Journal of Ethnopharmacology 252 (2020) 112496

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Green propolis increases myeloid suppressor cells and CD4$^+$Foxp3$^+$ cells and reduces Th2 inflammation in the lungs after allergen exposure

Annie R. Piñeros[a], Mikhael H.F. de Lima[a], Tamara Rodrigues[a], Ana Flávia Gembre[a], Thais B. Bertolini[a], Miriam D. Fonseca[d], Andresa A. Berretta[c], Leandra N.Z. Ramalho[b], Fernando Q. Cunha[d], Juliana I. Hori[c,1], Vânia L.D. Bonato[a,*,1]

[a] Basic and Applied Immunology Program, Department of Biochemistry and Immunology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil
[b] Department of Pathology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil
[c] Apis Flora Industrial e Comercial Ltda, Ribeirao Preto, Sao Paulo, Brazil
[d] Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil

ARTICLE INFO

Keywords:
Asthma
Propolis
PMN-MDSC
Treg cells

ABSTRACT

Ethnopharmacological relevance: Propolis is a natural product produced by honeybees used as a medicine at least to 300 BC. In the last decades, several studies showed biological and pharmacological properties of propolis, witch scientifically explains the empirical use for centuries. The anti-inflammatory activity of propolis with the purpose to reduce Th2 inflammation has been evaluated in allergic asthma. However, it remains to be determined how propolis negatively regulates the immune response after allergen re-exposure.

Aim of the study: We hypothesized that the anti-inflammatory activity of propolis is dependent on the induction of myeloid derived suppressor cells (MDSC) and regulatory T cells.

Materials and methods: To assess this hypothesis, we used an ovalbumin-induced asthma model to evaluate the effect of EPP-AF® dry extract from Brazilian green propolis.

Results: Propolis treatment decreased pulmonary inflammation and mucus production as well as eosinophils and IL-5 in the broncoalveolar lavage. Propolis enhanced also *in vitro* differentiation and *in vivo* frequency of lung MDSC and CD4$^+$Foxp3$^+$ regulatory T cells.

Conclusions: Together these results confirm the immunomodulatory potential of propolis during sensitization and challenge with allergen. In addition, the collecting findings show, for the first time, that propolis increases the frequency of MDSC and CD4$^+$Foxp3$^+$ regulatory T cells in the lungs, and suggest that it could be use as target for development of new immunotherapy or adjuvant immunotherapy for asthma.

1. Introduction

Asthma is a chronic inflammatory disease of inferior airways that causes bronchial hyperreactivity, mucus overproduction and airway narrowing (Lambrecht and Hammad, 2015; Kabata and Artis, 2019). Allergic asthma is mediated by Th2 inflammation, characterized by increased number of CD4$^+$ T cells that produce IL-4, IL-5 and IL-13 (Th2 cells) and induce high IgE levels, eosinophilic infiltration and mast cell activation (Robinson et al., 1992; Lambrecht and Hammad, 2013).

CD4$^+$Foxp3$^+$ regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) play a critical role in the down-modulation of the Th2 immune response (Huang et al., 2009; Arora et al., 2010, 2011; Gabrilovich et al., 2012; Josefowicz et al., 2012; Whitehead et al., 2012; Xu et al., 2012; Veglia et al., 2018). MDSC represent a heterogeneous population of immature myeloid cells described as polymorphonuclear (PMN-MDSC - CD11b$^+$Ly6G^{high}Ly6C^{low}) and monocytic (M-MDSC - CD11b$^+$Ly6G$^-$Ly6C^{high}) suppressor cells (Gabrilovich et al., 2012; Bronte et al., 2016; Fleming et al., 2018). The contact with high LPS

* Corresponding author. Av. Bandeirantes, 3900, Monte Alegre, Ribeirao Preto, SP, CEP: 14049-900, Brazil.
E-mail addresses: anniep93@gmail.com (A.R. Piñeros), mikhaelharuo@usp.br (M.H.F. de Lima), tamararodrigues@usp.br (T. Rodrigues), flagembre@yahoo.com.br (A.F. Gembre), thabertolini@gmail.com (T.B. Bertolini), miriamfarmacia@yahoo.com.br (M.D. Fonseca), andresa.berretta@apisflora.com.br (A.A. Berretta), lramalho@fmrp.usp.br (L.N.Z. Ramalho), fdqcunha@fmrp.usp.br (F.Q. Cunha), juliana.hori@apisflora.com.br (J.I. Hori), vlbonato@fmrp.usp.br (V.L.D. Bonato).
[1] JI Hori and VLD Bonato contributed equally to this study.

https://doi.org/10.1016/j.jep.2019.112496
Received 29 July 2019; Received in revised form 11 December 2019; Accepted 19 December 2019
Available online 21 December 2019
0378-8741/

dose increased $CD11b^+Gr1intF4/80^+$ lung cells resembling to MDSC that suppressed Th2 inflammation in a model of allergic asthma induced by house dust mite (Arora et al., 2010, 2011). The interaction of MDSC with Treg cells has been described and collected findings showed that activated MDSC cells produce TGF-β that induce the expansion of Treg cells (Ren et al., 2016; Wang et al., 2016).

Because asthma affects up to 235 million people in the world (WHO, 2019), allergen-specific and allergen-free immunotherapy strategies have been investigated to control pulmonary Th2 inflammation and to improve pulmonary function (Fonseca et al., 2012, 2015; Prado et al., 2015; Ortega et al., 2014). Although steroids are the most effective treatment to reverse the symptoms of asthma, these induce side effects and their prescriptions are not allowed for long time (Lambrecht and Hammad, 2015; Borish, 2016). Furthermore, asthma is a heterogeneous disease and the benefits of corticoids or immunotherapy are not observed in 30% of treated-asthmatic population (Borish, 2016). These clinical findings reinforce the importance to develop new immunotherapies.

Propolis is a natural product produced by honeybees through mixing saliva and beeswax with exudate from different botanical sources (AlGabbani et al., 2017; Franchin et al., 2018; Kitamura et al., 2018). It has been reported that propolis contains more than 300 different compounds among phenolic compounds as flavonoids, aromatic acids, benzopyranes, as well as essential oils (Sforcin, 2007; Hori et al., 2013; Miranda et al., 2015). However the composition of propolis is determined by both the geographic location (vegetation) and the bee's genetics (Bankova et al., 2014; Kitamura et al., 2018). Brazilian propolis is produced mainly by Africanized bees, which are hybrids between *Apis mellifera scutellata* and *Apis mellifera* and is rich in organic substances from *Baccharis dracunculifolia* specie of plant (Park et al., 2004; Lemos et al., 2007). Several studies have reported biological activities for green propolis such as anti-bacterial, anti-fungal, wound healing, anti-inflammatory, as well as immunomodulatory activity during asthma (Barros et al., 2007; Coelho et al., 2007; Castro et al., 2011; Machado et al., 2012; Hori et al., 2013; Franchin et al., 2018; Kitamura et al., 2018), witch scientifically explains its empirical use for centuries.

Here we used an ovalbumin-induced asthma model to investigate the anti-inflammatory role of the standardized brazilian green propolis extract – EPP-AF®. We confirmed that propolis reduced eosinophilic infiltration and Th2 inflammation, and for the first time, we showed that propolis increased PMN-MDSC and $CD4^+Foxp3^+$ cells in the lungs of mice exposed to allergen. Propolis increased the PMN-MDSC and Treg cells differentiation *in vitro*, suggesting that both leukocytes negatively regulate asthma associated Th2 inflammation.

2. Materials and methods

2.1. Obtention and chemical characterization of standardized green propolis EPP-AF®

Propolis EPP-AF® dry extract (batch 005900117, Apis Flora Indl. Coml. Ltda, Ribeirão Preto, SP, Brazil) was obtained by hydro alcoholic extraction using maceration followed by turbo extraction process. The dryness step was preceded according Marquiafável (2015) with some modifications. The chemical profile was obtained according Berretta et al. (2012) using HPLC system acopled to DAD-UV detector, using as reference standards: caffeic acid (Fluka, L. 43706045), p-coumaric acid (Fluka, L.3250759), 3,5-Dicaffeoylquinic acid (Phytolab, L. 13672946) and 4,5 dicaffeoylquinic acid (Phytolab, L. 13672903) (Berretta et al., 2012). The aromadendrin-4-O-etil ether, drupanin, artepillin C and baccharin were previously isolated, identified and donated by Sousa et al. Total flavonoid and total polyphenols were analyzed according Rocha et al. (2013).

2.2. Animals

C57BL/6 female mice (6–8 weeks old) were obtained from the breeding facility of Ribeirao Preto Medical School (FMRP - University of São Paulo, Ribeirao Preto, SP, Brazil). Mice were maintained in ventilated cages (Alesco, Monte Mor, SP, Brazil) under barrier conditions with free access to sterile food and water. Experiments were approved by the Animal Research Ethics Committee of the FMRP (protocol number 202/2017).

2.3. OVA-induced asthma model

Mice were sensitized with 1.6 mg of aluminum hydroxide adjuvant and 100 μg of OVA (Sigma, St. Louis, MO, USA) by subcutaneous (s.c.) route. Fourteen days after the first sensitization, the mice were injected with 50 μg of OVA in 100 μL of saline by intraperitoneal (i.p.) route. Seven days after the second sensitization, mice were challenged twice in a 7-day interval with 100 μg of OVA by intranasal (i.n) route. After three days, immune response and inflammation were assessed.

2.4. Propolis treatment

Mice were treated with 150 mg/Kg of propolis by gavage after the last OVA sensitization and during 17 or 22 days, every day (Fig. 1A and Supplemental Fig. 1A).

2.5. Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was obtained using saline sterile, as previously described by Piñeros et al. (2017).

2.6. Flow cytometry

Luns samples were collected and digested with 0.5 ug/mL of liberase and 25U/mL of DNAse (Roche Applied Science, Indianapolis, IN, USA) at 37 °C for 30 min to obtain single-cell suspension. All the samples were stained with Live/Dead APC.Cy7 BD Horizon™ Fixable Viability Stain (BD Biosciences, San Jose, CA, USA). For intracellular staining, lung cells were stimulated for 6 h with PMA (100 ng/mL), ionomycin (500 ng/mL) (Sigma-Aldrich) and Golgi Stop (BD Biosciences), at 37 °C and 5% CO_2. Cells were fixed in PBS containing 4% paraformaldehyde and permeabilized in PBS containing 1% FBS, 0.1% sodium azide, and 0.2% saponin. Intracellular staining was performed with specific antibody incubation for 20 min at 4 °C. Cells were washed and fixed in PBS containing 1% paraformaldehyde. Samples were acquired in FACSCanto II (BD Bioscience, Franklin Lakes, NJ, USA) and data obtained were analyzed by FlowJo 7.6.1 TM Software (Tree Star, Inc., Ashland, Oregon, USA). The following monoclonal antibody conjugates were used: F4/80 (BM8), CD11c (N418), CD11b (M1/70), iNOS (CXNFT), CD206 (C068C2), Siglec-F (E50-2440) Ly6G (1A8), Ly6C (AL-21). CD4 (RM4-5), CD44 (IM7), CD62L (MEL-14), TIGITII (1G9), Foxp3 (MF23). All antibodies were obtained from BD or eBioscience.

2.7. Cytokines

IL-5 and IL-13 were measured in BALF and in supernatant from mediastinal lymph nodes (mLN) cell culture using ELISA kits (BD *OptEIA Set*, BD Biosciences, San Diego, CA, USA). mLNs were sterile processed and cell suspensions ($2x10^6$/mL) were stimulated with OVA (100 μg/mL), Concanavalin A (ConA; 40 μg/mL) or left unstimulated. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum during 48 h, at 37 °C and 5% of CO_2 and then the supernatants were harvested to cytokines assay.

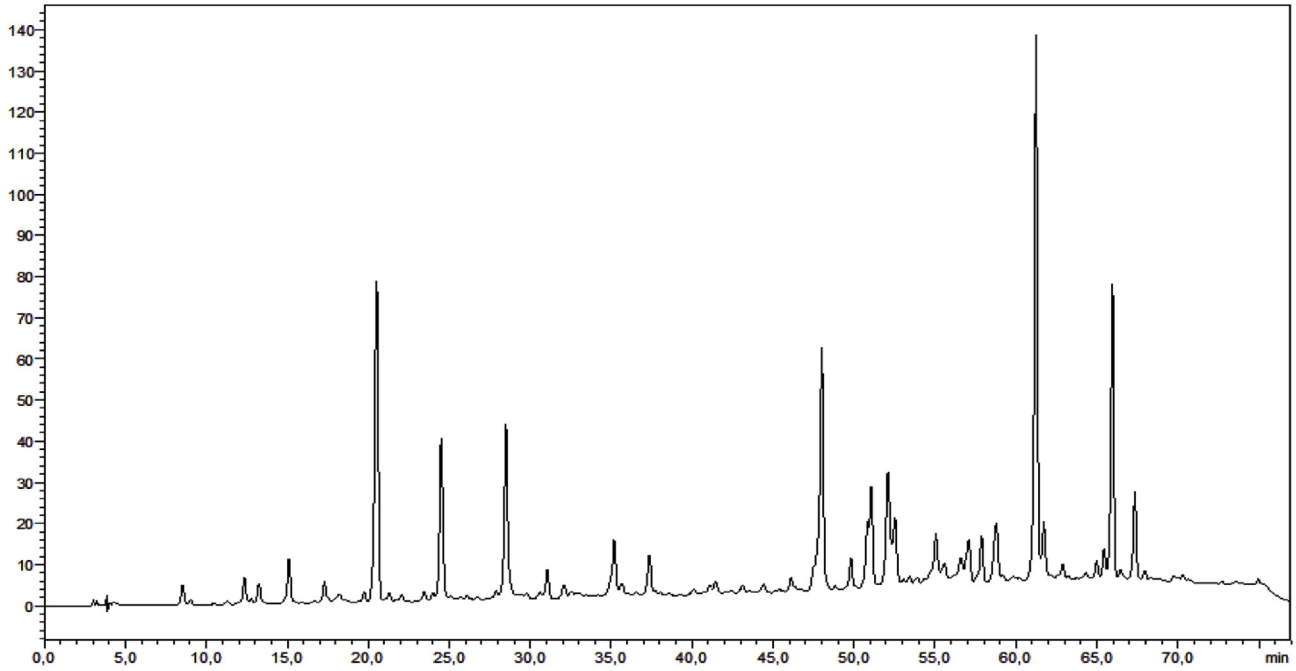

Fig. 1. Characterization of propolis extract. Fingerprint analysis of green propolis extract (EPP-AF). Chromatograms were plotted at 275 nm, using RP-HPLC equipment with C18 (shim-pack, CLC-ODS (M), 25 cm x 4.6) column and gradient elution with methanol and acidic water (pH = 2.7). Chromatographic profile includes the compounds: 1. Caffeic acid (around 15 min); 2. p-coumaric acid (around 20 min); 3. 3,5-Dicafeoyl quinic; 4. 4,5-Dicafeoyl quinic; 5. aromadendrin-4-O-methyl-ether; 6. Drupanin; 7. Artepillin C and 8. Baccharin.

2.8. Real time-PCR

RNA from the frozen lung samples was extracted with the TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and converted to cDNA using SuperScript II Reverse Transcriptase kit (Life Technologies). Quantitative mRNA analysis was performed with a StepOnePlus Real-Time PCR System (Life Technologies) using the SYBR Green fluorescence system. The forward and reverse primer sequences were used for detection of β-*Actin* (forward, 5′-CCC TAG GCA CCA GGG TGT GA-3′; reverse, 5′-GCC ATG TTC AAT GGG GTA CTT C-3′) and *Il-13* (forward, 5′-ACC AAC ATC TCC AAT TGC AA-3′; reverse, 5′-ATG CAA TAT CCT CTG GGT CC-3′).

2.9. MDSC cell differentiation

Bone marrow cell precursors ($5x10^5$/mL) from C57BL/6 naive mice were stimulated with GM-CSF (40 ng/mL) and IL-6 (40 ng/mL) during 4 days for MDSC differentiation, in the presence or absence (vehicle) of propolis (50 μg/mL). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum at 37 °C and 5% of CO_2.

2.10. Treg cell differentiation

Naive T cells ($CD4^+CD44^-CD62L^+$) were isolated from spleen and lymph node of C57BL/6 naive mice using FACS sorting (FACSAria III, BD Biosciences). To induce Treg differentiation, cells ($5x10^5$/mL) were stimulated with monoclonal antibodies anti-CD3 (1 μg/mL, BD Pharmingen™ 145-2C11), anti-CD28 (1 μg/mL, BD Pharmingen™ 37.51), and murine TGF-β recombinant (3 ng/mL, eBioscience), in presence or absence of propolis (50 μg/mL) in RPMI-1640 medium (Sigma-Aldrich) supplemented with 1% sodium pyruvate, 1% non-essential amino acids, and 10% fetal bovine serum. Cells were cultured during 96 h, at 37 °C and 5% of CO_2.

2.11. Statistics

Data are expressed as mean ± SEM and analyzed with StatSoft Inc. (2004) STATISTICA software, version 7. Significance between two groups was estimated using the *t*-test, 1-tailed for parametric data, and the Mann-Whitney *U* test for nonparametric data. Experiments with 3 or more groups were analyzed using 1-way ANOVA and corrected using the Tukey's comparison test and the Kruskal-Wallis test for nonparametric data. A p-value of less than 0.05 was considered significant.

3. Results

3.1. Chemical characterization of propolis extract (EPP-AF®)

Propolis standardized extract, EPP-AF®, was evaluated by HPLC and the fingerprint is represented on Fig. 1. The chemical characterization showed the presence of caffeic acid (1.90 ± 0.014 mg/g), p-coumaric acid (10.016 ± 0.028 mg/g), 3,5-dicafeoyl quinic (3,5-DCQ) (14.293 ± 0.081), 4,5-DCQ (18.364 ± 0.164), aromadendrin-4-O-methyl-ether (2.519 ± 0.023), drupanin (17.343 ± 0.072), artepillin C (50.299 ± 1.039) and baccharin (8.459 ± 0.281). Total flavonoid content as quercetin demonstrated 41.449 ± 0.425 mg/g and total poliphenol as gallic acid presented 150.37 ± 0.78 mg/g.

3.2. Propolis treatment ameliorates experimental allergic asthma

We and another groups have shown that EPP-AF® propolis extract decrease inflammation and improve tissue repair (Barud et al., 2013; Hori et al., 2013). However, the effect of EPP-AF® in asthma remains unknown. To determine the effect of propolis in experimental asthma, we used an ovalbumin (OVA)-induced allergy model. After the second OVA sensitization, animals were treated with 150 mg/Kg of EPP-AF® propolis extract by gavage daily (Fig. 2A). As expected, sensitization and challenge with allergen (OVA group), evaluated 72 h after the last OVA challenge, increased total cell number, frequency and number of eosinophils in the BALF compared to control animals (Naive group)

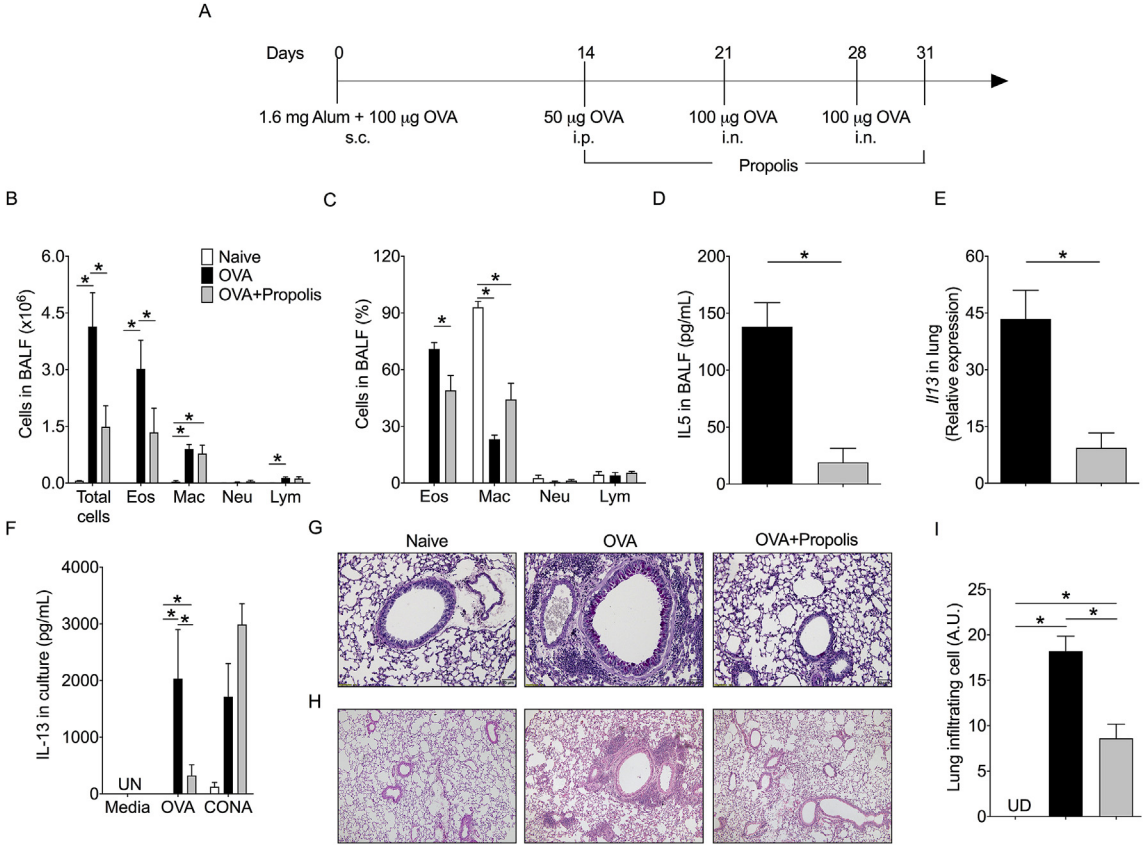

Fig. 2. Propolis treatment ameliorates experimental allergic asthma. (A) Female C57BL/6 mice were immunized by s.c. injection with a solution alum and 100 µg of OVA. After 14 days the mice were sensitized with 50 µg of OVA (i.p.) and treated with 150 mg/Kg of propolis by gavage every day during 17 days. Mice were challenged with OVA, 100 µg (i.n.), twice, 21 days and 28 days after the first immunization. Mice were euthanized three days after second challenge. (B) Total cell number, (C) frequency and (D) IL-5 production quantified in the BALF. (E) IL-13 secretion quantified in supernatants of mLN cell culture. (F) *il-13* gene expression in the lungs. (G, H) Photomicrographs of H&E and mucus staining. (I) Score of lung inflammation. Data are representative of 2 independent experiments expressed as mean ± SEM (n = 5–7 mice per group). *P < 0.05.
Naive = non OVA-sensitized, -challenged, non-treated with propolis.
OVA = OVA-sensitized and –challenged.
OVA + Propolis = OVA-sensitized and –challenged and treated with propolis.

(Fig. 2B, C). Propolis treatment (OVA + Propolis) reduced the total cell number into the BALF compared to allergic group (OVA) (Fig. 2B) and decreased the number and the frequency of eosinophils in the BALF (Fig. 2B, C). No difference was found in the number and frequency of neutrophils, macrophages and lymphocytes between OVA and OVA + propolis groups (Fig. 2B, C). Lower IL-5 levels in the BALF and lower il13 gene expression in the lungs of OVA + Propolis groups confirmed the attenuation of Th2 inflammation in animals treated with Propolis compared to OVA group (Fig. 2D, E). In addition, propolis treatment also induced a decrease of IL-13 production on lymph node cell culture re-stimulated with OVA compared to non-treated allergic mice (Fig. 2F). A significant decrease in the mucus production (Fig. 2G) as well as in the cellular infiltration (Fig. 2H, I) were found in propolis-treated allergic mice. Together these results show that propolis played an anti-inflammatory role in the OVA-induced Th2 inflammation.

3.3. Propolis decreases eosinophils and M2 macrophages in the lungs

Next, the frequency and number of eosinophils were evaluated in the lungs. Firstly we found that propolis treatment reduced the total cell numbers in the lungs of allergic mice compared to non-treated allergic animals (Fig. 3A, B). This reduction was followed by a decrease in the frequency and number of eosinophils, ($CD11b^+SiglecF^+$ cells) (Fig. 3C, D). Because differentiation of alternatively activated macrophages (M2) is induced by Th2 immune response and M2 macrophages act on tissue remodeling, we also quantified this population in the lungs. M2 macrophages, characterized as $F4/80^+CD11b^+CD206^+$ cells, were increased in frequency and number in the lungs of allergic animals (OVA group) compared to control animals (Naive group). Propolis treatment significantly reduced the number of M2 macrophages in the lungs (Fig. 3E, F). These findings show that propolis decreases eosinophils and M2 macrophages in the lungs.

3.4. Propolis increases PMN-MDSC and $CD4^+Foxp3^+$ cells in the lungs

To evaluate whether the anti-inflammatory effect of propolis could be mediated by MDSC, which negatively regulates Th2 immune response in the experimental asthma (Cao et al., 2019), we evaluate both M-MDSC ($CD11b^+Ly6G^{high}Ly6C^{low}$) and PMN-MDSC ($CD11b^+Ly6G^-Ly6C^{high}$) in the lungs. We found that propolis treatment induced a significant increase in frequency and number of PMN-MDSC, but not in M-MDSC in the lung of allergic mice when compared with non-treated allergic mice (Fig. 4A–C). In attempt to confirm whether propolis could directly induce the differentiation or expansion of PMN-MDSC, bone marrow cell precursors were stimulated with GM-CSF and IL-6 during 4 days for MDSC differentiation, in the presence or absence (vehicle) of propolis. We confirm that propolis treatment induced an increase of PMN-MDSC cells compared to control (vehicle) (Fig. 4D–E).

MDSCs have been described to induce Treg differentiation (Lee

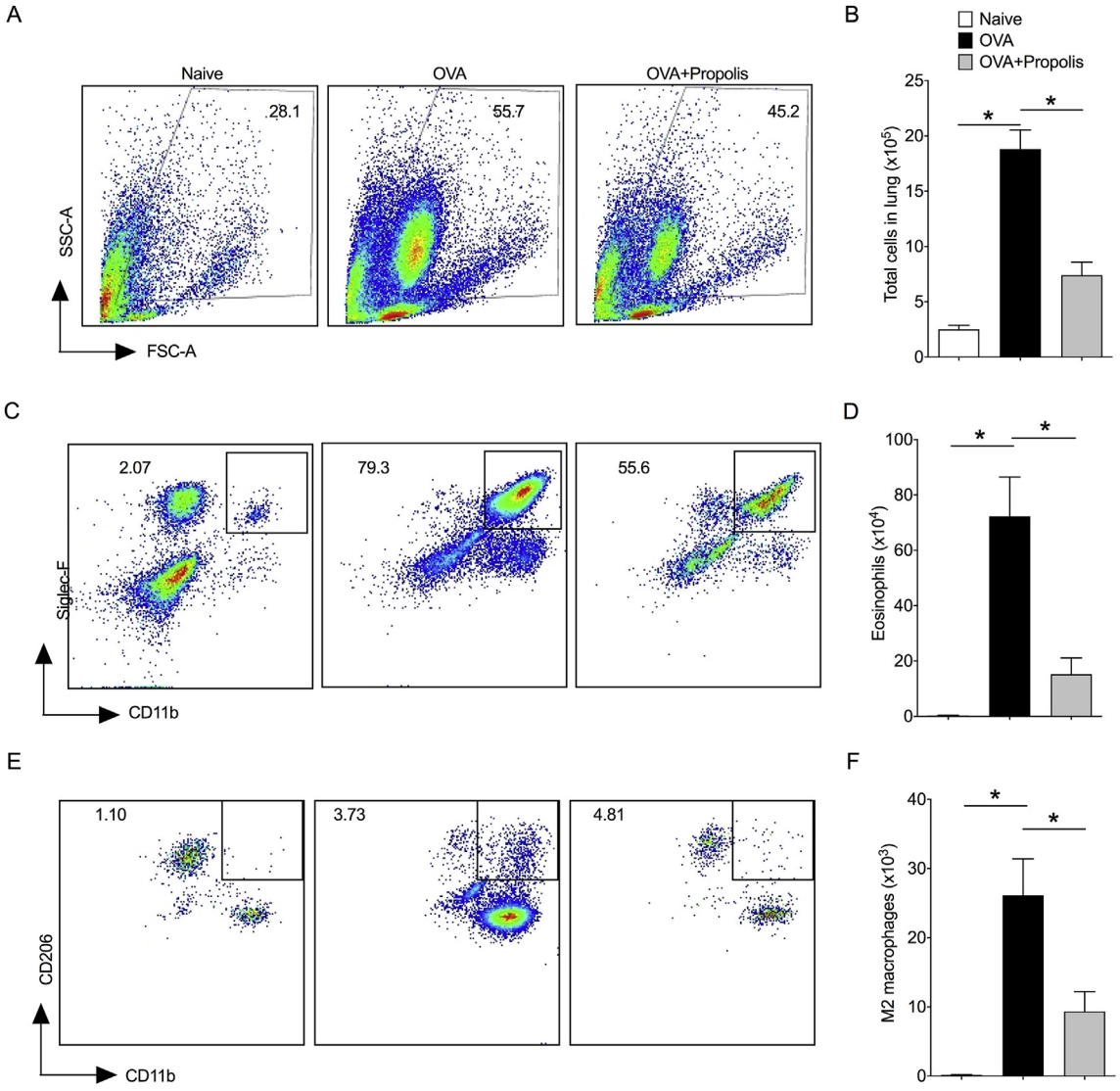

Fig. 3. Propolis decreases eosinophils and M2 macrophages in the lungs. Mice were treated with propolis as described in Figs. 1A and 72 h after OVA challenge, (A, B) total cell number, (C, D) eosinophils ($CD11b^+SinglecF^+$), and (E, F) M2 macrophages ($F4/80^+CD11b^+CD206^+$) were evaluated by flow cytometry. Data are representative of 2 independent experiments expressed as mean ± SEM (n = 5–7 mice per group). *$p < 0.05$.

et al., 2016). Because propolis enhances PMN-MDSC cell differentiation, we evaluated whether the increase of PMN-MDSC in the lungs of allergic mice treated with propolis would be associated with the increase of $CD4^+Foxp3^+$ Treg cells. We observed that propolis induced an increase of frequency and total number of $CD4^+Foxp3^+$ cells in the lungs of allergic mice compared to non-treated allergic group and naive group (Fig. 5A–C).

These collected findings show that propolis plays an additional effect in the differentiation/expansion of PMN-MDSC *in vivo* and *in vitro* and increase of Treg cells *in vivo*.

3.5. Propolis enhances differentiation of $CD4^+Foxp3^+$ cells

Next we evaluate whether propolis has a direct effect in the expansion of Treg cells. To address that, naive T cells isolated from spleen and lymph nodes were stimulated with TGF-β during four days in the presence or absence (vehicle) of propolis. Additional treatment with propolis significantly increased the differentiation of $CD4^+Foxp3^+$ cells *in vitro* (Fig. 6A, B). We also evaluated TIGIT (T cell Ig and ITIM domain receptor) at the surface of Treg cells since it was previously described that TIGIT-expressing Treg cells are no capable to suppress Th2 responses (Joller et al., 2014; Kourepini et al., 2016). Propolis reduced TIGIT expression on Treg cells (Fig. 6C, D). Together, these results showed that despite of propolis increase the differentiation of Treg cells *in vitro*, it attenuates the expression of TIGIT by these cells.

3.6. Propolis treatment during the challenge with allergen decreases Th2 immune response

Next to address the effect of propolis during established asthma, animals were treated with propolis after OVA challenges (Supplementary Fig. 1A). Propolis treatment reduced the frequency and the number of eosinophils in the BALF of allergic mice (Supplementary Figs. 1B and C). The decrease in the eosinophils was followed by reduction in IL-5 levels in the BALF (Supplementary Fig. 1D). Furthermore, we found a decrease in the secretion of IL-13 on OVA-stimulated cells obtained from lymph nodes of propolis treated animals (Supplementary Fig. E).

Collectively, these results show that EPP-AF® green propolis extract exhibits an immunomodulatory potential that ameliorates asthma-induced Th2 inflammation by increasing differentiation of PMN-MDSC and Treg cells.

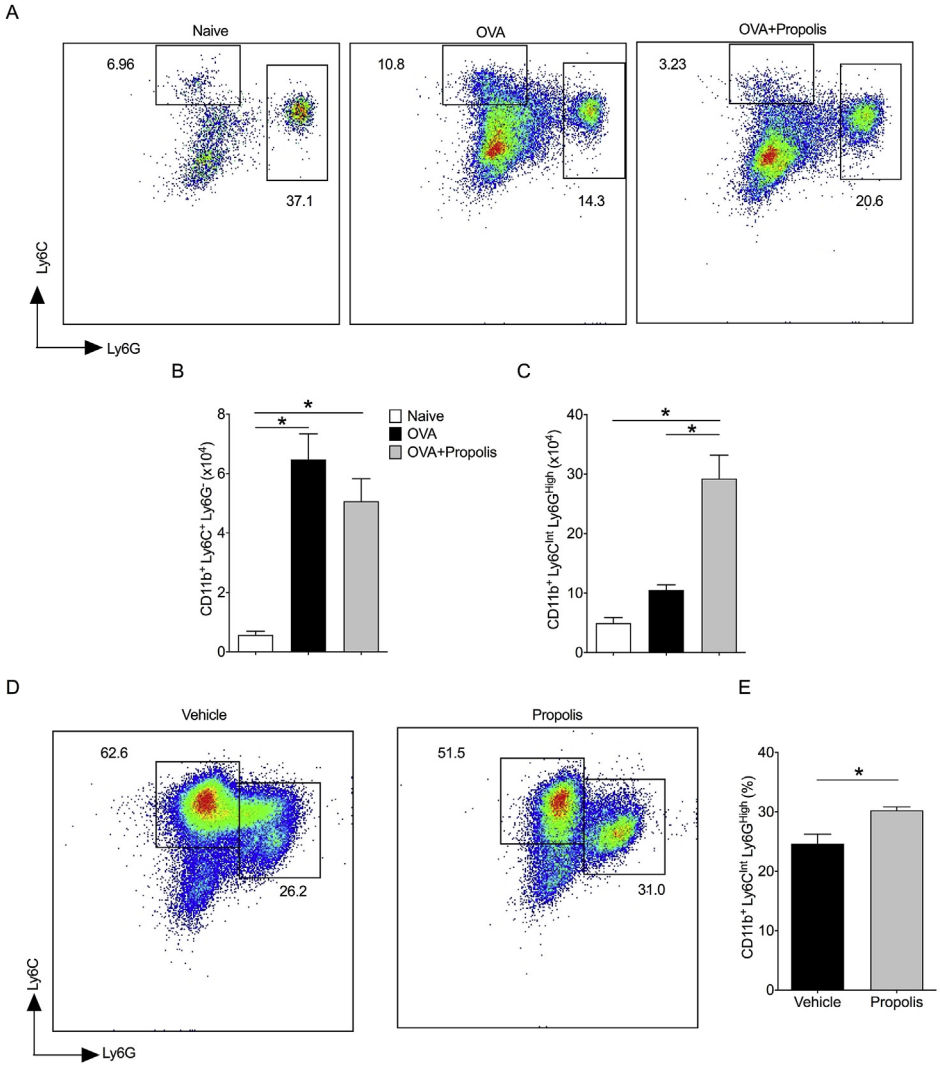

Fig. 4. Propolis enhances PMN-MDSC, but not M-MDSC cells, in the lung of allergic mice. Mice were treated with propolis as described in Figs. 1A and 72 h of OVA challenge, MDSC cells were evaluated in the lungs by flow cytometry. (A) Flow cytometry representative analysis. Total number of (B) M-MDSC cells ($CD11b^+Ly6C^+Ly6G^-$) and (C) PMN-MDSC ($CD11b^+Ly6G^{high}Ly6C^{low}$) cells in the lungs. (D) Flow cytometry representative analysis of MDSC subsets derived from bone marrow cells stimulated with GM-CSF and IL-6 in the presence or absence of propolis during 4 days. (E) Frequency of M-MDSC and PMN-MDSC cells. Data are representative of 2 independent experiments expressed as mean ± SEM (n = 3–5 mice per group). *P < 0.05.

4. Discussion

The study of natural products with medicinal properties has resulted in the discovery of different active compounds used for treatment of inflammatory disorders (Petrovska, 2012; Attiq et al., 2018). As reviewed by Sforcin (2007), propolis extract has been widely used, mainly for its anti-inflammatory effect, as a medicine in local and popular medicine, in ancient times by Egyptians, Greeks and Romans, and its use continues to this day in medicines and personal products. Was described that propolis decreases leukocyte infiltration and chemokine secretion in acute and chronic inflammatory diseases, such as arthritis and peritonitis (Franchin et al., 2016). In the context of asthma, propolis has also been shown to decrease polymorphnuclear cells in the BALF and in the peripheral blood, as well as decrease lung inflammation in experimental asthma model (Farias et al., 2014; El-Aidy et al., 2015; Ma et al., 2016).

Here we used the brazilian green propolis EPP-AF® and verified that there was an eosinophil reduction at the BALF and lungs of allergic mice after induction of experimental asthma. We showed for the first time that propolis treatment induces PMN-MDSC and $CD4^+Foxp3^+$ cells expansion, which are associated with the reduction of Th2 immune response.

M-MDSC and PMN-MDSCs, which accumulate in the blood, lymphoid organs and spleen during inflammatory conditions, down-modulate the immune response (Gabrilovich and Nagaraj, 2009; Solito et al., 2014). Under inflammatory conditions, PMN-MDSC produce arginase 1 and nitric oxide, which are involved in immune suppression or immune stimulation (Gabrilovich and Nagaraj, 2009; Lindau et al., 2013; Solito et al., 2014; Lee et al., 2016). Here we reported that propolis played an additional effect in the differentiation of PMN-MDSC *in vitro*, but not M-MDSC, and increased the number of PMN-MDSC, but not M-MDSC, into the lungs of animals exposed to the allergen.

Recently, it was reported that PMN-MDSCs, but not M-MDSCs, alleviated airway allergy by suppressing type 2 innate lymphoid cells (Cao et al., 2019). It remains to be investigated how PMN-MDSC could reduce Th2 inflammation induced by propolis treatment. Kitamura and coworkers showed that propolis induced MDSCs in the visceral adipose tissue of high fat diet fed mice and increase of IL-10 production (Kitamura et al., 2018). Therefore, the effect of propolis could be dependent on secretion of IL-10 by PMN-MDSC. Several studies have also shown that MDSC promote differentiation of Treg cells (Wang et al., 2016; Ren et al., 2017; Park et al., 2018). It also remains to be addressed whether PMN-MDSC would have a direct effect in the increase of $CD4^+Foxp3^+$ cells during propolis treatment.

The role of propolis in the expansion of Treg cells is still controversial. Rifa and Widodo (2014) described that propolis treatment increased Treg cells in naive mice while Kusnul and coworkers (2017) showed that propolis has not effect in the expansion of Treg cells in DMBA-induced Breast Cancer in rats. Recent studies showed that Treg cells are subpopulations that selectively regulate specific T cell responses (Chaudhry et al., 2009; Cipolletta et al., 2012; Burzyn et al., 2013a; Burzyn et al., 2013b). We suggest that propolis might selectively

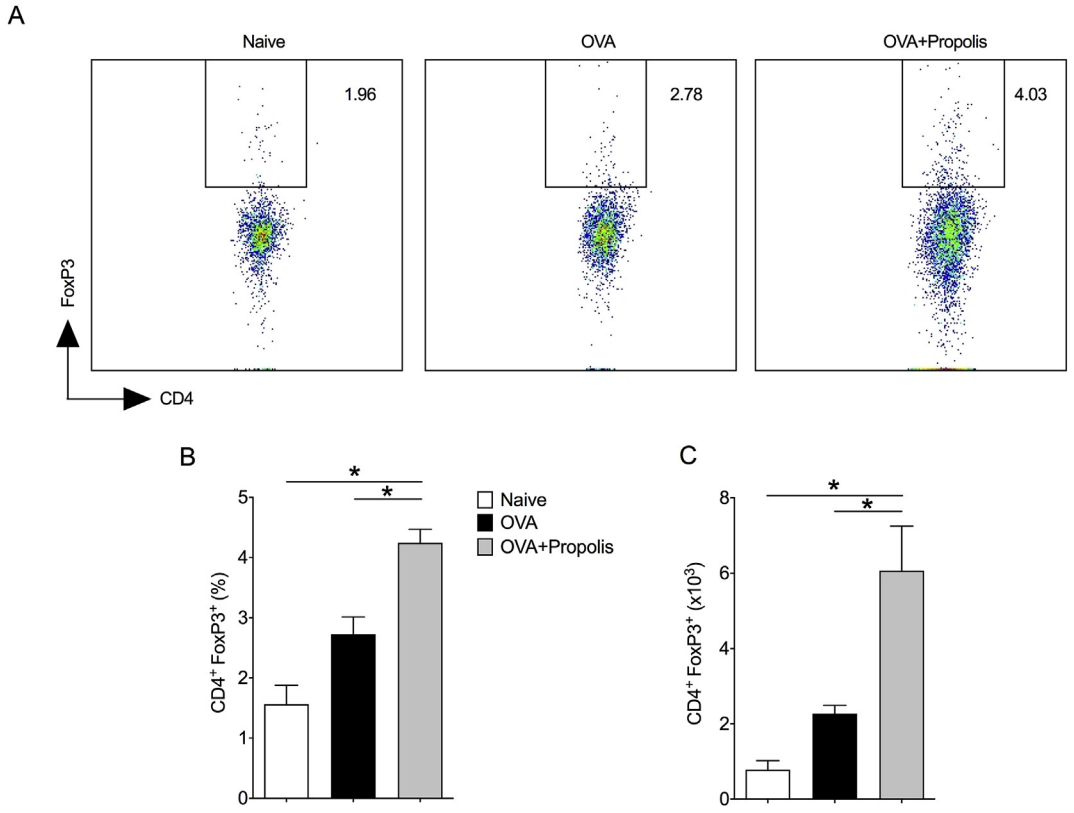

Fig. 5. Propolis enhances the frequency and number of $CD4^+Foxp3^+$ cells in the lungs. Mice were treated with propolis as described in Figs. 1A and 72 h after OVA challenge, Treg cells were evaluated in the lungs by flow cytometry. (A) Flow cytometry representative analysis. (B) Frequency and (C) total number of $CD4^+Foxp3^+$ cells in the lungs. Data are representative of 2 independent experiments expressed as mean ± SEM (n = 3–5 mice per group). *P < 0.05.

inhibit specific Treg cell subsets, as we showed the decrease of $CD4^+Foxp3^+TIGIT^+$. Joller and coworkers described that Treg cells expressing TIGIT inhibit Th1 and Th17, but not Th2 cells (Joller et al., 2014). Recently TIGIT was described to enhance Th2 immune response (Kourepini et al., 2016). Therefore, it is important to evaluate further the expression of TIGIT on $CD4^+IL\text{-}4^+$ or $CD4^+GATA3^+$ cells in lungs or draining lymph nodes of animals exposed to allergen and treated with propolis.

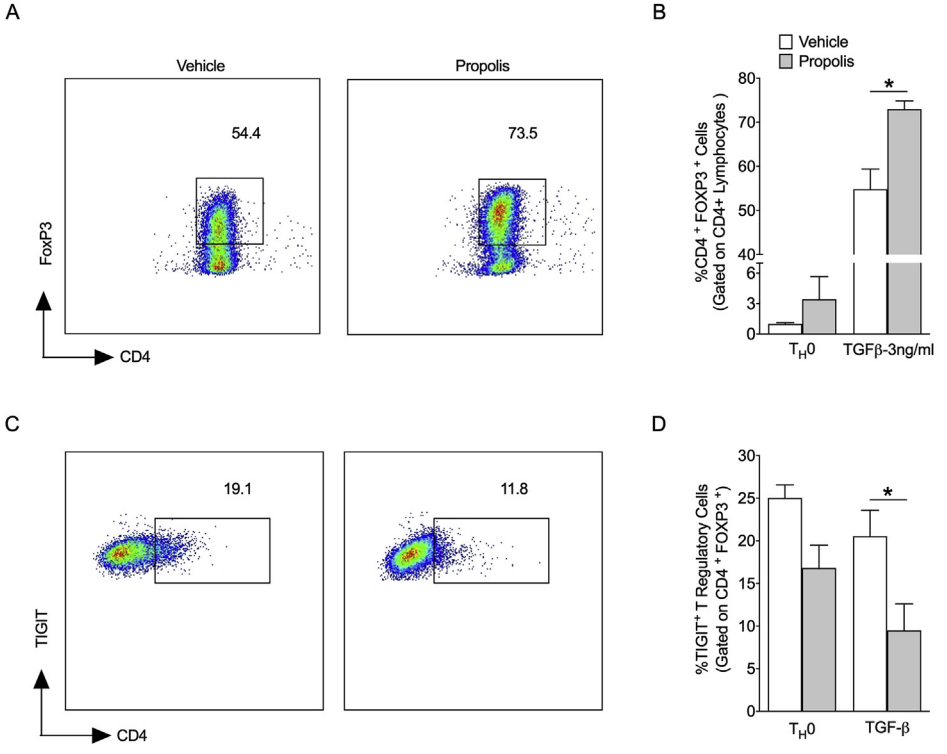

Fig. 6. Propolis enhances differentiation of $CD4^+Foxp3^+$ cells. Cells from spleen and mLNs were isolated from naive animals and cultured with 3 ng/mL of recombinant TGF-β. After 96 h, $CD4^+Foxp3^+$ cells were evaluated by flow cytometry. (A) Flow cytometry representative analysis. (B) Frequency of $CD4^+Foxp3^+$ cells. (C) Flow cytometry representative analysis. (D) Frequency of $TIGIT^+$-expressing Treg cells ($CD4^+Foxp3^+TIGIT^+$). Data are representative of 2 independent experiments expressed as mean ± SEM (n = 3 mice per group). *P < 0.05.

5. Conclusion

Our findings show that anti-inflammatory role of propolis in a model of airway Th2 inflammation is associated with increase of PMN-MDSC and $CD4^+Foxp3^+$ cells. Because propolis increases *in vitro* differentiation of PMN-MDSC and $CD4^+Foxp3^+$ cells, we suggest that propolis-mediated anti-inflammatory effect is dependent on suppressor myeloid cells and regulatory T cells. These results support the investigation of single bioactive components of EPP-AF® propolis extract as target for development of new immunotherapy or adjuvant immunotherapy for asthma.

Author contributions

A.R.P., J.I.H. and V.L.D.B. designed the research. A.R.P., M.H.F.L, T.R., A.F.G. and T.B.B. carried out the experiments. M.D.F. and A.A.B. performed chemical characterization of EPP-AF® extract. L.N.Z.R. performed histopathological analysis. A.R.P., J.I.H. and V.L.D.B. wrote the manuscript. F.Q.C. revised the manuscript. All the listed authors have read and approved the submitted manuscript.

Declaration of competing interest

The authors have no conflicts of interest to disclose.

Acknowledgments

We would like to thank Raul B. Ferreira from Apis Flora Company (Ribeirao Preto, Sao Paulo, Brazil) for providing the EPP-AF® propolis extract. The researchers leading to these results received funding from the São Paulo Research Foundation (FAPESP) under grant agreement n° 2011/09702-2, 2015/15963-4 and grant agreement n° 2013/08216-2 (Center for Research in Inflammatory Diseases - CRID) and from Coordination for the Improvement of Higher Education Personnel (CAPES).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2019.112496.

References

AlGabbani, Q., Mansour, L., Elnakady, Y.A., et al., 2017. Al-Quraishy S, Alomar S, Al-Shaebi EM, et al. In vivo assessment of the antimalarial and spleen-protective activities of the Saudi propolis methanolic extract. Parasitol. Res. 116 (2), 539–547.

Arora, M., Poe, S.L., Oriss, T.B., et al., 2010. TLR4/MyD88-induced CD11b+Gr-1 int F4/80+ non-migratory myeloid cells suppress Th2 effector function in the lung. Mucosal Immunol. 3 (6), 578–593.

Arora, M., Poe, S.L., Ray, A., et al., 2011. LPS-induced CD11b+Gr1(int)F4/80+ regulatory myeloid cells suppress allergen-induced airway inflammation. Int. Immunopharmacol. 11 (7), 827–832.

Attiq, A., Jalil, J., Husain, K., Ahmad, W., 2018. Raging the war against inflammation with natural products. Front. Pharmacol. 9, 976.

Bankova, V., Popova, M., Trusheva, B., 2014. Propolis volatile compounds: chemical diversity and biological activity: a review. Chem. Cent. J. 8, 28.

Barros, M.P., Sousa, J.P., Bastos, J.K., et al., 2007. Effect of Brazilian green propolis on experimental gastric ulcers in rats. J. Ethnopharmacol. 110 (3), 567–571.

Barud, H.A.S., de Araújo Júnior, A.M., Saska, S., et al., 2013. Mestieri LB, Campos JA, de Freitas RM, et al. Antimicrobial Brazilian Propolis (EPP-AF) Containing Biocellulose Membranes as Promising Biomaterial for Skin Wound Healing. Evid Based Complement Alternat Med 703024.

Berretta, A.A., Nascimento, A.P., Bueno, P.C., et al., 2012. Vaz MM, Marchetti JM. Propolis standardized extract (EPP-AF®), an innovative chemically and biologically reproducible pharmaceutical compound for treating wounds. Int. J. Biol. Sci. 8 (4), 512–521.

Borish, L., 2016. The immunology of asthma: asthma phenotypes and their implications for personalized treatment. Ann. Allergy Asthma Immunol. 117 (2), 108–114.

Bronte, V., Brandau, S., Chen, S.H., et al., 2016. Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat. Commun. 7, 12150.

Burzyn, D., Benoist, C., Mathis, D., 2013a. Regulatory T cells in nonlymphoid tissues. Nat. Immunol. 14 (10), 1007–1013.

Burzyn, D., Kuswanto, W., Kolodin, D., et al., 2013b. A special population of regulatory T cells potentiates muscle repair. Cell 155 (6), 1282–1295 2013.

Cao, Y., He, Y., Wang, X., Liu, Y., Shi, K., Zheng, Z., et al., 2019. Polymorphonuclear myeloid-derived suppressor cells attenuate allergic airway inflammation by negatively regulating group 2 innate lymphoid cells. Immunology 156 (4), 402–412. https://doi.org/10.1111/imm.13040. Epub 2019 Jan 17.

Castro, P.A., Savoldi, M., Bonatto, D., et al., 2011. Molecular characterization of propolis-induced cell death in Saccharomyces cerevisiae. Eukaryot. Cell 10 (3), 398–411.

Chaudhry, A., Rudra, D., Treuting, P., et al., 2009. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science 326 (5955), 986–991.

Cipolletta, D., Feuerer, M., Li, A., et al., 2012. PPAR-γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells. Nature 486 (7404), 549–553.

Coelho, L.G., Bastos, E.M., Resende, C.C., et al., 2007. Brazilian green propolis on Helicobacter pylori infection. a pilot clinical study. Helicobacter 12 (5), 572–574.

El-Aidy, W.K., Ebeid, A.A., Sallam, Al-R., et al., 2015. Evaluation of propolis, honey, and royal jelly in amelioration of peripheral blood leukocytes and lung inflammation in mouse conalbumin-induced asthma model. Saudi J. Biol. Sci. 22 (6), 780–788.

Farias, J.H., Reis, A.S., Araújo, M.A., et al., 2014. Effects of stingless bee propolis on experimental asthma. Evid Based Complement Alternat Med 951478.

Fleming, V., Hu, X., Weber, R., et al., 2018. Targeting myeloid-derived suppressor cells to bypass tumor-induced immunosuppression. Front. Immunol. 9, 398.

Fonseca, D.M., Wowk, P.F., Paula, M.O., Campos, L.W., et al., 2012. Recombinant DNA immunotherapy ameliorate established airway allergy in a IL-10 dependent pathway. Clin. Exp. Allergy 42 (1), 131–143.

Fonseca, D.M., Wowk, P.F., Paula, M.O., et al., 2015. Requirement of MyD88 and Fas pathways for the efficacy of allergen-free immunotherapy. Allergy 70 (3), 275–284.

Franchin, M., Cólon, D.F., Castanheira, F.V., et al., 2016. Vestitol isolated from Brazilian red propolis inhibits neutrophils migration in the inflammatory process: elucidation of the mechanism of action. J. Nat. Prod. 79 (4), 954–960.

Franchin, M., Freires, I.A., Lazarini, J.G., Nani, B.D., et al., 2018. The use of Brazilian propolis for discovery and development of novel anti-inflammatory drugs. Eur. J. Med. Chem. 153, 49–55.

Gabrilovich, D.I., Nagaraj, S., 2009. Myeloid-derived suppressor cells as regulators of the immune system. Nat. Rev. Immunol. 9 (3), 162–174.

Gabrilovich, D.I., Ostrand-Rosenberg, S., Bronte, V., 2012. Coordinated regulation of myeloid cells by tumours. Nat. Rev. Immunol. 12 (4), 253–268.

Hori, J.I., Zamboni, D.S., Carrão, D.B., et al., 2013. The inhibition of inflammasome by Brazilian propolis (EPP-AF). Evid Based Complement Alternat Med 418508.

Huang, M.T., Dai, Y.S., Chou, Y.B., 2009. Regulatory T cells negatively regulate neovasculature of airway remodeling via DLL4-Notch signaling. J. Immunol. 183 (7), 4745–4754.

Joller, N., Lozano, E., Burkett, P.R., et al., 2014. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. Immunity 40 (4), 569–581.

Josefowicz, S.Z., Niec, R.E., Kim, H.Y., et al., 2012. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature 482 (7385), 395–399.

Kabata, H., Artis, D., 2019. Neuro-immune crosstalk and allergic inflammation. J. Clin. Investig. 130.

Kitamura, H., Saito, N., Fujimoto, J., et al., 2018. Brazilian propolis ethanol extract and its component kaempferol induce myeloid-derived suppressor cells from macrophages of mice in vivo and in vitro. BMC Complement Altern. Med. 18 (1), 138.

Kourepini, E., Paschalidis, N., Simoes, D.C., et al., 2016. TIGIT enhances antigen-specific Th2 recall responses and allergic disease. J. Immunol. 196 (9), 3570–3580.

Lambrecht, B.N., Hammad, H., 2013. Asthma: the importance of dysregulated barrier immunity. Eur. J. Immunol. 43 (12), 3125–3137.

Lambrecht, B.N., Hammad, H., 2015. The immunology of asthma. Nat. Immunol. 16 (1), 45–56.

Lee, C.R., Kwak, Y., Yang, T., et al., 2016. Myeloid-derived suppressor cells are controlled by regulatory T cells via TGF-β during murine colitis. Cell Rep. 17 (12), 3219–3232.

Lemos, M., de Barros, M.P., Sousa, J.P., et al., 2007. *Baccharis dracunculifolia*, the main botanical source of Brazilian green propolis, displays antiulcer activity. J. Pharm. Pharmacol. 59 (4), 603–608.

Lindau, D., Gielen, P., Kroesen, M., et al., 2013. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology 138 (2), 105–115.

Ma, Y., Zhang, J.X., Liu, Y.N., et al., 2016. Caffeic acid phenethyl ester alleviates asthma by regulating the airway microenvironment via the ROS-responsive MAPK/Akt pathway. Free Radic. Biol. Med. 101, 163–175.

Machado, J.L., Assunção, A.K., da Silva, M.C., et al., 2012. Brazilian green propolis: anti-inflammatory property by an immunomodulatory activity. Evid Based Complement Alternat Med 157652.

Marquiafável, 2015. Development and characterization of a novel standardized propolis dry extract obtained by factorial design with high artepillin C content. In: P, A., SB, H., M-O, F., F, P., KB, J., B, A. (Eds.), Journal of Pharmaceutical Technology & Drug Research.

Miranda, M.M., Panis, C., Cataneo, A.H., et al., 2015. Nitric oxide and Brazilian propolis combined accelerates tissue repair by modulating cell migration, cytokine production and collagen deposition in experimental leishmaniasis. PLoS One 10 (5), e0125101.

Ortega, H.G., Liu, M.C., Pavord, I.D., et al., 2014. Mepolizumab treatment in patients with severe eosinophilic asthma. N. Engl. J. Med. 371 (13), 1198–1207.

Park, M.J., Lee, S.H., Kim, E.K., et al., 2018. Interleukin-10 produced by myeloid-derived suppressor cells is critical for the induction of Tregs and attenuation of rheumatoid inflammation in mice. Sci. Rep. 8 (1), 3753.

Park, Y.K., Paredes-Guzman, J.F., Aguiar, C.L., et al., 2004. Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern Brazilian

propolis. J. Agric. Food Chem. 52 (5), 1100–1103.
Petrovska, B.B., 2012. Historical review of medicinal plants' usage. Pharmacogn. Rev. 6 (11), 1–5.
Piñeros, A.R., Campos, L.W., Fonseca, D.M., et al., 2017. M2 macrophages or IL-33 treatment attenuate ongoing Mycobacterium tuberculosis infection. Sci. Rep. 7, 41240.
Prado, R.Q., Bertolini, T.B., Piñeros, A.R., et al., 2015. Attenuation of experimental asthma by mycobacterial protein combined with CpG requires a TLR9-dependent IFN-γ-CCR2 signalling circuit. Clin. Exp. Allergy 45 (9), 1459–1471.
Ren, J.P., Wang, L., Zhao, J., et al., 2017. Decline of miR-124 in myeloid cells promotes regulatory T-cell development in hepatitis C virus infection. Immunology 150 (2), 213–220.
Ren, J.P., Zhao, J., Dai, J., et al., 2016. Hepatitis C virus-induced myeloid-derived suppressor cells regulate T-cell differentiation and function via the signal transducer and activator of transcription 3 pathway. Immunology 148 (4), 377–386.
Rifa, 'i M., Widodo, N., 2014. Significance of propolis administration for homeostasis of CD4(+)CD25(+) immunoregulatory T cells controlling hyperglycemia. SpringerPlus 3, 526.
Robinson, D.S., Hamid, Q., Ying, S., et al., 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N. Engl. J. Med. 326 (5), 298–304.
Rocha, B.A., Bueno, P.C., Vaz, M.M., et al., 2013. Evaluation of a propolis water extract using a reliable RP-HPLC methodology and in vitro and in vivo efficacy and safety characterisation. Evid Based Complement Alternat Med 670451.
Sforcin, J.M., 2007. Propolis and the immune system: a review. J. Ethnopharmacol. 113 (1), 1–14.
Solito, S., Marigo, I., Pinton, L., et al., 2014. Myeloid-derived suppressor cell heterogeneity in human cancers. Ann. N. Y. Acad. Sci. 1319, 47–65.
Veglia, F., Perego, M., Gabrilovich, D., 2018. Myeloid-derived suppressor cells coming of age. Nat. Immunol. 19 (2), 108–119.
Wang, L., Zhao, J., Ren, J.P., et al., 2016. Expansion of myeloid-derived suppressor cells promotes differentiation of regulatory T cells in HIV-1+ individuals. AIDS 30 (10), 1521–1531.
Whitehead, G.S., Wilson, R.H., Nakano, K., et al., 2012. IL-35 production by inducible costimulator (ICOS)-positive regulatory T cells reverses established IL-17-dependent allergic airways disease. J. Allergy Clin. Immunol. 129 (1), 207–215 e1-5.
WHO, 2019. Asthma. https://www.who.int/respiratory/asthma/en/, Accessed date: 7 August 2019.
Xu, W., Lan, Q., Chen, M., et al., 2012. Adoptive transfer of induced-Treg cells effectively attenuates murine airway allergic inflammation. PLoS One 7 (7), e40314.