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Narcy Arizmendi ¹, Chenjie Hou ², Fujiang Guo ², Yiming Li ² and Marianna Kulka ^{1*}

¹Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, T6G 2E1, Canada

²School of Pharmacy, Shanghai University of Traditional Chinese Medicine Shanghai, 201203, China

Abstract

Background: Dendritic cell (DC) activation induces expression of co-stimulatory surface molecules, as well as migration into secondary lymphoid organs, where they activate naïve T cells. A family of plant derivatives, eremophilane-

Objective: Since mucosal DCs are central in innate immune responses, we hypothesized that eremophilane-type

Methods: This study assessed the bicyclic eremophilane-type petasite sesquiterpene compounds Fukinone and

Results: Effects on DC maturation were evaluated by surface expression of the co-stimulatory molecule CD86 by

Conclusion:

Keywords: In ammation; Plant derivatives; Transcription factor; Peroxisome proliferator activated receptor gamma

Abbreviations: BmDC: Bone-marrow derived Dendritic Cell; DC: Dendritic Cell; ELISA: Enzyme Immunosorbent Assay; LPS: Lipopolysaccharide; MFI: Median Fluorescence Intensity; TGZ: Troglitazone; 15d-PGJ2: 15d-PGJ2-15deoxy- 12,14-Prostaglandin J2; PGJ2.

Introduction

Sesquiterpenes have been known have anti-inflammatory activity in a variety of settings, showing inhibitory effects on nitric oxide production in lipopolysaccharide (LPS)-activated mouse macrophages [1-8]. Some sesquiterpenes inhibit inflammation by targeting dendritic cell (DC) maturation and activation. For example, a sesquiterpene glycoside isolated from *Kandelia candel* inhibited pro-inflammatory cytokine production from lipopolysaccharide (LPS)-stimulated bone marrow-derived DCs [9], and micheliolide, a sesquiterpene lactone, inhibits the production of IL-6 and TNF from LPS-stimulated primary DCs [10]. While some examples of the anti-inflammatory effects of sesquiterpene on DCs have been demonstrated, the molecular targets of specific sesquiterpenes and their interactions with endogenous inflammatory signaling pathways are unknown.

One possible target of sesquiterpenes in many inflammatory cells is the peroxisome proliferator-activated receptor (PPAR) pathway,

which plays an important role in several cellular functions, including maturation and differentiation. Three PPAR subtypes have been identified, alpha, delta and gamma, and are ligand activated nuclear receptors which can be activated by polyunsaturated fatty acids, eicosanoids and various synthetic ligands. PPAR gamma (PPAR γ) is primarily expressed in adipose tissue and, to a lesser extent, in the colon, immune system and the retina. PPAR was first identified as a regulator of adipogenesis, but also plays an important role in cellular differentiation, insulin sensitization, atherosclerosis and cancer. Several sesquiterpenes or terpenoid-like compounds have been shown to either directly activate PPAR or to modify its response to other ligands. For example, odoratin, an undecanortriterpenoid from *Chromolaena odorata*, moderately activates PPAR [11]; tirtundin and tagitinin

*Corresponding author: Marianna Kulka, National Institute for Nanotechnology, National Research Council Canada, 11421 Saskatchewan Drive, Edmonton, AB, T6G 2M9, Canada, Tel: 780-641-1687; E-mail: Marianna.kulka@nrc-cnrc.gc.ca
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Both sesquiterpene lactones, transactivate PPAR γ -dependent promoters, including PPRE (PPAR response element), SHP, and ABCA1 gene promoters in dose-dependent manner, and artemisinin, a bicyclic eremophilane-type sesquiterpene, reduces expression of PPAR γ in complete media alone were included as positive and negative controls for human adipose tissue-derived mesenchymal stem cells [12]. Altogether, these data suggest that sesquiterpenes may similarly influence DC function through the PPAR pathway. Recently, our group isolated two novel eremophilane-type sesquiterpene compounds from *Petasites tatewakianus*, characterized their inhibitory effects on the degranulation of antigen-activated mast cells (manuscript in preparation). We hypothesized that these novel sesquiterpenes would inhibit DC maturation and activation, and that this activity would be augmented in the presence of a PPAR γ agonist. In this study, we demonstrate, for the first time, that the novel bicyclic eremophilane-type petasite isolated sesquiterpenes have the ability to efficiently inhibit dendritic cell maturation and activation, and this inhibition is potentiated by the synthetic, as well as naturally occurring, nuclear peroxisome proliferator-activated receptor agonists.

Materials and Methods

Plant material

Bicyclic sesquiterpenes Fukinone (ZYFDC21), and 10 H-8, 12-Epidioxyeremophil-7(11)-en-8-ol (ZYFDC22) were isolated and purified from rhizome of *Petasites tatewakianus*, at the School of Pharmacy, Shanghai University of Traditional Chinese Medicine as previously described [13].

Generation of bone marrow DCs from C57BL/6 mice

Female C57BL/6 mice (6-10 wk old) were obtained from Jackson Laboratory. All mice were treated according with protocols approved by the University of Alberta Animal Care and Use Committee. Bone marrow derived DCs (BmDC) were generated using a standard protocol with little modification [14]. Briefly, bone marrow was flushed dispersed and collected from femurs and tibiae of female C57BL/6 mice, passed through a 70 μ m nylon mesh, and suspended in complete medium (RPMI 1640 containing 5 mM HEPES, 50 U Pen/Strep, 2 mM glutamine, 50 μ M 2-ME, 50 mM gentamycin sulfate, 10% FBS) in the presence of GM-CSF and IL-4, 10 ng/mL respectively (Peprotech, Rocky Hill, NJ, USA), and cultured in tissue culture dishes (Corning Fisher, Carlsbad, CA, USA), in a humidified atmosphere of 5% CO₂ in air at 37°C. All media components, except for GM-CSF and IL-4, were obtained from Gibco (Carlsbad, CA, USA). During culture, half of the media was replaced on days 3, and 6. On day 8, BmDC were harvested, and their phenotype was confirmed by microscopical analysis.

Effect of sesquiterpenes and PPAR γ agonists on BmDC

Initially, 0.2×10^6 BmDC/mL were deposited, per well, in a 12 well plate, and incubated with either eremophilane-type petasite sesquiterpene, ZYFDC21 (50 μ M), ZYFDC22 (25 μ M), in presence or absence of synthetic PPAR γ agonists troglitazone (TGZ) (5 μ M, or 10 μ M; Sigma-Aldrich Canada, Oakville, ON, Canada) or GW1929 (40 μ M; Cayman Chemical, Ann Arbor, MI, USA), or the physiologically relevant PPAR γ natural ligand 15d-PGJ2 (0.5 μ M, or 5 μ M; Cayman Chemical, Ann Arbor, MI, USA). Cells were then incubated for 20 h at 37°C, and 5% CO₂. Cell viability was assessed by trypan blue exclusion (Gibco Carlsbad, CA, USA). Cells exposed to the petasite sesquiterpenes and synthetic and natural PPAR γ ligands were $>90\%$ viable after treatment (data not shown). In order to evaluate if the PPAR pathway and/or the petasite sesquiterpenes were involved in the maturation and activation of DCs, BmDC were pre-incubated under

Flow cytometry of BmDC

After stimulation, 1×10^5 BmDC were analyzed by flow cytometry for the expression of CD80 and CD86 surface molecules. For this, we used FITC-Armenian Hamster IgG Anti-Mouse CD80 (A ymetrix eBioscience, USA), and APC-Rat anti mouse CD86 antibodies (BD Pharmingen, San Diego, CA, USA), along with their isotype controls: FITC-Armenian Hamster IgG (A ymetrix eBioscience, USA) and APC-Rat IgG2a k (BD Pharmingen, San Diego, CA, USA). Cells were incubated for 60 min at 4°C, washed twice and analyzed in a CytoFLEX flow cytometer (Beckman Coulter, USA). Gating was determined according to the APC-Armenian Hamster anti mouse CD11c (BD Pharmingen, San Diego, CA, USA) positive population, while Armenian Hamster IgG (A ymetrix eBioscience, USA) was included as isotype control. Cell analysis was performed using the FlowJo V10 LLC software (Ashland OR, USA). Results were expressed as the median of fluorescence intensity (MFI) \pm SEM.

Cytokine release analysis

Concentrations of TNF, IL-6 and IL-12p70 released in the cell-free supernatants were determined by ELISA according to the instructions of the commercial ELISA Kits (A ymetrix eBiosciences, Santa Clara, CA, USA).

Statistical analysis

Experiments were performed in triplicate, with BmDC obtained from at least three biological replicates (n = 3). Values are expressed as mean \pm standard error of the median (SEM). Statistical differences in the mean values among treatment groups were determined by using a one-way ANOVA test with post-hoc analysis with Tukey's multiple comparison tests. In all cases, a value for p < 0.05 was considered statistically significant.

Results

PPAR γ activation inhibits DC maturation

In order to determine whether PPAR γ agonists modified the maturation of DC, we first analyzed the surface expression of the maturation marker CD86 on BmDC by flow cytometry. BmDC were treated with the synthetic PPAR γ agonists TGZ (5 or 10 μ M), or GW1929 (40 μ M), and under these conditions, treatment with the PPAR γ agonists had no effect on the expression of CD86. Figure 1A shows that BmDC exposed to different concentrations of TGZ (5 and 10 μ M) did not show altered expression of CD86, exhibiting levels similar to control untreated cells; yet LPS stimulation induced upregulation of the co-stimulatory molecule CD86 on BmDC (MFI 31431 ± 7316 , n=5). Interestingly, when the BmDC were pre-treated with the synthetic PPAR γ agonist TGZ (5 or 10 μ M for 3 h) followed by 20 h stimulation with LPS, there was a significant inhibition in the CD86 surface expression on BmDC compared to the LPS stimulation alone (MFI 6563 ± 1938 and MFI 5989 ± 2072 for TGZ 5 or 10 μ M, respectively; n=5, Figure 1A). The same is true for BmDC exposed



Figure 1: \$ & ' VXUIDFH H[SUHVVLRQ LQ %P' & DIWHU K SUHLQFXEDWLRQ ZLWK 33\$5 DJRQLVW 7* = IRORZHG E\ /36 RYHUQLJKW VWLWXOWV DUH H[SUHVVHG DV GLIIHUHQFHV LQ 0), EHWZHHQ /36 DFWLYDWHG %P' & DQG 7* = ZLWK 33\$5 DJRQLVW *: IRORZHG E\ /36 RYHUQLJKW VWLWXOWV DUH H[SUHVVHG DV GLIIHUHQFHV LQ 0), EHWZHHQ /36 DFWLYDWHG %P' & DQG 7* = VXUIDFH H[SUHVVLRQ LQ %P' & DIWHU K SUHLQFXEDWLRQ ZLWK 7* = IRORZHG E\ /36 RYHUQLJKW VWLWXOWV DUH H[SUHVVHG DV GLIIHUHQFHV LQ 0), EHWZHHQ /36 DFWLYDWHG %P' & DQG *: " /36 'DWD DUH H[SUHVVHG DV PHDQV Q " 6

to GW1929 for 3 h, followed by 20 h stimulation with LPS, where we observed a 76% inhibition in the CD86 expression response compared to LPS alone (MFI 7459 ± 2317, n=5, Figure 1B). We also examined the expression of CD80 after BmDC were exposed to the synthetic PPAR agonists, followed by LPS overnight stimulation. We observed a 12-20% inhibition in the expression of CD80 when cells were pre-treated with TGZ, and this inhibition was dose-dependent (Figure 1C). Pre-treatment with GW1929 also promoted a 15% inhibition in the expression of CD80 (Figure 1D).

Natural PPARγ ligand 15d-PGJ2 modulates DC maturation

The cyclopentenone metabolite of PGJ2, 15d-PGJ2, is a naturally occurring derivative of prostaglandin D2 and has been shown to directly activate PPAR [15-17]

BmDC were preincubated with 15d-PGJ2 (0.5 and 5 μM), for 3 h and the expression of CD86 was evaluated by flow cytometry. As shown in Figure 2A, 15d-PGJ2 alone had no effect on the expression of CD86 at either of the concentrations tested. 15d-PGJ2 treatment for 3 h decreased LPS-induced expression of CD86 by BmDC by 60% and 50% (MFI 12166 ± 1138 at 0.5 μM, and 15147 ± 1376 at 5 μM; n=3, Figure 2). CD80 surface expression was also downregulated (9 to 17% for 0.5 μM and 5 μM, respectively), by LPS-stimulated BmDC that were pretreated for 3 h with the natural PPAR agonist 15d-PGJ2 (Figure 2B, n=3). BmDC stimulated with LPS or complete media were included as positive and negative controls respectively.

PPARγ activation promotes the inhibition of BmDC cytokine secretion

We analyzed the effects of the activation of PPAR on the cytokine secretion of TNF, IL-6 and IL-12p70 released in the cell-free supernatant of BmDC, after the 3 h treatment with TGZ (5 or 10 μM), or GW1929 (40 μM), by commercial ELISA. As shown in Figure 3A, treatment with both concentrations of TGZ slightly inhibited TNF release; pre-treatment with GW1929 significantly inhibited release of TNF (about 65 ± 5% compared to LPS, Figure 3B). However, under the same conditions, BmDC release of IL-6 was unaffected by treatment with the PPAR agonists, compared to LPS stimulation alone (Figure 3C and Figure 3D). The release of IL-12p70, the bioactive isoform of the cytokine, was also evaluated in the cell-free supernatants of BmDC exposed to 5 μM and 10 μM TGZ with and without LPS stimulation. We found that TGZ significantly inhibited (68 ± 1% and 66 ± 2%, respectively) IL-12p70 production, as shown in Figure 3E. Furthermore, we observed a modest 20 ± 5% inhibition of IL-12p70 released by BmDC exposed to the synthetic PPAR agonist GW1929 (Figure 3F).

PPAR ligation skews BmDC cytokine response

We were interested in studying the response of BmDC to the treatment with the natural PPAR ligand 15d-PGJ2 (0.5 and 5 μM), and we found that this molecule similarly inhibited TNF release by BmDC. BmDC treated for 3 h with 15d-PGJ2 plus LPS showed a 29 ± 9% and 33 ± 9% inhibition at concentrations of 0.5 and 5 μM of the PPAR ligand, respectively, in

Figure 2: \$ & ' VXUIDFH H[SUHVVLQR LQ %P' & DIWHU K SUHLQFXEDWLRQ ZLWK WKH QDWXUDO 33\$5 OLJD
E\ ARZ F\WRPHWU\ UHVXOWV DUH H[SUHVVHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG
SUHLQFXEDWLRQ ZLWK G 3*- UHVXOWV DUH H[SUHVVHG DV GLIIHUHQFHV LQ 0), EHWZHHQ /36 DFWLY
" 6(0 3 3

the presence of LPS compared to TNF release of BmDC treated with LPS alone (Figure 4A). Moreover, we observed a slight (10%) increase in IL-6 release in cell-free supernatants of BmDC exposed to 15d-PGJ2 followed by overnight LPS stimulation (Figure 4B) under both conditions, compared to the LPS positive control. IL-12p70 showed a 33 ± 5% and 27 ± 8% inhibition in release, at 0.5 and 5 µM respectively (Figure 4C). Cell-free supernatants of BmDC cultured in complete media were included as negative controls.

Bicyclic petasite eremophilane-type sesquiterpenes potentiate the effects of PPAR γ agonists on BmDC maturation and activation

Petasite sesquiterpenes have been shown to have anti-inflammatory activity in a variety of settings. We sought to assess the effects of two petasite eremophilane-type sesquiterpene compounds Fukinone (ZYFDC21), and 10-H-8,12-Epidioxeremophil-7(11)-en-8-ol (ZYFDC22), isolated from the rhizome of *Petasites tatewakianus*, on the maturation and activation of BmDCs. To evaluate the cytotoxic effects of the bicyclic compounds we performed dose-response assays with several cell lines, using the XTT assay kit (Roche, data not shown). We selected sub-toxic doses of ZYFDC21 (50 µM) and ZYFDC22 (25 µM) and further evaluated their cytotoxic effects on BmDC after 1, 3, 24 and 48 h incubation, measuring viability by trypan blue exclusion (data not shown). BmDC viability was 95% under all tested conditions and therefore, these concentrations were used for all experiments.

There is evidence that some sesquiterpenes exert anti-diabetic, anti-carcinogenic and anti-inflammatory effects, mediated by the PPAR pathway. We sought to identify whether the sesquiterpenes would inhibit BmDC maturation and activation, and if this inhibitory activity would be augmented by the presence of a PPAR synthetic agonist. For that purpose, BmDC were exposed to the synthetic PPAR agonists TGZ (5 or 10 µM) or GW1929 (40 µM), in presence or absence of the petasite sesquiterpenes ZYFDC21 (50 µM) or ZYFDC22 (25 µM) for 3 h, followed by the overnight LPS stimulation. Firstly, we assessed the effects of bicyclic sesquiterpenes on BmDC maturation by flow cytometry. The presence of the sesquiterpene ZYFDC21 (Figure 5A and Figure 5B) and ZYFDC22 (Figure 6A and Figure 6B) alone induced a noticeable increase in CD86 expression (MFI 6985 ± 1825 and 6882 ± 1274 respectively) compared to control, untreated BmDC (MFI 2073 ± 510). Interestingly, when BmDC were incubated with the petasite

ZYFDC22 (25 µM), was more effective in inhibiting CD86 expression, decreasing CD86 expression up to 25% in BmDC treated with ZYFDC22 alone. The combination of the sesquiterpene with TGZ (5 or 10 µM), followed by the overnight LPS activation, resulted in up to 85% inhibition in CD86 surface expression (Figure 6A and Figure 6B).

In regard to the synthetic PPAR agonist GW1929, we found that it also downregulated CD86 expression of BmDC after the 3 h pretreatment (40 µM) in combination with the sesquiterpenes ZYFDC21 (50 µM) or ZYFDC22 (25 µM), and following LPS overnight stimulation (80% reduction, Figure 7A and Figure 8A).

Secondly, we evaluated the activation of the immune response by the presence of the pro-inflammatory mediators TNF, IL-6 and IL-12p70 released in the cell-free supernatants of BmDC treated for 3 h with the synthetic PPAR agonists (TGZ or GW1929), in combination with the petasite sesquiterpene (ZYFDC21 or ZYFDC22), followed by LPS overnight stimulation. Synthetic PPAR agonist TGZ stimulated a differential response: BmDC exposed to TGZ in combination with petasite sesquiterpene ZYFDC21 (50 µM) for 3 h, followed by LPS overnight stimulation showed a dose-response inhibitory effect, when 5 µM of the PPAR agonist was used. As shown in Figure 5C and Figure 5E, we obtained 19% and 14% inhibition in TNF and IL-6 release, respectively; however, when we increased the concentration of the PPAR agonist to 10 µM, the observed inhibition was only 8% (Figure 5D and Figure 5F), for both cytokines.

In these studies, we found that IL-12p70, the bioactive isoform of IL-12, seems to be involved in the PPAR/petasite sesquiterpene pathway. In this regard, BmDC exposed to the sesquiterpene ZYFDC21 for 3 h, followed by overnight stimulation with LPS showed a 35% of IL-12p70 inhibition compared to LPS alone; when BmDC were pretreated with ZYFDC21 in combination with the synthetic PPAR agonist TGZ (5 or 10 µM), followed by LPS overnight stimulation, we found that almost 95% of the IL-12 response was suppressed (Figure 5G and Figure 5H).

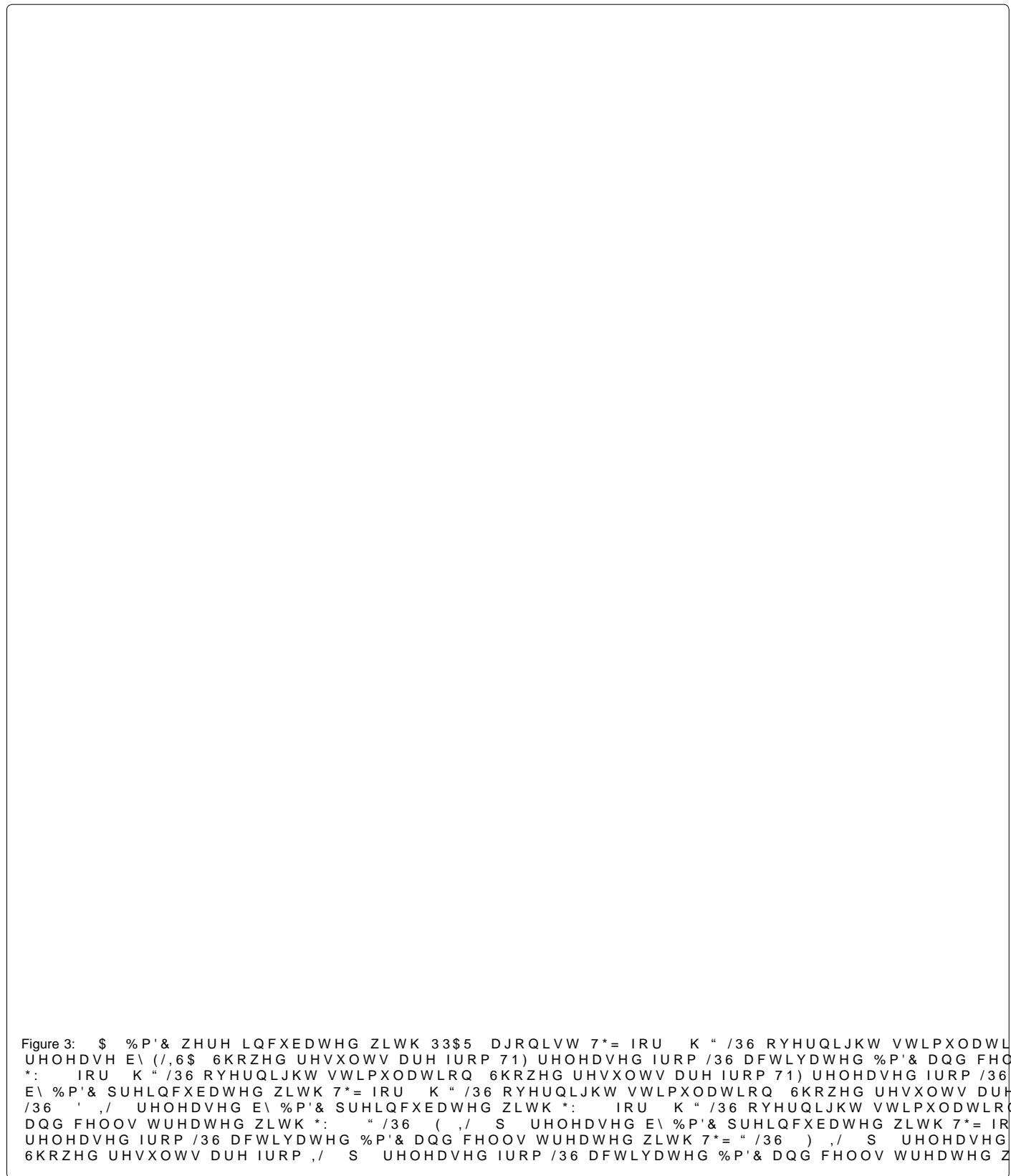


Figure 4: \$ %P' & ZHUH LQFXEDWHG WKH QDWXUDO 33\$5 OLJDQG IRU K " /36 RYHUQLJKW VWLPXODWLR UHOHDVH 6KRZHG UHVXOWV DUH IURP 71) UHOHDVHG IURP /36 DFWLYDWHG %P' & DQG FHOOV WUHDWH ZLWK G3*- IRU K " /36 RYHUQLJKW VWLPXODWLRQ Q & ./ S UHOHDVHG E\ %P' & SUHLQFXEDW DUH H[SUHVVG DV PHDQV " 6(0 3

The same response was seen for the sesquiterpene ZYFDC22 (25 μ M) for 3 h in combination with eremophilane sesquiterpenes however, in this case, the cytokine release inhibition was decreased by LPS overnight stimulation. We observed a robust inhibition as the concentration of the TGZ was increasing, with 3 and 10% TNF release inhibition (Figure 6C and Figure 6D), and 10 and 15% IL-6 release inhibition at 5 and 10 μ M, respectively (Figure 6E and Figure 6F). The IL-12p70 release profile was also inhibited by about 35% of 15d-PGJ2 (Figure 9A and Figure 9B respectively).

for ZYFDC22 followed by LPS overnight stimulation. Finally, the combination of sesquiterpene ZYFDC22 (25 μ M) with TGZ (5 or 10 μ M) followed by overnight LPS stimulation inhibited around 90% the IL-12 release (Figure 6G and Figure 6H).

As shown in Figure 7A and Figure 8A, when BmDC were treated with the PPAR agonist GW1929 (40 μ M), along with ZYFDC21 or ZYFDC22, results were similar, exhibiting downregulation in CD86 surface expression of CD86 (80% \pm 7%), as well as inhibition in release of TNF (43% and 37%, Figure 7B and Figure 8B), and IL-6 (33% and 25%, Figure 7C and Figure 8C). IL-12p70 release seemed to be affected by the combination of PPAR agonist/petasite sesquiterpenes resulting in around 75% inhibition when using ZYFDC21, and 64% for ZYFDC22 (Figure 7D and Figure 8D).

Petasite sesquiterpenes potentiate the effects of PGD2 metabolites on BmDC maturation and activation

BmDC were exposed to the natural PPAR ligand (15d-PGJ2, 0.5 μ M) for 3 h pre-treatment followed by overnight LPS stimulation. We observed a robust inhibition of CD86 surface expression (82 \pm 6%) with 0.5 and 5 μ M 15d-PGJ2 (Figure 10A and Figure 10B respectively). Similarly, BmDC activation was also affected by the treatment with the natural PPAR ligand, where TNF release by BmDC was inhibited after pre-treatment with the combination of the PPAR ligand (15d-PGJ2, 0.5 and 5 μ M) sesquiterpenes, plus overnight incubation with LPS (30% for ZYFDC21, Figure 9C and Figure 9D; and, 25 \pm 10% for ZYFDC22, Figure 10C and Figure 10D) compared to LPS alone. When BmDC were exposed to petasite sesquiterpenes plus LPS there was a 50 \pm 2% inhibition in the TNF release. In addition, the combination of the natural PPAR ligand pre-treatment plus petasite sesquiterpenes and LPS overnight stimulation promoted a modest IL-6 inhibition of 9%, and 18% for ZYFDC21 (Figure 9E and Figure 9F); but a solid 39, 60% IL-6 inhibition following a dose-dependent PPAR ligand pattern (Figure 10E and Figure 10F). Both sesquiterpenes have the ability to inhibit IL-6 release by BmDC by around 30% after 3 h pre-treatment

Figure 5: \$ %P' & ZHUH SUHWUHDWHG ZLWK WKH HUHPRSKLODQH W\SH VHVTLXWHUSHQH =<)'& " 33\$5 V\ /36 RYHUQLJKW VWLPXODWLRQ GHQGULWLF FHOOV ZHUH FROOHFWHG ¿[HG DQG DQDO\]HG E\ ÁRZ % 0), EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)'& 7*=" /36 RU FRPELQDWLRQ 7*=" =<)'& /36 & %P' & ZH DJRQLVW 7*=" -0 & RU -0 ' IRU K IROORZHG E\ /36 RYHUQLJKW VWLPXODWLRQ DQG FHOO IU 'LIIHUHQFHV LQ 71) UHOHDVHG DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & I %P' & ZHUH SUHWUHDWHG ZLWK WKH =<)'& " 33\$5 V\QWKHWLF DJRQLVW 7*=" -0 (RU -0) IRU LQ ,/ UHOHDVHG DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)'& 7 SUHWUHDWHG ZLWK =<)'& " 33\$5 V\QWKHWLF DJRQLVW 7*=" -0 * -0 + IRU K IROORZHG E\ / DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)'& 7*=" /36 RU FRPELQD "6(0 3 3 3

Figure 6: \$ %P' & ZHUH SUHWUHDWHG ZLWK WKH HUHPRSKLODQH WISH VHVTXLWHUSHQH =<)'& " 33\$5 V\ /36 RYHUQLJKW VWLPXODWLRQ GHQGULWLF FHOOV ZHUH FROOHFWHG ¿[HG DQG DQDO\]HG E\ ÀRZ % LQ 0), EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)'& 7* = " /36 RU FRPELQDWLRQ 7* = =<)'& /36 & % V\QWKHWLF DJRQLVW 7* = -0 & RU -0 ' IRU K IROORZH E\ /36 RYHUQLJKW VWLPXODWLRQ DQ ' 'LIIHUHQFHV LQ 71) UHOHDVHG DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' (%P' & ZHUH SUHWUHDWHG ZLWK WKH =<)'& " 33\$5 V\QWKHWLF DJRQLVW 7* = -0 (RU -0) I LQ ,/ UHOHDVHG DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)'& 7 SUHWUHDWHG ZLWK =<)'& " 33\$5 V\QWKHWLF DJRQLVW 7* = -0 * -0 + IRU K IROORZH E\ / DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)'& 7* = " /36 RU FRPELQD " 6(0 3 3



followed for LPS stimulation. The release of IL-12 was also inhibited (50 ± 10%) with the combination of sesquiterpene ZYFDC21 and both tested concentrations of the natural PPAR ligand (0.5 and 5 µM) plus LPS stimulation (Figure 9G and Figure 9H). However, when we tested the sesquiterpene ZYFDC22 in combination of 15-PJG2, we found that the inhibition was dose-dependent (30 ± 10% and 50 ± 10% IL-12 release inhibition at 0.5 and 5 µM 15-PJG2, respectively. Figure 10G and Figure 10H). Both sesquiterpenes were able to inhibit about 35% of IL-12 release after LPS stimulation.

Discussion

Peroxisome proliferator activator receptors (PPARs) were initially identified as receptors that controlled physiological responses to dietary intake of fatty acids [18,19], in particular, PPAR α is involved in adipocyte differentiation, and glucose metabolism [20]. It has been shown that PPAR ligands have anti-inflammatory effects on mast cells, monocytes, macrophages and dendritic cells, by modulating expression of costimulatory and adhesion molecules, altering their phenotype and leading to an impaired expression of pro-inflammatory cytokines/chemokine factors involved in T-cell activation and recruitment [21-25].

to promote the contact between dendritic cells and T cells, while costimulatory molecules signal T cells to proliferate and differentiate [26]. In the mouse, CD86 is the main activation marker of bone-marrow derived DC, being strongly up-regulated after maturation, while CD80 expression is less relevant for murine DC [26,27]. In this context, our studies demonstrated that LPS stimulation upregulated CD80 and CD86 expression on BmDC. We also confirmed that the use of synthetic (Troglitazone and GW1929), as well as natural (15d-PGJ2) PPAR ligands decreased the expression of CD80 and CD86 after LPS stimulation. Further, we observed a significant reduction on TNF cytokine release with the GW1929 and 15d-PGJ2. IL-6 release exhibited no change with all PPAR agonists used, while IL-12p70 production was attenuated by the PPAR agonist. All of these results were similar to previous reports [22,23,28-31].

Advances in the investigation of plant-derived chemicals used in alternative medicine for the treatment of several chronic diseases have shown that Petasite species from petasite sesquiterpenes possess anti-inflammatory properties [32-34]. Due to their anti-inflammatory effects mediated via leukotriene synthesis inhibition, sesquiterpenes have been used for the treatment of inflammatory diseases such as arthritis, migraine, as well as asthma and allergy [35-37].

Dendritic cells are the most potent antigen presenting cells (APCs), and are involved in initiating the adaptive immune responses. Due to their ability to block Ca²⁺ channels, decreasing intracellular Ca²⁺ concentration, inhibiting leukotriene B4 and cysteinyl leukotrienes synthesis in eosinophils and neutrophils [12,32,35,38-43]. The active



components are sesquiterpene esters of the caryophyllane-type, and lactones tirotundin and targitining A, isolated from *Tithonia diversifolia* against PPARs. For this, they used a transient transfection reporter assay with HepG2 cells, and found that tirotundin and extract of *Petasites japonicus* was petasine, which had inhibitory effects on leukotriene synthesis [38] and bronchoconstriction [47]. Another eremophilane-type sesquiterpene ketone, namely, fukinone (1 and 3) (Naya and Kotake), exerted suppressive mechanisms in type I hypersensitivity model in rats and IgE-sensitized RBL-2H3 cells, through inhibition of smooth muscle constriction and inhibition of degranulation, leukotriene release, and TNF production by mast cells [32,46]. In this context [32], also reported anti-allergic and anti-inflammatory effects of several compounds obtained from plants of the petasites genus in an ovalbumin-induced asthma model. Use of Bakkenolide B isolated from *P. japonicus* results in inhibited mast cell degranulation as well as, expression induction of inducible nitric oxide synthase and cyclooxygenase 2. The bronchoalveolar lavage fluid of their ovalbumin-induced asthma model showed that Bakkenolide B inhibited the migration of eosinophils, macrophages, and lymphocytes to the lungs. In this case, however, the authors mentioned that Bakkenolide B did not prevent maturation of immature dendritic cells. Previous studies from our lab showed that different extracts of petasites could inhibit type I and type IV hypersensitivity in mouse models of homogeneous and heterogeneous passive cutaneous anaphylaxis [48].

Elegant studies evaluated the agonistic activity of sesquiterpene components on PPARs. For this, they used a transient transfection reporter assay with HepG2 cells, and found that tirotundin and extract of *Petasites japonicus* was petasine, which had inhibitory effects on leukotriene synthesis [38] and bronchoconstriction [47]. Another eremophilane-type sesquiterpene ketone, namely, fukinone (1 and 3) (Naya and Kotake), exerted suppressive mechanisms in type I hypersensitivity model in rats and IgE-sensitized RBL-2H3 cells, through inhibition of smooth muscle constriction and inhibition of degranulation, leukotriene release, and TNF production by mast cells [32,46]. In this context [32], also reported anti-allergic and anti-inflammatory effects of several compounds obtained from plants of the petasites genus in an ovalbumin-induced asthma model. Use of Bakkenolide B isolated from *P. japonicus* results in inhibited mast cell degranulation as well as, expression induction of inducible nitric oxide synthase and cyclooxygenase 2. The bronchoalveolar lavage fluid of their ovalbumin-induced asthma model showed that Bakkenolide B inhibited the migration of eosinophils, macrophages, and lymphocytes to the lungs. In this case, however, the authors mentioned that Bakkenolide B did not prevent maturation of immature dendritic cells. Previous studies from our lab showed that different extracts of petasites could inhibit type I and type IV hypersensitivity in mouse models of homogeneous and heterogeneous passive cutaneous anaphylaxis [48].

On another hand [44], demonstrated that petasin derived from *P. japonicus* activates AMPK in the liver, skeletal muscle and adipose tissue of mice, via phosphorylation of AMPK. AMPK activation enhanced the transcription of the proliferator-activated receptor-coactivator-1 (PGC-1), which regulates the genes involved in energy metabolism including mitochondrial biogenesis.

Our studies showed that the eremophilane-type sesquiterpenes ZYFDC21 and ZYFDC22 noticeably increased CD80 and CD86 surface expression in non-stimulated BmDC. In contrast, when the cells were

Figure 9: \$ %P' & ZHUH SUHWUHDWHG ZLWK WKH HUHPRSKLODQH W\SH SHWDVLWH VHVTXLWHUSHQH =<)' & K IROORZHG E\ /36 RYHUQLJKW VWLPXODWLRQ DQG %P' & FHOOV ZHUH FROOHFWHG DQG DQDO\HG E GLIIHUHQFHV LQ 0), EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)' & 3*- " /36 RU FRPELQDWLRQ G 3*- =<)' G 3*- -0 & RU -0 ' IRU K IROORZHG E\ /36 RYHUQLJKW VWLPXODWLRQ DQG FHOV IUHH VXSHU LQ 71) UHOHDVHG DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)' & 3 ZHUH SUHWUHDWHG ZLWK =<)' & " G 3*- -0 (RU -0) IRU K IROORZHG E\ /36 RYHUQLJKW DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)' & 3*- " /36 RU FRPELQDWLRQ G 3*- =<)' & G 3*- -0 * RU -0 + IRU K IROORZHG E\ /36 RYHUQLJKW VWLPXODWLRQ + 'GLIIHUHQFHV LQ DFWLYDWHG %P' & DQG =<)' & 3-* " /36 RU FRPELQDWLRQ G 3*- =<)' & /36 Q YDOXHV DUH SUHVHQW

Figure 10: \$ %P' & ZHUH SUHWUHDWHG ZLWK WKH SHWDVLWH VHVTXLWHUSHQH =<)' & " G 3*- -0 \$
DQG %P' & FHOOV ZHUH FROOHFWHG DQG DQDO\JHG E\ ARZ % 'LIIHUHQFHV LQ WKH &' VXUIDFH H[SU
%P' & DQG =<)' & 3*- " /36 RU FRPELQDWLRQ G 3*- =<)' & /36 & %P' & ZHUH SUHWUHDWHG ZLWK W
-0 & RU -0 ' IRU K IROORZHG E\ /36 RYHUQLJKW VWLPXODWLRQ DQG FHOV IHH VXSHUQDWD
UHOHDVHG DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)' & 3*- " /3
SUHWUHDWHG ZLWK WKH SHWDVLWH VHVTXLWHUSHQH =<)' & " G 3*- -0 (RU -0) IRU K IRC
DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)' & 3*- " /36 RU FRPELQ
ZLWK WKH SHWDVLWH VHVTXLWHUSHQH =<)' & " G 3*- -0 * RU -0 + IRU K IROORZHG E\ /3
DUH UHSUHVHQWHG DV GLIIHUHQFHV EHWZHHQ /36 DFWLYDWHG %P' & DQG =<)' & 3*- " /36 RU FRPELQ
PHDQV " 6(0 3 3 3

pre-treated with sesquiterpene ZYFDC21 (Fukinone) followed by LPS. Petasite sesquiterpenes are compounds with significant potential value we observed a decrease in CD86 surface expression is inhibition for the treatment of inflammatory disorders.

was amplified up to 80% by the presence of the PPAR agonists TGZ, GW1929 and 15d-PGJ2. An inhibitory effect was also observed when we used ZYFDC22 (10 H-8,12-Epidioxyeremophil-7(11)-en-8-ol) in combination with TGZ, GW1929 or 15-d PGJ2, followed by LPS stimulation, where 85% of CD86 surface expression was significantly inhibited by PPAR agonist treatment. The absence of costimulatory molecules, such as CD86, influences dendritic cell function, altering their maturation, and varying the expression of the necessary signals required for the activation and differentiation of naive T cells into type 1 (IL-12, IFN γ) or type 2 (IL-4, IL-5, IL-10) cytokine producing cells.

In this context, our studies showed that both sesquiterpenes ZYFDC21 and ZYFDC22 inhibited the secretion of the soluble factors TNF and IL-6, after LPS stimulation. These results are comparable to those obtained by [50], who demonstrated that the sesquiterpene lactone parthenolide inhibited dendritic cell maturation and cytokine secretion induced by LPS.

The level of IL-12 secreted by dendritic cells induced by microbial pathogens, such as LPS, during the immunological synapse is a key factor in the outcome of immune responses. IL-12 is a critical 1-skewing cytokine that elicits IFN γ production by T cells and by NK cells [51], favoring a 2/3 response and inhibiting T cell recruitment [52]. PPAR is an important modulator on B and T lymphocytes as well as dendritic cells [22,53,54] and PPAR ligands include a class of antidiabetic drugs, thiazolidinediones (TZD); as well as naturally produced Prostaglandin D2 (PGD2) and its metabolite 15-dideoxy-PGJ2 (15d-PGJ2), which associate irreversibly to the receptor through covalent binding, mediating their effects by activation of PPAR-dependent and independent pathways [21,55]. Prostaglandins production result in activation of PPAR-mediated transcription, leading to the inhibition of differentiation, migration and cytokine secretion by antigen-presenting cells, such as dendritic cells or macrophages, hence, affecting the priming and effector functions of T lymphocytes [21].

Our studies showed for the first time, that dendritic cells exposed to the PPAR ligands TGZ, GW1929 and 15d-PGJ2, in presence of these novel isolated bicyclic eremophilane-type petasite sesquiterpenes ZYFDC21 and ZYFDC22 followed by LPS stimulation exhibited a significant reduction (up to 95%) in the production of the bioactive isoform of IL-12 (IL-12p70). In this regard, it has been documented that 15d-PGJ2 abrogates IL-12 production by directly inhibiting the function of I κ B kinase, therefore, preventing the translocation of NF- κ B to the nucleus [56-58]. Our results showed that sesquiterpenes reduced LPS-induced DC maturation, and inhibited TNF and IL-6 release, as well as the production of the bioactive isoform of IL-12p70, presumably through the direct activation of PPAR. Since it is well known that the transcription factor NF- κ B plays a key role in the activation of PPAR in the inflammatory response, it would be of interest to determine whether sesquiterpenes bind directly to the PPAR receptors, thereby inhibiting I κ B kinase (IKK), and to analyze the downstream signaling cascades that would prevent the translocation of NF- κ B to the nucleus, interfering with the inflammatory response.

Conclusions

In summary, our results suggest that the novel Fukinone and 10 H-8,12-Epidioxyeremophil-7(11)-en-8-ol sesquiterpenes derived from Petasite tatewakianus inhibit the maturation of dendritic cells, as well as the production of TNF, IL-6 and IL-12p70 after LPS stimulation. These events seem to be mediated and potentiated by the activation of PPAR

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Conflict of Interest Disclosure

7 KH DXWKRU V GHFODUH QR FRQÀLFW RI LQWHUHV

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2. *DR 6 ;LD * :DQJ / =KRX / =KDR) HW DO Curcuma wenyujin with their inhibitory activities on nitric oxide production in 5\$: FHOOV 1DW 3URG 5HV
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