streptomycin, kanamycin from Life Technologies; amphotericin B from Bristol-Myers Squibb; and ciprofloxacin from Fluka), MEM vitamin, L-glutamine, nonessential amino acids and sodium pyruvate (LifeTechnologies) was used for isolation of tonsil mononuclear cells (TMC) and cell cultures. The combination of anti-CD2 (clone 4B2 and 6G4), anti-CD3 (clone OKT3) and anti-CD28 monoclonal antibodies (clone B7G5; all from Sigma), type B CpG2006 (TLR9-L, Mycosynth), Lipopolysaccharide (LPS, Sigma) and the synthetic cannabinoid WIN55212-2 (Sigma) were used for cell cultures.

Isolation of tonsil mononuclear cells and purification of tonsil DC subsets

In this study, we used tonsil samples from healthy non-atopic donors previously characterized and storage at the Biobank in the Swiss Institute of Allergy and Asthma Research (SIAF). Non-inflamed tonsils from patients undergoing tonsillectomy were employed. Single-cell suspensions of tonsil mononuclear cells (TMC) were isolated by mechanical disruption. 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). Tonsil cDC2s were obtained from TMC by positive selection by using the CD1c (BDCA-1)⁺ dendritic cell isolation kit (Miltenyi Biotec) supplemented in the first incubation step with CD11c-biotin antibodies. The negative fraction was used to purify tonsil pDCs by positive selection using the CD304 (BDC4/Neuropilin-1) microbead kit (Miltenyi Biotec).

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription was performed with the Revert Aid M-MuLV Reverse Transcriptase (Fermentas) using random hexamer primers according to the manufacturers protocol. Gene expression was analyzed by quantitative CFX384 Real-Time PCR instrument (Biorad). The sequences of the employed primer pairs were: Elongation factor 1α (EF1α) (fwd, CTGAACCATCCAAT; rev, GCCGTGTGGCAATCCAAT); CB1 (fwd, AAGGTGACATGGCATCCAAAT; rev, AGGACGAGAGAGACTTGTGTAA); CB2 (fwd, CACTGATCCCCAATGACTAC; rev, CCACTCCGTAGAGCATAGAT); FAAH (fwd, GGCCACACCTTCCTACAGAA; rev, GTTTTGCGGTACACCTCGAT); MAGL (fwd, CAAGGCCCTCATCTTTGTGT; rev, ACGTGGGAAGTCAGACACTAC); IFN-γ (fwd,

TCTCGGAAACGATGAAATATACAAGTTAT; rev,
 GTAACAGCCAAGAGAACCCAAAA), IL-10 (fwd, GTGATGCCCCAAGCTGAGA; rev,
 CACGGCCTTGCTCTTGTTTT), IL-13 (fwd, GCCCTGGAATCCCTGATCA; rev,
 GCTCAGCATCCTCTGGGTCTT); IL-5 (fwd, GACCTTGGCACTGCTTTCTACTC; rev,
 TGTACAGGAACAGGAATCCTCAGA); and IL-17 (fwd,
 CCATCCCCAGTTGATTGGAA; rev, CTCAGCAGCAGTAGCAGTGACA); and. PCR
 conditions were 10 minutes at 95 °C followed by 40 cycles of 15 seconds at 90 °C and
 1 minute at 60 °C. Data were expressed as arbitrary units (A.U.), which are $2^{-(\Delta CT)}$
 values multiplied by 10^4 , where ΔCT correspond to the difference between the cycle
 threshold value for the gene of interest and the housekeeping gene, EF1 α .

Immunofluorescence

Palatine tonsil tissues 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 μ m) were blocked and sequentially stained with the following antibodies. For CB1
 staining, first, the slides were incubated with anti-human CD123 antibody (eBioscience)
 or anti-human CD1c antibody (Abcam), or the corresponding isotype control for 45 min.
 After washing with PBS-Tween, CD123- or CD1c-binding Abs were detected by using
 Alexa Fluor 546-conjugated goat anti-mouse antibody (Invitrogen) by incubation for 30
 min. The sections were washed with PBS-Tween and, then they were incubated with
 HU210-Alexa488 probe¹ or Alexa Fluor 488 alkyne for 45 min. For CB2 staining, first,
 the slides were incubated with anti-human CB2 or the corresponding isotype control for
 24 hours at 4°C. After washing with PBS-Tween, the slides were incubated with anti-
 human CD123 antibody or anti-human CD1c antibody or the corresponding isotype
 control for 45 min. Then, the slides were washed and incubated with secondary
 antibodies Alexa Fluor 546-conjugated goat anti-mouse antibody (for CD123 or CD1c)
 and Alexa Fluor 488-conjugated goat anti-rabbit antibody (CB2). In both cases, after
 staining, the sections were treated with 1% PFA, washed with PBS and mounted with
 ProLong Gold antifade reagent with DAPI (Invitrogen) for nuclei demonstration. Images
 were acquired and analyzed using the confocal microscope LMS 780 Zeiss (Carl Zeiss
 Microscopy GmbH, Oberkochen, Germany).

Cell cultures

TMC were seeded at a density of 10^6 cells/mL and stimulated with the following
 combination of monoclonal antibodies to induce T cell proliferation: anti-CD2 (0.5
 μ g/mL), anti-CD3 (0.5 μ g/mL) and anti-CD28 (0.5 μ g/mL) monoclonal antibodies in the

absence or presence of different doses of WIN55212-2 (1 or 10 μ M) for 3 days. After 3 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 [3H]-thymidine during the last 8 hours of culture. Cell viability was determined using trypan blue after the culture.

Purified pDCs or cDC2s were seeded at a density of 10^6 cells/mL and stimulated with TLR9-L (2 μ M) or LPS (0,1 μ g/mL), respectively in the presence or absence of WIN55212-2 (10 μ M) for 18 hours.

Luminex

Concentrations of human IFN γ , IL-5, IL-13, IL-17, IL-10, TNF α and IL-6 in cell-free supernatants were quantified by luminex magnetic bead immunoassays following the manufacturer's instruction (BioRad).

Flow cytometry

Cells were washed with PBS/EDTA (2 mM) 0.5% BSA and labelling of cell-surface markers with Violet 500 anti-human CD3, Brilliant Violet 786 anti-human CD4, PE anti-human CD127 and Brilliant Violet 421 anti-human CD25 antibodies was performed at room temperature for 15 minutes in darkness. Then, 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 antibody for 30 minutes in darkness at room temperature. Cells were diluted in 1 % PBS/PFA for flow cytometry analysis in the BD LSRFortessa Cell Analyzer (BD Biosciences).

Statistical Analysis

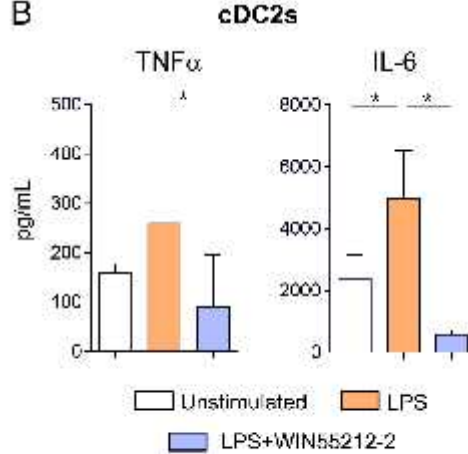
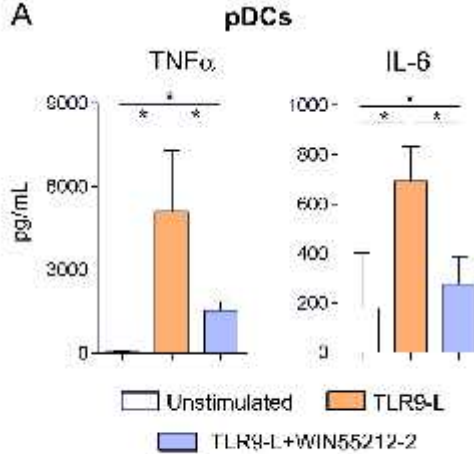
Statistical analyses were performed using GraphPad Prism software, version 6.0. All the data were expressed as mean $\pm$ SEM of the corresponding parameter. Statistical analysis was calculated using Paired t test. Differences were considered statistically significant when $P < 0.05$.

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FIGURE LEGEND

Supplementary Figure 1. The synthetic cannabinoid WIN55212-2 reduces cytokine production in stimulated pDCs and cDC2s. **A**, Cytokine production after stimulation of pDCs with medium (unstimulated), TLR9-L or TLR9-L plus WIN55212-2 for 18 hours (n = 4). **B**, Cytokine production after stimulation of cDC2s with medium (unstimulated), LPS or LPS plus WIN55212-2 for 18 hours (n = 3). * $P < 0.05$. Data represent the mean with SEMs.



Supplementary Figure 1. Angelina et al