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Cannabinoid WIN55212-2 impairs peanut-allergic sensitization and promotes the generation of allergen-specific regulatory T cells

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Abstract

Background: Cannabinoids are lipid-derived mediators with anti-inflammatory properties in different diseases. WIN55212-2, a non-selective synthetic cannabinoid, reduces immediate anaphylactic reactions in a mouse model of peanut allergy, but its capacity to prevent peanut-allergic sensitization and the underlying mechanisms remains largely unknown.

Objective: To investigate the capacity of WIN55212-2 to immunomodulate peanut-stimulated human dendritic cells (DCs) and peanut-allergic sensitization in mice.

Methods: Surface markers and cytokines were quantified by flow cytometry, ELISA and qPCR in human monocyte-derived DCs (hmoDCs) and T-cell cocultures after stimulation with peanut alone or in the presence of WIN55212-2. Mice were epicutaneously sensitized with peanut alone or peanut/WIN55212-2. After peanut challenge, drop in body temperature, haematocrit, clinical symptoms, peanut-specific antibodies in serum and $FOXP3^+$ regulatory (Treg) cells in spleen and lymph nodes were quantified. Splenocytes were stimulated *in vitro* with peanut to analyse allergen-specific T-cell responses.

Results: WIN55212-2 reduced peanut-induced hmoDC activation and promoted the generation of $CD4^+CD127^-CD25^+FOXP3^+$ Treg cells, while reducing the induction of IL-5-producing T cells. *In vivo*, WIN55212-2 impaired the peanut-induced migration of DCs to lymph nodes and their maturation. WIN55212-2 significantly reduced the induction of peanut-specific IgE and IgG_1 antibodies in serum during epicutaneous peanut sensitization, reduced the clinical symptoms score upon peanut challenge and promoted the generation of allergen-specific $FOXP3^+$ Treg cells.

Conclusions: The synthetic cannabinoid WIN55212-2 interferes with peanut sensitization and promotes tolerogenic responses, which might well pave the way for the development of novel prophylactic and therapeutic strategies for peanut allergy.

KEYWORDS
cannabinoids, dendritic cells, peanut allergy, regulatory T cells

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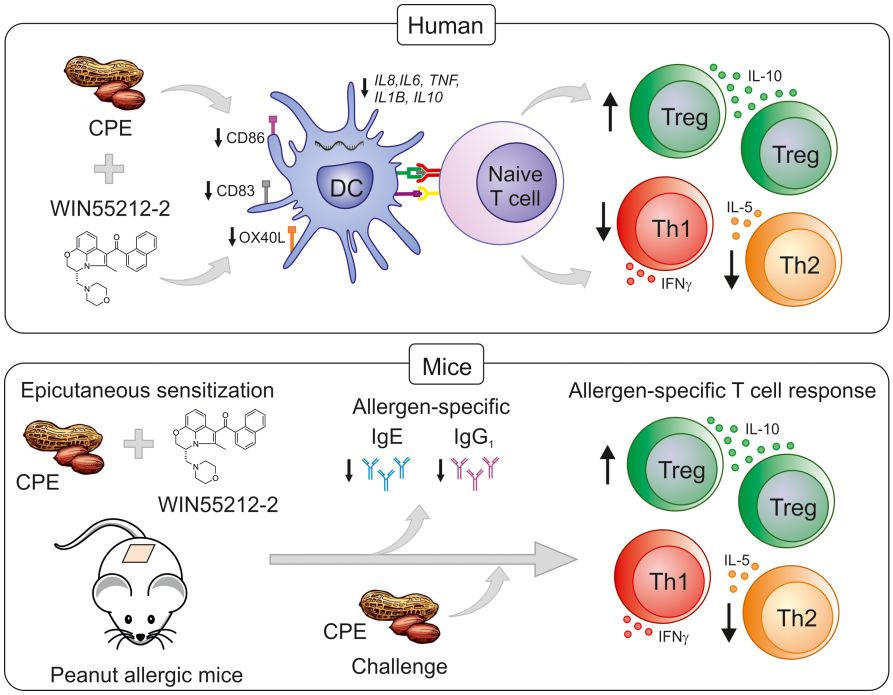

GRAPHICAL ABSTRACT

Peanut allergy represents the most common cause of food-induced anaphylaxis. We show that the synthetic cannabinoid WIN55212-2 reduces peanut-induced human dendritic cell maturation and promotes the generation of regulatory T cells (Tregs), while reducing IL-5-producing T cells. *In vivo*, the administration of WIN55212-2 during epicutaneous peanut sensitization in mice decreases the induction of peanut-specific IgE and IgG_1 antibodies in serum and promotes the generation of allergen-specific $FOXP3^+$ Tregs. Our data indicate that WIN55212-2 interferes with peanut sensitization promoting tolerogenic responses.

1 | INTRODUCTION

Food allergy is a worldwide health problem of increasing prevalence affecting 5–10% of the population in developed countries.[1–4] Although oral tolerance is the physiological response to ingested antigens, the breakdown of this tolerance leads to allergic sensitization.[5–7] The management and treatment of food allergy is mainly based on strict food avoidance and the use of epinephrine upon accidental ingestion. Peanut allergy constitutes the most common cause of food-induced anaphylactic reactions.[1,2,8] Different studies focused on the development of oral, sublingual and epicutaneous peanut-specific immunotherapy approaches, but currently only an oral immunotherapy product for peanut allergy is approved by FDA.[9–11] Therefore, the development of novel prophylactic and therapeutic interventions for peanut allergy is highly demanded.[12]

Cannabinoids are lipid-derived mediators with anti-inflammatory properties in different diseases.[13,14] The role of cannabinoids in allergy is not yet fully understood.[14] Some mouse studies demonstrated the protective role of the endocannabinoid system in contact allergy or allergen-induced airway inflammation.[15,16] In humans, cannabinoid receptor 1 (CB1)–mediated signalling suppresses the activation of skin and airway mast cells.[17,18] Other studies reported that cannabinoid receptor 2 (CB2) activation contributes to allergic exacerbations.[19] We previously demonstrated that WIN55212-2, a non-selective synthetic cannabinoid, restores airway epithelial barrier integrity during rhinovirus infection[20] and displays anti-inflammatory features.[21] We also showed that CB1 mRNA levels are significantly increased in tonsils and PBMC from peanut-allergic children.[22,23] The simultaneous administration of WIN55212-2 during peanut challenge reduces immediate anaphylactic reactions and promotes regulatory T (Treg) cell generation in peanut-allergic mice. Herein, we sought to investigate the potential capacity of WIN55212-2 to regulate peanut-stimulated human dendritic cells (DCs) and to prevent *in vivo* peanut-allergic sensitization in mice.

Key messages

- The synthetic cannabinoid WIN55212-2 increases the capacity of peanut-stimulated human DCs to polarize Treg cells.
- WIN55212-2 impairs *in vivo* peanut-induced migration of DCs from skin to draining lymph nodes in mice.
- WIN55212-2 reduces peanut-allergic sensitization and promotes the generation of peanut-specific Treg cells in mice.

2 | MATERIALS AND METHODS

2.1 | Material, media and reagents

We used RPMI 1640 medium (Lonza) supplemented (cRPMI) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone) 100 μg/ml normocin (InvivoGen), 50 μg/ml penicillin-streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mmol/L sodium pyruvate (all from Life Technologies). Crude peanut extract (CPE, Greer) and WIN55212-2 (Sigma) were used for cell culture and mouse models.

2.2 | HmoDCs generation and human naive CD4$^+$ T cells' purification

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. Immature hmoDCs were generated from blood monocytes obtained from total PBMC using anti-CD14 microbeads (Miltenyi Biotec) and cultured for 6 days with cRPMI medium containing 100 ng/mL of IL-4 and GM-CSF (PeproTech). The purity and phenotype of monocytes and generated immature hmoDCs were analysed by flow cytometry with lineage-specific markers. Purified naïve CD4$^+$ T cells were isolated from PBMC using the "Naïve CD4$^+$ T Cell Isolation Kit" (Miltenyi Biotec). All isolations were performed in autoMACS Pro according to manufacturer's protocol.

2.3 | Cell cultures

Immature hmoDCs from healthy donors (10^6 cells per ml) were stimulated with medium (unstimulated), CPE (100 μg/ml) or CPE plus WIN55212-2 (10 μM) for 4 or 18 h. Cells were used to analyse their phenotype by flow cytometry and qPCR.

2.4 | Coculture experiments

HmoDCs treated with medium (unstimulated), CPE (100 μg/ml) or CPE plus WIN55212-2 (10 μM) were cocultured with purified allogeneic naïve CD4$^+$ T cells (DC:T cell ratio of 1:5) for 5 days. IFNγ, IL-5 and IL-10 were quantified in cell-free supernatants by ELISA.

2.5 | Peanut epicutaneous sensitization

All the animal procedures employed in this study were approved by the Complutense University of Madrid and Comunidad Autónoma de Madrid "Experimental Animal Ethic Committees" (ref. PROEX 152/18).

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

2.6 | Inguinal lymph node processing

ILN were triturated between frosted slides in RPMI medium, washed and filtered (40 μM). Phenotype of DCs in ILN was analysed by flow cytometry.

2.7 | Peanut-induced anaphylaxis mouse model

2.7.1 | Epicutaneous sensitization

One hundred μg of CPE (10 mg/ml) in the presence or absence of 10 μg of WIN55212 was directly applied onto shaved and tape-stripped skin for 10 consecutive days.

2.7.2 | Challenge

2.5 mg of CPE in 500 μl of PBS was injected intraperitoneally 2 weeks after the last sensitization treatment. Mice were monitored for 40 min after challenge to assess anaphylactic response as reported[24]:

1. Clinical score. Anaphylactic symptom score was obtained by evaluating the following symptoms: 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.
2. Core body temperature. Rectal temperature readings were performed every 10 min with a rectal probe digital thermometer (VWR).
3. Haemoconcentration. Peripheral blood was collected 40 min after challenge by retro-orbital bleeding into microcapillaries. The tubes were centrifuged for 5 min by using a microhaematocrit centrifuge (QuercusLab). The haematocrit value was expressed as a percentage of cell volume.

2.8 | Serum peanut-specific immunoglobulins

Peanut-specific IgE and IgG_1 were measured by ELISA. High binding 96-well plates (Corning) were coated with CPE (20 μg/ml) in coating buffer at 4°C overnight. Coated plates were blocked with FBS (10%) in PBS for 2 h at room temperature. Plates were washed and incubated with serum samples diluted in PBS (IgE 1:2 and IgG1 $1:10^1$, $1:10^2$, $1:10^3$ and $1:10^4$) overnight at 4°C. After washing, detection antibody (rat anti-mouse IgE-HRP (Southern Biotech) or goat anti-mouse IgG_1-HRP (Thermo Fisher Scientific)) was added and incubated for 2 h at room temperature. *O*-phenylenediamine (OPD) was

used to develop the assay, and H_2SO_4 (3N) was added to stop the reaction for absorbance reading at 492 nm.

2.9 | Spleen processing and cell culture

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 splenocytes viability using Trypan Blue exclusion by light microscope. Triplicates of 0.8×10^6 splenocytes were cultured in medium alone or with CPE (250 μg/ml) in flat-bottom 96-well plates (Corning). After 5 days of culture at 37°C and 5% CO_2, the triplicates were pooled, cells were collected for FOXP3 staining, and cell-free supernatants were used to quantify IFNγ, IL-5 and IL-10 by ELISA.

2.10 | Peritoneal lavage

Peritoneal lavage fluid was collected 72 h after challenge and eosinophils were identified by flow cytometry as previously reported.[24]

2.11 | Flow cytometry

Cells were washed with PBS/EDTA (2 mM) 0.5% BSA, and labelling of cell-surface markers was performed at room temperature for 15 min in darkness with fluorescence-labelled antibodies (Table 1) or corresponding isotype controls. For analysis of FOXP3 expression in human T cells primed by DCs, cells were first subjected to surface staining. After fixation and permeabilization, cells were stained with anti-human FOXP3-Alexa Fluor 488, according to manufacturer's recommendations. The same protocol described above was carried out for the phenotypic characterization of $CD4^+CD25^{high}FOXP3^+$ Treg cells in freshly isolated mouse splenocytes.

2.12 | RNA extraction, cDNA synthesis and quantitative real-time PCR

RNA was isolated from harvested cells stimulated with the different stimulus for 4 h using the RNeasy mini kit (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 (Rox) (Roche). The sequences of the employed pair primers are shown in Table 2. Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems). Data were expressed as arbitrary units (A.U.), which are $2^{-(\Delta CT)}$ values multiplied by 10^4, where ΔCT corresponds to the difference between the cycle threshold value for the gene of interest and the housekeeping gene, EF1α. Data were represented as fold change relative to unstimulated condition.

TABLE 1 Flow cytometry antibodies

Name	Supplier	Clone	Conjugate
Human HLA-DR	Biolegend	L243	FITC
Human CD86	Miltenyi	FM95	PE
Human CD83	Miltenyi	HB15	APC
Human OX40L	BD Pharmingen	Ik–1	PE
Human CD4	Biolegend	OKT4	PerCP
Human CD127	Biolegend	A019D5	PE
Human CD25	Biolegend	BC96	APC
Human FOXP3	Biolegend	259D	Alexa488
Mouse CD11c	Biolegend	N418	APC
Mouse MHC II	Biolegend	M5/114.15.2	PE/Cy7
Mouse CD40	Biolegend	3/23	PE
Mouse CD80	Biolegend	16-10A1	FITC
Mouse OX40L	Biolegend	RM134L	APC
Mouse CD86	Biolegend	GL–1	BV605
Mouse CCR7	Biolegend	4B12	BV421
Mouse FOXP3	Biolegend	MF–14	Alexa 488
Mouse CD25	Biolegend	PC61	PE
Mouse CD4	Biolegend	GK1.5	PerCP
Mouse Siglec F	Biolegend	S1700L	PE
Mouse F4/80	Biolegend	BM8	BV421

TABLE 2 Primers for real-time qPCR

Gene	Forward	Reverse
EF1α	CTGAACCATCCAGGCCAAAT	GCCGTGTGCAATCCAAT
IL–8	GCAGCTCTGTGTGAAGG TGCAGTT	TTCTGTGTTGGCGCAGT GTGGTC
IL–6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTT TCAC
IL–1β	TTTTTGCTGTGAGTCCCGGAG	TTCGACACATGGGATAA CGAGG
IL–10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTG TTTT

2.13 | Cytokine quantification

Concentrations of IFNγ, IL-10 and IL-5 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences) following manufacturer's instructions.

2.14 | Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6.0. All the data were expressed as mean ± SEM of the corresponding parameter. Statistical analysis was calculated using one-way ANOVA or paired *t* test. Differences were considered statistically significant when $p < .05$.

3 | RESULTS

3.1 | The synthetic cannabinoid WIN55212-2 enhances the capacity of peanut-stimulated human DCs to polarize Treg cells

To assess whether WIN55212-2 could immunomodulate the functional properties imprinted by peanut in human DCs, we stimulated human monocyte-derived DCs (hmoDCs) from healthy donors with crude peanut extract (CPE) alone or in the presence of WIN55212-2. CPE stimulation significantly increased the expression of HLA-DR and the costimulatory molecules CD86, CD83 and OX40L in hmoDCs, which was significantly impaired by WIN55212-2 (Figure 1A). Interestingly, WIN55212-2 reduced both the expression of CPE-induced costimulatory molecules as well as the basal expression (Figure 1A). WIN55212-2 abolished the induction of IL-8, IL-6, TNFα, IL-1β and IL-10 at the mRNA level in CPE-stimulated hmoDCs (Figure 1B), demonstrating that WIN55212-2 inhibits peanut-induced maturation and activation of hmoDCs. To evaluate how WIN55212-2 could condition the capacity of CPE-stimulated hmoDCs to polarize T-cell responses, coculture experiments with allogeneic naïve $CD4^+$ T cells were performed. CPE-stimulated hmoDCs generated T cells producing significantly higher levels of IL-5 than unstimulated hmoDCs without significant changes for IFNγ and IL-10 production (Figure 1C), indicating that peanut stimulation of human DCs favours Th2 polarization as previously reported.[25] Remarkably, hmoDCs

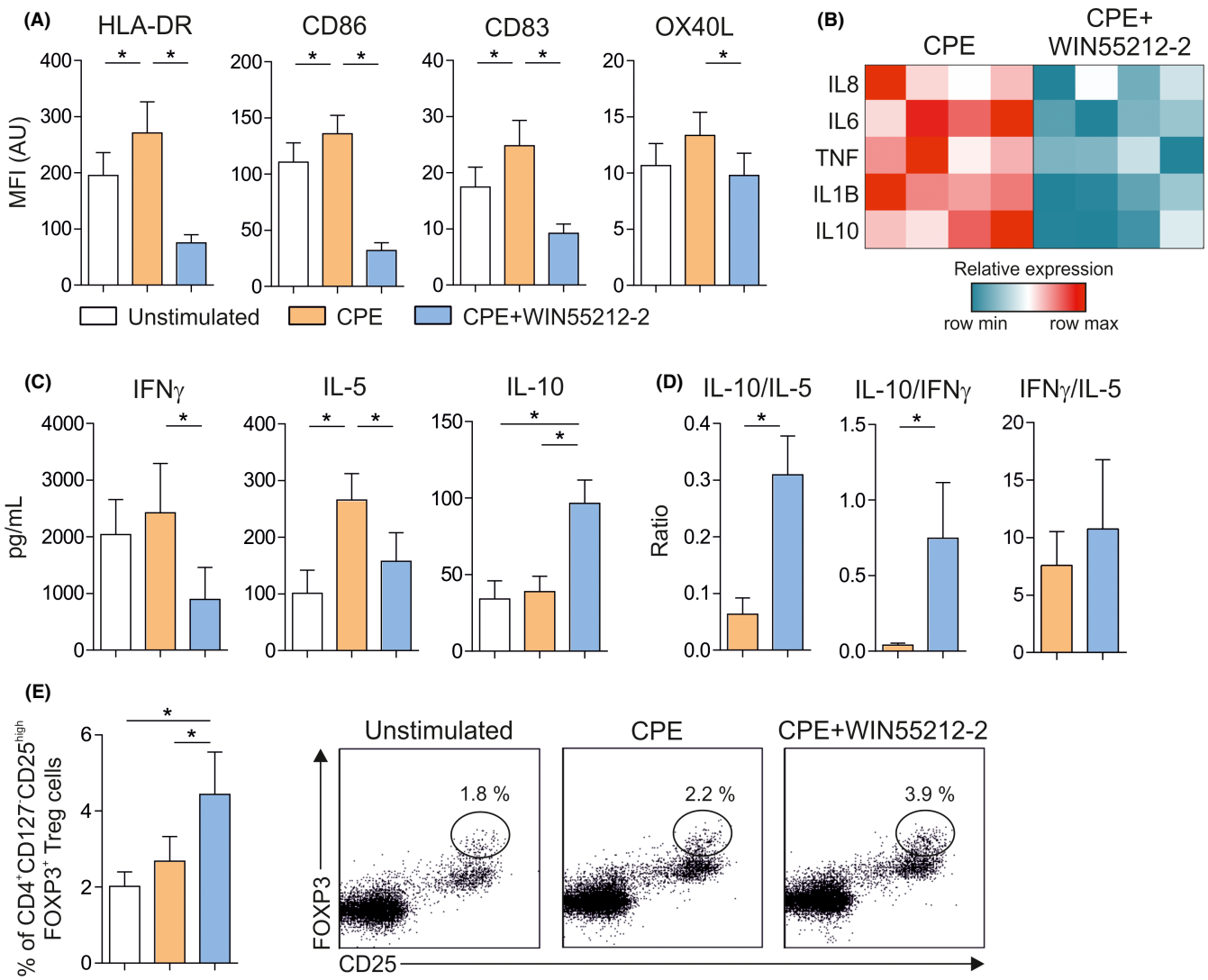

FIGURE 1 WIN55212-2 promotes $FOXP3^+$ Tregs cells under peanut-induced Th2-polarizing conditions. (A) Mean fluorescence intensity (MFI) of surface markers after stimulation of hmoDCs with medium (unstimulated), CPE (100 μg/ml) or CPE (100 μg/ml) plus WIN55212-2 (10 μM) for 18 h ($n = 7$). (B) Heatmap of cytokine gene expression after stimulation of hmoDCs with CPE or CPE plus WIN55212-2 for 4 h relative to unstimulated condition. (C) Cytokines produced by allogeneic naïve $CD4^+$ T cells primed by unstimulated, CPE or CPE plus WIN55212-2-stimulated hmoDCs after 5 days ($n = 7$). (D) Cytokine ratios by primed naïve $CD4^+$ T cells in the indicated conditions ($n = 7$). (E) Percentage of induced $FOXP3^+$ Tregs cells under the indicated conditions ($n = 7$). Flow cytometry representative dot plots are shown. Values are mean ± SEM. Statistical significance was determined using one-way ANOVA, $^*p < .05$

activated with CPE in the presence of WIN55212-2-generated T cells producing significantly lower levels of IFNγ and IL-5 and significantly higher levels of IL-10 than CPE-stimulated hmoDCs (Figure 1C). Accordingly, the IL-10/IL-5 and IL-10/IFNγ ratios were significantly higher when T cells were primed by CPE/WIN55212-2-stimulated hmoDCs than CPE alone (Figure 1D), suggesting the generation of Treg cells. In addition, the IFNγ/IL-5 ratio was slightly higher when T cells were primed by CPE/WIN55212-2-stimulated hmoDCs than CPE alone (Figure 1D). Supporting these data, the frequency of induced $CD4^+CD127^-CD25^+FOXP3^+$ Treg cells was significantly higher when hmoDCs were stimulated with CPE/WIN55212-2 than CPE alone (Figure 1E and Figure S1). Collectively, these results demonstrated that WIN55212-2 impairs the capacity of CPE-activated hmoDCs to polarize Th2 responses, while enhancing the generation of IL-10-producing $CD4^+$ T cells and $CD4^+CD127^-CD25^+FOXP3^+$ Treg cells, thus confirming that WIN55212-2 shifts CPE-stimulated human DCs from pro-allergic into tolerogenic.

3.2 | WIN55212-2 impairs *in vivo* peanut-induced migration of DCs from skin to draining lymph nodes in mice

We evaluated the ability of WIN55212-2 to prevent *in vivo* DC activation and migration in a preclinical model of peanut-allergic sensitization.[24] BALB/c mice were subjected to epicutaneous sensitization to CPE for 3 consecutive days in the absence or presence of WIN55212-2. One day after the last administration, mice were sacrificed and the frequency and activation status of DCs in draining lymph nodes characterized (Figure 2A). CPE-sensitized mice displayed a significantly higher percentage of total $MHCII^+CD11c^+$ DCs in the inguinal lymph nodes than naïve mice (Figure 2B). Interestingly, the presence of WIN55212-2 during CPE sensitization significantly reduced the migration of DCs to the draining lymph nodes (Figure 2B). We also quantified the expression of the activation or migration markers CD40, CD80, CD86, OX40L and CCR7. Mice sensitized with CPE increased the number of DCs expressing activation markers in inguinal lymph nodes (Figure 2C). However, mice sensitized in the presence of WIN55212-2 showed significantly lower number of mature DCs in the draining lymph nodes than CPE-sensitized mice (Figure 2C). Collectively, these data indicated that WIN55212-2 impairs *in vivo* the activation and migration of skin DCs to inguinal lymph nodes in a preclinical mouse model of peanut-allergic sensitization.

3.3 | WIN55212-2 reduces peanut-allergic sensitization and promotes the generation of peanut-specific Treg cells in mice

To further investigate the capacity of WIN55212-2 to control *in vivo* Th2 priming during CPE epicutaneous sensitization, we used a described model of peanut-induced anaphylaxis.[24] Mice were epicutaneously sensitized to peanut in the absence or presence of WIN55212-2 for 2 weeks (Figure 3A). Ten days later, the levels of peanut-specific IgE and IgG_1 antibodies in serum were quantified.

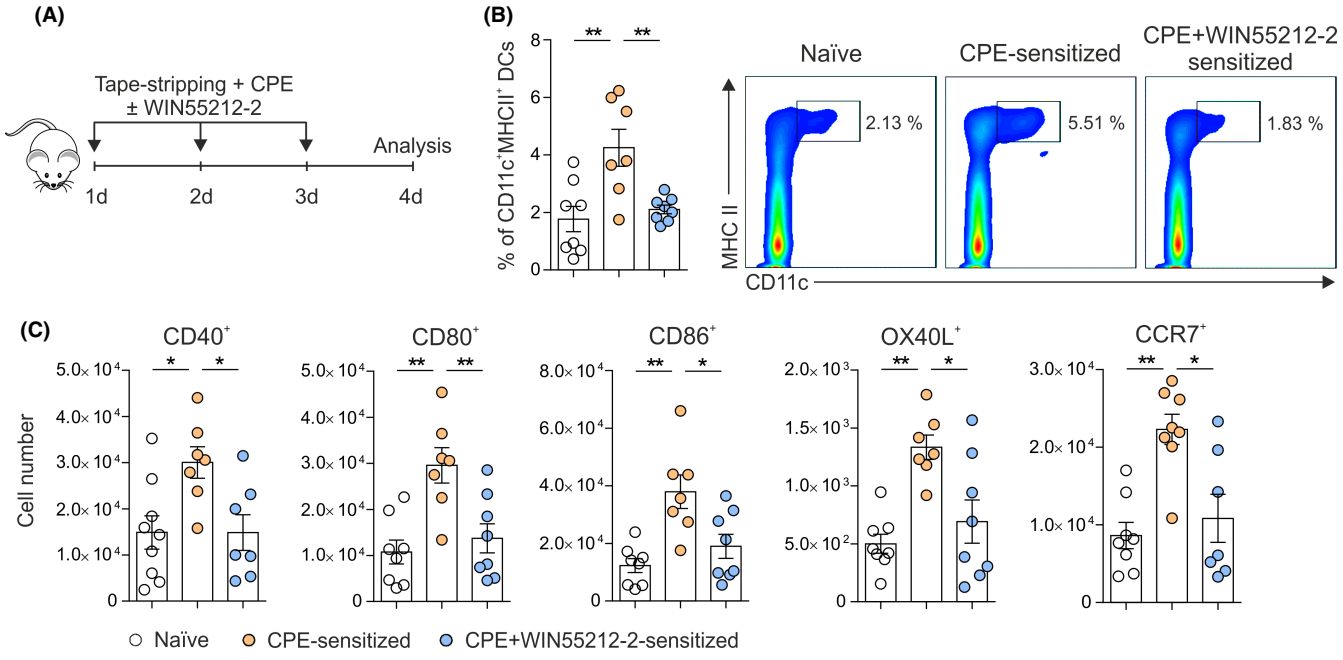

FIGURE 2 The synthetic cannabinoid WIN55212-2 impairs DC migration to inguinal lymph nodes during peanut epicutaneous sensitization. (A) Scheme of epicutaneous sensitization protocol. (B) Percentage of $CD11c^+MHCII^+$ DCs from inguinal lymph nodes of naïve, CPE-sensitized or CPE plus WIN55212-2-sensitized mice ($n = 8$). Flow cytometry representative dot plots are shown. (C) Cell number of positive cells for CD40, CD80, CD66, OX40L and CCR7 in DCs from inguinal lymph nodes of the indicated mice ($n = 7$–9). Values are mean ± SEM. Statistical significance was determined using one-way ANOVA, $^*p < .05$, $^{**}p < .01$

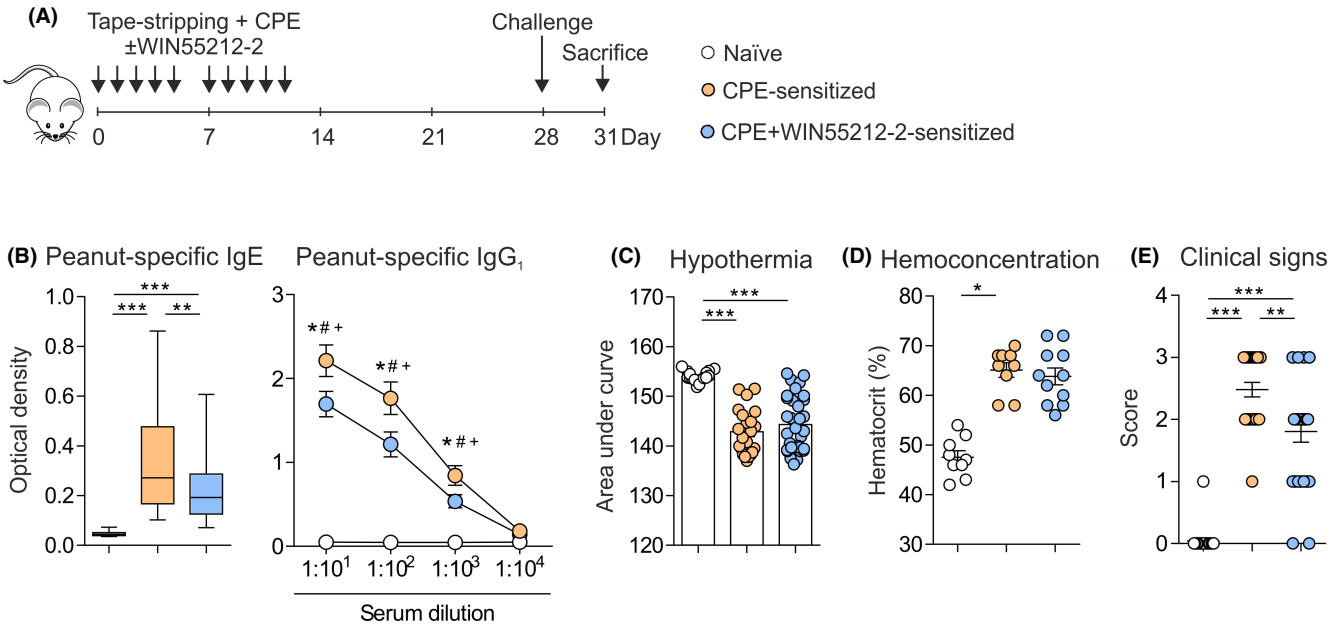

FIGURE 3 WIN55212-2 reduces peanut-allergic sensitization (A) Scheme of epicutaneous sensitization protocol. (B) Levels of peanut-specific IgE and IgG_1 measured at different dilutions of serum from naïve, CPE-sensitized or CPE plus WIN55212-2-sensitized mice ($n = 15–20$). (C) Changes in body temperature 40 min after challenge represented as area under curve is shown. (D) Haemoconcentration 40 min after challenge. (E) Clinical signs observed after peanut challenge in naïve, CPE or CPE plus WIN55212-2-sensitized mice. Values are mean ± SEM. Statistical significance was determined using One-way ANOVA, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

CPE-sensitized mice displayed significantly higher levels of peanut-specific IgE and IgG_1 antibodies in serum than naïve mice, which were significantly reduced by WIN55212-2 (Figure 3B). To assess the clinical effects of WIN55212-2 prophylactic treatment within the immediate phase reaction upon peanut re-exposure, mice were intraperitoneally challenged with CPE 2 weeks after the last sensitization (Figure 3A). Rectal temperature was measured every 10 min during the 40 min following challenge and haematocrit quantified as surrogate markers of peanut-induced anaphylaxis. Clinical symptoms were also carefully monitored after peanut challenge. Although we did not observe differences in body temperature drop and haematocrit when comparing CPE- or CPE/WIN55212-2-sensitized mice (Figure 3C and D), mice sensitized with CPE plus WIN55212-2 displayed significantly lower clinical signs score compare to mice sensitized with CPE alone (Figure 3E). Next, we analysed late phase response outcomes 72 h after challenge. As expected, CPE-sensitized mice showed higher percentage of eosinophils in the peritoneal cavity than naïve mice (Figure 4A). Interestingly, CPE/WIN55212-2-sensitized mice displayed significantly lower percentage of eosinophils than CPE-sensitized mice (Figure 4A), suggesting that WIN55212-2 could reduce the accumulation of anaphylactogenic mediators and impair the capacity of DCs to prime allergen-specific Th2 cells, thus ameliorating peanut-induced late phase responses. To further investigate these mechanisms, we quantified the generation of $FOXP3^+$ Treg cells in freshly isolated spleen and inguinal lymph nodes after 72 h of peanut challenge. Remarkably, 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 (Figure 4B and Figure S2), suggesting that WIN55212-2 might well regulate late T cell-mediated responses after CPE-induced anaphylaxis by the induction of Treg cells.

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 affecting IFNγ production (Figure 4C). Accordingly, IL-10/IL-5 and IFNγ/IL-5 ratios were significantly higher in splenocytes from mice sensitized with CPE/WIN55212-2 than CPE alone (Figure 4D). Finally, the increment in peanut-specific $FOXP3^+$ Treg cells after CPE stimulation was also significantly higher in splenocytes from mice sensitized with CPE plus WIN55212-2 than CPE alone (Figure 4E). Collectively, these data indicated that WIN55212-2 induced allergen-specific Treg cells that might well contribute to the suppression of peanut-induced late phase Th2-mediated responses in mice.

4 | DISCUSSION

In this study, we show that the synthetic cannabinoid WIN55212-2 impairs the capacity of peanut-stimulated human DCs to polarize Th2 responses while enhancing the generation of $FOXP3^+$ Treg cells. Our *in vivo* data also demonstrate that WIN55212-2 suppresses peanut-induced activation and migration of DCs during peanut-allergic epicutaneous sensitization in mice. WIN55212-2 prophylactic treatment during peanut sensitization significantly reduces the immediate clinical outcomes upon peanut challenge and late phase

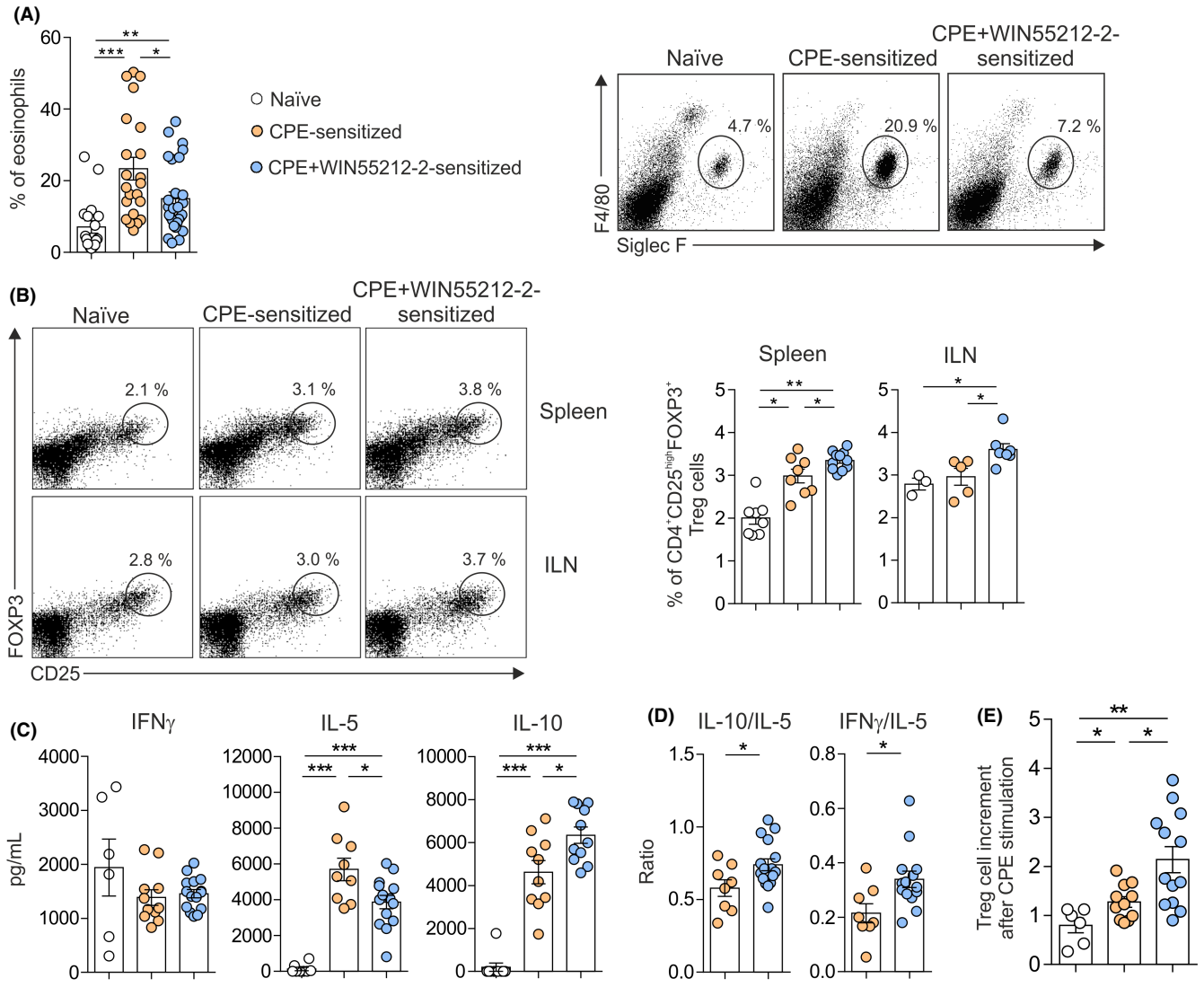

FIGURE 4 WIN55212-2 promotes the generation of peanut-specific Treg cells in mice (A) Percentage of eosinophils in peritoneal cavity 72 h after peanut challenge (n = 15–20). Flow cytometry representative dot plots are shown. (B) Percentage of $CD4^+$ $CD25^{high}FOXP3^+$ Tregs cells in spleen and inguinal lymph nodes (ILN) (n =5–10). Flow cytometry representative dot plots are shown. (C) Cytokine production and (D) cytokine ratios by splenocytes from the indicated mice stimulated *in vitro* with CPE (250 μg/ml) for 4 days (*n* = 6–10). (E) Increment of $FOXP3^+$ Tregs cells generation after *in vitro* CPE stimulation for 4 days relative to unstimulated condition (*n* = 6–10). Values are mean ± SEM. Statistical significance was determined using one-way ANOVA or paired t test (D), * $p < .05$, ** $p < .01$, *** $p < .001$

reactions by a mechanism presumably depending on the generation of peanut-specific Treg cells. It is well known that strong increases in IL-10/IL-5 ratios and Treg cell generation are associated with healthy immune responses to allergens and with successful allergen-specific immunotherapy.

Compelling experimental evidence demonstrated that proteins, sugars and lipids contained in peanut flour and CPE might well act as adjuvants that initiate type 2 immune responses by acting on DCs, thus leading to Th2 polarization and allergic sensitization.[25-32] In this study, we initially sought to assess the potential capacity of the synthetic cannabinoid WIN55212-2 to interfere with the polarizing effects imprinted by CPE on human DCs. For that, we employed a well-defined co-culture experimental model of hmoDCs and allogeneic naïve $CD4^+$ T cells, which allowed us to study how human DCs condition T-cell polarization in a non-antigen-specific manner.[33-35] WIN55212-2 significantly impairs the expression of costimulatory molecules and proinflammatory cytokines induced by CPE-stimulated hmoDCs. Interestingly, CPE/WIN55212-2-stimulated hmoDCs displayed lower levels of HLA-DR and the co-stimulatory molecules CD86 and CD83 than untreated hmoDCs, indicating the potent capacity of this synthetic cannabinoid to promote tolerogenic DCs. Supporting these data, we previously showed similar effects under Th1-polarizing conditions upon LPS stimulation.[21] Remarkable, WIN55212-2 enhanced the capacity of CPE-activated hmoDCs to generate IL-10-producing T cells and $FOXP3^+$ Treg cells while inhibiting both IFNγ- and IL-5-producing T cells. In line with these data, we previously showed using similar *in vitro* models that WIN55212-2 promotes tolerogenic human DCs

able to prime Treg cells and inhibit T effector cells under LPS-driven Th1 polarizing conditions.[21] Similarly, in mice the phytocannabinoid Δ^9-tetrahydrocannabinol (THC) suppresses IL-12 production and the expression of costimulatory molecules in DCs treated with *Legionella pneumophila*.[36,37] THC also reduces the capacity of mouse DCs to prime effector T cells in response to pro-inflammatory stimuli.[38] Our results provide novel insights into the capacity of synthetic cannabinoids to generate tolerogenic DCs under Th2-polarizing conditions in humans.

It has been described that epicutaneous peanut sensitization by the application of peanut allergens onto the tape-stripped skin leads to the migration and activation of DCs to the inguinal lymph nodes, thus indicating an ongoing immune response.[24] The presence of WIN55212-2 during *in vivo* peanut sensitization in mice impairs the activation and migration of skin DCs to inguinal lymph nodes. We previously showed that the administration of WIN55212-2 during peanut challenge reduces the immediate anaphylactic reaction and promotes Treg cells in allergic mice.[21] However, the ability of WIN55212-2 to interfere with peanut sensitization remained completely unknown. To shed light into this aspect, here we employed a well-defined peanut-allergic mice model generated by epicutaneous sensitization.[24] Administration of WIN55212-2 during epicutaneous sensitization significantly reduced the induction of peanut-specific IgE and IgG_1 antibodies in serum. Similarly, other cannabinoids such as THC, cannabinol or CB2 agonists attenuates serum IgE levels in ovalbumin-induced asthma in mice and rats.[39,40] At the late effector phase, the administration of WIN55212-2 during peanut sensitization reduced eosinophil infiltration and promoted the generation of allergen-specific Treg cells both in spleen and inguinal lymph nodes. Interestingly, *in vitro* expansion with CPE of splenic cells from the different mice showed significantly higher levels allergen-specific IL-10-producing T cells and $FOXP3^+$ Treg cells and lower levels of IL-5-producing T cells in mice treated with CPE/WIN55212-2 than CPE alone without significant changes observed in allergen-specific IFNγ-producing T cells. These data suggested that *in vivo* WIN55212-2 promoted allergen-specific Treg cells and reduced allergen-specific Th2 cells without affecting allergen-specific Th1 cells as demonstrated by the higher IL-10/IL-5 and IFNγ/IL-5 ratios in peanut-expanded splenic cells from mice treated with WIN55212-2/CPE than CPE alone. It is well established that increases in IL-10/IL-5 and IFNγ/IL-5 ratios together with the induction of allergen-specific Treg cells are essential mechanisms to induce tolerance to allergens during allergen-specific immunotherapy.[41–43]

In conclusion, our findings showed for the first time that the synthetic cannabinoid WIN55212-2 interferes with peanut sensitization and promotes tolerogenic responses, which might well contribute to pave the way for the development of future novel prophylactic and therapeutic strategies for peanut allergy.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

O.P. conceived and designed the study. A.A., R.J.-S., M.P.-D., A.M. and B.R. performed the experiments. M.M.-F., M.A., C.A. and O.P. provided reagents. A.A., R.J.-S-, M.P.-D., A.M., B.R., M.A., M.M.-F., C.A. and O.P. analysed and discussed the data. O.P. and A.A. wrote the paper. All the authors revised the manuscript, contributed with revisions and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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