

Original Paper

Protein-Bound Polysaccharides from *Coriolus Versicolor* Fungus Disrupt the Crosstalk Between Breast Cancer Cells and Macrophages through Inhibition of Angiogenic Cytokines Production and Shifting Tumour-Associated Macrophages from the M2 to M1 Subtype

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Key Words

Coriolus versicolor • Protein-bound polysaccharides • Breast cancer cells • Cytokines • Macrophage polarization

Abstract

Background/Aims: The tumour microenvironment is rich in multiple cells that influence cancer development. Among them, macrophages are the most abundant immune cells, which secrete factors involved in carcinogenesis. Since protein-bound polysaccharides (PBP) from the *Coriolus versicolor* fungus are believed to inhibit the growth of cancers, in the present study, we investigated whether these PBP influence crosstalk between triple-negative 4T1 breast cancer cells and RAW 264.7 macrophages. **Methods:** 4T1 cells were cultured in conditioned media (CM) collected after: stimulation of the macrophages with PBP (CM-PBP) or incubation of non-treated macrophages (CM-NT). A co-cultured model of both cell lines was also employed to investigate the crosstalk between the cells. Cell viability was measured using the MTT assay. The levels of cytokines and chemokines were determined by ELISA methods. Commercial assay kits were used to assess the activity of both arginase 1 and inducible nitric oxide synthase (iNOS) and the level of cell migration. **Results:** The results revealed that CM-NT promotes proliferation and migration of 4T1 cells, and increases the secretion of pro-angiogenic factors (VEGF, MCP-1) by cancer cells. In contrast, CM-PBP inhibits 4T1 cell growth and migration, decreases the secretion of pro-angiogenic factors (VEGF, MCP-1) and upregulates

the production of pro-inflammatory mediators (IL-6, TNF- α) with certain anti-tumoral properties. Moreover, PBP-treated CM significantly decreases the level of M2 macrophage markers (arginase 1 activity, IL-10 and TGF- β concentrations), but upregulates iNOS activity and IL-6 and TNF- α production, which are M1 cell markers. **Conclusion:** The results suggest that PBP suppress the favourable tumour microenvironment by inhibiting the crosstalk between 4T1 cells and macrophages through the regulation of production of angiogenic and inflammatory mediators, and modulating the M1/M2 macrophage subtype.

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Introduction

Triple-negative breast cancer (TNBC) is defined by the lack of protein expression of the oestrogen receptor (ER) and progesterone receptor (PR) and the absence of HER2 protein overexpression. TNBC is characterized by its unique molecular profile, aggressive behaviour, distinct patterns of metastasis and lack of targeted therapies [1]. It has been well-established that the tumour microenvironment secretes various signalling molecules, such as cytokines and chemokines that have a critical effect on the recruitment of various immune cells that contribute to carcinogenesis and that provoke the resistance of cancer cells to the immune response [2-3]. In the tumour microenvironment, malignancies recruit, among others, circulating monocytes by producing tumour-derived chemotactic factors, such as macrophage chemoattractant protein-1 (MCP-1) or vascular endothelial growth factor (VEGF), and then induce monocytes to differentiate into tumour-associated macrophages (TAMs) [4]. Many studies have demonstrated that TAMs are associated with poor prognosis for breast cancer patients [5-6], which means that TAM-targeted agents have largely focused on macrophage depletion as an anticancer strategy. However, it is becoming increasingly evident that TAM re-education may represent a more effective approach. These strategies are because that TAMs are not a single uniform population, instead, they are composed of multiple distinct pro- and anti-tumour subpopulations with overlapping features depending on a variety of external factors [7]. TAMs display a phenotypic plasticity with two main types of macrophages, M1 and M2, which usually have contrasting effects on tumour cells [8-9]. M1 macrophages highly express inducible nitric oxide synthase (iNOS) and they induce adaptive immune responses by producing pro-inflammatory cytokines, such as tumour necrosis factor (TNF) α and interleukin (IL) 6 with certain anti-tumour properties [10-11]. In contrast, M2 macrophages display enhanced expression of arginase 1 and produce a large amounts of anti-inflammatory cytokines, such as IL10 and transforming growth factor β (TGF- β) [12]. Furthermore, Owen and Mohamadzadeh [13] have found that M2 cells also promote tumour growth by stimulating angiogenesis and suppressing the adaptive immune response.

Since several studies have shown that TAMs produce cytokines that promote tumour growth, the inhibitors of these negative processes are urgently sought [14]. Previously, we showed that protein-bound polysaccharides (PBP), natural compounds isolated from Chinese fungus *Coriolus versicolor* (CV), decreased the viability of the oestrogen-receptor-positive human MCF-7 breast cancer cells [15] and the human SKMel-188 melanoma cell line [16]. Their anti-tumour properties are mediated not only through direct cytotoxic effects on tumour cells, but also by immunomodulatory regulation [15, 17]. Therefore, in the present study, we have continued our research to verify whether PBP may regulate the immune response in both triple negative breast cancer cells (the 4T1 cell line) and RAW 264.7 macrophages that co-exist in the tumour microenvironment. These interactions were investigated using 4T1 cells cultured in RAW 264.7 macrophage-conditioned media and the co-cultures of the both cell lines in the same well. To the best of our knowledge, this is the first study to evaluate the anti-tumour and immunomodulatory properties of PBP using not only single cell lines, but also a co-culture system.

Materials and Methods

Cell culture

The murine breast cancer 4T1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), and the murine macrophage RAW 264.7 cell line was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK). Both cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS), 100 µg/mL streptomycin and 100 IU/mL penicillin (all compounds from Sigma-Aldrich, Darmstadt, Germany) at 37°C in a humidified atmosphere with 5% CO₂. The culture medium was changed every 2-3 days. Macrophages were passaged using a cell scraper, whereas 4T1 cells were passaged using 0.25% trypsin-EDTA solution (Sigma-Aldrich) when reaching 70-80% of confluency.

Preparation of PBP from CV extract

C. versicolor capsules were purchased from MycoMedica Company (Czech Republic). Protein-bound polysaccharides were extracted following protocols described previously [15-16, 18]. The final concentration of PBP in the stock solution was determined based on the manufacturer's Certificate of Analysis (MycoMedica Company) and was tested with the CP2010-UV method.

Generation of RAW-conditioned medium

RAW 264.7 macrophages, 2.5×10^4 cells/well, were seeded in 6-well plates containing 3 mL of DMEM medium supplemented with 10% FBS and pre-incubated for 24 h. The cells were then cultured for another 24 h in the presence or absence of 50 µg/mL PBP in DMEM medium containing 1% FBS. The highest concentration of PBP that did not reduce the viability of 4T1 cells during 24-hour stimulation (50 µg/mL) was used to prepare CM-PBP. Supernatants were collected and cell debris was removed by centrifugation (2000 x g, 5 min.). Conditioned media were stored at -80°C for further experiments.

Cell viability assay

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma Aldrich) tests were performed to evaluate the viability of 4T1 cancer cells after PBP stimulation as well as RAW-conditioned media treatment. The cells were seeded into 96-well plates at a density of 2×10^3 cells/well and pre-incubated for 24 h. In the first experiment, the cells were stimulated with PBP solution at the final concentrations of 50, 100, 200 and 300 µg/mL for 24, 48 and 72 h. After determining the highest non-toxic dose of PBP, in the separate experiments 4T1 cells were treated in media containing 10, 25, 50 and 75% non-treated (CM-NT) or PBP-stimulated (CM-PBP) conditioned medium and 1% FBS/DMEM medium for 24, 48 and 72 h. After treatment, the cells were incubated in MTT/culture medium (5 mg/mL of MTT reagent in phosphate-buffered saline) solution for 3 h at 37°C. Subsequently, the supernatants were aspirated and 100 µL of DMSO was added to dissolve the formazan crystals. The plate was mixed horizontally for 15 min and the optical density was measured at 570 nm (with a reference wavelength of 630 nm) using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). In the case of PBP stimulation, the results were expressed as a percentage of untreated control cells. The viability of CM-treated cells is presented as the percentage of the cells incubated in complete DMEM medium containing 10, 25, 50 or 75% of culture medium supplemented with 1% FBS (control group).

Transwell migration assay

Transwell cell migration assays were performed using a CytoSelect™ 24-well cell migration assay (8 µm, Colorimetric Format, Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, the cells at a density of 5×10^4 were placed in the insert and allowed to migrate for 24 h in serum-free medium. The lower wells of the migration plate were filled with 500 µl of media supplemented with 10% FBS and containing 10, 25, 50 and 75% non-treated (CM-NT) or PBP-treated (CM-PBP) conditioned medium. In the case of the control 4T1 cells, the lower chambers were filled with 500 µl of 10% FBS/DMEM medium containing 10, 25, 50 or 75% culture medium supplemented with 1% FBS. After incubation, non-migrating cells on the upper surface of the insert were removed with cotton swabs. Then, the cells that migrated through the polycarbonate membrane were incubated with crystal violet and subsequently extracted using 10% acetic acid. Finally, 100 µL from each sample was transferred to a 96-well microtiter

plate and the optical density was measured at 560 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments). The level of migration of CM-treated cells was expressed as a fold change relative to equivalent control cells incubated in complete DMEM medium containing 10, 25, 50 or 75% culture medium supplemented with 1% FBS.

Co-cultures of 4T1 cancer cells and RAW 264.7 macrophages

To define the role of the mammary microenvironment in tumorigenesis, the experimental models consisted of 4T1 murine breast cancer cells cultured either alone in RAW-CM or co-cultured with RAW 264.7 macrophages in non-treated (CM-NT) or PBP-treated (CM-PBP) RAW-CM. To mimic a physiological environment where macrophages infiltrate into the areas surrounding breast cancer cells, RAW 264.7 and 4T1 cells were co-cultured in the same well of 6-well plates at densities of 1×10^5 and 4×10^5 cells/well, respectively. First, the cells were pre-incubated in 10% FBS/DMEM culture medium. Then, the cells were maintained in 1% FBS/DMEM media containing 50% non-treated (CM-NT) or 50% PBP-stimulated (CM-PBP) RAW-CM for 72 h. The following control cell cultures were also carried out: RAW 264.7 macrophages alone, 4T1 cells alone, stimulation only with PBP at a dose of 25 $\mu\text{g}/\text{mL}$ (PBP), which was 50% the concentration of CM-PBP used in the experiments (prepared CM contained PBP at a concentration of 50 $\mu\text{g}/\text{mL}$), and cells co-cultured in fresh 1% FBS/DMEM medium (vehicle). Culture supernatants were harvested and stored at -80°C until the chemokine and cytokine levels were measured by ELISA.

Analysis of cytokine production

First, 4T1 cells were seeded in 24-well tissue culture plates at a density of 4×10^4 cells/well in 1 mL of culture growth medium and pre-incubated for 24 h. Then, the cells were treated in media containing 50% non-treated (CM-NT) or 50% PBP-stimulated (50 $\mu\text{g}/\text{mL}$) (CM-PBP) RAW-CM for 72 h. In a separate group, control cells were stimulated with PBP at a dose of 25 $\mu\text{g}/\text{mL}$. Culture supernatants were collected, and the levels of pro-inflammatory and pro-angiogenic cytokines, including IL-6, TNF- α (both kits were obtained from R&D Systems Minneapolis, MN, USA), VEGF and MCP-1 (both kits were purchased from Biorbyt, Cambridge, UK), were determined by standard ELISA kits according to the manufacturer's instructions. The concentrations of cytokines in the CM-NT only and CM-PBP only (without cells) groups were also assessed. RAW-CM only allowed background levels of the mediators in the original conditioned medium to be measured. The levels of IL-6, TNF- α , VEGF and MCP-1 were also measured in the culture supernatants harvested from the co-cultures described above. Colorimetric changes in the assays were detected using Synergy HT Multi-Mode Microplate Reader (BioTek Instruments).

Determination of the M1/M2 phenotype of RAW 264.7 macrophages

To determine the M1/M2 RAW 264.7 cell subtype in co-culture systems with 4T1 cancer cells, the cell were incubated in 1% FBS/DMEM media containing 50 % non-treated (CM-NT) or PBP-treated (CM-PBP) RAW-CM for 72 h and the appropriated assays were performed. M1 macrophage polarization was assessed using the following markers: determination of IL-6 and TNF- α levels in the culture media was performed using standard ELISA kits and inducible nitric oxide synthase (iNOS) activity was measured by a Nitric Oxide Synthase Detection System (Fluorometric, FCANOS1-1KT, Sigma Aldrich) according to the manufacturer's instructions. Upregulation of IL-10 and TGF- β concentrations in the culture media determined by standard ELISA kits (both purchased from Biorbyt, Cambridge, UK) and increased arginase 1 activity measured by an Arginase Activity Assay Kit (Colorimetric, Cambridge, UK) according to the manufacturer's instructions were indicative of M2 macrophage polarization. The concentration of iNOS and arginase 1 activity were normalized to the protein concentration determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Statistical analysis

GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical comparisons between different values. Data are expressed as the means \pm standard error mean (SEM) and were analysed by analysis of variance (one-way ANOVA) followed by Tukey's HSD test with the level of significance set at $P < 0.05$.

Results

PBP decrease the viability of 4T1 cancer cells

To determine the highest non-toxic dose of PBP, 4T1 cancer cells were treated with different concentrations of PBP for 24, 48 and 72 h. As shown in Fig. 1, PBP decreased the viability of cancer cells only at the two highest concentrations of PBP (100 and 200 µg/mL) in a dose-dependent manner. The PBP concentrations in the range of 10-50 µg/mL had no effect on the number of metabolically active cells. This effect was observed at each time point used in the experiment. The highest cytotoxic effect of PBP was observed at the concentration of 100 µg/mL after 72 h of treatment, when the viability of 4T1 cells was reduced to $80.2 \pm 1.7\%$, and at the concentration of 200 µg/mL, the cell viability decreased to $76.7 \pm 2.5\%$. Based on these results, PBP at a concentration of 50 µg/mL was selected as the highest non-toxic dose, which was used for the preparation of PBP-stimulated conditioned medium (PBP-CM). This dose of PBP also did not reduce the viability of RAW 264.7 macrophages during 24 h of treatment (Supplementary Fig. S1 – for all supplemental material see www.cellphysiolbiochem.com).

PBP-treated conditioned medium inhibited 4T1 breast cancer cell proliferation and migration

To study the effects of macrophage mediators on 4T1 cell viability, 4T1 breast cancer cells were cultured in RAW 264.7 cell-conditioned media (RAW-CM). The cancer cells were cultured in different concentrations of RAW-CM in the presence (CM-PBP) or absence (CM-NT) of PBP, and the cell viability was assessed using MTT assays. A progressive increase in the number of 4T1 cells occurred with an increase in the concentration of CM-NT. This increase in the amount of metabolically active cells, compared to the control (0% RAW-CM), occurred in a dose-dependent manner, which was observed after 72 h of incubation. The viability of 4T1 cells increased from $138.1 \pm 3.6\%$ to $195.8 \pm 2.7\%$ for CM-NT concentrations of 25% and 75%, respectively (Fig. 2A).

The opposite results were observed when 4T1 cells were cultured in PBP-stimulated RAW-CM (CM-PBP), where 4T1 cell viability was significantly decreased, especially after 48 and 72 h of incubation. This decrease in cell survival was inversely related to the concentration of CM-PBP. During the 72 h incubation, the viability of cells cultured in media containing 75% and 10% CM-PBP decreased from $82.1 \pm 2.4\%$ to $39.2 \pm 4.8\%$, respectively (Fig. 2B).

The potential of 4T1 cancer cells to metastasize was analysed using transwell cell migration assays. As shown in Fig. 3, increasing the concentration of CM-NT stimulated the migration of 4T1 cells in a dose-dependent manner (from a 1.54 ± 0.09 to a 4.49 ± 0.19 -fold increase for the cells cultured in CM-NT in the range of 10-75%, respectively). Similarly, the conditioned medium collected from PBP-stimulated cells also provoked an increase in cell migration level cells in a dose-dependent manner (from a 1.27 ± 0.07 to a 3.33 ± 0.05 -fold increase). However, the concentration of CM-PBP in the range of 25-75% significantly inhibited cell migration in comparison with 4T1 cells incubated in the respective dose of CM-NT.

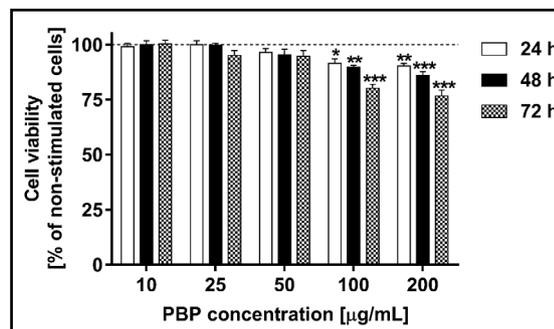
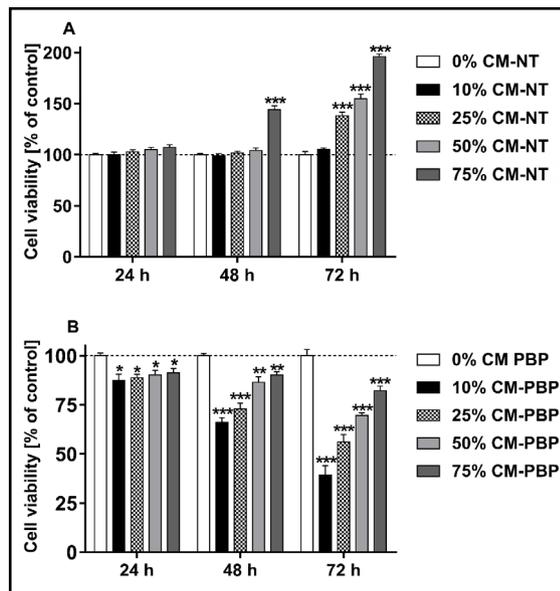


Fig. 1. Cell viability of murine breast cancer 4T1 cells stimulated with different concentrations of protein-bound polysaccharides (PBP) for 24, 48 and 72 h. Cell viability was assessed by the MTT colorimetric method. The data are shown as the means \pm SEM of three independent experiments with six wells for each experiment. The results are expressed as a percentage of control non-stimulated cells (served as 100%; horizontal line). Asterisks show significant differences between the PBP-treated cells compared with non-stimulated cells (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Fig. 2. Viability of 4T1 cells cultured in different amounts of RAW 264.7 macrophage-conditioned media: non-treated (CM-NT) (A) or PBP-treated (CM-PBP) (B). The cells were cultured in CM for 24, 48 and 72 h. Cell viability was measured using the MTT assay. The reported data are the means \pm SEM of three independent experiments with six wells for each experiment. Viability of the cells is presented as the percentage of cells incubated in complete DMEM medium containing 10, 25, 50 or 75% culture medium supplemented with 1% FBS (control group). Asterisks show significant differences between the cells cultured in CM-NT or CM-PBP compared with 0% conditioned-medium (** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).



PBP-treated conditioned medium upregulated secretion of the pro-inflammatory cytokines and decreased the production of angiogenic cytokines by 4T1 cells

Cytokines related to inflammation (IL-6 and TNF- α) and angiogenesis (MCP-1 and VEGF) in the cultured supernatants were measured using ELISA assays. CM-NT and CM-PBP only allowed background levels of mediators in the original conditioned medium to be measured. As shown in Fig. 4, treatment of cells with CM-NT and CM-PBP significantly increased the production of pro-inflammatory cytokines by 4T1 breast cancer cells compared with control cells (vehicle). However, secretion of both IL-6 and TNF- α were significantly higher when the 4T1 cells were cultured in 50% CM-PBP than in 50% CM-NT (127.1 ± 5.0 pg/mL vs. 85.4 ± 1.1 pg/mL for IL-6; $P < 0.05$, and 807.6 ± 9.2 pg/mL vs. 188.1 ± 7.8 pg/mL for TNF- α ; $P < 0.001$, respectively). Furthermore, both tested conditioned media samples increased the secretion of pro-angiogenic cytokines compared with vehicle. Importantly, in contrast to the measurement of pro-inflammatory mediators, these results showed that the presence of PBP in CM significantly decreased the production of MCP-1 (6.3 ± 0.08 ng/mL) and VEGF (568.7 ± 20.9 pg/mL) in comparison with the 4T1 cells treated with CM-NT (7.2 ± 0.06 ng/mL and 834.7 ± 25.8 pg/mL, respectively; $P < 0.001$). Stimulation of 4T1 only with PBP (25 μ g/mL) did not affect the levels of the tested mediators in the cultured supernatants.

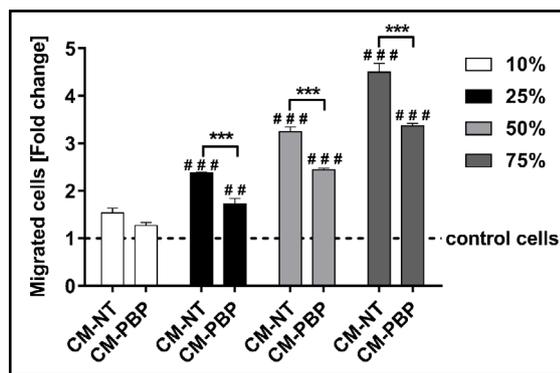
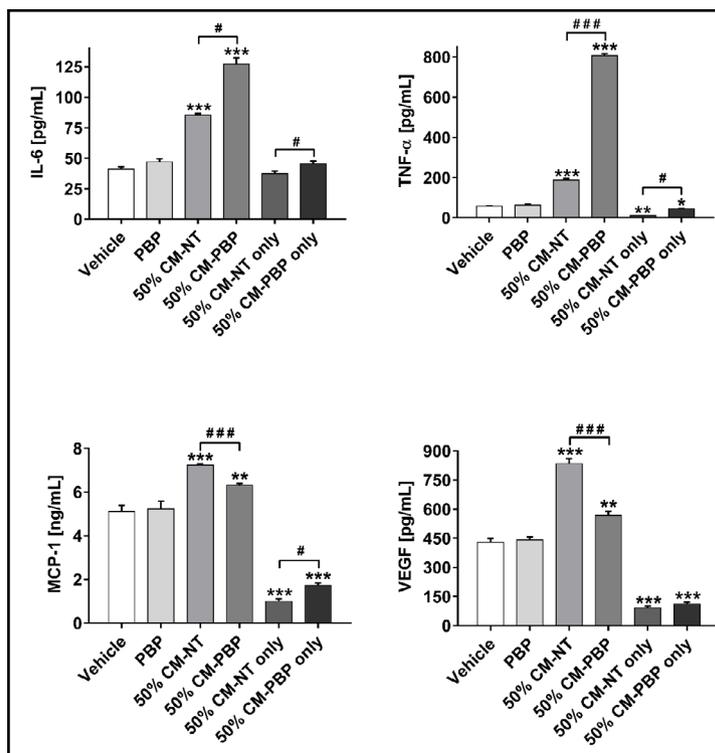


Fig. 3. Migration of 4T1 cells in different amounts of RAW 264.7 macrophage-conditioned media: non-treated (CM-NT) or PBP-treated (CM-PBP). Cells were cultured in CM-NT or CM-PBP for 24 h. Migration patterns of 4T1 cells were assessed using the colorimetric transwell cell migration assay. The level of migration of CM-treated cells was expressed as a fold change relative to equivalent control cells incubated in complete DMEM medium containing 10, 25, 50 or 75% of 1% FBS/DMEM medium. Data are from three independent experiments and presented as the mean \pm SEM. Asterisks show significant differences between the cells cultured in CM-NT compared with the CM-PBP-treated cells (** $P < 0.001$). Hash marks denote differences in the migration level between cells cultured in the suitable conditioned medium and control cells (horizontal line) (### $P < 0.001$; ## $P < 0.01$).

Fig. 4. Concentrations of pro-inflammatory (IL-6 and TNF- α) and pro-angiogenic (MCP-1 and VEGF) cytokines produced by 4T1 breast cancer cells cultured in RAW 264.7 macrophage-conditioned media: non-treated (CM-NT) or PBP-treated (CM-PBP). Cells were cultivated in 50% conditioned media for 72 h, and then the levels of cytokines in the supernatants were measured by ELISA methods. Concentrations of cytokines in original RAW 264.7 macrophage-conditioned media (CM-NT only and CM-PBP only), supernatants collected after cell stimulation with only PBP (PBP) or incubation of cells in control medium (vehicle) for 72 h was also assessed. Data are shown as the mean \pm SEM of six independent experiments. Asterisks show significant differences between the vehicle and all other tested supernatants (*** P <0.001; ** P <0.01, * P <0.05). Hash marks indicate significant differences between CM-NT and CM-PBP or CM-NT only and CM-PBP only, respectively (### P <0.001; # P <0.05).



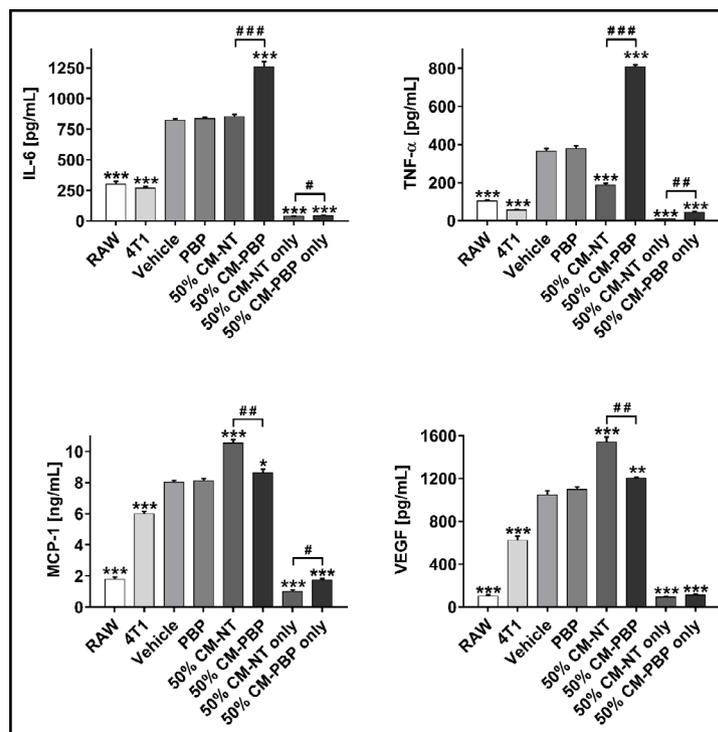
PBP-stimulated conditioned medium interfered with the production of pro-inflammatory and pro-angiogenic cytokines in co-cultures of breast cancer cells and macrophages

To further confirm that PBP affect the production of potential mediators of interactions between cells, 4T1 cells and RAW 264.7 macrophages were co-cultured with each other in the same well to mimic the pathophysiology of the tumour microenvironment. When both cell lines were treated with CM-NT, there was significant inhibition of TNF- α production and upregulation of pro-angiogenic cytokine secretion (MCP-1 and VEGF) compared with vehicle. In contrast, treatment of the cells with CM-PBP induced the highest production of IL-6 and TNF- α (P <0.001). Moreover, RAW 264.7 and 4T1 cells co-cultured in CM-PBP produced less MCP-1 (8.62 ± 0.2 ng/mL) and VEGF (1202.1 ± 10.3 pg/mL) than those co-cultured in CM-NT (10.5 ± 0.2 ng/mL and 1541.3 ± 46.3 pg/mL, respectively; P <0.01). There were only low levels of all cytokines in individual RAW 264.7 cell supernatants. Individually cultured 4T1 cells produced higher amounts of MCP-1 and VEGF than macrophages. The concentrations of all tested cytokines in the cultured media collected after the stimulation of cells with only PBP were comparable to those estimated for the control co-culture (Fig. 5).

PBP-stimulated conditioned medium regulated M1 and M2 macrophage polarization in co-cultures of breast cancer cells and macrophages

In the co-culture system, we determined whether the presence of PBP in conditioned medium affect M1/M2 macrophage polarization based on enzyme activities and cytokine concentrations. The M1 subpopulation was assessed by determining IL-6 and TNF- α levels and iNOS activity. Increasing concentrations of IL-10 and TGF- β and upregulation of arginase 1 activity are indicative of the M2 macrophage subtype. To present the results of the experiments more clearly, the concentrations of IL-6 and TNF- α shown in Fig. 5 as pro-inflammatory cytokines, they are again presented in Fig. 6 as M1 cell markers.

Fig. 5. Concentrations of pro-inflammatory (IL-6 and TNF- α) and pro-angiogenic (VEGF and MCP-1) cytokines in the supernatants collected after co-culture of 4T1 cells and RAW 264.7 macrophages. The cells were co-cultured in conditioned media containing 50% non-treated (CM-NT) or PBP-stimulated (CM-PBP) RAW-CM for 72 h. Cell cultures of the following control groups were also performed: RAW 264.7 macrophages alone (RAW), 4T1 cells alone (4T1), cells stimulated only with PBP (PBP) and cells co-cultured in control medium (vehicle). The levels of mediators in original RAW 264.7 macrophage-conditioned media (CM-NT only and CM-PBP only) were also assessed. Data are shown as the mean \pm SEM of six independent experiments. Asterisks show significant differences between the vehicle and all other tested supernatants (*** P <0.001; ** P <0.01; * P <0.05). Hash marks indicate significant differences between CM-NT and CM-PBP or CM-NT only and CM-PBP only, respectively (### P <0.001; ## P <0.01, # P <0.05).

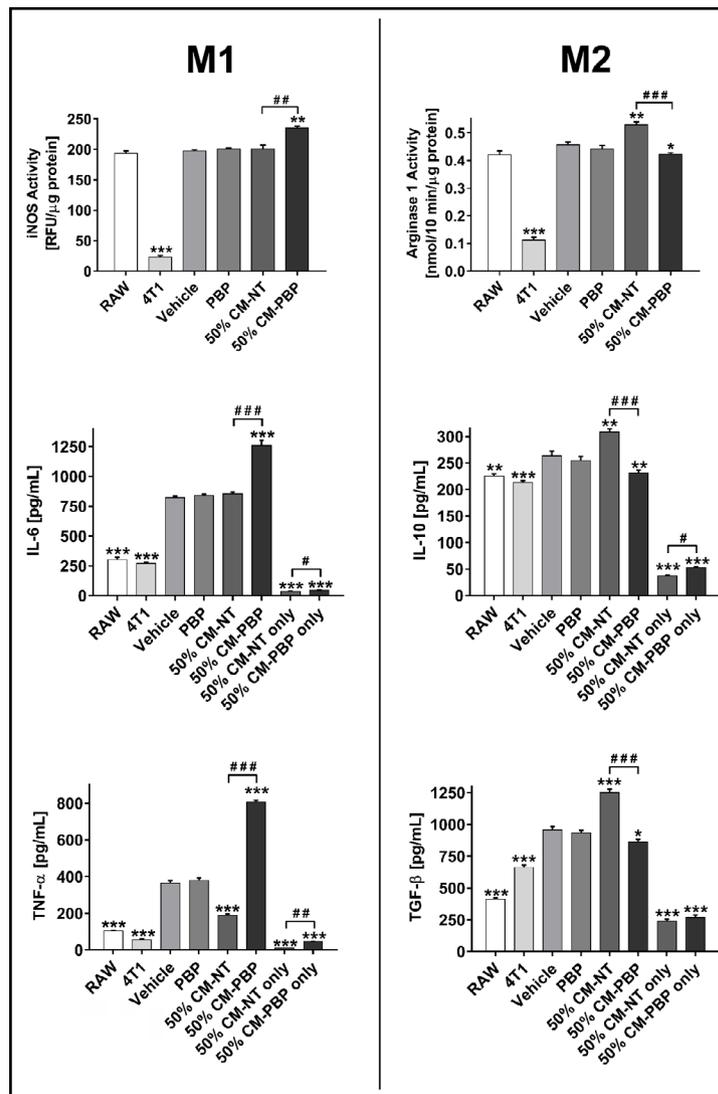


As shown in Fig. 6, treatment of co-culture with CM-PBP significantly increased the levels of all M1 cell markers compared with CM-NT-stimulated cells and vehicle treatment. When RAW 264.7 cells were co-cultured with 4T1 cells in CM-NT, iNOS activity (193.5 ± 2.3 RFU/ μ g protein) and IL-6 levels (853.3 ± 14.6 pg/mL) did not differ compared with vehicle (201.0 ± 6.4 RFU/ μ g protein and 822.3 ± 10.7 pg/mL, respectively), whereas the TNF- α concentrations significantly decreased (364.7 ± 14.7 pg/mL vs. 188.1 ± 7.8 pg/mL, respectively; P <0.001). The levels of the tested markers in the supernatants collected after co-culture of 4T1 and RAW 264.7 cells in the presence of only PBP at a dose of 25 μ g/mL were comparable to those measured for the control co-culture. Analysis of M2 cell markers showed that stimulation of the co-culture with CM-NT significantly upregulated arginase 1 activity and IL-10 and TGF- β concentrations compared with vehicle treatment. When the cells were co-cultured in the presence of CM-PBP, the levels of these markers significantly decreased compared with not only the cells treated with CM-NT, but also the control co-culture.

Discussion

The triple negative form of breast cancer is associated with a poor prognosis due to a lack of identifiable, unique cellular markers [19]. Moreover, tumour-associated macrophages infiltrating the tumour microenvironment secrete many cytokines that stimulate cancer growth and angiogenesis [20-21]. Therefore, effective agents that are able to disrupt this crosstalk are urgently sought. In the present study, protein-bound polysaccharides from *C. versicolor* fungus were determined to be a potential chemopreventive agent with anti-angiogenic properties in the tumour microenvironment created using RAW-CM and co-cultures of RAW 264.7 macrophages and 4T1 cells. The results from the MTT assay showed that PBP decreased the viability of 4T1 breast cancer cells in a dose-dependent man-

Fig. 6. Effects of RAW 264.7 macrophage-conditioned media: non-treated (CM-NT) or PBP-treated (CM-PBP) on the M1/M2 macrophage subtype following co-culture with 4T1 cells. Cells were co-cultured in 50% CM for 72 h. Cell cultures of the following control groups were also performed: RAW 264.7 macrophages alone (RAW), 4T1 cells alone (4T1), cells co-cultured in fresh 1% FBS/DMEM medium (vehicle), cells stimulated with only PBP (PBP) and original RAW 264.7 macrophage-conditioned media (CM-NT only and CM-PBP only for cytokine measurements). M1 macrophage polarization was assessed using the following markers: determination of IL-6 and TNF- α levels and activity of inducible nitric oxide synthase (iNOS). The concentrations of IL-10 and TGF- β , and arginase 1 activity were indicative of M2 macrophage polarization. Data are shown as the mean \pm SEM of six independent experiments. Asterisks indicate significant differences between the vehicle and the all other tested groups (** P <0.001; * P <0.01, * P <0.05). Hash marks show significant differences between CM-NT and CM-PBP or CM-NT only and CM-PBP only, respectively (### P <0.001; ## P <0.01, # P <0.05).



ner. These findings increase knowledge about the anti-cancer activity of PBP, indicating that PBP may be an effective agent not only in the treatment of the oestrogen-receptor-positive breast cancer cells [15] but also TNBC. Based on the results from the MTT assay, PBP at a concentration of 50 μ g/mL was selected as the highest non-toxic dose, which was used for the preparation of PBP-stimulated conditioned medium (CM-PBP) to exclude PBP toxicity during stimulation of 4T1 cells with CM-PBP.

It is well established that tumorigenesis accompanies macrophage infiltration [20]. Conditioned media derived from macrophages that were used in our experiments mimicked a pathophysiological environment where macrophages infiltrate into the areas surrounding breast cancer cells. This experimental approach is more appropriate for testing the immune response in the tumour microenvironment because it contains multiple mediators produced by cancer cells and macrophages. Moreover, it directly shows how PBP may modulate communication between macrophages and cancer cells by affecting the secretion of pro-inflammatory and angiogenic mediators. We demonstrated that CM derived from non-treated macrophages (CM-NT) promoted 4T1 breast cancer cell proliferation and migration. In contrast, the culture of 4T1 cells in the PBP-stimulated CM not only inhibited cell proliferation but also caused a decrease in the number of metabolically active cells compared with control cells.

Moreover, CM-PBP significantly inhibited 4T1 cell migration, indicating that PBP interfere with crosstalk between cancer cells and macrophages, thus inhibiting cancer cell growth and migration. The disturbance of communication between these cells is important since the results of other researchers have shown that mediators released by macrophages induce the migration, invasion and metastasis of breast, colorectal and gastric cancer cells [22-24]. Although, PBP influence the migration of 4T1 cells in presence of macrophages and *vice versa* is still unknown, there are some experimental data showing the direct inhibitory effect of CV extract containing PBP on the migration and invasion of certain cancer cells. Luo et al. [25] demonstrated that CV aqueous extract inhibited 4T1 breast cancer cell invasion and migration *in vitro*. These results were reflected *in vivo* when mice injected with 4T1 cells showed decreased growth of lung but not liver metastases in response to CV extract treatment. The anti-migratory activity of extracts from *Trametes versicolor* (also known as *Coriolus versicolor*) was also demonstrated in several human colon cancer cell lines [26-27] and melanoma cells [28]. The proposed mechanism for this action of CV extract is its ability to reduce expression of matrix metalloproteinases (MMPs) in cancer cells. Several studies showed that the activity of MMP-2 and/or MMP-9 was significantly reduced after CV extracts treatment in breast cancer cells [25] and colony cancer cells [26-27] that correlated with a decrease in cell migration. Matrix metalloproteinases have an important role during the degradation of the extracellular matrix when cancer cells move from the primary lesion to interstitium and enter the vasculature. These enzymes can influence the tumour environment by promoting angiogenesis, tumour growth, and metastasis [29]. Among TAMs, M2-like phenotype cells are characterized by high expression of MMPs, including MMP-2, MMP-7, MMP-9, and MMP-12 [30]. Although, the effect of PBP on the MMP expression in macrophages is still unknown, however, our results indicate that PBP altering TAMs from M2 to M1 subtype, they can indirectly reduce the amount of MMPs in tumour microenvironment. However, further research is needed to confirm this thesis. There are also no evidences that PBP can influence macrophages migration in tumour microenvironment and new research is needed to investigate this phenomenon. However, preclinical and human level studies show that CV extract is able to reduce the depression of immune cells and immune cell activity following chemotherapy. This includes, increasing the number of macrophages, lymphocytes and neutrophils. Moreover, CV extract also induces the tumouricidal activities of macrophages, such as presentation of tumour antigen and production of reactive nitrogen intermediates, reactive oxygen intermediates and TNF- α [31].

Cancer development is a result of the secretory action of both cancer and immune cells [32]. Therefore, in the present study, we examined the concentrations of pro-inflammatory and pro-angiogenic cytokines that were produced by 4T1 cells cultured in CM-NT or CM-PBP. Our results demonstrated that the presence of PBP in RAW-CM significantly inhibited the production of angiogenesis-related factors (MCP-1 and VEGF) and, in contrast, stimulated the secretion of pro-inflammatory mediators (IL-6 and TNF- α) by 4T1 cancer cells. These findings clearly indicate that PBP from *CV fungus* are able to disrupt the crosstalk between 4T1 breast cancer cells and RAW 264.7 macrophages.

MCP-1 is a well-known chemokine in macrophage-related migration that is also involved in breast cancer progression through stimulation of cancer cells migration and mediation of the recruitment of specific monocyte populations that support the establishment of metastatic disease [33]. VEGF is a cytokine that is critical for the proliferation, angiogenesis and metastasis associated with tumour progression [34]. It has been demonstrated that blocking MCP-1 as well as VEGF signalling notably inhibited 4T1 cell migration [35]. Therefore, we presume that PBP-treated CM may also decrease the migration of 4T1 cells by interfering with the interactions related to the MCP-1 and VEGF secretion by macrophages and cancer cells, thus inhibiting tumorigenic signals.

The role of pro-inflammatory cytokines, such as IL-6 and TNF- α , in the tumour microenvironment is ambiguous and controversial and has been presented in the review papers. On the one hand, there is evidence that shows that IL-6 and TNF- α stimulate the growth of tumour cells by inducing angiogenesis or inhibiting anti-tumour immune responses, respec-

tively [36-37]. However, numerous reports have revealed that both cytokines are involved in tumour-preventing processes. IL-6 is able to shift the T cell immune response from a suppressive to a responsive state that can effectively act against tumours [36], and it also stimulates vessel sprouting with defective pericyte coverage, potentially contributing to abnormal tumour vasculature [38]. The anti-tumour role of TNF- α involves immune responses that prevent tumour formation, including the promotion of tumour stroma destruction by cytotoxic T lymphocytes, tumour infiltrating macrophages and dendritic cells [37]. Moreover, we previously showed that treatment with PBP induced a decrease in breast cancer MCF-7 cell growth, which was a TNF- α -dependent phenomenon [15]. In the present work, we demonstrate that CM-PBP induced an increase in TNF- α production by 4T1 cells, which inversely referred to a decrease in the number of metabolically active cancer cells. All these findings indicate that an increase in the production of TNF- α and IL-6 by cancer or immune cells may inhibit the tumour growth.

Macrophages can be divided into two distinct phenotypes, M1 and M2, which have opposite roles in tumour growth and metastasis. M1 macrophages foster an inflammatory response against tumour cells, whereas M2 cells tend to exert an immune suppressive phenotype, favouring tumour progression [39]. Therefore, we examined the effect of CM-NT and CM-PBP on M1/M2 macrophage polarization following co-cultures with 4T1 cells. Categorizing M1 and M2 macrophages using phenotypic surface markers is difficult given the potential for intermediates. Alternatively, assessment of functional markers aids in the identification of M1 and M2 phenotypes. These include upregulation of iNOS activity and increased production of IL-6 and TNF- α for M1 cells and high expression of arginase 1 and increased secretion of IL-10 and TGF- β for M2 cells [40]. Our results revealed that CM-PBP treatment decreased M2 marker expression, whereas co-culture of macrophages with 4T1 cells in CM-NT significantly upregulated these markers. Comparing these results with the findings from MTT and migration tests suggests that the suppressive microenvironment created by CM-NT promotes the growth of breast cancer cells. In contrast, non-treated CM did not change the level of M1 markers except for lowering the TNF- α level. Importantly, the presence of PBP in CM upregulated the activity of iNOS and the production of IL-6 and TNF- α . These results indicate that PBP promote the M1 macrophage phenotype in the neoplastic microenvironment and inhibit the immunosuppressive response elicited by M2 cells. Since Engström et al. [41] demonstrated that CM from M1 macrophages, but not the M2 phenotype, inhibits the proliferation of the colon cancer cell lines HT-29 and CACO-2, we concluded that the presence of PBP in RAW-CM decreases the viability of 4T1 cancer cells by inducing the M1 phenotype.

Conclusion

Accumulating evidence clearly indicates that macrophages play a crucial role in the tumour microenvironment, which includes intricate crosstalk involving a series of chemokines and cytokines secreted from neoplastic cells and infiltrating macrophages. Since TAMs resemble M2 macrophages and exert pro-tumour functions through immunosuppressive actions [4], the suppression of TAM recruitment, switching from the M1 to M2 phenotype, and production of associated mediators have been proposed as cancer therapeutic strategies [42]. The results of the present study show that PBP interfere with the crosstalk between 4T1 breast cancer cells and macrophages by changing communication through pro-inflammatory and angiogenic mediators and modulating M1/M2 macrophage subtypes. All these findings suggest that PBP have chemopreventive properties and they are a promising agents to prevent TNBC progression. This has a valuable significance since the treatment of TNBC patients is limited due to a lack of identifiable, unique cellular markers.

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Author Contributions

T.J.: provided the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, revised it critically for important intellectual content; M.P. and J.S.: analysis and interpretation of data for the work; S.W.: revising the work critically for important intellectual content. All authors approved the final version of the manuscript.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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