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Mitraphylline inhibits lipopolysaccharide-mediated activation of primary human neutrophils

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ABSTRACT

Background: Mitraphylline (MTP) is the major pentacyclic oxindolic alkaloid presented in *Uncaria tomentosa*. It has traditionally been used to treat disorders including arthritis, heart disease, cancer, and other inflammatory diseases. However, the specific role of MTP is still not clear, with more comprehensive studies, our understanding of this ancient herbal medicine will continue growing.
Hypothesis/Purpose: Some studies provided its ability to inhibit proinflamatory cytokines, such as TNF-α, through NF-κB-dependent mechanism. TNF-α primes neutrophils and modulates phagocytic and oxidative burst activities in inflammatory processes. Since, neutrophils represent the most abundant pool of leukocytes in human blood and play a crucial role in inflammation, we aimed to determine the ability of MTP to modulate neutrophil activation and differentially regulate inflammatory-related cytokines.
Methods: To determine the mechanism of action of MTP, we investigated the effects on LPS-activated human primary neutrophils responses including activation surface markers by FACS and the expression of inflammatory cytokines, measured by real time PCR and ELISA.
Results: Treatment with MTP reduced the LPS-dependent activation effects. Activated neutrophils ($CD16^+CD62L^-$) diminished after MTP administration. Moreover, proinflamatory cytokines (TNF-α, IL-6 or IL-8) expression and secretion were concomitantly reduced, similar to basal control conditions.
Conclusion: Taken together, our results demonstrate that MTP is able to elicit an anti-inflammatory response that modulates neutrophil activation contributing to the attenuation of inflammatory episodes. Further studies are need to characterize the mechanism by which MTP can affect this pathway that could provide a means to develop MTP as new candidate for inflammatory disease therapies.

Introduction

Uncaria tomentosa (Willdenow ex Roemer & Schultes) DC. (Rubiaceae) is a Peruvian thorny liana, traditionally used in folk medicine to deal with many ailments, such as viral infections, gastric illnesses (gastric ulcers), arthritis and other inflammatory disorders as well as, antitumoral agent (Dietrich et al., 2014; Rojas-Duran et al., 2012; Muller and Kanfer, 2011; Rosenbaum et al., 2010; Garcia-Prado et al., 2007). *U. tomentosa's* bark contains a series of secondary metabolites, such as oxindole alkaloids and polyphenols (flavonoids, proanthocyanidins, tannins) and small concentrations of other secondary metabolites, such as quinovic acid glycosides, polyhydroxylated triterpenes and saponins (Laus, 2004; Aquino et al., 1990, Aquino et al., 1989).

Most of the alkaloids contained in *U. tomentosa* have been well recognized as phytochemical markers due to their pharmacological activities (Heitzman et al., 2005). Mitraphylline (MTP) is an oxindole alkaloid and the most ubiquitous alkaloid being present in *Uncaria* species (Heitzman et al., 2005). Most of the pharmacological studies have been generally focused on the fractions of either plant species or "crude drug", considered as a preparation from either a single or a mixture of *Uncaria* plants. However, little is known about the bioactivity of isolated compound(s), specifically MTP. We have recently tested the anti-inflammatory and immuno-modulatory action of the MTP on human circulating monocytes (Montserrat-de la Paz et al., 2015); therefore more research is needed to understand completely the

Abbreviations: MTP, Mytraphilline; IL-6, Interleukin 6; IL-8, Interleukin 8; TNF-α, Tumor necrosis factor-α; LPS, lipopolysaccharide.

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role of MTP on leukocytes modulation and its anti-inflammatory properties.

Neutrophils represent the most abundant pool of leukocytes in human blood and play a crucial role in inflammation, stand as the first line of defence of the innate immune system (Mantovani et al., 2011; Brown et al., 2006). Neutrophil-mediated inflammation is the subject of extensive research (Nathan, 2002). Influx of neutrophils into tissues begins by rolling, which is mediated via surface CD62L (L-selectin) interacting with complementary ligands on endothelial cells, and is followed by firm neutrophil adhesion to the endothelium and consequently, transmigration (Lerman and Kim, 2015). Once activated, neutrophils recognize, phagocyte, and kill invading microorganisms, and this function is achieved via release of proteolytic enzymes, pro-inflammatory cytokines as TNF-α, IL-6 or IL-8 and generation of NO (Zimmermann et al., 2015; Jyoti et al., 2014; Wright et al., 2010; Chatterjee et al., 2009). Following killing, it is required an effective resolution of inflammation, and defects of this process is implicated in the pathogenesis of numerous disorders (Roberts et al., 2013; Wright et al., 2010), chronic inflammation (Berry et al., 2010), autoimmunity (García-Romo et al., 2011) and cancer (Kuang et al., 2011).

Therefore, to take advantage of the beneficial effects of MTP from *Uncaria tomentosa*, we evaluated the ability of MTP to modulate LPS-mediated activation on primary human neutrophils. To approach this, we analysed gene and surface markers expression, in addition to the release of pro-inflammatory mediators such as IL-6, IL-8 and TNF-α, in primary human neutrophils, which are strongly involved in the pathogenesis of numerous inflammatory disorders.

Materials and methods

Bark extract

The plant material was collected in the Peruvian forest and was provided by Dr Carlos S. González and was identified in the Botanic Department of the San Lorenzo Chemical Science School from the Asuncion University in Paraguay. The plant extract was obtained and the compound MTP identified as previously described (García-Prado et al., 2007). Briefly, (Supplemental Fig. 1), 500 g of *U. tomentosa* dried inner bark was treated with ammonium hydroxide and extracted with 500 ml of dichloromethane for 3 times. After filtration, the obtained solution was concentrated in vacuo to afford a residue, which was dissolved in a hydrochloric acid solution (3%). Ammonium hydroxide and dichloromethane were added again. After concentration in vacuum, the purified alkaloid fraction was obtained as a brown residue and the yield was 0.1%.

Gas-chromatography/mass-spectrometry (GC/MS) analysis of the alkaloid fraction

Gas-chromatography/mass-spectrometry analysis was performed by using a CARLO ERBA/KRATOS MS 80 RFA apparatus. Helium (99.99%) was the carrier gas (1 ml/min). 1 µl of the sample was dissolved into dichloromethane and injected into the gas chromatograph. The injector and detector temperatures for the gas chromatograph were 275 °C and 325 °C, respectively. The column oven temperature was increased linearly from 230 °C to 300 °C (4 °C/min). The ionization mode was electron impact (EI). NBSL 1 B2 library was used to recognize all derivatives found in the fraction.

Isolation and identification of MTP

The dried residue of alkaloid fraction (0.5 g) was subjected to a silica gel column chromatography, compacted with silica gel with silica gel 0.063–0.2 mm (0.8 × 25 cm), followed by a gradient elution with various mixtures of *n*-hexane, dichloromethane, and methanol (Table 1). All the collected eluents were monitored by thin layer chromatography (TLC). Twenty-five fractions of 15 ml each were collected and 200 mg of white crystal was obtained from the fractions 14–16 and thereafter eluted with a mixture of dichloromethane/methanol (9.5:0.5), which corresponded to 87.2% of the total alkaloids. An isolated compound was obtained by the TLC method (SiO_2, CH_2Cl_2/ MeCO (5:4) Rf: 0.83; ethyl ether/ EtOAc (1:1) Rf: 0.73; CH_2Cl_2/ EtOH (95:5) Rf: 0.68) and visualized as a brown and orange spot with the reagents sulphuric acid/CH3COOH/H2O (1:20:4).

Table 1
Development of the solid–liquid chromatography column corresponding to fraction of the total alkaloids from *U. tomentosa*.

Fractions	Eluents	Eluates
1–4	*n*-Hexane	Mixtures
5–10	Dichloromethane	Mixtures
11–13	*n*-Hexane/Dichloromethane (5:5)	Total alkaloids and mixtures
14–16	Dichloromethane/Methanol (9.5:0.5)	Total alkaloids
17–19	Dichloromethane/Methanol (9:1)	Total alkaloids and mixtures
20–25	Dichloromethane/Methanol (8:2)	Mixtures

EIMS and ^{1}H and ^{13}C NMR experiments were carried out for alkaloid identification by using an AVANCE 500 spectrophotometer. The following 2D NMR experiments COSY-DQF, (^{1}H–^{13}C)–HSQC, (^{1}H–^{13}C)–HMBC, and NOESY correlation was used to elucidate its structure. Furthermore, the ^{15}N chemical shifts of the isomeric oxindole alkaloids, (^{1}H–^{15}N)–HMBC, was necessary to facilitate its characterization. The solvent used for 70 NMR spectra was CDCl3 (García-Gimenez et al., 2010).

Blood collection and neutrophil isolation

This study was conducted according to the guidelines of good clinical practice. Peripheral venous blood was isolated from healthy adult volunteers (< 35 years old) at the University Hospital Virgen del Rocio at Seville. The investigation conformed to the principles outlined in the Helsinki Declaration of the World Medical Association. Neutrophils were isolated by dextran sedimentation in a Ficoll Histopaque gradient (Sigma-Aldrich Chem., St. Louis, MO, USA) and erythrocytes were removed by hypotonic lysis. The purity of neutrophils preparation was > 97% by trypan blue exclusion. Following isolation, the cells were suspended in a RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 1% heat-inactivated fetal bovine serum. Neutrophils were seeded at a density of 3×10^6 cells/ml. Cells were treated with 0.1 µg/ml LPS from *E. Coli 055:B5* (Sigma-Aldrich®, St Louis, MO, USA) in presence or absence of the MTP (25 µM) for 6 h.

Cytotoxicity assay

Neutrophils seeded in 96-well plates (1×10^5 cells/well) were incubated in presence or absence of different MTP concentrations for 6 and 24 h. At the end of the exposure time, the effect on cell growth/viability was analyzed by MTT colorimetric assay (Montserrat-de la Paz et al., 2012). Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).

Nitrite and cytokine production

Cells in 24-well plates were treated (or untreated) with MTP (25 µM), and 30 min later stimulated with LPS (0,1 µg/ml) for 6 h.

The culture supernatants (100 μl) were transferred to a 96-well assay plate mixed with Griess reagent (Sigma-Aldrich Chem) and incubated for 15 min at room temperature. The amount of nitrite, as an index of NO generation (Csonka et al., 2014), was determined by the absorbance at 540 nm in an ELISA reader (BioTek, Bad Friedrichshall, Germany). After the extrapolation from a standard curve with sodium nitrite, the results were expressed as the percentage of nitrite compared with that of cells treated with only LPS. L-NAME (1 mM, Sigma-Aldrich Chem) was used as negative control.

Flow cytometry

Membrane expression of CD16 (PE anti-human CD16), CD62L (FITC anti-human CD62L) on neutrophils was assessed by flow cytometry. According to the manufacturer's instructions, 10^6 of purified neutrophils, after *in vitro* stimulation with or without MTP (25 μM) in the presence or absence of LPS (0.1 μg/ml) for 6 h, were incubated with antibodies at room temperature for 15 min. Then, erythrocytes were lysated with 2X volume of Fluorescence Activated Cell Sorting (FACS) lysing solution (BD Bioscience, San Jose, CA, USA). Fluorescence intensity was measured by FACS Canto II (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and calibrated using FACS Canto II Cell analyzer software (Becton Dickinson). Mean fluorescence intensity (MFI) of 10^4 counted cells was measured in each sample. Neutrophils were gated as forward scatter^{high} (FSC^{high})-side scatter^{high} (SSC^{high})-CD16^{high} cells. Expression levels were presented as MFI corrected for nonspecific binding of isotype control antibodies on neutrophils from the same donor.

RNA isolation and qRT-PCR analysis

Total RNA was extracted by using Trisure Reagent (Bioline GmbH, Berlin, Germany), following the manufacturer's instructions. RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA (1 μg) was subjected to reverse transcription (iScript, Biorad, Madrid, Spain) according to the manufacturers' protocol. An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined by real-time PCR in a MX3000P system (Stratagene, La Jolla, CA). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (BioRad, CA, USA) containing the primer pairs for either gene [TNFα (NM_000594) Forward, TCCTTCAGACACCCTCAACC, Reverse, AGGCCCCAGTTTGAATTCTT]; [IL-6 (NM_000600) Forward, TACCCCCAGGAGAAGATTCC, Reverse TTTTCTGCCAGTGCCTCTTT]; [IL-8 (NM_000584.3) Forward TAGCAAAATTGAGGCCAAGG, Reverse, AAACCAAGGCACAGTGGAAC] or for glyceraldehyde 3-phosphate dehydrogenase [GAPDH (NM_001289746), Forward, CACATGGCCTCCAAGGAGTAAG, Reverse, CCAGCAGTGAGGGTCTCTCT] and β-actine [(NM_001101) Forward, CGCAAAGACCTGTATGCCAA, Reverse, CACACAGAGTACTTGCGCTC] as housekeeping genes. All amplification reactions were performed in triplicate, and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (GAPDH and β-actine) gene content and expressed as percentage of controls.

Quantification of cytokines levels

The cytokines levels of IL-6, IL-8 and TNF-α released to the cell supernatants, were measured by enzyme-linked immunosorbent assay (ELISA), following the indications of the manufacturer (Diaclone).

Statistical analysis

All values in the Figures and text are expressed as arithmetic means ± standard error (S.E.M). Experiments were carried out in quadruple. Data were evaluated with Graph Pad Prism® Version 5.0 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA); using Bonferroni as post hoc test. P values of < 0.05 were considered statistically significant. In the flow cytometry experiments, the figures shown are representative of at least three different experiments

Results

Analysis of the sample composition

In order to obtain and identify efficiently the alkaloid fraction, 500 g of *U. tomentosa* dried inner bark was treated as explained above, to obtain a brown residue yield up to 0.1%. As explained above, the purified alkaloid fraction obtained as a brown residue was subjected to silica gel column chromatography followed by elution with different solvents. The chromatographic study of the mobile phase showed the presence of 5 compounds whose rf values and different color are summarized in Supplemental Fig. 2.

The nature of the alkaloids presented in the extract was visualized qualitatively with Dragendorff's reagent. Alkaloids belonged to the group of pentacyclic oxindole as they fluorescence at 254 nm and react with a pink color in the presence of ferric chloride 0.2 M in hydrochloric acid at 35%. Alkaloids have been mainly separated and identified by HPLC (Ganzera et al., 2001), but we used GC/MS to recognize the alkaloid profile of a purified alkaloid fraction as previously described by Phillipson and Hemingway (Phillipson and Hemingway, 1975). In the gas-chromatogram, a main peak was observed, at retention time of 24.33 min. This compound represented 87.3% of the total alkaloid fraction (Fig. 1). Its mass spectrum and molecular formula was determined to be $C_{21}H_{24}N_2O_4$. The majority compound was obtained as a solid. EIMS and ^1H and ^{13}C NMR experiments were realized for its identification using an AVANCE 500 spectrophotometer. The following 2D NMR experiments COSY-DQF, (^1H–^{13}C)-HSQC, (^1H–^{13}C)-HMBC (Fig. 2A), and NOESY (Fig. 2B) correlation were used to elucidate its structure. Furthermore, data from the (^1H-^{15}N)-HMBC spectrum (Fig. 2A) allowed us to confirm main alkaloid due to its structural characteristics of MTP, which has already been used by other authors to distinguish between stereoisomeric pairs of oxindole alkaloids (Paradowska et al., 2008; Muhammad et al., 2001; Seki et al., 1993). The structure of the alkaloid is illustrated in Fig. 2C. These chemical pentacyclic types of alkaloids are considered to be biochemical marker of *U. tomentosa*.

Effect of MTP on cell viability in Human Neutrophils

To conduct the experimentation outlined below, the effect on cell growth/viability was analysed by MTT colorimetric assay to ensure maximal efficiency with minimal toxicity. Determinations of IC50 at different MTP concentrations (0, 15, 25, 35, 45, 75 and 100 μM) were evaluated in circulating human neutrophils for 6 and 24 h. Staurosporine (1 nM) was used as reference control. The calculated MTP concentration required for a 50% reduction in neutrophil (IC50) was 50 μM at 6 h and 35 μM at 24 h (data not shown). 25 μM was the concentration selected to carry out the rest of the experimental in vitro assays as a non-toxic concentration.

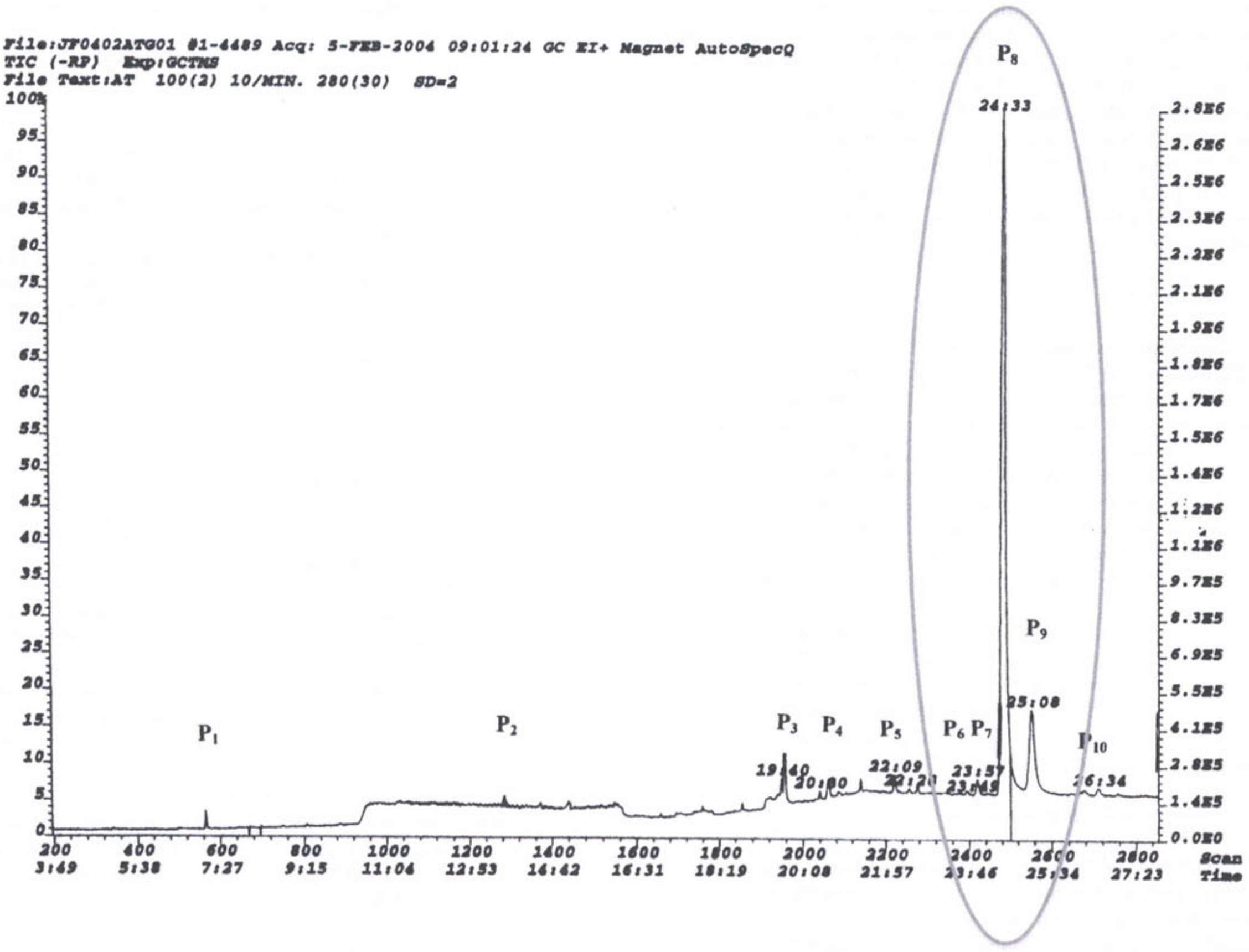

Fig. 1. GC fraction of total alkaloids from *U. tomentosa*. Gas-chromatogram, main peak (Mitraphylline, P8) was observed at retention time of 24.33 min.

Nitrite Production by LPS-stimulated Human Neutrophils

We determined the ability of neutrophils to produce nitrite ion, which is detected and analyzed by formation of a red pink color upon treatment of a NO_2^--containing supernatant with the Griess reagent. A significant increase was found in LPS-activated neutrophils (Fig. 3A) compare to control (+73%, $p < 0.001$), therefore, incubation with MTP (25 μM) diminished significantly the NO_2^- production in those LPS-activated cells (–54%, $p < 0.05$), approaching to the data achieved with the NOS inhibitor, L-NAME. Next, neutrophils LPS-activated showed high mRNA expression levels of inducible nitric oxide synthase (iNOS) (Fig. 3B) compare to non-LPS activated samples (+56%, $p < 0.01$), but marked downregulation of iNOS mRNA expression was observed in LPS-activated neutrophils in the presence of MTP (25 μM), (–67%, $p < 0.01$). These data provide evidence that MTP may ameliorate the pro-oxidative state on LPS-stimulated neutrophils by regulating NO production.

IL-8, IL-6 and TNF-α Production by LPS-stimulated in Human Neutrophils

Likewise, we investigated the effect of MTP on neutrophils activation by determining its ability to modulate pro-inflammatory cytokine gene expression in response to LPS. Neutrophils were exposed to 0.1 μg/ml LPS in the absence or presence of 25 μM of MTP for 6 h. Gene expression analysis showed that pro-inflammatory cytokines such as IL-6 and IL-8 and TNF-α were significantly downregulated by MTP in LPS-treated neutrophils (–83%, –37%, –53% respectively, $p < 0.05$) (Fig. 4A–C). Same pattern was observed with the level of secreted cytokines, which were strongly diminished in the presence of MTP (–62%, –79%, –65%, respectively, $p < 0.05$) (Fig. 4D–F). We therefore hypothesized that MTP may act modulating neutrophil to a less inflammatory phenotype.

MTP triggers Neutrophils Changes toward Anti-inflammatory Phenotype

Herein, we determined the degree of activation on human neutrophils through measuring membrane markers by flow cytometry after LPS incubation in the presence or absence of 25 μM of MTP. The down panel of Fig. 5 depicts a flow cytometer dot plot, showing uniform expression of CD16 and CD62L by normal granulocytes before and after LPS administration. The control pool of neutrophils consisted of a clearly marked phenotype with 95% of $CD16^+CD62L^{++}$ (Fig. 5A). The $CD16^+CD62L^{++}$ cells were almost depleted after LPS administration (–98%, $p < 0.001$) (Fig. 5B). At this time point, banded ($CD16^+CD62L^+$) neutrophils slightly appeared after LPS-MTP co-incubation and these populations comprised 10–15% of total neutrophils ($p < 0.05$). $CD16^+CD62L^{++}$ was the most abundant neutrophils population, although did not reach control levels (Fig. 5C). MTP administration seems to returning neutrophils phenotype to basal conditions.

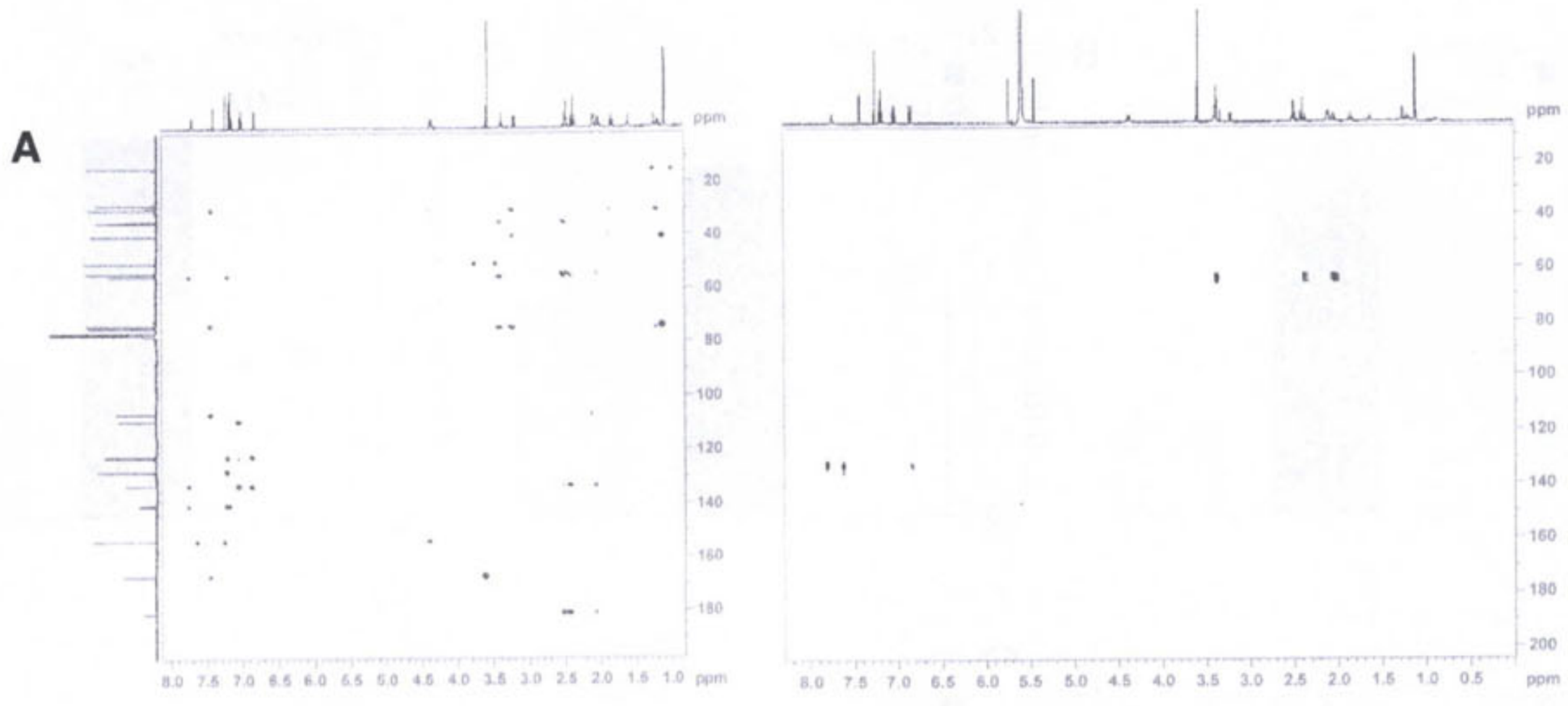

Fig. 2. RMN spectra for identification of MTP. (A) (^{1}H–^{13}C)- HMBC and (^{1}H-^{15}N)- HMBC spectra of MTP, (B) NOESY spectra of MTP, (C) Spatial chemical structure of the alkaloid mitraphylline and isomitraphylline.

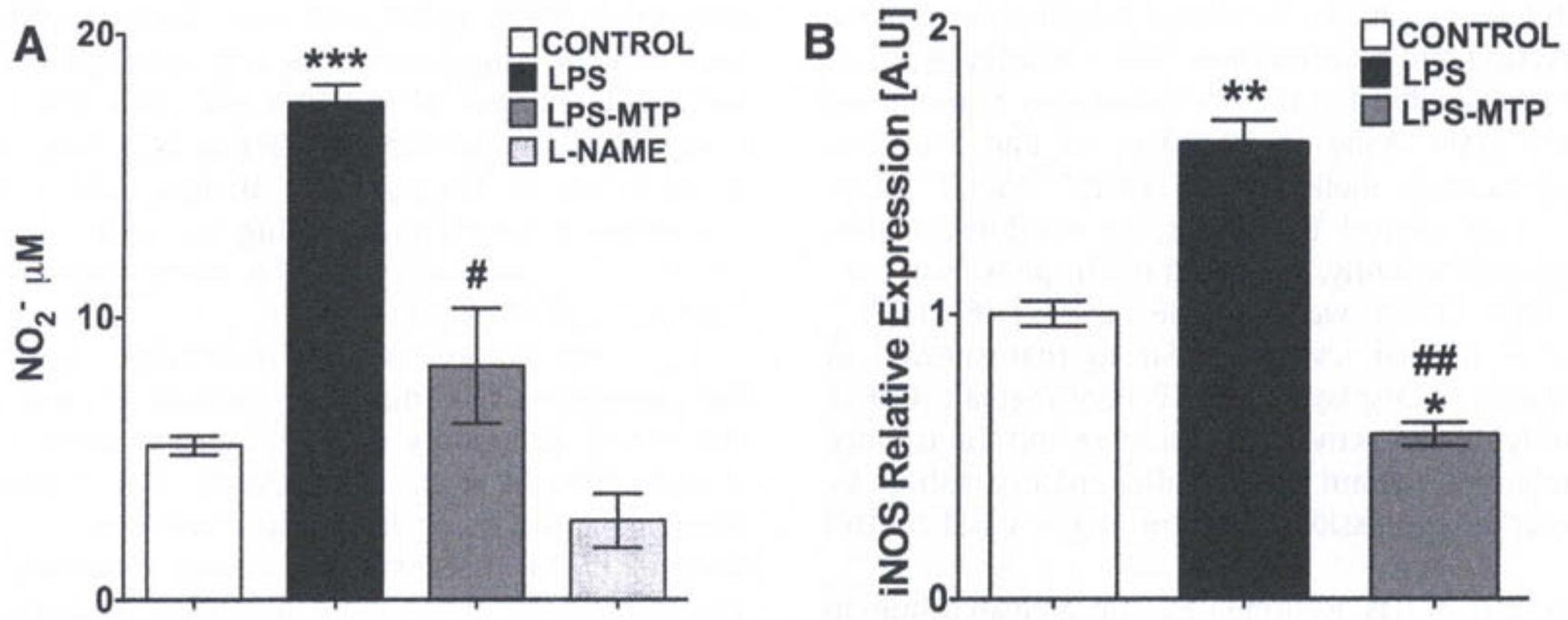

Fig. 3. MTP reduces NO production in human LPS-activated neutrophils. NO2-production (A) and iNOS gene expression (B) in human neutrophils. Values marked with * and # are significantly different ($P < 0.05$) and n=4.

Discussion

U. tomentosa has proven to be a very valuable genus to the discovery and utilization of medicinal natural products that includes alkaloids. MTP is the most ubiquitous and abundant alkaloid from *U. tomentosa*, and recognized molecule with potent anti-inflammatory and immunomodulatory effects (Domingues et al., 2011). We are the first to demonstrate the potential modulator MTP on leukocyte activation (Montserrat-de la Paz et al., 2015), and specifically herein shown, on neutrophils. Neutrophils are the first line of innate immune defense against infectious diseases. Influx of neutrophils into tissues begins by rolling, which is mediated via surface CD62L (L-selectin) interacting with complementary ligands on endothelial

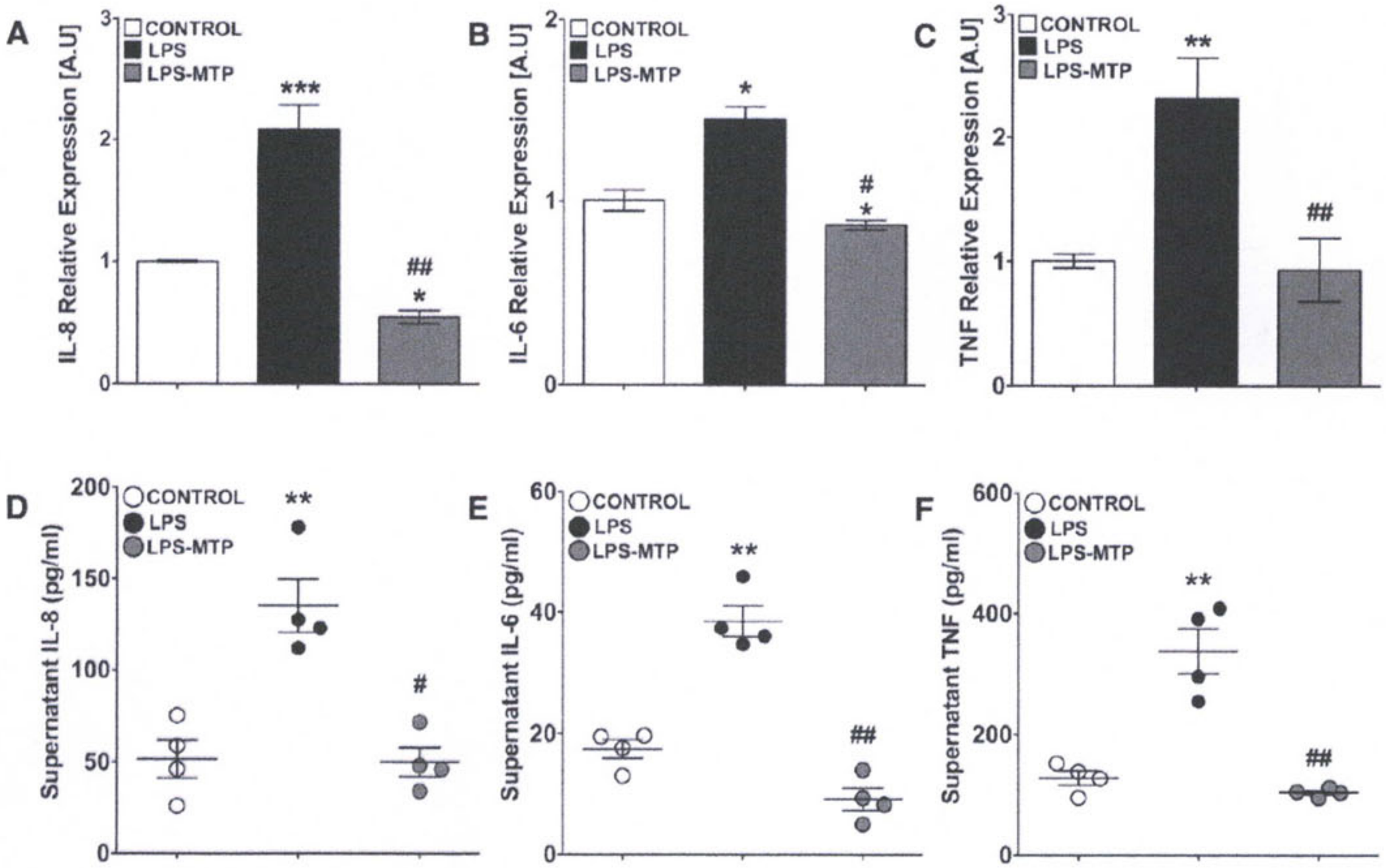

Fig. 4. Anti-inflammatory effect of MTP on cytokines production. IL-8 (A), IL-6 (B), and TNF-α (C) mRNA expression; IL-8 (D), IL-6 (E) TNF-α (F), cytokine production expressed in pg/ml in human neutrophils exposed to LPS 0.1 μg/ml in the absence or presence of 25 μM MTP for 6 h. Values marked with $*$ and # are significantly different ($P < 0.05$) and n = 4.

cells, and is followed by firm neutrophil adhesion to the endothelium and transmigration. Upon activation, neutrophils shed CD62L and increase other surface expression markers, which is accompanied by degranulation, respiratory burst (Van Ziffle and Lowell, 2009, Uddin et al., 2007). Activated neutrophils provide signals for the activation and maturation of other leukocytes, making them partially responsible for the spreading and maintaining of the inflammatory state (Yang et al., 2011). In line, our data showed that LPS-activation abolished the $CD16^{+}CD62L^{++}$ initial population appeared in control conditions, by shedding CD62L surface markers and consequently, activating neutrophils. However, after exposure to MTP, CD62L was upgraded and $CD16^{+}CD62L^{++}$ population returns to normal levels, similar to that showed in control assays. It seems to display that MTP may regulate CD62L shedding, thus modifying LPS-activated phenotype and the balance of neutrophil populations toward a less inflammatory subset by increasing CD62L marker expression and returning to basal control conditions.

Great body of evidences has accumulated that activated human neutrophil is both target and source of several proinflammatory cytokines (Nauseef and Borregaard, 2014; Nauseef, 2007). Interestingly, IL-8 is not only the most abundantly secreted cytokine by neutrophils, but also neutrophils are the primary cellular target of IL-8 (Nauseef and Borregaard, 2014), which perpetuates neutrophils activation by inducing chemotaxis, triggering burst, degranulation and other proinflamatory cytokines production (Takami et al., 2002). Accordingly, neutrophil activation by LPS mediated IL-8 over-expression and over-secretion; therefore gene expression and cytokine secretion were markedly attenuated after exposure to MTP, achieving values even lower than those observed for control. Additionally, we found that MTP was also able down-regulated key pro-inflammatory cytokines such as TNF-α and IL-6, in LPS-activated human neutrophils. Previous research have demonstrated that resting neutrophils did express lower amounts of iNOS mRNA and thus did not produce NO, however, after *in vitro* stimulation with LPS, neutrophils become activated and highly express iNOS mRNA and thus produce NO (Tsukahara et al., 2001). An additional effect of MTP have been encountered in our research, the ability to abolish nitric oxide production in LPS-activated neutrophils, giving an added value that expands the current knowledge on the antioxidant effects of Uncaria (Gonçalves et al., 2005).

It is well recognized that neutrophils have a pivotal role in the pathogenesis of numerous human chronic diseases such as obstructive pulmonary disease, Behçet's disease or inflammatory arthritis (Wright et al., 2010). Despite that classical medications is frequently prescribing for chronic and degenerative inflammatory diseases, it is also necessary to expand knowledge about the mechanisms of action of the active principles in alternative natural therapies to fully understand their therapeutic benefits, with minimal side effect. *U. tomentosa* features a long history of traditional use. It is believed to have immunomodulatory and anti-inflammatory effects and has been utilized to deal with arthritis, inflammation, and cancer and also to encourage injury healing. Therefore, little is known about how *U. tomentosa* and more specifically the isolated compounds would work in the treatment of inflammatory diseases. We previously have identified one of these compounds, MTP, as an important player with anti-inflammatory properties, highlighting its role as master regulator on monocyte/macrophages activation (Montserrat- de la Paz et al., 2015).

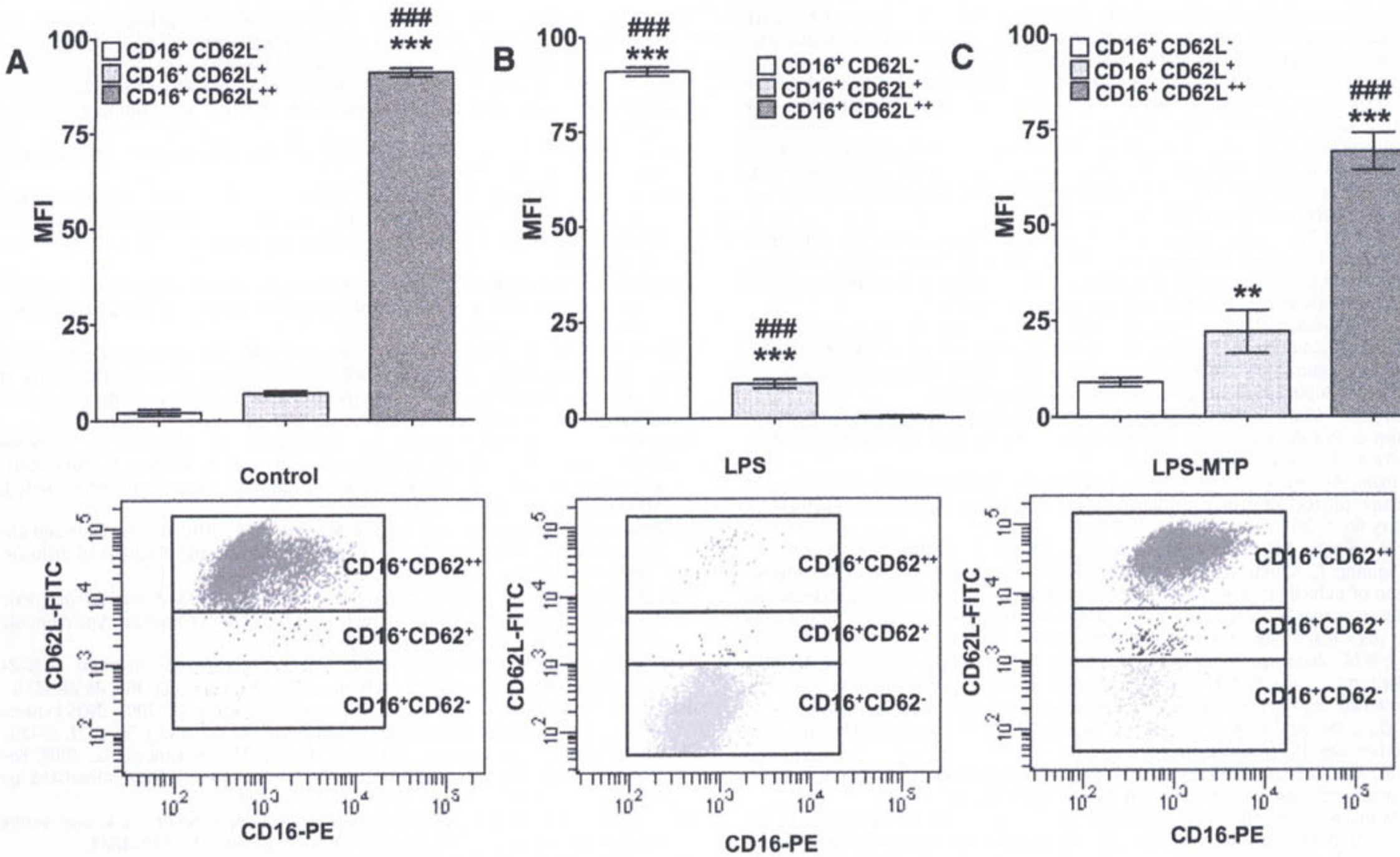

Fig. 5. MTP modulates neutrophil activation markers in human neutrophils. Mean fluorescence intensity (MFI) of neutrophil surface markers CD16 and CD62L, quantification and representative pictures of flow cytometry analysis after incubation with LPS in presence or absence of MTP (A, B and C) after 6 h in primary human neutrophils. Values marked with ∗ and # are significantly different ($P < 0.05$) and n = 4.

Herein, our results have also shown a beneficial effect mediated by MTP in LPS-activated human neutrophils. Suggesting that MTP not only regulates the activation of monocytes but is also committed to neutrophil homeostasis, which indicates that MTP could be a mediator in the general activation of the immune system Nevertheless, more research would be needed to establish guidelines and mechanism of actions of MTP to strengthening the current knowledge concerning the main biological properties attributed to *U. tomentosa*.

Collectively, our data demonstrated that MTP, one of the major alkaloids of *U. tomentosa* and partly responsible for the anti-inflammatory activity of Uncaria's extracts (Aguilar et al., 2002), being able to switch activated phenotype after LPS exposure toward less inflammatory neutrophils population. Nevertheless, more research is needed to achieve full knowledge of the mechanism of action of MTP and its use as a phytodrug to overcome inflammatory diseases.

Conclusion

In conclusion, our data demonstrate that MTP may play an important role in neutrophil activation contributing to the attenuation of proinflamatory episodes that could be considered as a new strategy for chronic inflammatory diseases.

Conflicts of interest

We declare that we do not have any financial or other relationships that might lead to a conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2015.12.015.

References

Aguilar, J.L., Rojas, P., Marcelo, A., Plaza, A., Bauer, R., Reininger, E., Klaas, C.A., Merfort, I., 2002. Anti-inflammatory activity of two different extracts of Uncaria tomentosa (Rubiaceae). J. Ethnopharmacol. 81, 271–276.

Aquino, R., De Simone, F., Pizza, C., Conti, C., Stein, M.L., 1989. Plant metabolites. Structure and in vitro antiviral activity of quinovic acid glycosides from Uncaria tomentosa and Guettarda platypoda. J. Nat. Prod. 52, 679–685.

Aquino, R., De Simone, F., Pizza, C., Vincieri, F., Gacs Baltz, F.E., 1990. New polyhydroxylated triterpenes from Uncaria tomentosa. J. Nat. Prod. 53, 559–564.

Berry, M.P., Graham, C.M., McNab, F.W., Xu, Z., Bloch, S.A, Oni, T., Wilkinson, K.A., Banchereau, R., Skinner, J., Wilkinson, R.J., Quinn, C., Blankenship, D., Dhawan, R., Cush, J.J., Mejias, A., Ramilo, O., Kon, O.M., Pascual, V., Banchereau, J., Chaussabel, D., O'Garra, A., 2010. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 466, 973–977.

Brown, K.A., Brain, S.D., Pearson, J.D., Edgeworth, J.D., Lewis, S.M, Treacher, D.F., 2006. Neutrophils in development of multiple organ failure in sepsis. Lancet 368, 157–169.

Chatterjee, M., Saluja, R., Tewari, S., Barthwal, M.K., Goel, S.K, Dikshit, M., 2009. Augmented nitric oxide generation in neutrophils: oxidative and pro-inflammatory implications in hypertension. Free Radic. Res. 43, 1195–1204.

Csonka, C., Páli, T., Bencsik, P., Görbe, A., Ferdinandy, P., Csont, T., 2014. Measurement of NO in biological samples. Br. J. Pharmacol. 726, 1620–1632.

Dietrich, F., Kaiser, S., Rockenbach, L., Figueiró, F., Bergamin, L.S., da Cunha, F.M., Morrone, F.B., Ortega, G.G., Battastini, A.M., 2014. Quinovic acid glycosides purified fraction from Uncaria tomentosa induces cell death by apoptosis in the T24 human bladder cancer cell line. Food. Chem. Toxicol. 67, 222–229.

Domingues, A., Sartori, A., Valente, L.M., Golim, M.A., Siani, A.C., Viero, R.M., 2011. Uncaria tomentosa aqueous-ethanol extract triggers an immunomodulation toward a Th2 cytokine profile. Phytother. Res. 25, 1229–1235.

Ganzera, M., Muhammad, I., Khan, R.A., Khan, I.A., 2001. Improved method for the determination of oxindole alkaloids in Uncaria tomentosa by high performance liquid chromatography. Planta Med. 67, 447–450.

García-Gimenez, M.D., Garcia-Prado, E., Saenz-Rodríguez, T., Fernandez-Arche, A., De la Puerta, R., 2010. Cytotoxic effect of the pentacyclic oxindole alkaloid mitraphylline isolated from Uncaria tomentosa bark on human Ewing's sarcoma and breast cancer cell lines. Planta Med. 76, 133–136.

García-Prado, E., García-Gimenez, M.D., De la Puerta-Vázquez, R., Espartero-Sánchez, J.L., Sáenz-Rodríguez, M.T., 2007. Antiproliferative effects of mitraphylline, a pentacyclic oxindole alkaloid of Uncaria tomentosa on human glioma and neuroblastoma cell lines. Phytomedicine 14, 280–284.

Garcia-Romo, G.S., Caielli, S., Vega, B., Connolly, J., Allantaz, F., Xu, Z., Punaro, M., Baisch, J., Guiducci, C., Coffman, R.L., Barrat, F.J., Banchereau, J., Pascual, V., 2011. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci. Transl. Med. 3 73ra20.

Gonçalves, C., Dinis, T., Batista, M.T., 2005. Antioxidant properties of proanthocyanidins of Uncaria tomentosa bark decoction: a mechanism for anti-inflammatory activity. Phytochemistry 66, 89–98.

Heitzman, M.E., Neto, C.C., Winiarz, E., Vaisberg, A.J., Hammond, G.B., 2005. Ethnobotany, phytochemistry and pharmacology of Uncaria (Rubiaceae). Phytochemistry 66, 5–29.

Jyoti, A., Singh, A.K., Dubey, M., Kumar, S., Saluja, R., Keshari, R.S., Verma, A., Chandra, T., Kumar, A., Bajpai, V.K., Barthwal, M.K., Dikshit, M., 2014. Interaction of inducible nitric oxide synthase with rac2 regulates reactive oxygen and nitrogen species generation in the human neutrophil phagosomes: implication in microbial killing. Antioxid. Redox Signal. 20, 417–431.

Kuang, D.M., Zhao, Q., Wu, Y., Peng, C., Wang, J., Xu, Z., Yin, X.Y., Zheng, L., 2011. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. J. Hepatol. 54, 948–955.

Laus, G., 2004. Advances in chemistry and bioactivity of the genus Uncaria. Phytother. Res. 18, 259–274.

Lerman, Y.V., Kim, M., 2015. Neutrophil migration under normal and sepsis conditions. Cardiovasc. Hematol. Disord. Drug Targets 15, 19–28.

Mantovani, A., Cassatella, M.A., Costantini, C., Jaillon, S., 2011. Neutrophils in the activation and regulation of innate and adaptative immunity. Nat. Rev. Immunol. 11, 519–531.

Montserrat-de la Paz, S., Fernández-Arche, A., Angel-Martín, A., García-Giménez, M.D., 2012. The sterols isolated from evening primrose oil modulate the release of proinflammatory mediators. Phytomedicine 19, 1072–1076.

Montserrat-de la Paz, S., de la Puerta, R., Fernandez-Arche, A., Quilez, A.M., Muriana, F.J., Garcia-Gimenez, M.D., Bermudez, B., 2015. Pharmacological effects of mitraphylline from Uncaria tomentosa in primary human monocytes: skew toward M2 macrophages. J. Ethnopharmacol. 170, 128–135.

Muhammad, I., Dunbar, D.C., Khan, R.A., Ganzera, M., Khan, I.A., 2001. Investigation of Uña De Gato I. 7-Deoxyloganic acid and 15 N NMR spectroscopic studies on pentacyclic oxindole alkaloids from Uncaria tomentosa. Phytochemistry 57, 781–785.

Müller, A.C., Kanfer, I., 2011. Potential pharmacokinetic interactions between antiretrovirals and medicinal plants used as complementary and African traditional medicines. Biopharm. Drug. Dispos. 32, 458–470.

Nathan, C., 2002. Points of control in inflammation. Nature 420, 846–852.

Nauseef, W.M., Borregaard, N., 2014. Neutrophils at work. Nat. Immunol. 15, 602–611.

Nauseef, W.M., 2007. How neutrophils kill and degrade microbes: an integrated view. Immunol. Rev. 219, 88–102.

Paradowska, K., Wolniak, M., Pisklak, M., Gliński, J.A., Davey, M.H., Wawer, I., 2008. 13C, 15 N CPMAS NMR and GIAO DFT calculations of stereoisomeric oxindole alkaloids from Cat's Claw (Uncaria tomentosa). Solid State Nucl. Magn. Reson. 34, 202–209.

Phillipson, J.D., Hemingway, S.R., 1975. Chromatographic and spectroscopic methods for the identification of alkaloids from herbarium samples of the genus Uncaria. J. Chromatogr. 105, 163–178.

Roberts, K.J., Cross, A., Vasieva, O., Moots, R.J., Edwards, S.W., 2013. Inhibition of pre-B cell colonoy-enhancing factor (PBEF/NAMPT/visfatin) decreases the ability of human neutrophils to generate reactive oxidants bur does not impair bacterial killing. J. Leucok. Biol. 94, 481–492.

Rojas-Duran, R., González-Aspajo, G., Ruiz-Martel, C., Bourdy, G., Doroteo-Ortega, V.H., Alban-Castillo, J., Robert, G., Auberger, P., Deharo, E., 2012. Anti-inflammatory activity of Mitraphylline isolated from Uncaria tomentosa bark. J. Ethnopharmacol. 143, 801–804.

Rosenbaum, C.C., O'Mathúna, D.P., Chavez, M., Shields, K., 2010. Antioxidants and antiinflammatory dietary supplements for osteoarthritis and rheumatoid arthritis. Altern. Ther. Health Med. 16, 32–40.

Seki, A., Takayama, H., Aimi, N., Salai, S.L, Ponglux, D., 1993. A nuclear magnetic resonance study on the eleven stereoisomers of heteroyohimbine-type oxindole alkaloids. Chem. Pharm. Bull. 41, 2077–2086.

Takami, M., Terry, V., Petruzzelli, L., 2002. Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1. J. Immunol. 168, 4559–4566.

Tsukahara, Y., Morisaki, T., Kojima, M., Uchiyama, A., Tanaka, M., 2001. iNOS expression by activated neutrophils from patients with sepsis. ANZ J. Surg. 71, 15–20.

Uddin, M., Seumois, G., Lau, L.C., Rytila, P., Davies, D.E., Djukanovic, R., 2007. Enhancement of neutrophil function by the bronchial epithelium stimulated by epidermal growth factor. Eur. Respir. J. 31, 714–724.

Van Ziffle, J.A., Lowell, C.A., 2009. Neutrophil-specific deletion of Syk kinase results in reduced host defense to bacterial infection. Blood 114, 4871–4882.

Wright, H.L., Moots, R.J., Bucknall, R.C., Edwards, S.W., 2010. Neutrophil function in inflammation and inflammatory diseases. Rheumatology (*Oxford* 49, 1618–1631.

Yang, S., Liu, F., Wang, Q.J., Rosenberg, S.A., Morgan, R.A., 2011. The shedding of CD62L (L-selectin) regulates the acquisition of lytic activity in human tumor reactive T lymphocytes. PLoS One 6 e22560.

Zimmermann, M., Aguilera, F.B., Castellucci, M., Rossato, M., Costa, S., Lunardi, C., Ostuni, R., Girolomoni, G., Natoli, G., Bazzoni, F., Tamassia, N., Cassatella, M.A., 2015. Chromatin remodelling and autocrine TNFα are required for optimal interleukin-6 expression in activated human neutrophils. Nat. Commun. 6, 6061.