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In Vitro and *In Vivo* Study of *Pandanus conoideus* Oil Extract in the Maturation of Mouse Peritoneal Macrophages

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Abstract

BACKGROUND: Pandanus conoideus (PC) is a traditional medicinal plant with an interesting effect on inflammatory pathways, especially in the maturation of peritoneal macrophage (PM). PM is involved in the pathogenesis of several types of human inflammatory diseases and abdominal cancers, which can be targeted as a potential therapeutic strategy.

AIM: We aim to explore the role of PC oil extract (PCOE) in the maturation of PM, which may influence the effectiveness of an inflammation.

METHOD: This study is a true-experimental *in vitro* and *in vivo* laboratory study using CH3 mice. To explore the role of PCOE on PM maturation, experiments were carried out *in vitro* (detection of nitrite oxide [NO] levels, detection of interleukin (IL)-1β levels, analysis of PM phagocytosis and proliferation, and flow cytometry analysis) and *in vivo* (analysis of PM phagocytosis and flow cytometry analysis). The data were analyzed using an analysis of variance, followed by Bonferroni *post hoc* test to compare the differences.

RESULT: PCOE substantially enhanced NO and IL-1β production from mouse PM in a dose-dependent manner ($p < 0.05$), upregulated CD68 and CD14 expressions on mice, and promoted mouse PM phagocytic activity *in vitro* and *in vivo* ($p < 0.01$). However, PCOE did not affect the proliferation and major histocompatibility complex expressions of mouse PM.

CONCLUSION: PCOE functions in PM maturation by increasing the production of NO and IL-1β, enhancing the expression of CD14 and CD68, and promoting PM phagocytic activity.

Introduction

Herbal medicines are widely utilized for their therapeutic qualities and have grown in popularity across the world. They often have fewer adverse effects than synthetic drugs, and their efficacy can be enhanced using contemporary pharmacological techniques [1], [2]. Several natural products used in traditional medicine have been shown to influence immune response [3]. *Pandanus conoideus* (PC), often known as the red Papua fruit, is a member of the *Pandanaceae* family and is well-known as a traditional medicinal plant from Papua.

Various studies have used various models to investigate the mechanism of PC extract [4], [5]. The extract of these fruits is reported to possess antioxidant, antipyretic, antiulcerogenic, and especially anti-inflammatory properties [4], [5]. In this study, we are interested in exploring the anti-inflammatory properties of PC, particularly in the maturation of peritoneal macrophage (PM). PM is a type of peritoneal cell that plays a role in many aspects of innate and acquired immunity in the peritoneal cavity, including the development of various forms of human inflammatory illnesses and abdominal malignancies [6], [7]. Due to its critical function, targeted therapy aimed at modulating PM maturation that has gained a lot of attention as a potential therapeutic strategy for human diseases [7]. However, no studies have reported the effect of PC oil extract (PCOE) on the maturation activities of PM. We hypothesized that PCOE could affect PM maturation, thereby influencing the effectiveness of inflammation.

Materials and Methods

Study design and ethical declaration

This study is a true-experimental *in vitro* and *in vivo* laboratory study using CH3 mice. This

study was conducted at the Molecular Biology and Proteomics Core Facilities, Faculty of Medicine, Universitas Indonesia, from December 2020 to June 2021. Female C3H mice (8 weeks old) were utilized in this experiment, and they were acquired from the Laboratory of Experimental Pathology, Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia (Jakarta, Indonesia). Food control (Indonesia) aged 4–6 weeks, with bodyweight ranging from 60 g to 80 g. To explore the role of PCOE on PM maturation, experiments were carried out *in vitro* (including detection of nitrite oxide [NO] levels, detection of interleukin [IL]-1β levels, analysis of PM phagocytosis, analysis of PM proliferation, and flow cytometry analysis) and *in vivo* (including analysis of PM phagocytosis and flow cytometry analysis).

For the *in vitro* study, the sample used was PM cultured from one mouse. For the *in vivo* study, 18 mice were used for PM phagocytosis analysis (six mice in each control group, PCOE 30 ng/200 μl, and PCOE 60 ng/200 μl), and 12 mice were used for CD14 and CD68 expressions analysis (six mice in each control group and PCOE 30 ng/200 μl). The sample size in each group has been calculated with the Federer formula before the experiment begins, where the minimum number of samples in each group is four mice. In this study, six mice were used for each group. All samples were randomly assigned in each group using a random number generator. To avoid bias, the grouping data was only accessed by one researcher (E.W.). Other researchers do not know the groups from each sample until the process is complete.

Apart from the given experimental treatment, all samples received the same treatment and maintenance by the Guide for the Care and Use of Laboratory Animals by the Animal Care and Use Committee [8], namely, by maintaining the temperature of 25°C, 12 h of the light-dark cycle, 55% humidity, and standard food and drink. At the end of the study, all animals were anesthetized and euthanized. Anesthesia and euthanasia procedures were performed according to the American Veterinary Medical Association Guidelines for the Euthanasia of Animals [9]. Anesthesia was performed with ketamine (Merck, NMID686C) at 75–100 mg/kg body weight (BW) and xylazine (Merck, X1126) at a dose of 10 mg/kg BW intraperitoneally. All efforts were made to ameliorate any suffering of the animals. All results were reviewed and reported following the reporting guidelines for animal preclinical studies, namely, the Animal Research: Reporting of *in vivo* Experiments (ARRIVE guideline) [10].

Preparation of PCOE

PCOE was extracted using 0.5% PC oil, 6 mL ethanol (Merck, 443611), and butylated hydroxytoluene (Merck, 817074) in a ratio of 1:1, shaken for 10 s, then incubated in an 85°C water bath for 6 min. The samples were shaken for 3 min and vortexed for 10 s. Saponification was accomplished by increasing the concentration of potassium hydroxide (Merck, 105033). The supernatant was supplemented with petroleum ether: diethyl ether (2:1) before analyzed using a thin-layer chromatography (TLC). A carotenoid had been condensed and then passed through a TLC plate. Eluent petroleum ether: diethyl ether: acetone (40:10:10) (v/v) was added to the chamber and incubated for 10 min. After seeing several layers on the plate, lifting, and drying them, the retention factor was determined. The preparative technique was then carried out by separating each layer and carrying it over a centrifuge tube with enhanced petroleum ether: diethyl ether: acetone (8 ml:2 ml:2 ml), spinning at 1500 rpm for 10 min. The supernatant was dried and the weight was calculated.

NO and IL-1β levels in vitro assay from cultured mouse PM

We adopted and modified the method carried out by Wang *et al.* [11] The PM from mice was collected from the mice's peritoneal cavity using 5 mL Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Aldrich, R8758) and it is cultured for 1 h in a 10% fetal calf serum (FCS)-RPMI 1640 medium in a 37°C humidified incubator with 5% CO_2. After incubation, non-adherent cells were washed, and 12-well plates were used to seed the adherent cells at a density of 1×10^6 cells/well and cultured for 24 h in 5% FCS-RPMI 1640 medium under different circumstances (no PCOE, PCOE 1 ng/ml, 5 ng/ml, 15 ng/ml, or 30 ng/ml). As per the manufacturer's instructions, the NO kit was used to measure the NO levels (Abcam, ab65328). The microtiter plate reader assessed the absorbance at 540 nm. We determined the nitrite concentration by comparing it to a standard curve of sodium nitrite. Following the manufacturer's instructions, IL-1 levels were quantified in the culture supernatant using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, ab197742). This was followed by measuring the absorbance at 450 nanometers to determine the amount of IL-1.

Mouse PM phagocytosis in vitro assay

We adopted and modified the method carried out by Wang *et al.* [11] Chicken red blood cells (cRBC) were utilized to test the phagocytic capacity of mouse PM. Various PCOE concentrations (none, 15 or 30 ng/ml) were applied to mouse PM cultured in 5% FCS-RPMI 1640 medium for 24 h at 37°C, followed by 1 h of incubation with 5% FCS-RPMI 1640 media containing 0.5% cRBC at 37°C. Phosphate buffer saline solvent (PBS) 0.01 mol/L at pH 7.4 (Abcam, ab270748) was used to rinse the PM. Wright-Giemsa

dye (Abcam, ab245888) was used to color the cells, after they had been fixed with 4% paraformaldehyde and dried. A minimum of 200 PM was detected in each well of phagocytosed cRBCs. Both the phagocytosis ratio (PR) and the phagocytosis index (PI) of PM were determined as follows: PR = (number of PM phagocyting cRBC)/(number of PM), while PI = (number of cRBC phagocytosed by PM)/(number of PM) [11].

Mouse PM phagocytosis in vivo assay

We adopted and modified the method carried out by Wang *et al.* [11] The mice were intraperitoneally injected with PBS 200 l, PCOE 30 ng/200 l, or PCOE 60 ng/200 l. RBC (2%) was injected into the mice's abdominal cavity 30 min, before they were euthanized the next day. A 1 ml wash of PBS was then administered to the peritoneum under aseptic circumstances to collect PM. On a glass slide, the PMs were incubated in a humidified incubator for 30 min with 5% CO2. Light microscopy was used to view adherent cells, after they had been treated in a 4% paraformaldehyde solution. In addition, the PR and PI were both calculated.

Flow cytometric analysis for in vitro experiment

We adopted and modified the method carried out by Wang *et al.* [11] For major histocompatibility complex (MHC) I and MHC II expression assay *in vitro*, mouse PM was treated overnight in the absence (control) or presence of PCOE 15 ng/ml. Flow cytometry was used to examine cells that had been centrifuged, resuspended in 2% FCS-PBS, and treated with anti-mouse MHC I or II antibodies which conjugated with FITC (Biolegend, 107605). The samples were examined using FITC-conjugated IgG (Abcam, ab6785) as a control. The data were analyzed using Cell Quest software to assess the fraction of fluorescence-positive cells present. Meanwhile, mouse PM was incubated overnight *in vitro* in the absence (control) or presence of PCOE 30 ng/ml for CD14 and CD68 expression assay. The cells were centrifuged, resuspended in 2% FCS-PBS, and treated with 1 g of FITC-conjugated anti-mouse CD14 or CD68 antibody (Biolegend, 137006) before being subjected to the flow cytometry analysis. FITC-conjugated IgG was used to evaluate the controls. The information was gathered and processed using Cell Quest software.

Flow cytometric analysis for in vivo experiment

We adopted and modified the method carried out by Wang *et al.* [11] The mice were injected intraperitoneally with 30 ng PCOE/200 l PBS or 200 l PBS (control) for CD14 and CD68 expression analyses. The peritoneal cavity was cleaned with 2 ml of PBS the next day to collect PM. The cells were centrifuged, resuspended in 2% FCS-PBS, and treated with 1 g of FITC-conjugated anti-mouse CD14 or CD68 antibody (Biolegend, 137006) before being subjected to flow cytometry analysis. FITC-conjugated IgG was used to evaluate the control. The information was gathered and processed using Cell Quest software to determine the proportion of fluorescence positive cells.

Mouse PM proliferation analysis

We adopted and modified the method carried out by Wang *et al.* [11] Mouse PM was seeded at a density of 2×10^5 cells/ml into 96-well culture plates and cultured for 24 h at 37°C in 200 L of 5% FCS-RPMI 1640 media under various circumstances (no PCOE, PCOE 1 ng/ml, 5 ng/ml, 15 ng/ml, or 30 ng/ml). After 4 h, the live cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Merck, 298931). After removing the media, the formazan crystal was dissolved in 200 L of dimethylsulfoxide (DMSO) (Sigma-Aldrich, 276855). The absorbance at 570 nm was then measured to evaluate cell viability.

Statistical analysis

Data collection was entered into the main table using Microsoft Excel 2013 (Microsoft Corp, Redmond, WA, USA) before analysis. The tabulated data were analyzed using the Statistical Package for the Social Sciences/SPSS version 20 (IBM Corp, Armonk, NY, USA) and visualized using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). The data were analyzed using an analysis of variance, followed by Bonferroni *post hoc* test to compare the differences. The differences of $p < 0.05$ are considered statistically significant.

Results

PCOE stimulates the production of NO and IL-1 in mouse PM

In response to inflammatory stimuli, PM had been demonstrated to activate and release a range of inflammatory mediators [12]. As shown in Figure 1, PCOE significantly increased NO and IL-1 production from mouse PM in a dose-dependent manner. These data indicated that PCOE might directly increase mouse PM activity.

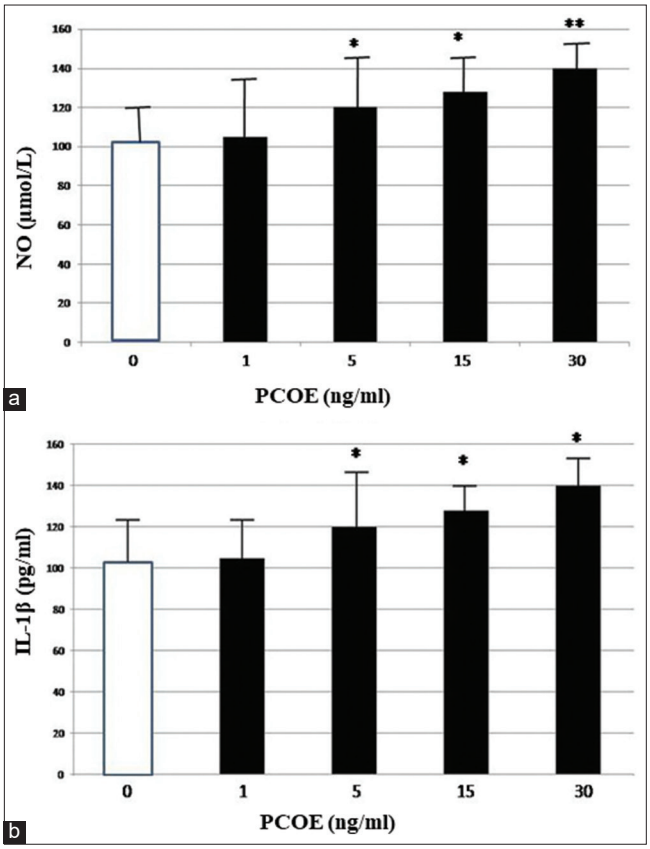

*Figure 1: Nitrite oxide (a) and IL-1 (b) levels in cultured macrophages were measured. All values are presented as mean ± SD. *p < 0.05, **p < 0.01, compared with control*

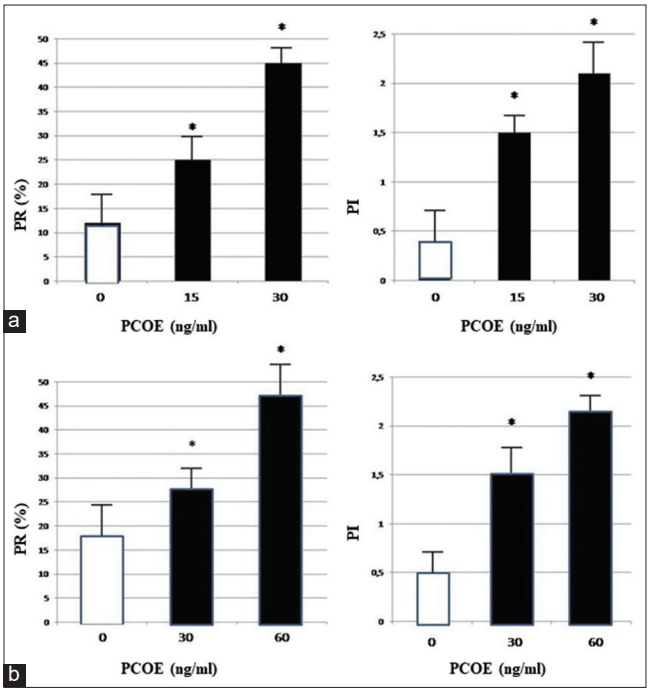

*Figure 2: In vitro and in vivo studies of mouse peritoneal macrophage (PM) phagocytosis. (a) Mouse PM was tested in vitro for phagocytosis of chicken red blood cells (cRBC) after being treated with or without Pandanus conoideus oil extract (PCOE). (b) Phagocytosis of mouse PM to cRBC was investigated in vivo following PCOE treatment. All values are presented as mean ± SD. *p < 0.01, compared with control*

PCOE increases mouse PM phagocytic activity

To determine the direct effect of PCOE on macrophage phagocytosis, macrophage phagocytosis of cRBC was studied *in vitro* and *in vivo*. Figure 2 shows that PCOE significantly increased the phagocytic activities of mouse PM *in vitro* and over the course of the experiment. PCOE increased phagocytic activity *in vivo*, according to our finding.

PCOE did not affect the expression of the MHC I and II molecules

MHC I and MHC II are the primary macrophage surface molecules that play a role in presenting both foreign and endogenous antigens [13]. Flow cytometry was utilized to analyze MHC I and MHC II expression on the mouse PM. According to the findings shown in Figure 3, PCOE treatment had no effect on the expression of MHC I and MHC II in mouse PM. PCOE could not influence mouse PM's capacity to transfer antigens.

PCOE increases the CD68 and CD14 expressions on mouse PM

In the present study, flow cytometry was utilized to examine the expression of mature macrophage surface markers CD68 and CD14 on mouse PM. It was discovered that when mouse PM was treated with PCOE *in vitro* and *in vivo*, their expression was up-regulated, as shown in Figure 4. PCOE appeared to enhance the activation and development of mouse PM *in vitro* and *in vivo*, according to these findings.

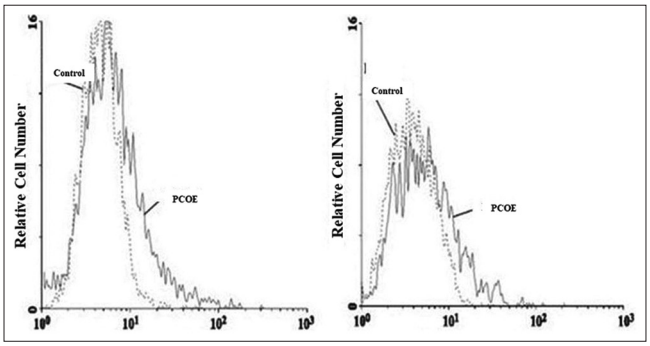

Figure 3: Major histocompatibility complex (MHC) I (left) and MHC II (right) expression based on flow cytometry assay of mouse peritoneal macrophage

PCOE did not influence mouse PM proliferation

The proliferation of mouse PM was investigated using the MTT colorimetric technique to further investigate the functions of PCOE in the control of mouse PM activities. Figure 5 shows that there was no significant change in the proliferation of mouse PM after 24 h of PCOE treatment. These data show that PCOE

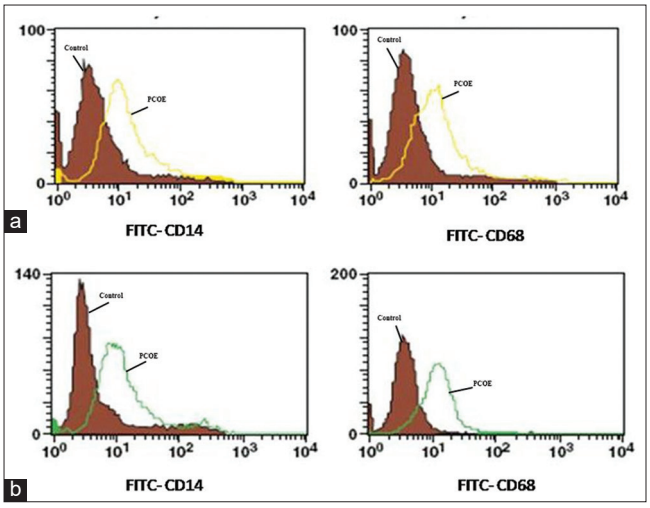

Figure 4: (a and b) CD14 and CD68 expressions based on flow cytometry of mouse peritoneal peritoneal macrophage s in vitro and in vivo

causes mouse PM to become activated and mature, but has no effect on macrophage proliferation.

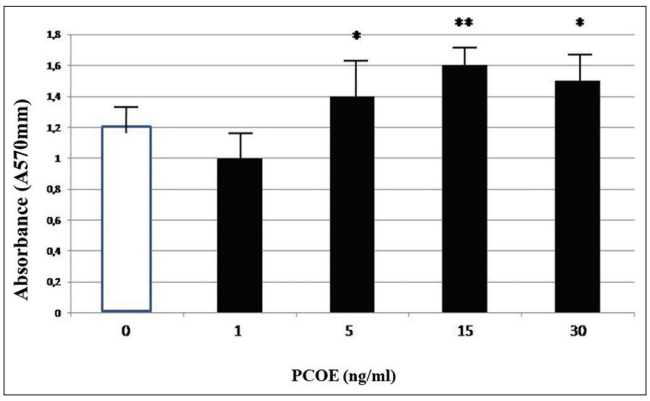

*Figure 5: Mouse peritoneal macrophage proliferation assay. All values are presented as mean ± SD. *p < 0.05, **p < 0.01, compared with control*

Discussion

In this study, the role of PCOE on PM maturation was observed through *in vitro* and *in vivo* experiments, showing an increased level of NO and IL-1β, enhanced phagocytic activities, and upregulated CD68 and CD14 expressions. No significant effect on MHC I and MHC II molecules expressions and PM proliferation was observed. Macrophage maturation is a process of differentiation into a spectrum of activation from inflammatory to non-inflammatory differentiation. The maturation of macrophages toward inflammatory differentiation leads to a pathogen-killing mechanism, while non-inflammatory differentiation leads to tissue healing and growth [7]. Based on these known characteristics of macrophages differentiation, this study shows that PCOE does affect PM maturation to both inflammatory and anti-inflammatory responses.

NO and IL-1β levels are significantly increased in a dose-dependent manner after *in vitro* PCOE administration to mouse PM group. In the environment of tissue injury or acute inflammation, macrophages maturation shifts to inflammatory differentiation and escalating inflammatory response by secreting inflammatory mediators including NO and IL-1β. A similar study by Wang *et al.* showed an increase in NO and IL-1β production by PM after being induced by TGF- β inducer Activin A [11]. However, a previous study by Rhee *et al.* showed that in the infection setting, PC reduced NO and other inflammatory cytokine levels. The LPS-induced murine macrophage cell line was used in this study, simulating late-stage inflammation, in which anti-inflammatory macrophages must dominate the condition [14]. These findings implicate that PCOE could induce the maturation of PM to a broad spectrum of differentiation based on the environment.

Both *in vitro* and *in vivo* experiments showed increased phagocytic activities of PM to cRBC after PCOE administration. Macrophages could be highly phagocytic and able to eat up microorganisms [15]. Another *in vitro* study by Ratnawati *et al.* showed a similar result, in which macrophage phagocytosis activity significantly increased after red fruit exposure in 10, 30, and 60 min with an optimal dose of 0,25 μg/dL [16]. Meanwhile, Jaggi *et al.* showed that phagocytosis ability was higher in the non-inflammatory type macrophages [17]. Therefore, PCOE administration could benefit the phagocytosis ability of PM in both inflammation and homeostasis environments.

PCOE administration *in vivo* and *in vivo* has also shown an upregulation of CD68 and CD14 expression on PM. Expressions of CD14 and CD68 are markers for macrophage maturation. In the maturation process, CD68 and CD14 are expressed in a wide range of macrophage differentiation. Still, a high level of CD68 is associated with a higher non-inflammatory reaction, with the latter being higher in the inflammatory condition [15], [18]. Another study by Duran *et al.* showed that CD14 supports inflammatory response through bacterial LPS co-receptor activation when upregulated [19]. To sum up, PM maturation induced by PCOE administration is confirmed through the upregulation of CD68 and CD14 as macrophage maturation markers.

No significant effect on PM proliferation and both MHC I and MHC II expression was seen after the PCOE administration. Even though some tissue-resident macrophages could express MHC II and act as an antigen-presenting cell, the role of maturated macrophages which already differentiated into M1 or M2-like type is insignificant. Macrophage proliferation is also mainly dependent on the environment, in which the level is increased in acute inflammatory conditions and is reduced in wound recovery or some conditions like tumors [15]. With that being said, insignificant effect on PM proliferation and MHC expression by PCOE

administration has a small relation to PM maturation, making PCOE stay as a potential inducer of PM maturation.

Although PM maturation through PCOE administration could benefit the inflammatory environment, macrophage maturation in the cancer environment is yet to be explored. In a cancer environment, macrophages, including PM, could backfire as tumor-associated macrophages (TAMs). TAMs are associated with polarization to non-inflammatory macrophages and promote almost all cancer growth hallmarks [20].

NO production induced by PCOE could display both beneficial and harmful effects on tumors based on their concentration. A high concentration of NO plays a significant role in tumor cell apoptosis, while a low concentration of NO shows pro-angiogenic properties and supports tumor growth and metastasis [21]. On the other hand, although IL-1β is secreted as an acute inflammatory and anti-tumor, chronic inflammatory conditions of late-stage tumors result in a tumor-promoting environment and sustaining TAMs activity. In this region, inducing NO and IL-1β through PCOE administration could benefit tumor cells destruction in the early-stage cancer.

Other parameters of PM maturation through PCOE administration might have a deleterious effect in the cancer environment. Researchers are currently investigating TAM's phagocytosis ability to engulf cancer cells as a target for cancer immunotherapy. On the other hand, cross-reaction to this target with cancer treatments has been associated with a pro-tumor event [22]. A study by Ciucci *et al.* found that fewer grades of ovarian cancer are associated with a lower density of tumor-infiltrating CD68+ macrophages [23]. In addition, Ni *et al.* found that high density of CD68+ macrophage is related to high lymph node metastasis, high Ki67, poor histological grade, and hormonal receptor negativity [18]. MHC I and II expressions are essential in the anti-tumor function of macrophages, especially in the M1 type, by recruiting CD8+ cytotoxic T cells [24]. Alternatively, PM proliferation toward anti-tumor M1-like macrophages and depleting TAMs could be used for cancer therapy [15]. Therefore, increased PM phagocytosis and CD68+ and CD14 expressions by PCOE administration might have promoted cancer growth while showing no effect on MHC expression and PM proliferation as a potential cancer therapy.

Several limitations need to be mentioned in this study. First, this study has successfully shown several crucial parameters to measure mouse PM maturation progress after PCOE administration. However, a study on another parameter is yet to be further explored. Other pro-inflammatory cytokines such as TNF- α, anti-inflammatory cytokines such as IL-4, IL-10, and TGF- β, for example, will provide a better knowledge of the polarization of PM maturation following PCOE treatment [25], [26]. Second, this study was conducted both *in vitro* and *in vivo*. Therefore, the *in vitro* model might not account for all the interactions between cells and biochemical processes during turnover and metabolism. With more resources, this study could be done on a bigger scale of an *vivo* study and with great hope for its application in clinical settings.

Conclusion

PCOE functions in PM maturation by increasing the production of NO and IL-1β, enhancing the expression of CD14 and CD68, and promoting PM phagocytic activity. Further studies involving more parameters are needed, especially to strengthen the potential application of PCOE in clinical settings.

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