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Medicinal Plant *Tinaspora Cordifolia* Polarizes Primary Human Macrophages into an M1 Phenotype

Tıbbi Bitki ***Tinaspora Cordifolia*** Primer İnsan Makrofajlarını M1 Fenotipine Polarize Eder

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Abstract

Objective: *Tinospora cordifolia* is a plant with several medicinal properties. However, the effect of *T. cordifolia* extract on macrophage polarization is not known. Here we report a detailed analysis of the impact of *T. cordifolia* extract on the polarization of primary human monocyte-derived macrophages.

Materials and Methods: Macrophages, derived from human peripheral blood mononuclear cells monocytes (M0 macrophages), were either left untreated or pretreated with *T. cordifolia* extract at doses of 50 µg/mL, 100 µg/mL, 500 µg/mL, or 1.000 µg/mL for 2 h. Macrophages were then polarized into M1 (LPS, IFNγ), M2a (IL-4), or M2c (IL-10) macrophages for 22 h. M1 (HLA-DR, CD64, CD86) and M2 (CD200R, CD206, CD163) surface markers were analyzed by flow cytometry. The M1 cytokine tumor necrosis factor (TNF) was analyzed by Enzyme-linked Immunosorbent Assay.

Results: Our data demonstrated that in M0 macrophages, *T. cordifolia* extract treatment increased the expression of the M1 marker CD86 ($p=0.0036$), while it decreased the expression of the M2a marker CD200R ($p=0.0059$). In M2a and M2c macrophages, *T. cordifolia* extract decreased the expression of the M2a marker CD200R ($p=0.0098$) and the M2c marker CD163 ($p=0.0173$), respectively. Interestingly, after treatment with *T. cordifolia* extract, the phagocytic receptors CD64 ($p<0.0001$) and CD206 ($p<0.0001$) were upregulated in M0, M1, M2a, and M2c macrophages. Finally, *T. cordifolia* extract treatment enhanced the production of TNF in M0, M2a, and M2c macrophages.

Conclusion: Overall, our data suggest that *T. cordifolia* extract shifts the polarization of primary human macrophages into a pro-inflammatory M1 phenotype, with an upregulation of the phagocytic receptors CD64 and CD206.

Keywords: Medicinal plants, macrophages, inflammation

Öz

Amaç: *Tinospora cordifolia*, çeşitli tıbbi özelliklere sahip bir bitkidir. Ancak *T. cordifolia* ekstraktının makrofaj polarizasyonu üzerindeki etkisi bilinmemektedir. Bu çalışmada, *T. cordifolia* ekstraktının primer insan monosit türevli makrofajların polarizasyonu üzerindeki etkisi ayrıntılı olarak incelenmiştir.

Gereç ve Yöntem: İnsan periferik kan mononükleer hücrelerinin monositlerinden türetilen makrofajlar (M0 makrofajlar), muamele edilmeden bırakıldı, ya da 50 µg/mL, 100 µg/mL, 500 µg/mL veya 1.000 µg/mL dozlarında *T. cordifolia* ekstraktı ile 2 saat boyunca önceden uyarıldı. Makrofajlar daha sonra 22 saat boyunca M1 (LPS, IFN-γ), M2a (IL-4) veya M2c (IL-10) makrofajlarına polarize edildi. M1 (HLA-DR, CD64, CD86) ve M2 (CD200R, CD206, CD163) yüzey belirteçleri akan hücre ölçer sistemi ile, M1 sitokin TNF, ELISA ile analiz edildi.

Bulgular: Verilerimiz, M0 makrofajlarında, *T. cordifolia* ekstraktı muamelesinin, M1 belirteci olan CD86'nın ekspresyonunu artırırken ($p=0.0036$), M2a belirteci olan CD200R'nin ekspresyonunu azalttığını gösterdi ($p=0.0059$). M2a ve M2c makrofajlarında, *T. cordifolia* ekstraktı, sırasıyla

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M2a belirteci olan CD200R (p=0.0098) ve M2c belirteci olan CD163'ün ekspresyonunu (p=0.0173) azalttı. İlginç olarak, *T. cordifolia* ekstraktı muamelesinden sonra, fagositik belirteçler CD64 (p<0.0001) ve CD206'nın (p<0.0001) ekspresyonları, M0, M1, M2a ve M2c makrofajlarında arttı. Son olarak, *T. cordifolia* ekstraktı muamelesi, M0, M2a ve M2c makrofajlarında TNF üretimini artırdı (p<0.05).

Sonuç: Sonuç olarak verilerimiz, *T. cordifolia* ekstraktının, CD64 ve CD206 fagositik belirteçlerini yükseltmesi ile birlikte, birincil insan makrofajlarını pro-enflamatuvar M1 fenotipine yönlendirdiğini göstermektedir.

Anahtar Kelimeler: Tıbbi bitki, makrofajlar, enflamasyon

Introduction

The prophylactic and therapeutic benefits of plants and their products are illustrated by many disease studies ranging from cancer to many infectious pathogens, including Avian influenza, Zika, and Ebola virus (1-6). *Tinospora cordifolia* (*T. cordifolia*) is a large deciduous perennial climbing plant, also known as "amrutha, "guduchi," and "giloy" in different languages, found in China and India (7-9). Its containing compounds, such as alkaloids, flavonoids, steroids, terpenes, and glycosides (10,11) have numerous medicinal properties (12). The plant is used to relieve symptoms of diseases like cold, fever, pharyngitis, headache, digestive disorders, diabetes, and ulcers (7,9). Macrophages are a versatile cell population that can adapt to microenvironmental signals (13) and are involved in various physiological functions including inflammation, antimicrobial defense, tissue remodeling, wound healing, and tumor responses (14-16). They are generally classified into two main groups as M1 or pro-inflammatory and M2 or anti-inflammatory macrophages (17-20). M1 macrophages are polarized *in vitro* by TLR4-ligand lipopolysaccharide (LPS) and by inflammatory cytokines such as IFN-γ or tumor necrosis factor (TNF) (21,22). Pro-inflammatory cytokines e.g. interleukin IL-12, IL-6, IL-1β, TNF, and chemokines e.g. CXCL9 and CXCL10, as well as reactive oxygen species, which all activate Th1 polarized immune responses (21) are primarily produced by the M1 macrophages. They also express co-stimulatory molecule CD86 and MHC class II molecules (23-25). Additionally, Fc gamma receptor CD64 has been defined as a specific M1 polarization marker (18,26). Anti-inflammatory M2 macrophages are commonly divided into 3 subgroups, M2a, M2b, and M2c, according to their stimulating factors and the markers they express (21,22). M2a macrophages are induced *in vitro* by the cytokines IL-4 and/or IL-13 (27,28). They express cell surface markers such as CD206 (MRC1), CD200R as well as the chemokines CCL17 and CCL22 (21,29-32). M2b macrophages are polarized by immune complexes and FcR/TLR agonists (27,28) and they produce cytokines including IL-6, TNF, and IL-10 (21,29,33). Finally, M2c macrophages are polarized by the cytokines IL-10 and TGF or glucocorticoids (27,28) and express CD163, IL-10, and TGF (29-31,34). The M1 and M2 phenotypes are highly dynamic, meaning that they can switch between polarization states according to the factors in the environment (35-37). Conflicting data on the impact of *T. cordifolia* products on macrophages have been reported. Sleep-deprived rats treated with the extract of *T. cordifolia* showed decreased expression profile of the inflammation markers CD11b/c, MHC class I, and pro-inflammatory cytokines (38). In autoimmune arthritis, *T. cordifolia* has been demonstrated to decreases pro-inflammatory cytokine levels, namely IL-17, IL-12, IL-1β, and TNF (39). On the other hand, the treatment of mouse macrophages with *T. cordifolia* extract *in vitro* increased their phagocytic activity against non-infectious (heat-killed) yeast and infectious (live) *E. coli*, along with myeloperoxidase, NADH-oxidase, and NADPH-oxidase levels, which cause macrophage activation (40). *In vivo* treatment of CCl4 poisoned mice peritoneal macrophages with *T. cordifolia* extract restored the decreased pro-inflammatory functions such as bactericidal capacity, phagocytosis, and NO production (41). Pro-inflammatory cytokine levels of GM-CSF and IL-1β were elevated in the splenocytes, and macrophages of Swiss albino mice administered intraperitoneal *T. cordifolia* extract (42). Furthermore, immunomodulatory protein (ImP), which is found only in the root of *T. cordifolia*, causes lymphocyte proliferation and macrophage activation (43). Also, another protein, G1-4A derived from *T. cordifolia*, acts as a non-microbial TLR4 receptor agonist leading to macrophage activation (44). Collectively, previous studies concerning *T. cordifolia* and macrophages were limited to mice and reported conflicting results. However, to our best knowledge, the effect of *T. cordifolia* on human macrophage polarization has not been investigated. Here we report a detailed analysis of the impact of *T. cordifolia* extract on human primary monocyte-derived macrophages (MDMs) at the protein level by flow cytometry and Enzyme-linked Immunosorbent Assay (ELISA). *T. cordifolia* extract upregulated the phagocytic receptors of M0, M1, M2a, and M2c macrophages and shifted M2a and M2c macrophages towards M1 polarization.

Materials and Methods

Generation of Human MDMs

The "Non-Interventional Research Ethics Committee" of the Izmir Biomedicine and Genome Center (iBG) provided ethical permission for the use of buffy coats (approval number: 2021-042), which were obtained from healthy donors after written consent from the Dokuz Eylul University Blood Bank (Izmir, Turkey). Gradient centrifugation was used to isolate human monocytes from the buffy coats: Peripheral blood mononuclear cells (PBMCs), and following peripheral monocytes were isolated from the buffy coats using Ficoll-Paque (GE healthcare) and Percoll (GE healthcare), respectively (45). RPMI-1640 medium (Gibco) completed with 1% Penicillin-Streptomycin (Gibco) and 5% heat-inactivated fetal bovine serum (Gibco) (R5 medium) was used as cell culture media. MDMs (M0 macrophages) were generated after 7 days of treatment of monocytes with 10 ng/mL recombinant human M-CSF (PeproTech) in ultra-low attachment six-well plates (corning) (46). The mature macrophages were collected on day 7 and the purity was verified by staining with pan macrophage marker CD68 to be more than 90% by flow cytometry.

Preparation of *T. cordifolia* Extracts

T. cordifolia/Amruth powder was kindly provided by Dr. Ravi Kumar Reddy from Sri Sri Tattva, (Bengaluru, Karnataka, India). *T. cordifolia* in powder form was dissolved in sterile dimethyl sulfoxide (DMSO) at 100 mg/mL as a stock concentration. The solution was shaken gently for 6 h at room temperature (RT), left to settle upright at RT for the next 18 h, then centrifuged at 200 g for 5 min. The supernatants were collected and stored at -80^{o}C for further use.

Treatment of *T. cordifolia* Extracts and Polarization of Human MDMs

Human MDMs were seeded at 6x10^{5} cells/mL/well density into 24-well plates and rested overnight at 37°C, 5% humidity before stimulation. Then, these M0 macrophages were either pre-treated with *T. cordifolia* extract at doses of 50 μg/mL, 100 μg/mL, 500 μg/mL, or 1.000 μg/mL for 2 h. Some macrophages were then polarized into M1 macrophages with 100 ng/mL Ultrapure LPS (InvivoGen) +20 ng/mL IFN-γ (R&D), or into M2a macrophages with 20 ng/mL IL-4 (R&D) or into M2c macrophages with 20 ng/mL IL-10 (R&D) for 22 h or left unstimulated (26,47-49).

ELISA

Supernatants from treated and stimulated MDMs were used to determine TNF levels by ELISA (BioLegend) according to the manufacturer's guidelines.

Flow Cytometry

Accutase (Thermo Fisher) was used to detach the macrophages from the culture plates. PBS completed with 1% bovine serum albumin and 0.1% sodium azide was used to stain single-cell suspensions. Cell viability is determined by Zombie UV Fixable Viability Kit (BioLegend). After blocking Fcγ receptors using Fc receptor blocking solution (BioLegend) for 15 min, cell surface markers were stained for 45 min at 4°C with indicated anti-human antibodies (BioLegend): HLA-DR-APC-Cy7 (1:400), CD86-BV605 (1:200), CD64-PerCP-Cy5.5 (1:200), CD206-AF700 (1:200), CD200R-PE Dazzle594 (1:400), and CD163-PE-Cy7 (1:200). Fluorescence analysis was performed using an LSR Fortessa (BD Biosciences) and FlowJo software (TreeStar), excluding doublets using forward and side-scatter parameters.

Statistical Analysis

The results are given as mean ± SEM. Data were analyzed using One-Way ANOVA with Sidak's multiple comparison post-hoc test to compare group means of experimental data with their corresponding controls at each concentration. Statistical significance was defined as p-values less than 0.05 and calculated as two-tailed. Bar graphs were generated, and statistical analyses were conducted with Graph Pad Prism 9.

Results

The Effect of *T. cordifolia* Treatment on the Viability and Surface Expression of M1/M2 Markers in Human M0 MDMs

First, to determine the impact of *T. cordifolia* extract on cell viability, human MDMs were either treated with *T. cordifolia* extract at 50 μg/mL, 100 μg/mL, 500 μg/mL, or 1000 μg/mL doses for 24 hours or left untreated. After that, the cells were stained with a fixable viability kit (Ghost UV) and analyzed by flow cytometry. As presented in Figure 1, *T. cordifolia* extract at doses of 50 μg/mL, 100 μg/mL, 500 μg/mL did not affect cell viability. A statistically significant (p=0.0060a) but slight decrease was observed after 1.000 μg/mL treatment. An average of 84.1% of the human MDMs were viable after *T. cordifolia* treatment (Figure 1).

To investigate the impact of *T. cordifolia* on the polarization of primary human M0 macrophages, the expression of the common markers of M1/M2 macrophage phenotypes (18,21,22,37,50) was analyzed at the protein level by flow cytometry. As illustrated in Figure 2, the protein expression of the M1 markers CD86 (p=0.0420; p=0.0036, respectively) and CD64 (Fc gamma receptor I) (p<0.0001) were significantly enhanced after 500 μg/

mL and 1.000 µg/mL *T. cordifolia* extract treatment in M0 macrophages (Figure 2A, C). M1 marker MHC class II molecule HLA-DR expression was diminished (p=0.0016; p=0.0031, respectively) after 500 µg/mL and 1000 µg/mL *T. cordifolia* extract treatment (Figure 2B). Moreover, CD206 expression was significantly enhanced in 500 µg/mL (p=0.0007) and 1000 µg/mL (p<0.0001) *T. cordifolia* extract treated M0 macrophages (Figure 2D). Even though CD206 is considered an M2a polarization marker (21,29), this mannose scavenger receptor also plays a significant role in phagocytosis (51-53); along with CD64 which is considered an M1 marker, CD64 (54,55). However, another M2a marker, CD200R was decreased (p=0.0059) after 1.000 µg/mL *T. cordifolia* treatment (Figure 2E). The M2c marker CD163 showed no significant change (p>0.05) after *T. cordifolia* treatment (Figure 2F). Together these data suggest that *T. cordifolia* treatment activates primary human MDMs, shown by the increase of the co-stimulatory molecule CD86 (Figure 2A) and the phagocytic receptors CD64 (Figure 2C) and CD206 (Figure 2D) expressions in M0 macrophages.

The Effect of *T. cordifolia* Treatment on Surface Expression of M1/M2 Markers in Polarized Human M1 MDMs

Next, we investigated the impact of *T. cordifolia* extract on M1 polarized primary human macrophages. To this end, the human MDMs were either pre-treated with four different doses of *T. cordifolia* extract for 2 h and then polarized into M1 with 100 ng/mL LPS and 20 ng/mL IFN-γ for 22 h (24-hour stimulation in total) or left unstimulated. Common M1 and M2 surface markers were analyzed by flow cytometry. The expression of the M1 marker CD64 was significantly enhanced after 500 µg/mL (p<0.0001) and 1000 µg/mL (p<0.0001) *T. cordifolia* extract treatment in M1 macrophages (Figure 3C). There was no significant change in the expression of CD86 or HLA-DR (p>0.05) (Figure 3A, B). Furthermore, there was a significant upregulation in CD206 expression in 500 µg/mL (p=0.0028) and 1000 µg/mL (p<0.0001) *T. cordifolia* extract treated M1 macrophages (Figure 3D), similar to the observation in M0 macrophages (Figure 2D). There was no significant change detected with the M2a marker CD200R and the M2c marker CD163 (p>0.05) (Figure 3E-3F). Together these data suggest that *T. cordifolia* treatment enhances the surface expression of the phagocytic receptors CD64 (Figure 3C) and CD206 (Figure 3D) in M1 macrophages.

Effect of *T. cordifolia* Treatment on the Surface Expression of M1/M2 Markers in M2a Polarized Human MDMs

Next, we investigated the effect of *T. cordifolia* extract on M2a polarized human MDMs. Therefore, the cells were either pre-treated with four doses of *T. cordifolia* extract for 2 h and then polarized to M2a with 20 ng/mL IL-4 for 22 h or left unstimulated. Common M1 and M2 surface markers were analyzed by flow cytometry. The CD64 expression was significantly increased after 500 µg/mL (p<0.0001) and 1000 µg/mL (p<0.0001) *T. cordifolia* extract treatment in M2a macrophages (Figure 4C), similar to the observation in M0 and M1 macrophages (Figure 2C, 3C). The slight increase in CD86 expression was not statistically significant (p>0.05) (Figure 4A). However, HLA-DR expression was significantly decreased after 1.000 µg/mL *T. cordifolia* treatment (p=0.0022) (Figure 4B). Although the surface expression of the M2a marker CD206 tended to be higher after *T. cordifolia* extract treatment, this did not achieve statistical significance (p>0.05) (Figure 4D). M2a marker CD200R expression was significantly decreased in 1.000 µg/mL *T. cordifolia* treated M2a macrophages (p=0.0098) (Figure 4E). These data suggest that *T. cordifolia* treatment shifts M2a macrophages toward an M1 phenotype, shown by the increase in the expression of the M1 marker CD64

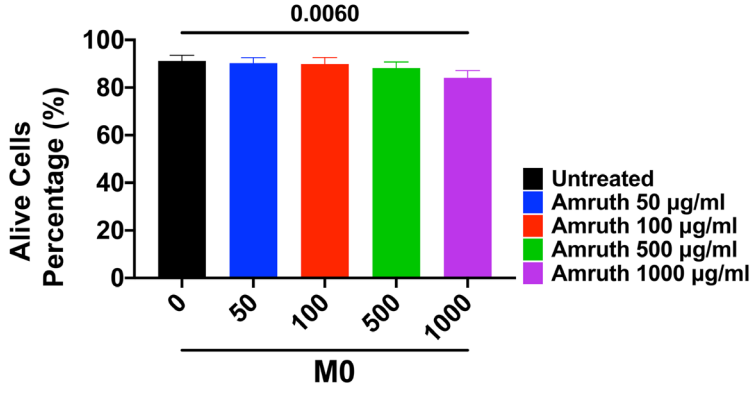

Figure 1. The effect of *T. cordifolia* extract treatment on the viability of human MDMs. Primary human MDMs (M0) were either treated with *T. cordifolia* extract at doses of 50 µg/mL, 100 µg/mL, 500 µg/mL, or 1.000 µg/mL for 24 h or left untreated. Macrophages were stained with a ghost UV fixable viability kit and analyzed by flow cytometry. The bar graphs indicate the biological replicates of 10 independent donors shown as the mean ± SEM. One-Way ANOVA was performed for the statistical analysis. Significant p-value is given on the graph.

(Figure 4C) and by the decrease in the expression of the M2a marker CD200R (Figure 4E). Moreover, *T. cordifolia* upregulated the phagocytic receptors CD64 and CD206 (Figure 4D) in M2a macrophages, similar to M0 and M1 macrophages.

The Effect of *T. cordifolia* Treatment on Surface Expression of M1/M2 Markers in M2c Polarized Human MDMs

Next, to investigate the impact of *T. cordifolia* extract on M2c polarized human MDMs, the macrophages were either pre-treated with four doses of *T. cordifolia* extract for 2 h. The cells were then stimulated with 20 ng/mL IL-10 for M2c polarization for 22 h or left unstimulated. Common M1 and M2 surface markers were analyzed by flow cytometry. As illustrated in Figure 5A, there was no significant change in CD86 expression on M2c macrophages after *T. cordifolia* treatment ($p>0.05$). After treatment with 500 µg/mL and 1.000 µg/mL of *T. cordifolia* extract, the expression of HLA-DR was decreased significantly ($p=0.0053$; $p=0.0027$, respectively) (Figure 5B), while

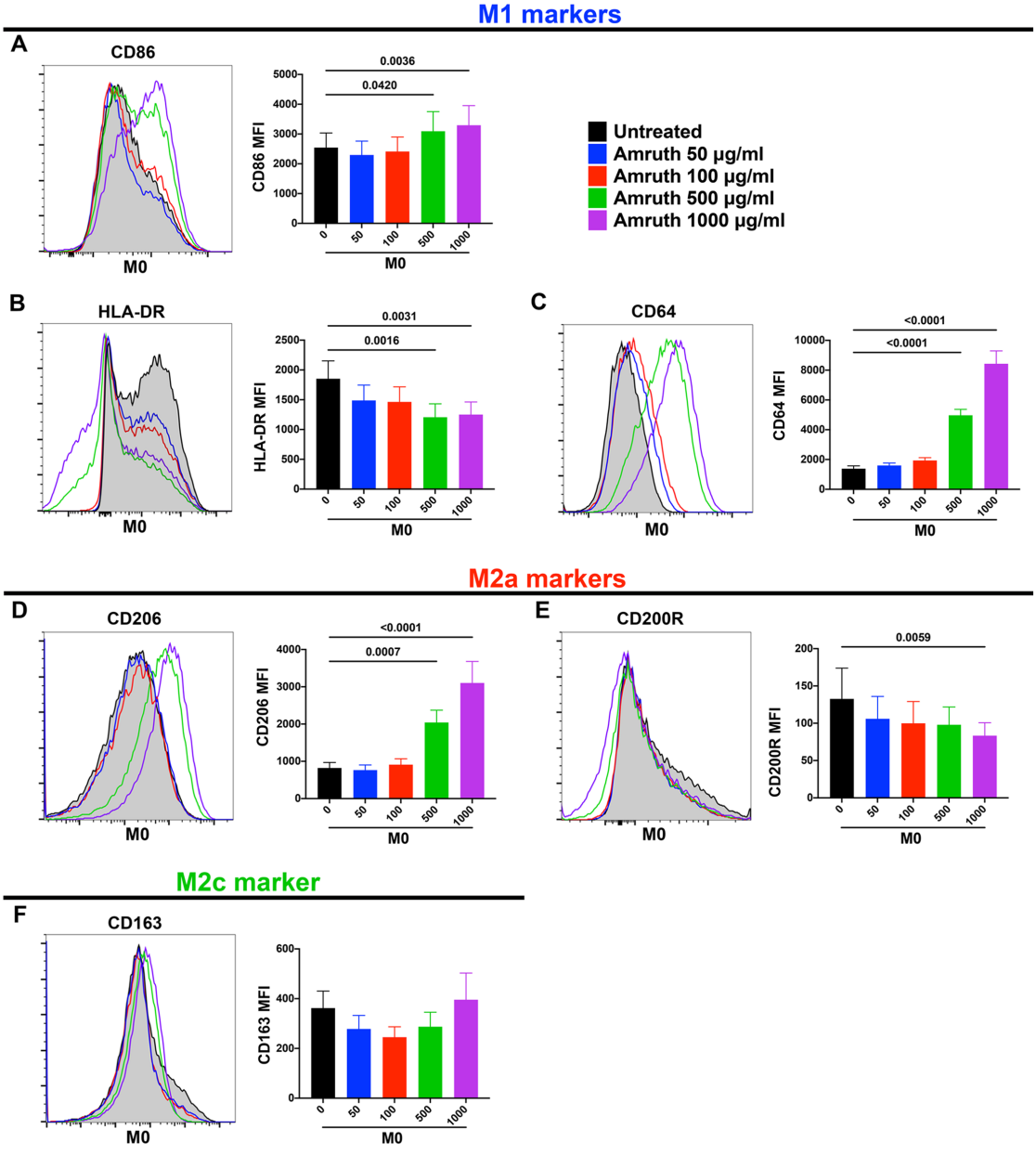

Figure 2. The effect of *T. cordifolia* extract treatment on the surface expression of M1/M2 markers in M0 human MDMs. Primary human MDMs (M0) were either treated with *T. cordifolia* extract at doses of 50 µg/mL, 100 µg/mL, 500 µg/mL, or 1.000 µg/mL for 24 h or left untreated. Surface marker expression of the (A-C) CD86, HLA-DR, and CD64-M1 markers- (D-E) CD206 and CD200R-M2a markers- and (F) CD163-M2c marker- were analyzed by flow cytometry. Histograms are given as representative, and the bar graphs indicate the biological replicates of 10 independent donors shown as the mean ± SEM. One-Way ANOVA was performed for the statistical analysis. Significant p-values are given on the graph.

the expression of CD64 (Figure 5C) and ($p<0.0001$) and CD206 (Figure 5D) ($p<0.0001$) was substantially increased in M2c macrophages. Furthermore, M2c marker CD163 expression was significantly decreased on 1.000 µg/mL *T. cordifolia* treated M2c macrophages ($p=0.0173$) (Figure 5E). These data suggest that *T. cordifolia* treatment shifts M2c macrophages toward an M1 phenotype, shown by the increase in the expression of the M1 marker CD64 (Figure 5C) and by the decrease in the expression of the M2c marker CD163 (Figure 5E). Moreover, *T. cordifolia* increased the expression of the phagocytic receptors CD64 and CD206 (Figure 5D) in M2a macrophages, similar to M0, M1, and M2a macrophages.

The Effect of *T. cordifolia* Treatment on TNF Production by Unpolarized and M1/M2 Polarized Human MDMs

Next, to investigate the impact of *T. cordifolia* on macrophage function, the production of the M1 cytokine TNF by unpolarized (M0) and M1, M2a, and M2c polarized human MDMs was analyzed by ELISA (Figure 6). As the changes in M1/M2 surface marker

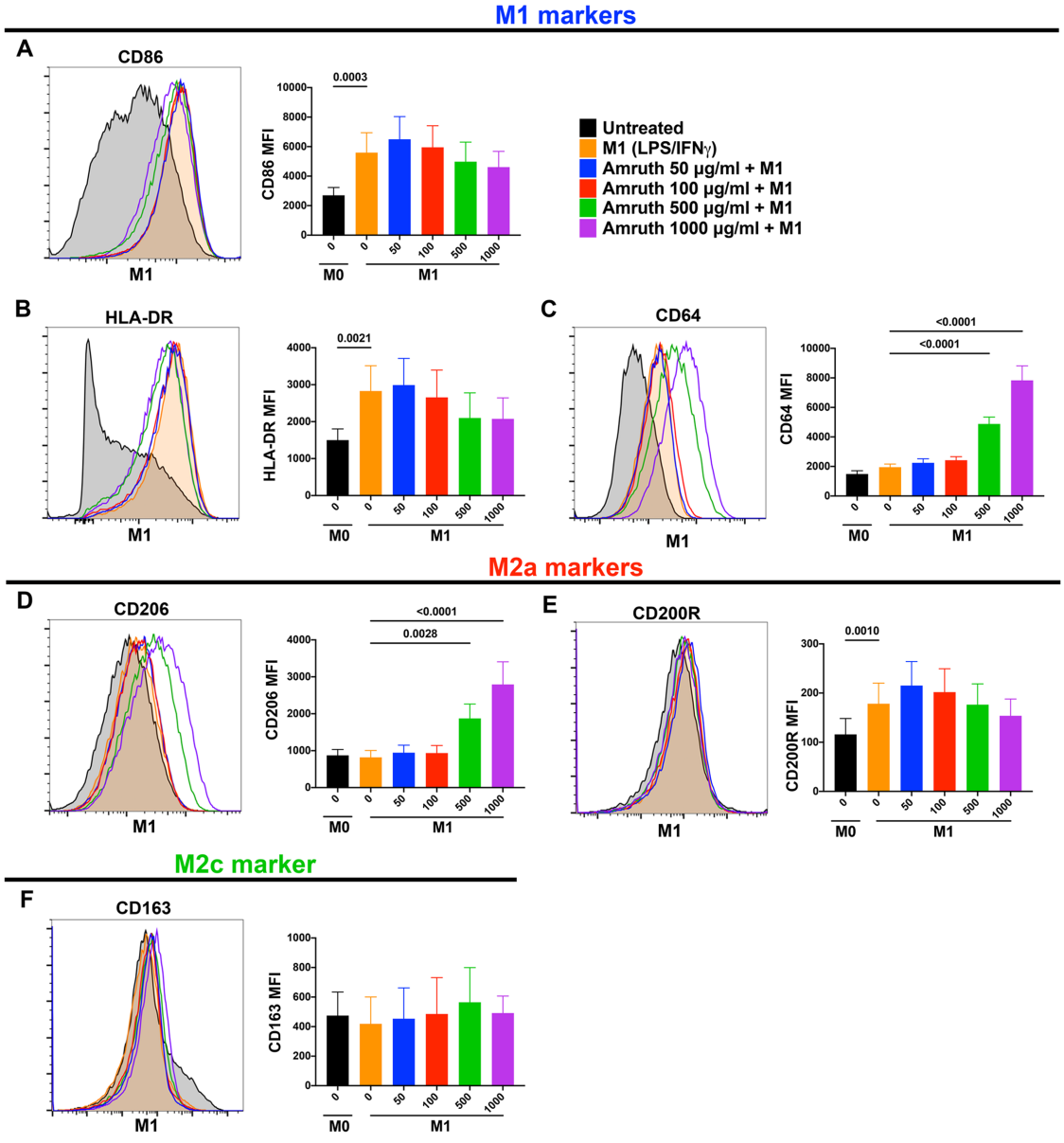

Figure 3. The effect of *T. cordifolia* extract treatment on the surface expression of M1/M2 markers in M1 polarized human MDMs. Primary human MDMs (M0) were either treated with *T. cordifolia* extract at doses of 50 µg/mL, 100 µg/mL, 500 µg/mL, or 1.000 µg/mL for 2 h and then stimulated with M1 100 ng/mL LPS and 20 ng/mL IFN-γ for M1 polarization for 22 h or left unstimulated. Surface marker expression of the (A-C) CD86, HLA-DR, and CD64-M1 markers- (D-E) CD206 and CD200R-M2a markers- and (F) CD163-M2c marker- were analyzed by flow cytometry. Histograms are given as representative, and the bar graphs indicate the biological replicates of 8 independent donors shown as the mean ± SEM. One-Way ANOVA was performed for the statistical analysis. Significant p-values are given on the graph.

expressions were most prominent with 500 µg/mL and 1.000 µg/mL of *T. cordifolia* extract (Figures 2-5), these two concentrations were chosen here. As shown in Figure 6, 500 µg/mL and 1.000 µg/mL *T. cordifolia* extract treatment enhanced the production of TNF in M0, M2a, and M2c macrophages ($p \leq 0.0001$), while it did not further increase the production of TNF by M1 macrophages. These data suggest that *T. cordifolia* treatment triggers TNF production in M0, M2a, and M2c polarized macrophages.

Discussion

Medicinal plants are an integral part of traditional medicine. Scientific research, especially in the last 20 years, supported the prophylactic and therapeutic benefits of plant products, for example, for cancer and infectious diseases. Studies with the medicinal plant *T. cordifolia* demonstrated various immune-modulatory, anti-bacterial, anti-diabetic, and therapeutic effects (7,9,12). Here, we investigated the *in vitro* impact of a *T. cordifolia* extract on the polarization of human primary macrophages. The four doses of *T. cordifolia* were chosen based on the literature (9,56). As *T. cordifolia* contains various compounds such as terpenes, glycosides, alkaloids, steroids, and flavonoids (8,10,11), we dissolved the extracts in DMSO, due to its ability to dissolve both polar and non-polar compounds (57).

First, we analyzed the expression of common M1 and M2 markers in unpolarized and M1/M2 polarized macrophages after *T. cordifolia* extract treatment by

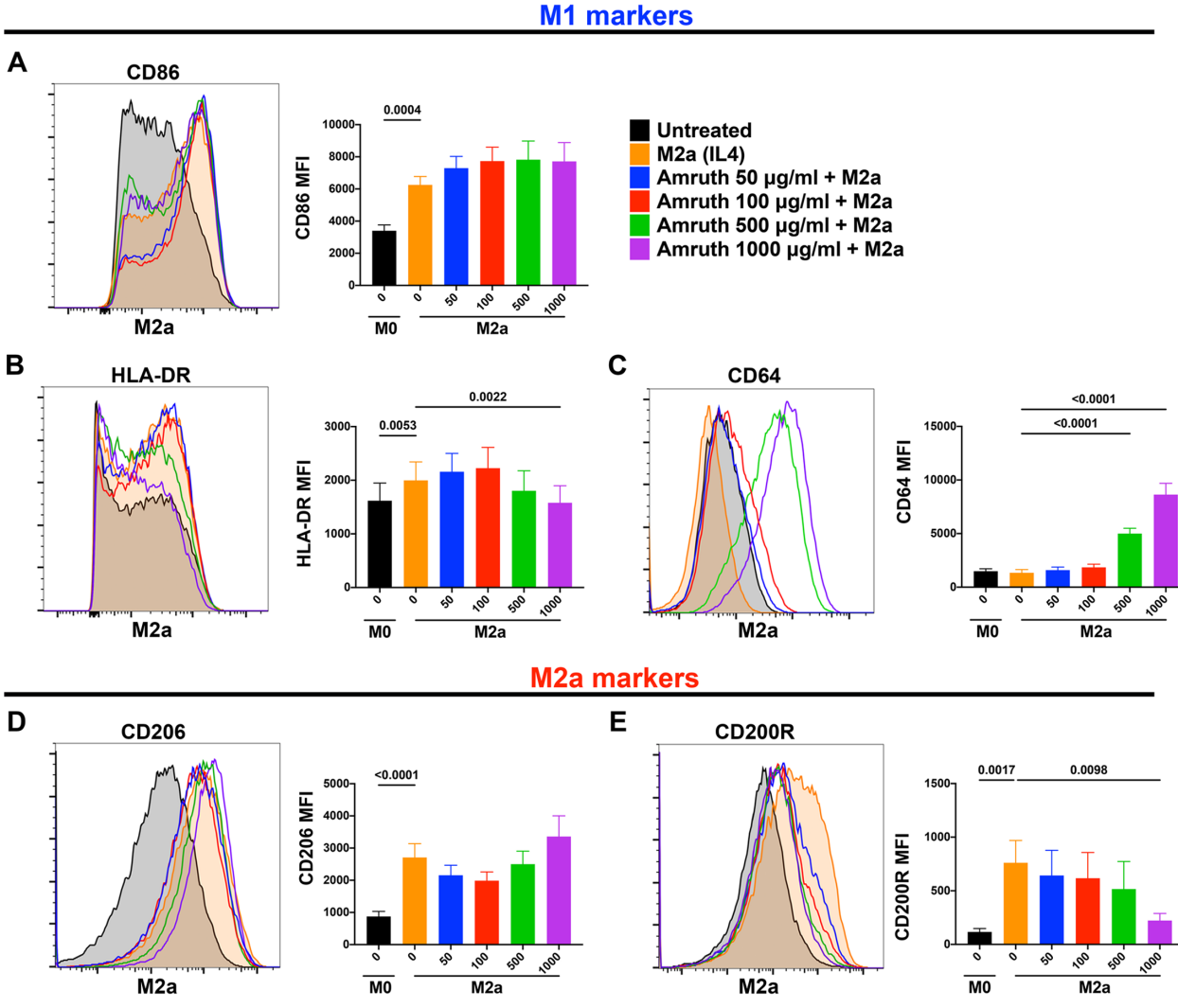

Figure 4. The effect of *T. cordifolia* extract treatment on surface expression of M1/M2 markers in M2a polarized human MDMs. Primary human MDMs (M0) were either treated with *T. cordifolia* extract at doses of 50 µg/mL, 100 µg/mL, 500 µg/mL, or 1.000 µg/mL for 2 h and then stimulated with 20 ng/mL IL-4 for M2a polarization for 22 h or left unstimulated. Surface marker expression of the (A-C) CD86, HLA-DR, and CD64-M1 markers- and (D, E) CD200R and CD206-M2a markers- were analyzed by flow cytometry. Histograms are given as representative, and the bar graphs indicate the biological replicates of 8 independent donors shown as the mean ± SEM. One-Way ANOVA was performed for the statistical analysis. Significant p-values are given on the graph.

flow cytometry. In M0 macrophages, incubation with *T. cordifolia* extract enhanced the expression of the M1 markers CD86 (Figure 2A) and CD64 (Figure 2C), while it downregulated the expression of the M2a marker CD200R (Figure 2E). This suggests that *T. cordifolia* extract polarizes macrophages to an M1-like phenotype. In M1 macrophages, the CD64 expression was increased after *T. cordifolia* extract treatment, while no change was observed in the expression of the M1 markers CD86 (Figure 3A) and HLA-DR (Figure 3B). As M1 polarization with LPS and IFNγ alone significantly increases CD86 and HLA-DR expressions (e.g. Figure 3A, B), no further changes by *T. cordifolia* extract might be possible. Moreover, in M2a macrophages, the expression of CD64 was increased (Figure 4C), while CD200R expression was decreased (Figure 4E) after *T. cordifolia* extract treatment. In M2c macrophages, similar to M2a macrophages, an upregulation of CD64 was observed (Figure 5C), while the M2c marker CD163 was downregulated (Figure 5E). These data suggest that *T. cordifolia* extract treatment shifts M2a and M2c macrophages towards an M1 phenotype. The expression of CD86, which is one of the common M1 markers (23,25,58) was increased with the incubation of 500 µg/mL and 1000 µg/mL *T. cordifolia* extract (Figure 2A). Whereas the expression of HLA-DR, another M1 marker, showed a significant decrease at the same concentrations of *T. cordifolia* extract (Figure 2B). We recently established that the optimum expression of HLA-DR can be observed 12 h after stimulation, followed by a down-regulation

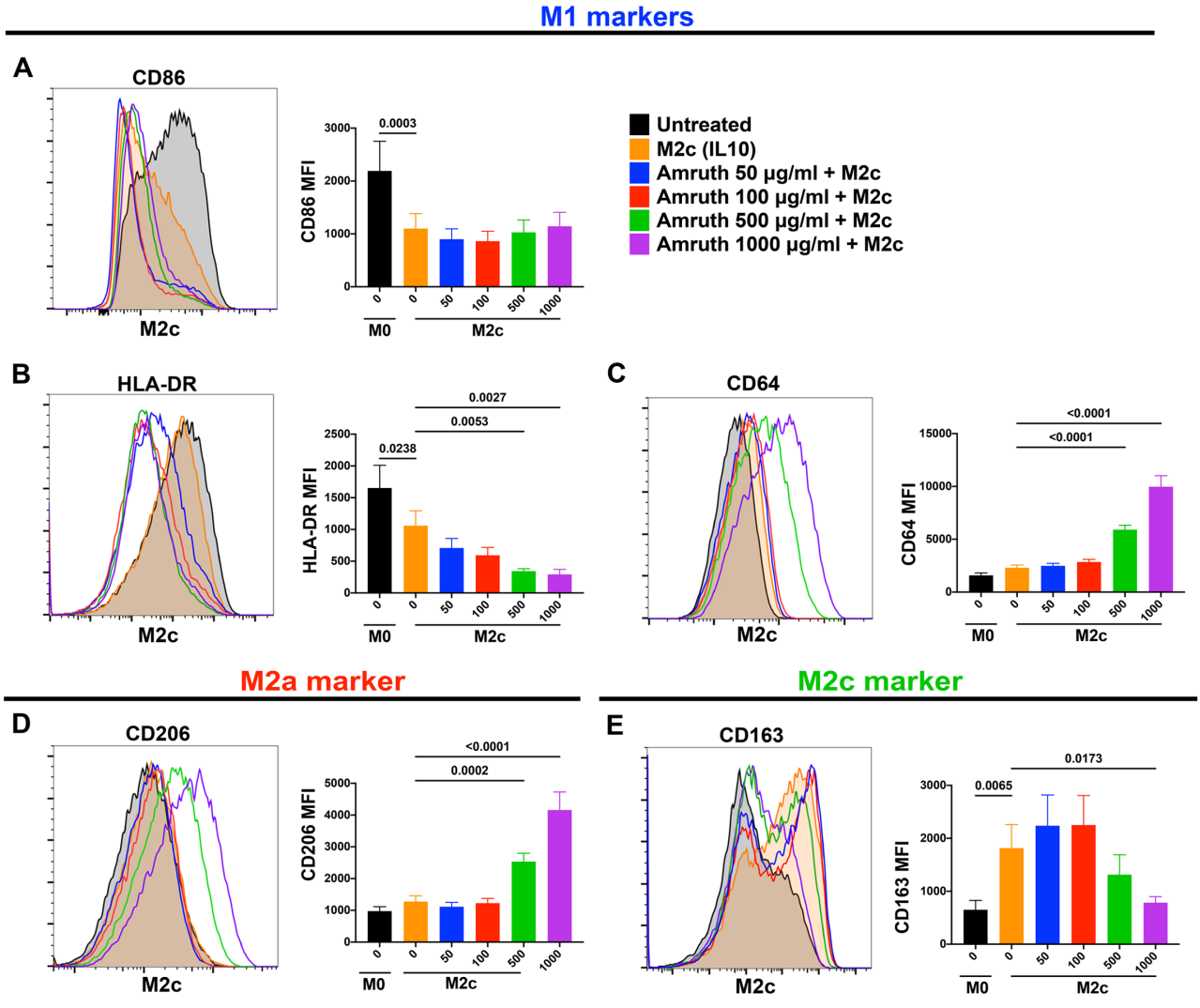

Figure 5. The effect of *T. cordifolia* extract treatment on the surface expression of M1/M2 markers in M2c polarized human MDMs. Primary human MDMs (M0) were either treated with *T. cordifolia* extract at doses of 50 µg/mL, 100 µg/mL, 500 µg/mL, or 1.000 µg/mL for 2 h and then stimulated with 20 ng/mL IL-10 for M2c polarization for 22 h or left unstimulated. Surface marker expression of the (A-C) CD86, HLA-DR, and CD64-M1 markers- and (D) CD163-M2c marker- were analyzed by flow cytometry. Histograms are given as representative, and the bar graphs indicate the biological replicates of 8 independent donors shown as the mean ± SEM. One-Way ANOVA was performed for the statistical analysis. Significant p-values are given on the graph.

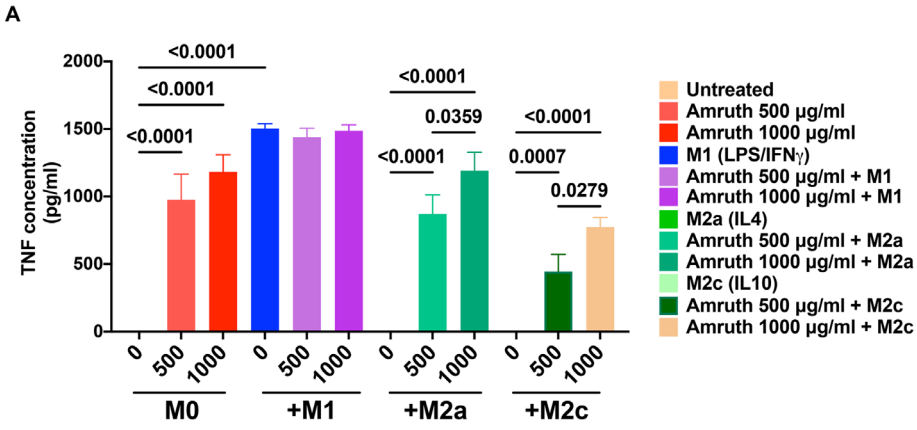

Figure 6. The effect of *T. cordifolia* extract treatment on TNF production by unpolarized and M1/M2 polarized human MDMs. Primary human MDMs (M0) were either treated with *T. cordifolia* extract at doses of 50 µg/mL, 100 µg/mL, 500 µg/mL, or 1.000 µg/mL for 2 h and then stimulated with 100 ng/mL LPS and 20 ng/mL IFNγ for M1, with 20 ng/mL IL-4 for M2a, or 20 ng/mL IL-10 for M2c polarization for 22 h or left unstimulated. Production of the M1 cytokine TNF was analyzed by ELISA. The bar graph indicates the biological triplicates of 6 independent donors shown as the mean ± SEM. One-Way ANOVA was performed for the statistical analysis. Significant p-values are given on the graph.

at 24 to 72 h (59). Hence, the decreased expression of HLA-DR we observed here (Figure 2B) could be due to the suboptimal time point of analysis. However, we chose this timing, as 24-hour stimulation is the best time point to analyze most M1/M2 markers (59). *T. cordifolia* extract treatment significantly enhanced CD64 and CD206 expressions in M0 macrophages, which are classified as M1 (23,26,60) and M2a (21,29,61) marker, respectively (Figure 2C, D). However, CD206, which is the mannose scavenger receptor, also plays a significant role in phagocytosis (51-53); along with the Fc gamma receptor I (FcγRI) CD64 (54,55). Interestingly, a similar increase in the expression of both phagocytic receptors was observed in M1 (Figure 3C, D), M2a (Figure 4C, D), and M2c (Figure 5C, D) polarized macrophages.

T. cordifolia extract was shown to increase the phagocytic activity of the J774 murine macrophage cell line against non-infectious (heat-killed) yeast and infectious (live) *E. coli in vitro* (40,62), as well as of human polymorphonuclear neutrophils against live yeast (63). Additionally, the reduced phagocytic activity of peritoneal macrophages isolated from carbon tetrachloride (CCl4)-treated mice against heat-killed *Staphylococcus aureus* was restored when the mice were additionally fed with *T. cordifolia* extract (41). Together with our results, this indicates that *T. cordifolia* increases the phagocytic activity of human macrophages.

We also examined the production of the M1 cytokine TNF. Control M1 macrophages, but not M2a and M2c macrophages, produced high amounts of TNF (Figure 6), in accordance with the literature (21,26,49,58). Since most of the surface marker changes were observed after 500 µg/mL and 1.000 µg/mL *T. cordifolia* extract treatment, we investigated the TNF production at these doses. Incubation with 500 µg/mL or 1000 µg/mL *T. cordifolia* extract, *T. cordifolia* extract led to significant TNF production by M0, M2a, and M2c macrophages, without a further increase in M1 macrophages (Figure 6). Conflicting data on the impact of feeding rats with *T. cordifolia* extract on TNF production *in vivo* were reported (38,39). *In vitro*, *T. cordifolia* extract could upregulated NADH-oxidase, NADPH-oxidase, and myeloperoxidase and TNF production of murine J774A macrophage cell line (64). Also, *T. cordifolia* extract recovered the pro-inflammatory capabilities, such as bactericidal activity, phagocytosis, and NO generation of peritoneal macrophages purified from CCl4-treated mice (41). Also, pro-inflammatory cytokines, namely IL-1β and GM-CSF levels were elevated in splenic macrophages when Swiss albino mice received intraperitoneal *T. cordifolia* extract (42). Our analysis of M1/M2 markers and TNF production indicates that *T. cordifolia* extract favors M1 polarization. The analysis of additional pro-inflammatory cytokines in future studies would help to support this conclusion.

Conclusion

We report here the impact of the medicinal plant *T. cordifolia* extract on M1 and M2 marker expression and the production of the cytokine TNF-α by unpolarized and polarized primary human macrophages. Altogether, these data suggest that *T. cordifolia* extract treatment shifts the polarization of primary human macrophages to an M1 phenotype, phenotype, with the upregulation of the phagocytic receptors CD64 and CD206. To the extent of our knowledge, this is the first study investigating the effect

of *T. cordifolia* extract on primary human macrophage polarization and our data provide useful information on the immunomodulatory abilities of *T. cordifolia* extracts.

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Ethics

Ethics Committee Approval: The "Non-Interventional Research Ethics Committee" of the Izmir Biomedicine and Genome Center (iBG) provided ethical permission for the use of buffy coats (approval number: 2021-042).

Informed Consent: Buffy coats were obtained from healthy donors after written consent from the Dokuz Eylul University Blood Bank (Izmir, Turkey).

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: D.S., Design: A.K., D.S., Data Collection or Processing: A.K., Analysis or Interpretation: A.K., D.S., Literature Search: A.K., Writing: A.K., D.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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