IMMUNOMODULATION OF *Uncaria tomentosa* OVER DENDRITIC CELLS, IL-12 AND PROFILE TH1/TH2/TH17 IN BREAST CANCER

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ABSTRACT

Objetives. This study aimed to research the *in vitro* immunomodulatory effects of an *Uncaria tomentosa* hydroalcoholic extract standardized (5.03%, pentacyclic oxindole alkaloids) (UT-POA) on the immunophenotype of dendritic cells (DC) subsets, Th1, Th2, Th17 and IL-12 cytokines from patients with stage II breast cancer (BCII) and healthy women (H). ***Materials and methods.*** Blood of 11 H and 7 BCII was obtained, PBMC were isolated and cultured for 2h with/without various concentrations of UT-POA and stimulated or not with LPS for 24h. PBMC were labeled with specific antibodies for DC and in the supernatant we measured Th1/Th2/Th17 cytokines, both by flow cytometry. Furthermore IL-12 was measured by ELISA. ***Results.*** UT-POA did not alter DC or accessory molecules expression in BCII. However, H exhibited a decrease in the percentage of mDC (myeloid DC) and an increase in HLA-DR and CD86 expression at 1000 µg/mL. IL-12 secretion was modified only in the H group, increasing p70 subunit and decreasing p40 subunit. UT-POA increased Th1 (IFN-γ and IL-2), Th2 (IL-4) and Th17 (IL-17) secretion in both groups. ***Conclusions.*** UT-POA increased the production of cytokines related with anti-tumoral response at concentrations of 500-1000 µg/mL. This positive effect should be evaluated not only systemically but also in the tumor microenvironment in further studies. UT-POA may be a useful phytochemical in chemoprevention and in the alternative use in cancer therapies.

Key words: Uncaria tomentosa; Dendritic cells; IL-12; Breast cancer (source: MeSH NLM).

INTRODUCTION

Uncaria tomentosa (Willd.) DC. (Rubiacaceae) (UT) or cat's claw is a vine that grows in the Peruvian jungles and in tropical areas of South and Central America. Indigenous groups such as the Asháninkas used this plant to improve their health [(1)]. UT is used as a contraceptive, an anticancer drug, and to treat inflammatory and gastrointestinal disorders. It is consumed as tea [(3)]. Sandoval-Chacón *et al*. and Aguilar *et al*. demonstrated its anti-inflammatory activity *in vitro* [(4,5)]. Aquino *et al*. did the same *in vivo* in murine inflammation models [(2)]. Dreifuss *et al*. showed its antitumor and antioxidant effects *in vivo* in a rat model of carcinoma [(6,7)].

The role of chronic inflammation during the development and progression of a tumor is known; therefore, the link between inflammation and cancer is not surprising [(8,9)]. UT has been shown to inhibit the translocation of NF-κB [(4,5)], a factor that activates genes involved in the anti-inflammatory response and in apoptosis regulation. In healthy cells, activation of NF-κB promotes

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apoptosis, but in malignant cells, it promotes survival, whereas inhibition of its activity may reduce tumor growth [10]. Allen-Hall L *et al*. showed that treatment with UT inhibits lipopolysaccharide (LPS)-dependent activation of specific components of the AP-1 signaling pathway [11]. An effective antitumor response requires active participation of antigen-presenting cells (APCs) responsible for presentation of tumor antigens [12]. APCs are important because defects in the function of lymphocytes that infiltrate the tumor in a patient with cancer (and T lymphocytes [TLs] of mice with tumors) can be completely reversed when an effective antigen presentation takes place and these lymphocytes are provided with exogenous IL-2. Dendritic cells (DCs) are the most powerful APCs [13,14]. DCs play a central role in antitumor immunity because they process tumor antigens and stimulate antigen-specific TLs [15,16]. An optimal costimulatory signal between the two cell types is necessary for effective presentation of antigens [12]. A population of isolated DCs from peripheral blood of patients with breast or head and neck cancer shows a reduced ability to group and stimulate allogeneic antigen-specific responses by TLs. Isolated DCs of these patients show a lower level of MHC II (HLA-DR) expression and of DC costimulatory molecules in comparison with healthy donors [17]. Our team has demonstrated that a hydroalcoholic UT extract reduces the proportion (%) of myeloid DCs (mDCs) and upregulates HLADR and CD86 in a dose-dependent manner in the peripheral blood mononuclear cells (PBMCs) of patients with rheumatoid arthritis, thus exerting an immunomodulatory effect [18].

The new paradigm of the immune balance not only involves CD8+ cytotoxic TLs, CD4+ TLs subsets, regulatory lymphocytes, γδ lymphocytes, natural killer cells, natural killer T cells, macrophages, DCs, and other innate-immunity cells but also proinflammatory cytokines and Th1, Th2, Th17, and immunoregulatory cytokines. IL-12 favors the Th1 profile, and Th1 is one of the most effective responses against tumors. Macrophages and DCs participate in the antitumor response by producing IL-12, among other functions inherent in innate immunity. In an *in vitro* study, we found that UT increases expression of IL-12p70 in PBMCs of healthy subjects (unpublished data); this result suggests that UT can promote a Th1 response. Domingues A. *et al*. demonstrated in healthy BALB/c mice that UT promotes the Th2 profile (IL-4, IL-5) in a dose-dependent manner but inhibits the Th1 profile (IL-2, TNF-α, IFN-k) [19]. Fazio A.L. *et al*. showed that in the peritoneal macrophages of C57/BL6 mice with B16/BL6 melanoma, UT downregulates IL-6 and NO but not TNF-α [20]. Urdanibia *et al*. uncovered an anti-inflammatory and antitumor effect of another species of the genus *Uncaria*, *U. guianensis,* when they observed inhibition of the growth of a breast tumor (4T1); this effect is probably related to downregulation of inflammatory mediators such as TNF-α and IL-6 via inhibition of the NF-κB pathway [21]. In the present study, we report *in vitro* antitumor activity of a UT hydroalcoholic extract as well as an immunomodulatory effect on DCs and on IL-12 and on Th1, Th2, and Th17 cytokines according to analysis of human PBMCs.

MATERIALS AND METHODS

HUMAN SUBJECTS

The participants were 10 healthy women (group H) with mammograms negative for breast adenocarcinoma (age 46.6 ± 9.1 years) and seven women with a histological diagnosis of stage II breast adenocarcinoma (age 54.7 ± 12.7 years; group BCII). The inclusion criteria were as follows: no history of treatment with any chemo- or radiotherapy or surgical intervention, no history of any other type of cancer, immunodeficiencies, and/or autoimmune diseases; no cytostatic drugs in the last 12 months, and no chronic diseases. Peripheral-blood samples were collected via venipuncture (36 ml).

HYDROALCOHOLIC UT EXTRACT STANDARDIZED AT 5.03% PENTACYCLIC OXINDOLE ALKALOIDS (UT-POA)

The extract was provided by Peruvian Heritage SAC. A hydroalcoholic extract was prepared from the bark of *U. tomentosa,* using the method of decoction with ethanol and water in the 7:3 ratio for 1 hour at 20°C, with subsequent spray drying to obtain a powder. The concentration of pentacyclic oxindole alkaloids was 5.03%, verified by highperformance liquid chromatography (HPLC) according to Dreifuss A.A, *et al*. A stock solution of UT-POA was prepared by diluting it to 30 g/L with double-distilled water; this solution was boiled for 30 minutes. After stationary incubation, the solution that was clear of solids was decanted and filtered twice through Whatman No. 3 paper and then microfiltered (pore size 0.22 µm). The stock solution was stored at –70°C.

ISOLATION of PBMCs

These cells were isolated by density gradient centrifugation with 1.077 g/mL Histopaque (Sigma, St. Louis, MO, USA). Beforehand, tubes had been prepared with 10 mL of Histopaque. The blood samples were

diluted 1:1 with complete RPMI 1640 (Sigma). The diluted blood (30 mL) was added onto the Histopaque and centrifuged at 400 × *g* for 30 minutes. The PBMCs were collected and stored on ice.

CELL CULTURE AND TEST GROUPS

The PBMCs were cultured in the RPMI 1640 medium supplemented with 10% bovine fetal serum (Hyclone), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, St Louis, MO, USA). We placed 10^6 PBMCs in cytometry tubes and incubated them at 37°C and 5% CO_2 for 2 hours with/without UT-POA. *E. coli* lipopolysaccharides were either added or not added (Sigma, St Louis, MO, USA) (LPS 1 µg/mL), standardized in our lab, and the cells were incubated at 37°C and 5% CO_2 for 24 hours. The test groups for BCII and H patients were as follows: A) Basal = PBMCs plus UT-POA vehicle; B) LPS = PBMCs plus vehicle and stimulated with LPS (1 µg/mL); C) UG50 = PBMCs plus UT-POA (50 µg/mL) and stimulated with LPS; D) UG500 = PBMCs plus UT-POA (500 µg/mL) and stimulated with LPS; and E) UG1000 = PBMC plus UT-POA (1000 µg/mL) and stimulated with LPS.

FLOW CYTOMETRIC ANALYSIS OF DCs AND HLA-DR/CD86

Prior to the flow cytometry, the culture supernatants were collected for quantification of the cytokines, and the cells were washed and resuspended in a cell wash solution (1% fetal bovine serum in PBS [pH 7.4]). The cells were immediately labeled with specific antibodies: anti-Lin1–FITC, anti-HLA-DR–PerCP, anti-CD11c–APC/anti-CD123–APC, and anti-CD86–PE (Becton Dickinson, San Jose, CA, USA). The labeled cells were incubated for 30 minutes at 2–8°C and washed twice with 1 mL of the cell wash solution with centrifugation at 400 × g for 5 minutes.

The cells were resuspended in 500 µL of the cell fixative (1% paraformaldehyde in PBS, pH 7.4) and stored at 4°C until analysis on a FACSCanto™ II flow cytometer (BD Immunocytometry Systems, USA). The data were analyzed in the Summit 4.3 software (Dako Colorado, Inc., USA). The percentages of myeloid DCs (mDCs; CD11c+HLADR+Lin-1-) and plasmacytoid dendritic cells (pDCs; CD123+HLA-DR+Lin-1-) were determined. The mean intensity of fluorescence (MIF) of HLA-DR and CD86 was measured in each subset.

QUANTIFICATION OF TH1, TH2, and TH17 CYTOKINES BY MEANS OF A CYTOMETRIC BEAD ARRAY (CBA)

A CBA kit was used to quantify the Th1, Th2, and Th17 cytokines (BD, San Jose CA, USA) in 50 µL of the cell culture supernatant. The following proteins were analyzed: interferon γ (IFN-γ), interleukin 2 (IL-2), tumor necrosis factor α (TNF-α), IL-4, IL-6, IL-10, and IL-17A. The manufacturer's recommendations were followed strictly. The data were acquired on a FACSCanto™ II flow cytometer (BD Immunocytometry Systems, USA) and analyzed in the BD™ Cytometric Bead Array Software, version 1.4.

MEASUREMENT OF IL-12 AND IL-6 LEVELS BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

We used ELISA kits (EIAOpt BDBiosciences, San Diego, CA, USA) for quantification of IL-12p40, IL-12p70, and IL-6. The manufacturer's recommendations were followed strictly. The IL-6 levels that were determined by CBA were above the upper boundary of the detection limit (5000 pg/mL); therefore, it was necessary to dilute the samples to analyze them with an ELISA.

STATISTICAL ANALYSIS

The results are expressed as mean ± standard error of the mean. Kruskal-Wallis nonparametric tests were performed and followed by Dunn's multiple-comparison test and the *t* test followed by the Mann-Whitney (two-tailed) rank-sum test in the GraphPad Prism software, version 6.00 for Mac (GraphPad Software, La Jolla, CA, USA). Differences with $p < 0.05$ were considered statistically significant.

ETHICAL CONSIDERATIONS

The Human Ethics Institutional Committee of the Cayetano Heredia Peruvian University approved this study's protocol (registration code 57825), and each participant signed an informed consent form.

RESULTS

mDCs, pDCs, and HLA-DR/CD86

The quantification of mDCs, pDCs, and of expression of HLA-DR and CD86 was performed on 10^6 PBMCs. After treatment with UT-POA, a dose-dependent decrease in % of mDCs in group H was observed; this effect was significant at doses 500 and 1000 µg/mL ($p < 0.05$; Figure 1). Group BCII showed a significant decrease in % of mDCs at 1000 µg/mL UT-POA ($p < 0.05$; Figure 1A). UT-POA significantly decreased % of pDCs at 1000 µg/mL ($p < 0.05$) in group H (Figure 1B).

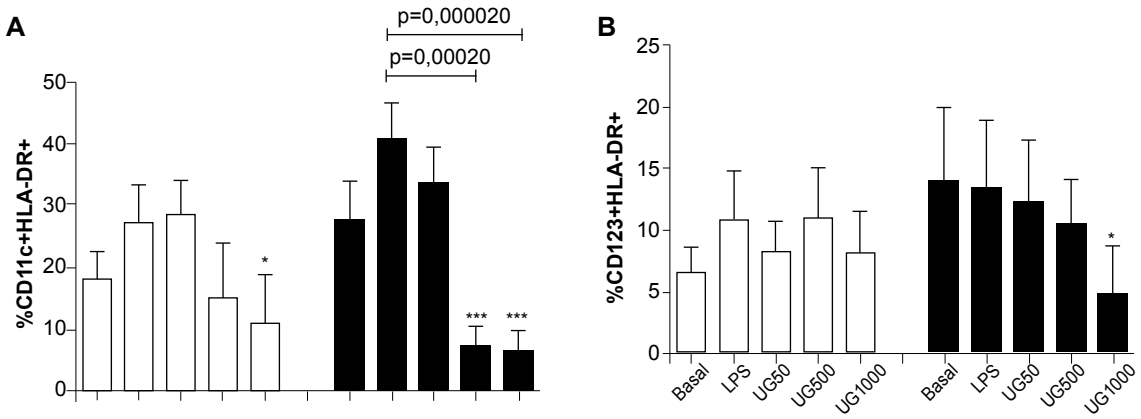

Figure 1. Effects of UT-POA on the percentage of A) mDCs (CD11c+HLA-DR+) and B) pDCs (CD123+HLA-DR+). We cultured 10^6 PBMCs per tube and incubated them for 2 hours with UT-POA (50, 500, or 1000 µg/mL) except for the Basal and LPS groups, which received vehicle. Later, all groups except Basal were stimulated with LPS (1 µg/mL) for 24 hours. Finally, the cells were labeled with DC-specific antibodies and analyzed by flow cytometry. The data were acquired, and the cell population percentages were quantified by means of Summit 4.3 software. The data represent mean ± standard error of the mean for group BCII (n = 7) and group H (n = 10). The Kruskal-Wallis test was performed, followed by Dunn's multiple-comparison test and the *t* test followed by the Mann-Whitney (two-tailed) test (GraphPad Prism v. 6.00). Differences with $p < 0.05$ relative to group LPS were considered significant. BCII = patients with stage II breast cancer; H = healthy women with mammograms negative for cancer; UT-POA = *Uncaria tomentosa* hydroalcoholic extract with 5.03% pentacyclic oxindole alkaloids; mDCs = myeloid dendritic cells; pDCs = plasmacytoid dendritic cells; and PBMCs = peripheral blood mononuclear cells; *$p < 0.05$; ***$p < 0.001$.

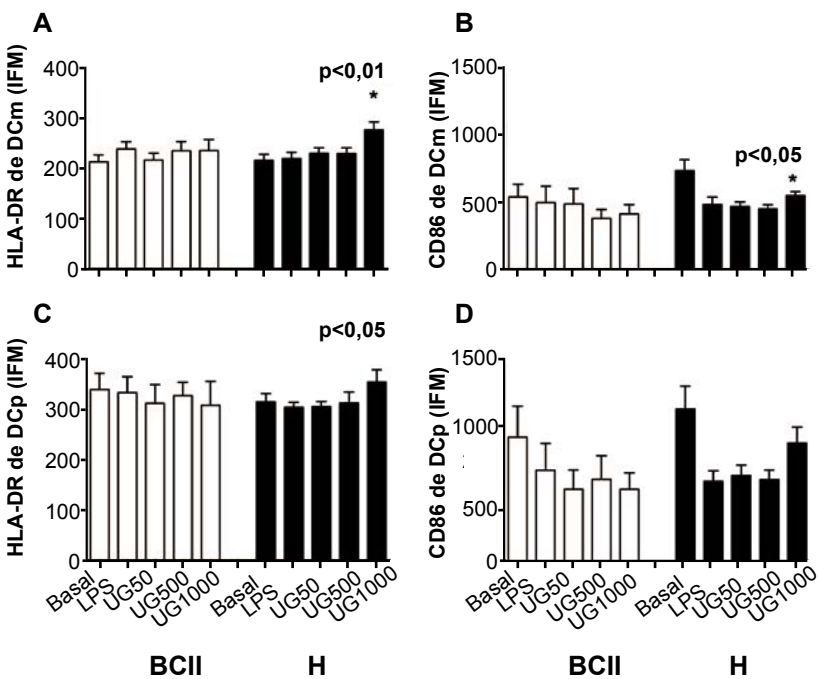

Figure 2. Effects of UT-POA on the expression of DC maturity molecules and DC activation molecules. We cultured 10^6 PBMCs per tube and incubated them for 2 hours with UT-POA (50, 500, or 1000 µg/mL) except for the Basal and LPS groups, which received vehicle. Later, all groups except Basal were stimulated with LPS (1 µg/mL) for 24 hours. Finally, the cells were labeled with antibodies specific to HLA-DR and CD86 and were analyzed by flow cytometry. The data were acquired, and the molecules' MIF was analyzed in Summit 4.3. A) The level of HLA-DR expression in mDCs; B) expression of CD86 in mDCs; C) the level of HLA-DR expression in pDCs; D) levels of CD86 expression in pDCs. The data represent mean ± standard error of the mean of group BCII (n=7) and group H (n=10). The Kruskal-Wallis test was performed followed by Dunn's multiple comparison test and the *t* test followed by the Mann-Whitney (two-tailed) test (GraphPad Prism v. 6.00). Differences with $p < 0.05$ relative to the LPS control were considered significant. BCII = patients with stage II breast cancer; H = healthy women with mammograms negative for cancer; UT-POA = *Uncaria tomentosa* hydroalcoholic extract with 5.03% pentacyclic oxindole alkaloids; HLA-DR = human leukocyte antigen, DR haplotype or type II major histocompatibility complex; CD86 = costimulatory molecule; MIF = mean fluorescence intensity; PBMCs = peripheral blood mononuclear cells, *$p < 0.05$.

The HLA-DR molecule in mDCs and pDCs was upregulated significantly ($p < 0.05$) in group H at the UT-POA dose 1000 µg/mL, whereas no change was detected in group BCII (Figure 2A and 2C). No changes in CD86 expression were observed (Figure 2B and 2D).

IL-12p70 and IL-12p40

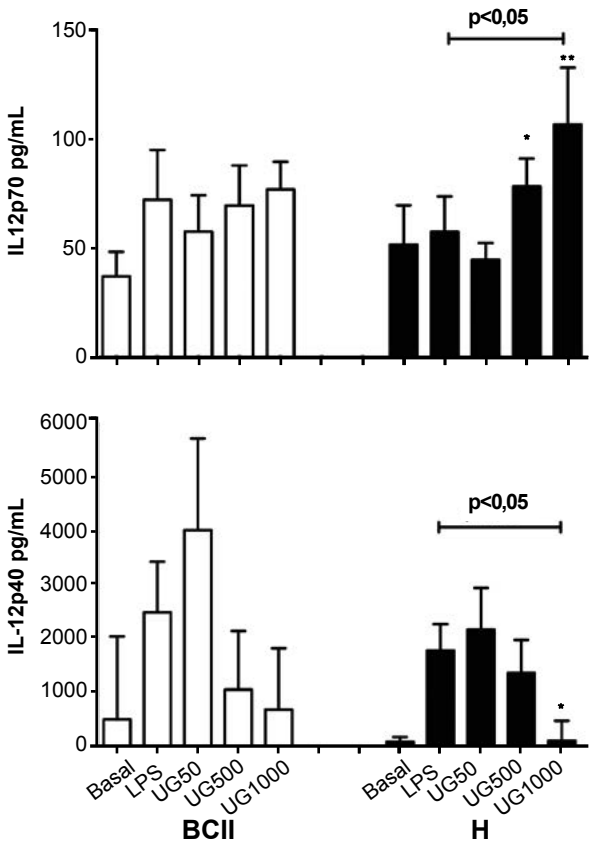

Figure 3. Measurement of IL-12p70 and IL-12p40 levels (pg/mL). After the isolation and culture of PBMCs for the flow cytometry, the culture supernatants were collected to quantify the two IL-12 isoforms by an ELISA. A) Increased levels of IL-12p70 that were significant relative to group H at the UT-POA dose 1000 µg/mL. B) Reduced levels of IL-12p40 that were significant relative to group H at the UT-POA dose 1000 µg/mL. The data represent mean ± standard error of the mean of 7 datapoints in group BCII and 10 datapoints in group H. The Kruskal-Wallis nonparametric test was performed, followed by Dunn's multiple comparison test. Differences with $p < 0.05$ relative to the LPS control were considered significant. BCII = patients with stage II breast cancer; H = healthy women with mammograms negative for cancer; PBMCs = peripheral blood mononuclear cells; *$p < 0.05$; **$p < 0.01$.

IL-12p70 was upregulated significantly in group BCII in response to 1000 µg/mL UT-POA ($p < 0.05$); however, it increased in a dose-dependent manner in group H (significantly in response to doses 500 and 1000 µg/mL; $p < 0.05$; Figure 3A). In both groups, IL-12p40 showed dosedependent downregulation, significant only in group H at the UT-POA dose 1000 µg/mL in comparison with LPS ($p < 0.05$; Figure 3B).

Th1 CYTOKINES

In groups BCII and H, IFN-γ and IL-2 levels increased in a dose-dependent manner at UT-POA doses 500 and 1000 µg/mL ($p < 0.05$) as compared to LPS treatment. Higher levels of IFN-γ and IL-2 were observed in group H than in group BCII (Figure 4A and 4B). The levels of TNFα did not change in any group (Figure 4C).

Th2 CYTOKINES

There was a dose-dependent increase in IL-4 levels in groups BCII and H ($p < 0.05$) at UT-POA doses 500 and 1000 µg/mL as compared to LPS treatment. We also found that group H produced more IL-4 than group BCII did (Figure 4D). IL-6 and IL-10 levels did not change (Figure 4E and 4F). In contrast, IL-6 levels decreased significantly at the UT-POA dose 50 µg/mL in group H; these IL-6 concentrations later remained at the same level as those in the LPS group (Figure 4F). There was a dose-dependent trend toward an increase in IL-10 levels, with a higher concentration of this cytokine observed in group BCII (Figure 4E).

Th17 CYTOKINES

There was a dose-dependent increase in IL-17 levels in groups BCII and H, and this effect became statistically significant at the UT-POA dose 1000 µg/mL ($p < 0.05$; Figure 4G).

DISCUSSION

UT is an immunomodulatory, anti-inflammatory and antioxidant phytochemical. In other studies, researchers found correlations of the concentration of pentacyclic oxindole alkaloids (POAs) in its leaves, bark, and roots with the immunomodulatory properties of this plant [22]. Several phytochemicals that were prepared from the ethanolic extracts of the bark and from alkaloid-rich parts of UT have been standardized by their oxindole alkaloid content [1,23]. Nevertheless, this situation does not mean that the POAs are the cause of UT's effects because there are products such as C-Med 100®—a water-soluble and ultra-filtered UT patented extract (for removal of high-molecular-weight conjugates; >10 kDa)—which contains carboxy alkyl esters (CAEs) as active ingredients (8–

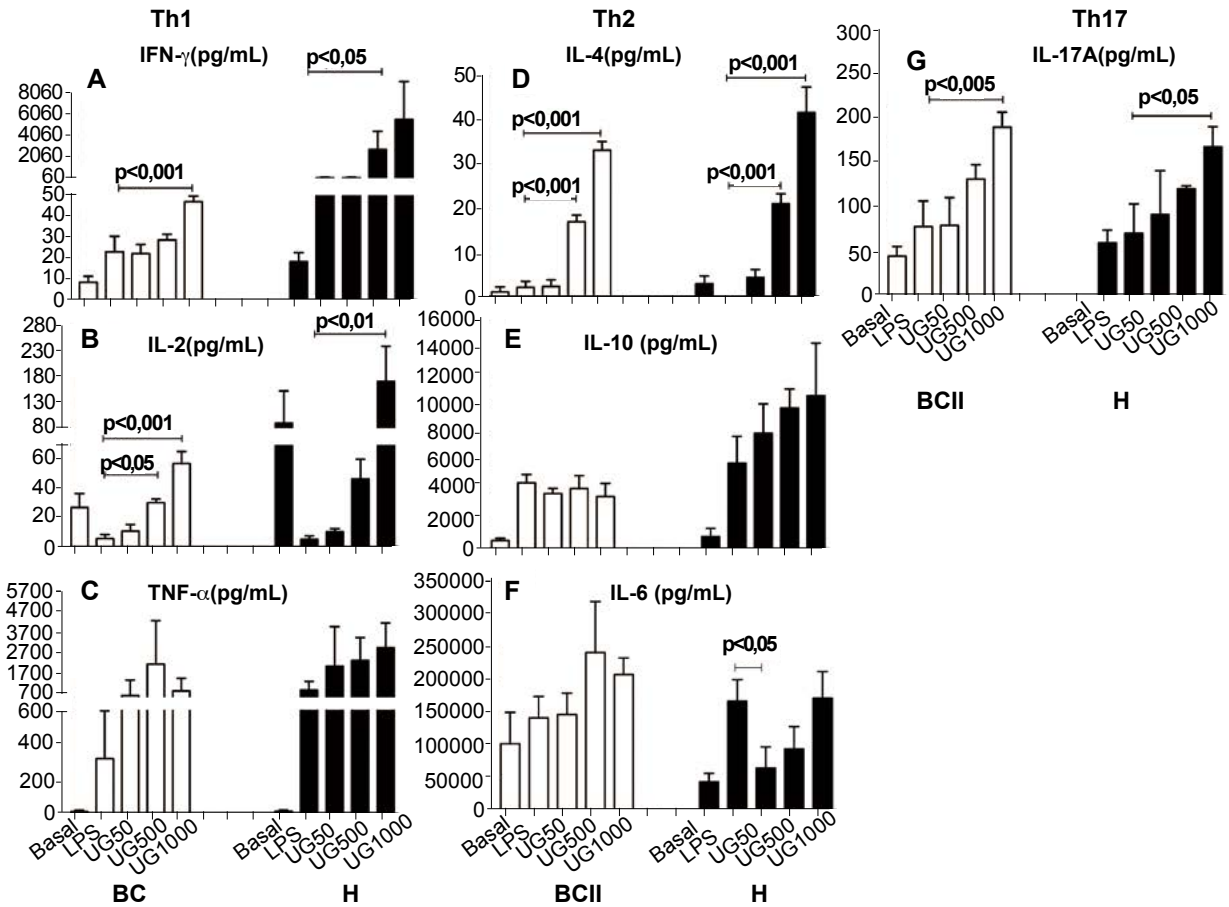

Figure 4. Measurement of cytokines from the pathways Th1, Th2, and Th17. After the isolation and culture of PBMCs for the flow cytometric analysis, the culture supernatants were collected to quantify the Th1, Th2, or Th17 cytokines by CBA. A) IFN-γ; B) IL-2; C) TNF-α; D) IL-4; E) IL-10, and F) IL-6. The data represent mean ± standard error of the mean of 7 datapoints in group BCII and 10 datapoints in group H. The Kruskal-Wallis test was performed, followed by Dunn's multiple-comparison test and the *t* test followed by the Mann-Whitney (two-tailed) test (GraphPad Prism v. 6.00). Differences with $p < 0.05$ relative to the LPS control were considered significant. BCII = patients with stage II breast cancer; H = healthy women with mammograms for negative cancer: CBA = cytometric bead array; PBMCs = peripheral blood mononuclear cells; *p < 0.05; **p < 0.01; ***p < 0.001.

10%) and is almost free of oxindole alkaloids (≤0.05%). Yet this product has immunomodulatory effects [24]. Therefore, it is possible that various active ingredients of this plant work in synergy.

We tested whether UT is capable of modifying the immunophenotype of DCs and can influence production of IL12 and Th1, Th2, and Th17 cytokines *in vitro* by human PBMCs. We found that UT-POA apparently has opposite immunomodulatory effects on DCs and on their HLA-DR marker: in the case of DCs, UT-POA upregulates these cell-specific markers; as for HLA-DR, UT-POA increases the expression of this maturity-related molecule. UT-POA did not induce activation of these cells because it has no effect on the costimulatory molecule CD86. There are few studies on the effects of UT on human DCs. Kim K.S. *et al*. tested other species such as *Uncaria rhynchophylla*, and in particular uncarinic acid (URC), and found that the DCs isolated from healthy human PBMCs increase the expression of CD1A, CD38, CD40, CD54, CD80, CD83, CD86, HLA-DR, and DC-Lamp after treatment with URC in the range 0.001 to 10 μM [25]. In the case of UT, Nunez C. *et al*. and Lozada-Requena *et al*. showed that the proportion (%) of mDCs among PBMCs of patients with rheumatoid arthritis and in healthy subjects decreases in a dose-dependent manner, whereas HLA-DR and CD86 are upregulated in patients with arthritis but undergo no changes in healthy subjects [18,26]. We confirmed the findings of Lozada-Requena *et al*. regarding the decrease in % of mDCs in group H, and we observed the same result in group BCII. Nevertheless, in the case of % of pDCs, our

results were the opposite because we actually observed downregulation of this subset in group H. It is possible that these patients' disease has a protective/blocking effect toward UT-POA, via typical evasion mechanisms of tumors and via factors released by tumors (or some other unknown mechanism). Accordingly, we did not detect any immunomodulatory effect of UT-POA on the pDCs and HLA-DR/CD86 cells of patients with breast cancer, except for a decrease in the number of mDCs at high doses. The systemic downregulation of this mDC subset should not be necessarily viewed as a bad prognosis. This is because DCs represent 0.1–1.0% of blood immunocompetent cells, and migration of DCs to the tumor microenvironment is possible. Additional studies are needed with standardized extracts at different doses and in the tumor microenvironment.

The production of IL-12p70 increased while that of IL-12p40 decreased. IL-12p70 is responsible for the DC-mediated Th1 polarization. Our findings show that PBMCs that are stimulated *in vitro* with UT-POA produce greater amounts of cytokines of the types Th1 (IFN-γ and IL-2), Th2 (IL-4), and Th17 (IL-17) in groups BCII and H. Although these effects are observed at high doses, we must take into account that the 50% inhibitory concentration (IC_{50}) of UT for human PBMCs is 16 mg/mL, which indicates that UT does not affect cellular viability of these PBMCs [18]. We propose measuring metabolic viability of these cells in terms of UT. It should also be noted that during the preparation of UT, there are filtering steps that may dilute the extract; therefore, the concentrations that we used can serve as a reference.

We demonstrated that expression of neither IL-12p70 nor the IL-12p40 subunit was changed by UT-POA in group BCII. In contrast, IL-12p70 was upregulated and IL-12p40 was downregulated in group H. A possible explanation is that a part of IL-12p40 dimerizes with IL-12p35 to form IL-12p70. It is well known that the IL-12 family consists of IL-12, IL-23, and IL-27, and that IL-12p40 not only generates IL-12 but also forms IL-23 by joining with IL-12p19. Therefore, it is possible that a part of IL-12p40, which was downregulated in group H, dimerized with the subunit IL-12p19 [27.28]. This hypothesis should be tested by measuring IL-23. According to our findings, at least in healthy subjects, UT-POA promotes production of IL-12. Patients (group BCII) may have a PBMC disorder that inhibits a favorable response to treatment with the UT-POA extract. The reason for this lack of response is the same as the reason we proposed for DCs, that is, tumor evasion or other unknown mechanisms.

Regarding the Th1/Th2/Th17 profile, we found that UT-POA increased expression of cytokines of the types Th1 (IFN-γ and IL-2), Th2 (IL-4), and Th17 (IL17A) in groups BCII and H. There are few studies on the effects of UT on Th1, Th2, and Th17 cytokines. Domingues A. *et al*. reported that the splenocytes of mice treated with a hydroalcoholic cat's claw extract have a Th2 profile characterized by an increase of IL-4 and IL-5 levels at the dose 500 µg/mL, whereas the same concentration produced a reduction in concentrations of Th1 cytokines (IFN-γ, IL-2, and TNF-α) [19]. Fazio A.L. *et al.* found that a Th1 response (TNF-γ) and Th2 response (IL-6) were reduced in the supernatant of peritoneal macrophage cultures treated with UT after LPS stimulation. Those authors also demonstrated that this reduction correlates with a deficiency in the NF-κB pathway [20]. Although those studies were performed on animal models, we demonstrated that cat's claw can have different effects on the expression of different cytokines, and that the results may not be extrapolated from mice to humans. We showed induction of a Th1 response in group H, probably due to the IL-12p70 downregulation, and this change can therefore explain the upregulation of IFN-γ and IL-2. It is possible, however, that in group BCII, UT-POA directly stimulates expression of IFN-γ and IL-2 via mechanisms that need to be researched further. Both cytokines are important for the immune response, whereas IL-2 works as a growth factor necessary for the survival and differentiation of TLs and is produced mainly by CD4+ TLs. This observation can explain why the presence or upregulation of IL-2 can improve an adaptive antitumor response. IFN-γ performs a direct function in antitumor immunity, not only in adaptive immunity but also in innate immunity, because this cytokine can have different effects on endothelial and stromal components in the tumor microenvironment. These effects may induce chemokines that attract effector cells from both arms of the immune system and downregulate angiogenesis and adhesion molecules of endothelial cells.

As for TNF-α, inhibition of its production by UT is well known. Various studies have shown an anti-inflammatory effect of UT in a variety of *in vitro* and *in vivo* models. Our results do not show TNF-α changes in the test groups. It is possible that unknown mechanisms prevent its inhibition or that our duration of treatment with UT-POA and duration of the LPS stimulation were not sufficient, and that these variables should be standardized for this particular model. Furthermore, the other studies were performed on standardized cell lines or on specific cell lines such as peritoneal macrophages. In contrast, we used PBMCs, which produce other leukocytes, which may cause greater variability in the measurements.

UT-POA increased the IL-4 levels in both groups. TLs, mast cells, and other cell populations are the source of IL-4, and it is required for Th2 differentiation. The use of PBMCs implies that the main source of IL-4 is TLs. Although a reduced Th2 profile can be expected due to a prevalent Th1 profile, UT-POA also enhanced the Th2 (IL-4) profile, showing once again its immunomodulatory effect. Despite the weak relevance of IL-4 to antitumor immunity, because this cytokine can induce polarization of M2 or TAM (tumor-associated macrophages) that are more protumor, IL-4 is expected to collaborate with B lymphocytes that produce IgG and IgE, and thereby to increase the expression of MHC. Therefore, these effects should also improve activation of TLs and the function of eosinophils [8].

Some antibodies against molecules present in cancer cells may inhibit oncogenic signals and promote tumor destruction by binding to Fc receptors of macrophages, granulocytes, and NK cells. Antibodies may also promote presentation of tumor antigens by APCs via formation of an immune complex [8]. For these reasons, the role Th2 (IL-4) differentiation in the antitumor response should not be ignored.

We showed that IL-10 was not affected by UT-POA in either group BCII or group H. In contrast, production of IL-6 decreased at UT-POA dose 50 µg/mL initially, and then in a dose-dependent manner, it reached the levels of the LPS group. Lemaire *et al.* demonstrated an inhibitory effect of two UT aqueous extracts on IL-6 in rat alveolar macrophages [29], but here, we demonstrated that UT-POA can have different effects at different concentrations. These findings underscore the importance of determining the optimal concentration at which these effects occur.

Th17 lymphocytes were recently identified in humans and are reported to play a proinflammatory role in autoimmune diseases. Th17 lymphocytes are also necessary for the antitumor response because they induce production of chemokines by primary tumors, which attract DCs, TLs, and NK cells to the tumor microenvironment [30]. On the other hand, there is also evidence of a correlation between regulatory TLs and LTh17 in patients with invasive breast cancer, suggesting that upregulation of these subsets is the result of conditions that favor aggressiveness of the tumor and poor prognosis. In other tumors, such as ovarian tumors, Th17 cells induce Th1-type chemokines and the recruitment of effector cells. Thus, Th17 cells and regulatory TLs can play a dual role in the tumor microenvironment [31]. These apparent discrepancies involving Th17 and IL-17A should be elucidated as soon as possible. Currently there are no studies that assess the effects of UT-POA on the expression of IL-17. Our results show that there is a dose-dependent increase in the production of IL-17A in groups H and BCII. The production of IL-17A was found to be always higher in group H than in group BCII, suggesting that although breast cancer reduces the secretion of IL-17A, this process is restored by UT-POA.

The data obtained in the PBMC experiments involving patients with breast cancer point to a clinically important immunomodulatory effect of cat's claw on the expression of cytokines of the types Th1 (IFN-γ and IL-2), Th2 (IL-4), and Th17 (IL-17A). This effect needs further research to confirm the cytoprotective activity of this phytochemical during an antitumor response.

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