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Effects of Early Stage Second-Degree Burn Blister Exudate on Macrophage Polarization and *In Vitro* Activity Against CT-26 Colon Cancer Cells

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Abstract

Background: Human second-degree burns can form blisters that allow burn wound microenvironment (WME) fluid to accumulate, which leads to inflammation. Different types of cells are present in burn WME, including macrophages; these innate immune cells are present in tumor microenvironments (TMEs) and burn WMEs. They adapt their phenotypes according to environmental stimuli, which vary from pro-inflammatory (M1) to anti-inflammatory (M2). It is evident that these microenvironments share some similarities in terms of macrophage plasticity; therefore, this study examines whether burn blister exudate (BBE) can enhance macrophage activity against CT-26 cancer cells by macrophage polarization.

Methods: Real-time PCR and ELISA were used to examine the effects of human BBE on untreated and M2 macrophages. As part of the immune response assessment, yeast phagocytosis was conducted. The impact of BBE-induced macrophages on CT-26 cancer cell survival and migration was assessed using MTT proliferation assay and scratch wound healing assay, respectively.

Results: According to the results, tumor necrosis factor-alpha, interferon regulatory factor 5, induced nitric oxide synthase, and CD86 were upregulated as M1-related markers and cytokines, and M2-associated cytokines and markers, transforming growth factor beta, IL-10, Fizz1, Arginase-1, and CD206, were downregulated in untreated and M2 macrophages treated with BBE. BBE also enhanced the phagocytic capacity of untreated and M2 macrophages. Furthermore, the incubated CT-26 cell line with conditioned medium of BBE treatment groups suppresses proliferation and impedes migration of cancer cells.

Conclusion: we found that BBE-treated macrophages possess an M1-like phenotype and inhibit the proliferation and motility of CT-26 cancer cells.

Keywords: Burn wound, M1 macrophages, Macrophage polarization, Tumor microenvironment.

Introduction

Macrophages play crucial roles in the innate arm of the immune system, acting as sentinel cells that identify pathogens based on their recognition patterns, demonstrating versatility and functionality under a variety of physiological and pathological conditions (1). These cells exhibit a remarkable degree of phenotypic plasticity and can reprogram their gene expression in response to external stimuli, which allows them to exist in different

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polarization states (2). In response to various environmental cues, these cells can switch between M1 and M2 activation phenotypes, adopting distinct polarization states. The polarization of macrophages into classically activated M1 or alternatively activated M2 subsets is a well-known phenomenon triggered by various external stimuli (3). As they polarize toward M1 or M2 phenotypes in response to microenvironmental signals, such as interleukin-4 (IL-4), interferon- γ (IFN-γ), and many other cytokines (4) macrophages can perform diverse functions in anti-inflammatory regulation and tissue homeostasis (5, 6). The balance between these two macrophage phenotypes needs to be maintained in a number of diseases, including cancer and burn wounds (7).

There is a highly structured ecosystem called the tumor microenvironment (TME), which comprises cancer cells surrounded by non-malignant cells. TMEs consist primarily of immune cells, cancer-associated fibroblasts (CAFs), endothelial cells (ECs), pericytes, and other cell types that are specific to each tissue (8). In the TME, macrophage polarization plays a critical role in determining tumor progression and immune responses. The polarization states of tumor-associated macrophages (TAMs) significantly contribute to the formation of the TME, which has an important impact on tumor growth and metastasis (9). There has been evidence that M2-polarized TAMs are associated with promoting the growth of tumors and have an immunosuppressive effect in the TME. On the other hand, M1-polarized macrophages within the TME may exhibit antitumor effects and contribute to the host's defense mechanisms (10).

Seconds-degree burns, also called partial-thickness burns, are characterized by damage that extends beyond the epidermis and into the dermis (11). This type of burn can be classified into superficial and deep second-degree burns, with the latter having a higher risk of chronic inflammation and scarring due to its reticular dermal layer involvement (12). When a second-degree burn is severe, blisters appear as a protective mechanism, allowing fluid to accumulate. As a result of the separation of the epidermis from the dermis, blisters form, which are often accompanied by inflammation (13). Among the critical components of burn blister exudate (BBE) are cytokines and signaling molecules that promote inflammation and tissue repair. It has been shown that blister exudate contains elevated levels of pro-inflammatory cytokines such as IFN- γ and tumor necrosis factor-α (TNF-α) (14). Proteins like matrix metalloproteinases (MMPs), which play an essential role in the remodeling of extracellular matrix components (15), and immunoglobulins and prostaglandins, which contribute to inflammatory responses (16). In addition to attracting immune cells to the site of injury, these cytokines and proteins promote angiogenesis and facilitate the process of re-epithelialization (17). These cytokines and proteins in blister exudate indicate an active inflammatory response. Therefore, we can conclude that The BBE represents the burn wound microenvironment (WME) (16, 18). There are three primary phases of burn injury. The inflammatory phase, lasts approximately 3 to 7 days. It is characterized by rapidly recruiting immune cells, including macrophages, to the injury site. At first, macrophages are activated into the M1 phenotype, which secretes pro-inflammatory mediators like TNF-α, interleukin-1 (IL-1), and interleukin-6 (IL-6). The proliferative phase, which typically lasts one to three weeks (19). As the inflammation progresses, macrophages shift towards the M2 phenotype, which mainly produces anti-inflammatory mediators like interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (20). Just like in tumors, in burn injuries, the wound microenvironment (WME) is complex (21). It is composed of a specific group of immune cells as well as stromal cells, each of which contributes an array of growth factors and cytokines. After thermal injury, macrophages are the most prominent cells in the burned area (22). Wound macrophages can express both M1 and M2 markers. In general, tissue-

resident macrophages are M2-like and reparative, whereas macrophages expressing the M1 phenotype are pro-inflammatory and originate from monocytes. Although the M2 macrophage is dominant in normal human skin, the pro-inflammatory M1 macrophage is dominant in the initial response to burns (23). These M1 cells secrete mediators such as nitric oxide (NO), TNF-α, IL-1, and IL-6, which serve as cytotoxins against damaged cells and mediate resistance against infections. At later time points, macrophage populations adopt an anti-inflammatory, proliferative, and remodeling profile dominated by the M2 phenotype (24). These M2 cells reduce inflammation at the site of the injury and release growth factors to promote proliferation and angiogenesis (25).

In 1986, Harold F. Dvorak, Professor of Pathology at Harvard Medical School, regarded tumors as "wounds that do not heal" due to their common stroma and vascular permeability (26-28). Based on the presence of both macrophage phenotypes in these microenvironments, it is evident that these microenvironments share some similarities in terms of macrophage plasticity. Therefore, in the current study, we aim to investigate the effect of human second-degree BBE as the burn WME (16, 18), on macrophage polarization to determine whether it can enhance macrophage activity against CT-26 colon cancer cells through macrophage polarization.

Materials and Methods

Second-degree burn blister exudate (BBE) collection

Burn blister exudate (BBE) were collected at the Taleghani Hospital of the Ahvaz Jundishpur University of Medical Sciences, Iran. from five patients admitted to the burn emergency room. Physicians determined the severity of burns, and the total body surface area burned (TBSA%). The hospital ethics board of research accepted the study, and all patients provided informed consent for BBE collection. This study was approved by the Biomedical Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences under the approval ID: IR.AJUMS.ABHC.REC.1402.401.

It was determined that the following inclusion criteria were to be met to collect BBEs:

1. Male and female patients must be between 18 and 50 years of age.
2. Patients must not be on antibiotic therapy.
3. No HIV or HBV infection.
4. No prior disease such as diabetes, immunodeficiency, or cancer.
5. Patients must be admitted within 48 hours of blister formation, representing the early stage of burn injury.

Patients who refused to participate in this study after BBE collection, or a patient who developed an infection after 72 hours of BBE collection, were excluded from participation. As part of the BBE collection process, all guidelines for burn management were followed (29). Blisters were disinfected with a 0.05% chlorhexidine solution and rinsed extensively with 0.9% NaCl. The BBE was aspirated using a conventional needle and syringe. To eliminate all debris and cells from the fluid after the samples were collected, and centrifuged (10 minutes at 700 RCF). The collected supernatants were stored at -80 °C until further analysis. A bicinchoninic acid (BCA) protein assay kit (Parstous Biotechnology, Iran) was employed to measure BBE protein concentration for future experiments. Although BBE is bacteriologically sterile, a 72-hour aerobic bacterial culture at 37 °C was carried out to verify the sterility of the aspiration method.

Animals and harvesting of peritoneal macrophages

Female Balb/c mice were purchased from the Ahvaz Jundishapur University of Medical Sciences Center for Reproduction and Maintenance of Laboratory Animals (Ahvaz, Iran). The Balb/c mice were housed in the animal maintenance center under a 12-hour light/dark cycle and received standard chow and water ad libitum. This study used mice weighing 27–31 g at 7–9 weeks of age. The Animal Care and Use Committee of Ahvaz

Jundishapur University of Medical Sciences approved the animal handling protocols.

Briefly, to stimulate macrophage recruitment into the peritoneal cavity, female Balb/c mice were injected intraperitoneally (IP) with 2 mL of 3% thioglycolate broth (Mirmedia microbiological, Iran). At the end of day four, the animals were sacrificed by cervical dislocation, and the peritoneal cavity was washed with 3% fetal bovine serum (FBS) (Biosera, France) enriched phosphate-buffered saline (PBS) (Bioidea, Iran). The gathered fluid was centrifuged for 10 minutes at speed of 300 RCF at 4 °C and the resulting pellet was numbered in a Neubauer chamber. For the next step, 2×10^6 cells were seeded in Roswell Park Memorial Institute 1640 (RPMI 1640) media (Biosera, France) supplemented with 12% FBS (Biosera, France) and 1% Penicillin/Streptomycin (Pen strep) (Biosera, France). After 4 h of incubation at 37° C with 95 % humidity and 5% CO2, the unattached cells were removed after media refreshment. Isolated macrophage characterization was determined using flow cytometry and antibodies targeting CD80, CD68, and surface markers.

Induction of M1 and M2 macrophages

The characterized macrophage population was treated with 100 ng/ml of lipopolysaccharide (LPS) to induce M1 phenotype and 100 ng/mL of IL-4 to induce M2 phenotype in three time periods of 6 hours, 12 hours, and 24 hours. The most appropriate time point was selected for further experiments. Afterward, the gene expression of M2-specific markers CD206, Fizz-1, and Arginase-1 (Arg-1) and M1-specific inducible nitric oxide synthase (iNOS), interferon regulatory factor 5 (IRF5), and CD86 markers were assessed utilizing SYBR Green real-time polymerase chain reaction (RT-PCR). Untreated macrophages were considered as a control group.

Treatment of macrophages with burn blister exudate

The characterized macrophage population was treated with BBE at three concentrations: 5%, 10%, and 15% of the culture media (30), in three time intervals: 6 hours, 12 hours, and 24 hours, to determine the optimal concentration and time frame for treatment of BBE. The LPS-induced M1 and IL-4-induced M2 phenotype groups served as control groups. After finding the optimal time and concentration, BBE treatment was performed on untreated macrophages, as well as on LPS-induced M1 and IL-4 induced M2 phenotypes. Subsequently, SYBR Green RT-PCR was used to analyze the expression of M1- and M2-associated markers.

RNA extraction and Real- time PCR

RNA extraction was performed using a SinaPure Total RNA Extraction Kit (Tehran Cavosh-Clon, Iran). Subsequently, Nanodrop equipment (Micro Digital Co., Ltd., South Korea) was used in order to determine RNA concentration and purity by measuring absorbance at 260 and 280 nm, and calculating the 260/280 ratio. Complementary DNA (cDNA) synthesis was performed using the First Strand cDNA Synthesis Kit (Tehran Cavosh Clon, Iran) according to manufacturer's protocol.

The effect of BBE on macrophage polarization was evaluated the gene expression of M2-associated markers (CD206, Fizz-1, and Arg-1) and M1-associated markers (CD86, iNOS, and IRF5) using specific primers (Table 1), under optimal cycle and temperature conditions, with SYBR Green Master Mix (Ampliqon, Denmark). Beta-2 microglobulin (β2m) was used as the housekeeping gene, and relative expression levels were calculated using the 2^-ΔΔCT method.

Phagocytosis assay

The yeast-based phagocytosis assay was conducted to evaluate the phagocytic capabilities of the treated macrophages. Macrophages from different treatment groups were incubated in RPMI1640 (Biosera, France) and yeast particles in a 10:1 ratio (yeast: macrophage) for 2 hours. Subsequently, non-ingested yeast particles were washed off with PBS (Bioidea, Iran), and

the cells were stained by Giemsa dye. Phagocytosis was visualized under an inverted microscope (Optika, Italy), where phagocytes were defined as macrophages that had ingested at least one yeast particle. For each sample, 100 cells were counted across five random fields, and the percentage of phagocytic cells was calculated.

Table 1. Sequence of specific primers used for SYBR Green real-time PCR analysis.

Gene Name	Forward	Reverse	Accession number
Arg-1 [a]	5′-CTCCAAGCCAAAGTCCTTAGAG-3′	5′-AGGAGCTGTCATTAGGGACATC-3′	NM_007482.3
Fizz-1 [a]	5′-AGGAGCTGTCATTAGGGACATC-3′	5′-CCAGTAGCAGTCATCCCAGC-3′	NM_020509.4
CD206 [a]	5′-CTCTGTTCAGCTATTGGACGC-3′	5′-CGGAATTTCTGGGATTCAGCTTC-3′	NM_008625.2
IRF5 [b]	5′-GGTCAACGGGGAAAAGAAACT-3′	5′-CATCCACCCCTTCAGTGTACT-3′	NM_001252382.1
CD86 [b]	5′-TCAATGGGACTGCATATCTGCC-3′	5′-GCCAAAATACTACCAGCTCACT-3′	NM_019388.3
iNOS [b]	5′-GTTCTCAGCCCAACAATACAAGA-3′	5′-GTGGACGGGTCGATGTCAC-3′	NM_001313921.1
β2m [c]	5′-TTCTGGTGCTTGTCTCACTGA-3′	5′-CAGTATGTTCGGCTTCCCATTC-3′	NM_009735.3

[a] CD206, Fizz-1, and Arg-1 are M2 phenotype specific markers, [b] CD86, iNOS, and IRF5 are M1 specific markers, [c] β2m is considered as housekeeping gene.

Enzyme-linked immunosorbent assay (ELISA)

The conditioned media from macrophages in different treatment groups were collected to measure cytokine levels. According to the manufacturer's instructions, ELISA kits (Karmania Parsgene, Iran) were used to assess the production of M2-related cytokines, transforming growth factor beta (TGF-β) and IL-10, and M1-related cytokines, IL-1 and TNF-α.

CT-26 tumor cell line culture

The CT-26 mouse colon carcinoma cancer cells were acquired from the National Cell Bank, Institute Pasteur of Iran (Tehran, Iran), cultured in RPMI 1640 (Biosera, France) enriched with 15% FBS (Biosera, France) and 1% Pen-Strep (Biosera, France), and incubated at 37 °C with 5% CO2 and 95% humidity to reach confluency. Furthermore, the medium was refreshed every three days, and Trypsin-EDTA (0.25 %) (Bioidea, Iran) was used to passage the cells.

Scratch wound healing assay

A scratch wound healing test was performed to determine whether treated macrophages inhibit the migratory capacity of CT-26 cells. A linear scratch was created using a pipette tip to wound the confluent monolayer of CT-26 cells in each well. In a subsequent step, the cells were washed three times with PBS (Bioidea, Iran) to remove unattached cells, and the conditioned media from the different groups of treated macrophages was added to wells for 48 hours. After scratching, inverted microscopy (Optika, Italy) was used to monitor the migration process at three different time points (0, 24, and 48 h). Lastly, the images were analyzed using the Wound Healing Size Tool, an ImageJ® plugin (31), The percentage of wound healing was calculated using the equation 1.1:

$$\left[Wound\ closure(\%) = \left(1 - \left(\frac{Wound\ Area\ at\ Ta_n}{Wound\ area\ at\ Ta}\right)\right) \times\ 100\right] \quad 1.1$$

The Ta parameter represents the time immediately following the creation of the wound, and the Ta_n parameter defines the time after the wounding (n = 24 and 48 h), with the wound area measured in μm^2.

MTT assay

To evaluate the potential effect of treated macrophages on CT-26 cell proliferation, the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was employed on confluent CT-26 cells using an MTT assay kit (Anacell, Iran) following the manufacturer's instructions. The optical density (OD) was measured at 570 nm with an

ELX-808 microplate reader (BioTek, USA) after 24 h, 48 h, and 72 h of treatment with the collected conditioned media of different groups of treated macrophages. The mean OD of CT-26 cells was then measured.

Statistical analysis

The statistical analysis and graph drawing were performed using GraphPad Prism version 10 (GraphPad, USA). Data are presented by the mean ± standard deviation (SD). The Shapiro-Wilk test was used to assess the normality of the data distribution. Where appropriate, one-way ANOVA followed by Dunnett's multiple comparisons test and two-way ANOVA followed by Tukey's multiple comparisons test was applied to evaluate statistical significance. A p-value of <0.05 was considered statistically significant.

Results

Patients and burn blister exudate properties

As a result of the physical examination of the patients, demographic and clinical data including sex, age, and burn characteristics were recorded. All consenting patients were male, with a mean age of 34.8±8.3 years. The color of the collected BBEs ranged from pale yellow to dark yellow, and the protein concentrations are summarized in Table 2. Bacteriological cultures of all five samples were negative for microbial growth.

Successful characterization of macrophages

Flow cytometry confirmed macrophages that were isolated from peritoneal cavity highly expressed CD68 and CD80, which are as macrophage-specific surface markers (Fig. 1).

Table 2. Clinical characteristics of burn patients and Exudate properties.

Patients	Sex	Age (yrs)	Burn Characteristic		Exudate volume (mL)	Exudate protein Concentration(mg/mL)
			Cause	TBSA (%)		
1	M	25	Boiled water	9	9	2.99
2	M	28	Boiled water	8	4	2.96
3	M	48	Boiled water	15	5	2.91
4	M	33	Boiled water	10	7	2.93
5	M	40	Boiled water	7	6	2.89

*After the admission of the burn patients at the Taleghani Hospital burn emergency room, sex (M, male) and age were recorded. Physicians evaluated the total body surface area (TBSA) and the burn's severity. Burn blister exudates (BBE) were collected using a conventional needle and syringe within 48 hours following blister formation, as indicated in Materials and Methods.

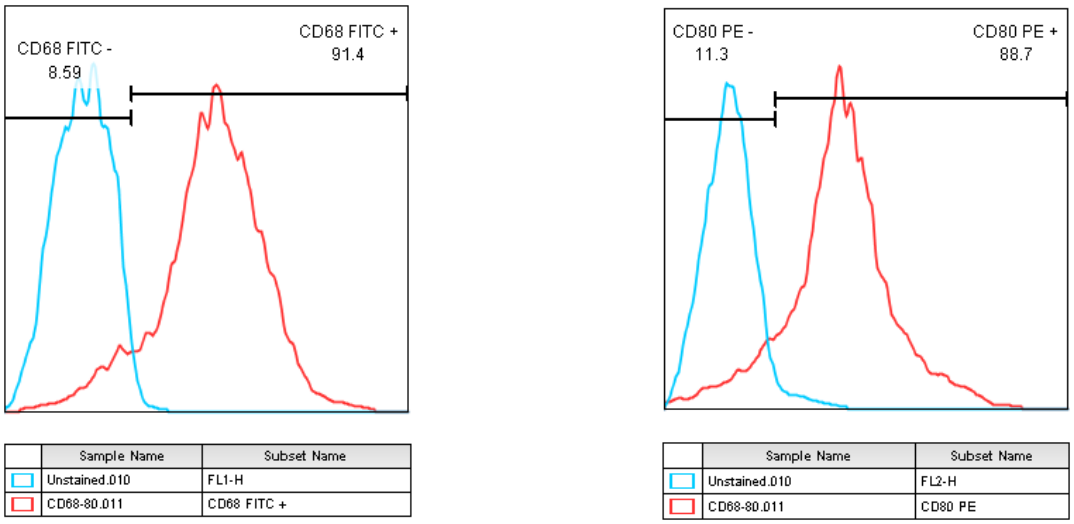

Fig. 1. In isolated cells analyzed by flow cytometry, CD68 and CD80 are highly expressed.

Successful polarization of LPS induced M1 and IL-4 induced M2 macrophages

To induce M1-like and M2-like phenotypes, characterized peritoneal macrophages were treated with 100 ng/ml LPS or 100 ng/ml IL-4 for 6, 12, and 24 hours, respectively. Based on marker expression analysis, the 6-hour time was identified as the most effective. LPS-treated macrophages exhibited increased expression of M1-specific markers, including IRF-5, iNOS, and CD86, while the relative expression of M2-specific markers, such as CD206, Arg-1, and Fizz-1 was decreased compared to untreated macrophages. Conversely, IL-4-treated macrophages showed decreased expression of M1-associated markers, and increased expression of M2-associated markers compared to untreated macrophages (Fig. 2). Furthermore, to confirm successful polarization of M1-like and M2-like macrophages by LPS and IL-4, ELISA assay was used to measure the secretion of IL-10 and TGF-β (M2-realated cytokines) and IL-1 and TNF-α (M1-related cytokines). It was observed that macrophages treated with LPS showed an increased level of M1 cytokines and a decreased level of M2 cytokines, while macrophages treated with IL-4 showed an increased level of M2 cytokines and a decreased level of M1 cytokines, compared to untreated macrophages (Fig. 3). Therefore, treatment with LPS and IL-4 effectively induced M1 and M2 polarization, respectively.

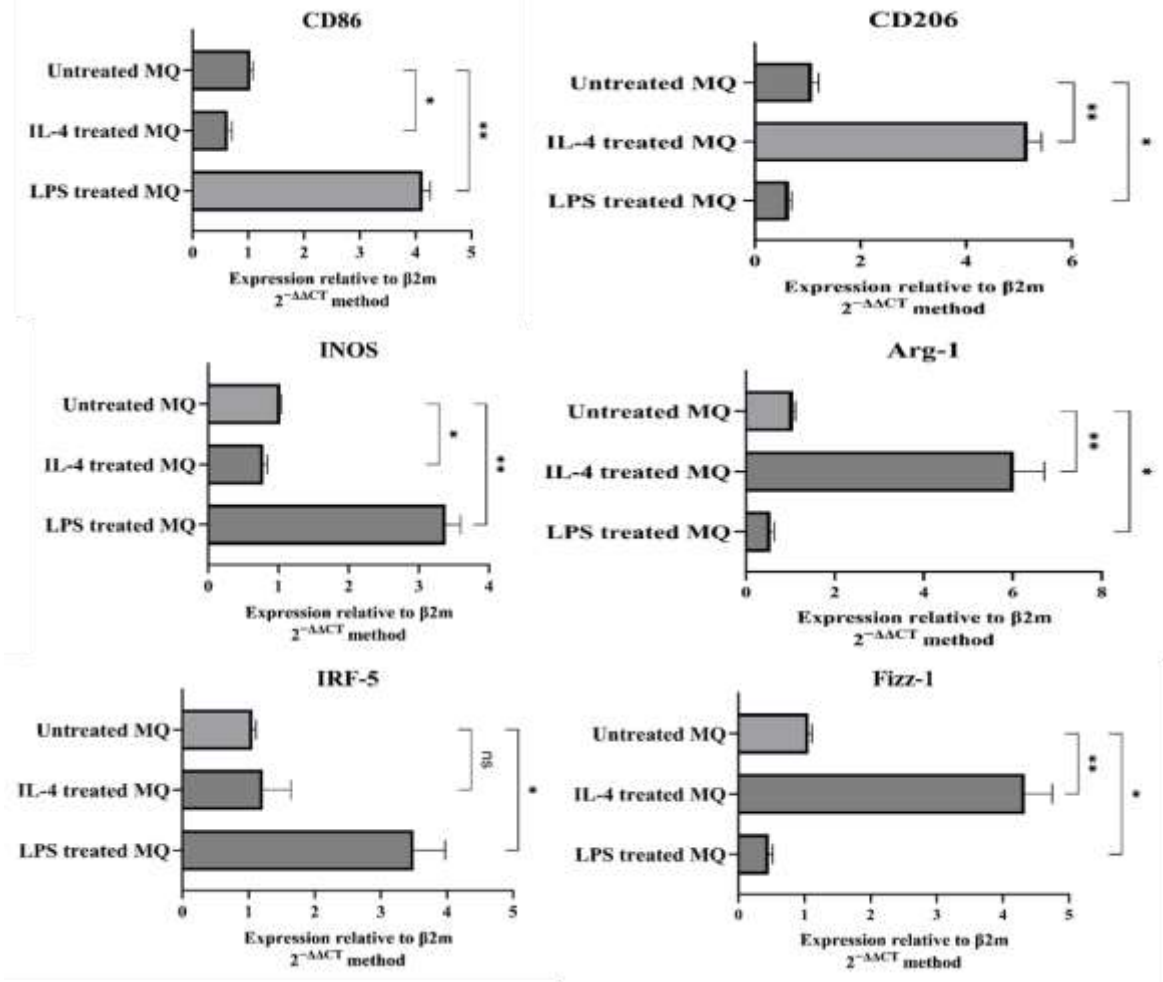

Fig. 2. In response to LPS and IL-4 treatment, M1 polarization and M2 polarization were successfully achieved. The relative expression of Fizz-1, Arg-1, and CD206 as M2-associated markers and CD86, IRF5, and iNOS as M1-associated markers in peritoneal macrophages that have been exposed to LPS (100 ng/ml) and IL-4 (100 ng/ml) for 6 hours. Data is shown as fold changes relative to β2m. Data presented are mean ± SD (n=3). * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ and ns = nonsignificant. Macrophages (MQ).

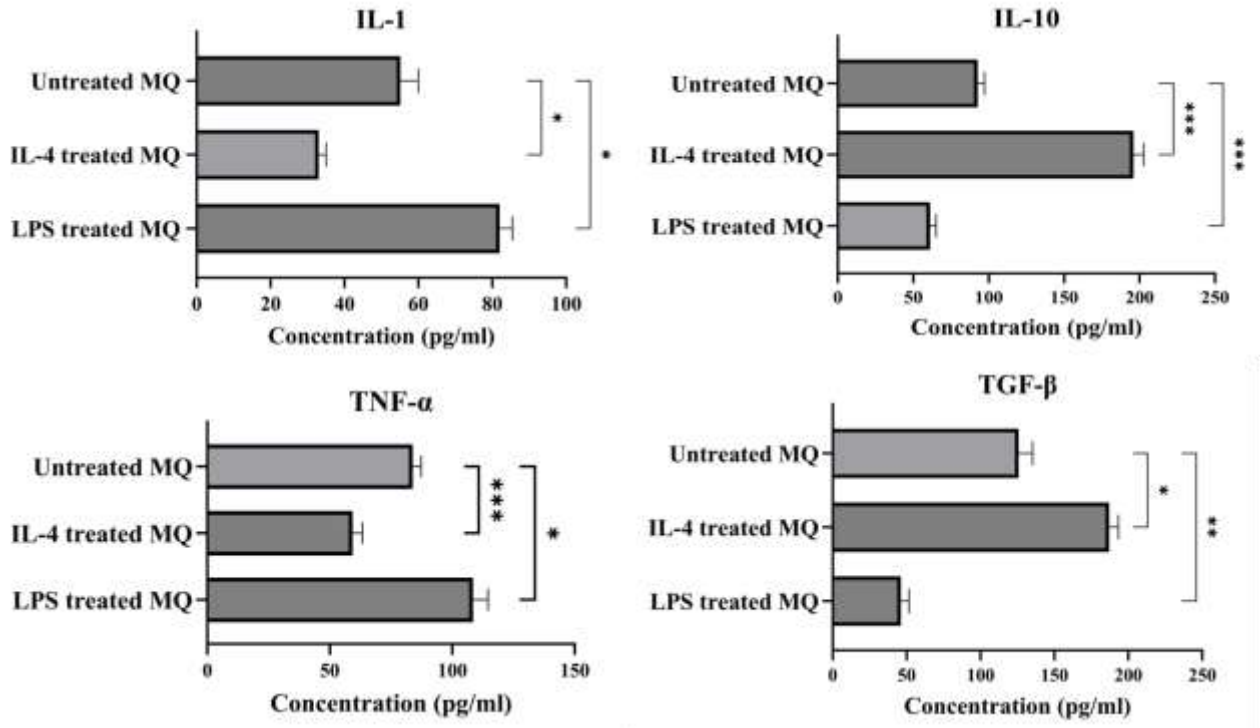

Fig. 3. In response to LPS and IL-4 treatment, M1 polarization and M2 polarization were successfully achieved, IL-10 and TGF-β secretion (M2 markers) decreased and IL-1 and TNF-α secretion (M1 markers) increased in LPS treated macrophages. While IL-1 and TNF-α secretion (M1 cytokines) decreased and IL-10 and TGF-β secretion (M2 cytokines) increased in IL-4 treated macrophages compared to untreated macrophages. The data is presented as concentration (pg/ml). Data presented are mean ± SD (n=3). * p< 0.05, ** p< 0.01, *** p< 0.001 and ns = nonsignificant. Macrophages (MQ).

Burn blister exudate induced M1-like phenotype

To assess the effects of BBE and establish the optimal concentration for macrophage treatment, characterized, untreated macrophages were exposed to BBE at concentrations of 5% (250 µg/ml), 10% (550 µg/ml), and 15% (1 mg/mL) of the culture media. These treatments were administered over three-time intervals: 6, 12, and 24 hours. The results indicate that the most effective concentration was 15% BBE (1 mg/mL) in culture medium applied for 6 hours. Subsequently, to evaluate the impact of BBE on M1 and M2 macrophage phenotypes, untreated macrophages, LPS-induced M1 macrophages, and IL-4-induced M2 macrophages were treated with the optimal concentration and time point. Our results demonstrated a decrease in the relative expression of M2- specific markers (Fizz- 1, Arg-1, and CD206) in the BBE-treated groups compared to the IL-4-induced M2 phenotype. Conversely, the expression levels of M1-markers (CD86, iNOS, and IRF-5) were upregulated in the treatment groups related to the IL-4-induced M2 phenotype (Fig. 4).

To further confirm the effects of BBE on different macrophage states, an ELISA assay was employed to quantify the levels of M2 -associated cytokines (IL-10 and TGF-β) as well as M1- associated cytokines (IL-1 and TNF-αs). In the BBE-treated groups, an increase in M1-specific cytokines was observed compared to IL-4-induced M2 macrophages, whereas M2-specific cytokines were decreased relative to the IL-4-induced M2 macrophages (Fig. 5). Based on these findings, we conclude that BBE promotes polarization toward an M1-like phenotype.

Burn blister exudate enhanced phagocytic competence of macrophages

Following the exposure of the treatment groups to yeast particles at a macrophage-to-yeast ratio of 1:10, the phagocytic capacity of the cells was assessed. The BBE-treated groups demonstrated an enhanced ability to phagocytose yeast compared to cells with an IL-4-induced M2 phenotype (Fig. 6). This increase in yeast phagocytosis in the BBE-treated Groups may indicate a shift towards a more active immune response and polarization toward an M1-like phenotype.

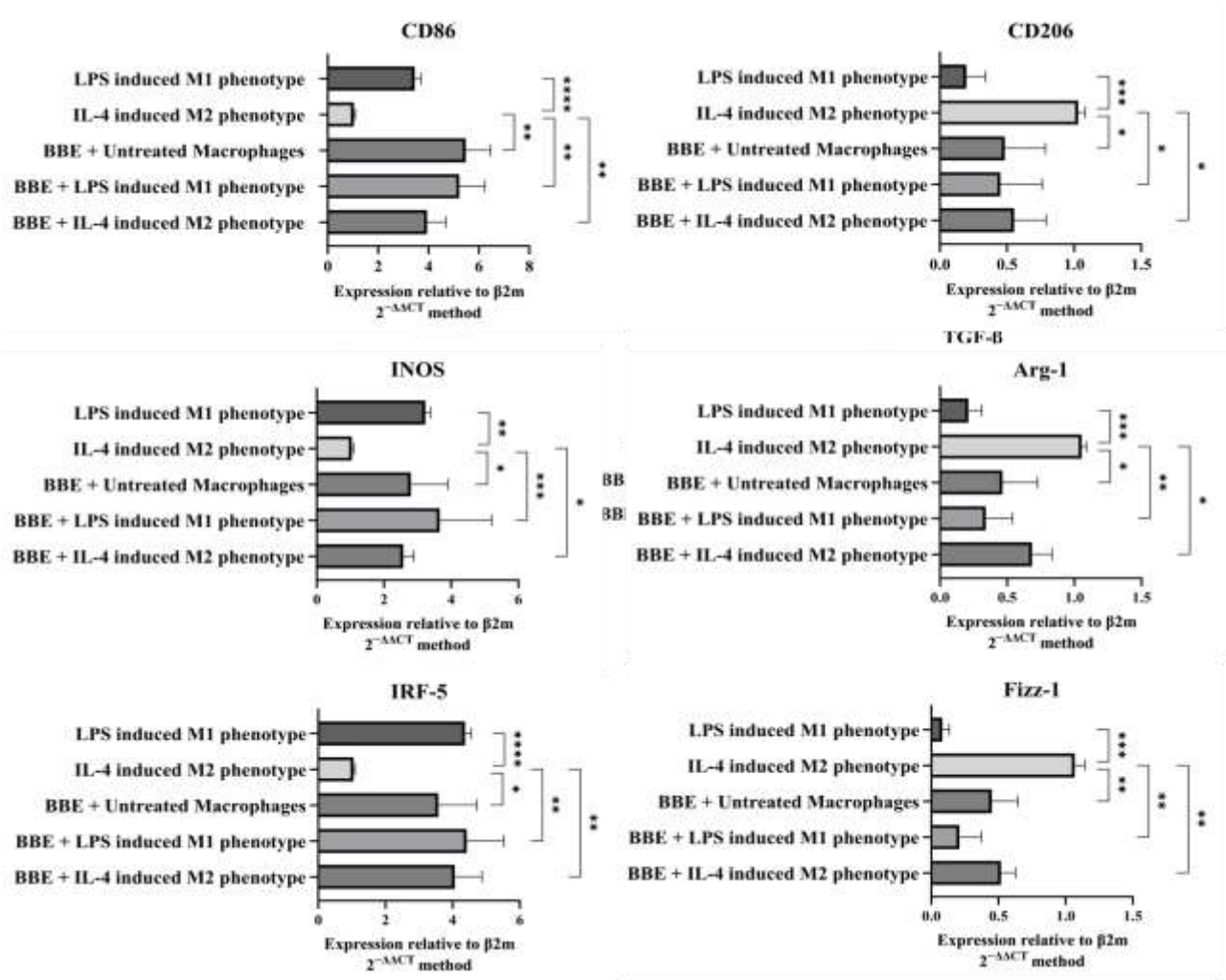

Fig. 4. Burn blister exudate (BBE) promoted macrophage polarization toward an M1-like phenotype. a) Gene expression analysis revealed upregulation of M1-associated markers (CD86, IRF5, iNOS) and downregulation of M2-associated markers (Fizz-1, Arg-1, CD206) in the BBE-treated groups compared to the IL-4 induced M2 phenotype. Data are shown as fold changes relative to β2m. Data presented are mean ± SD (n=3). Statistical significance: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.

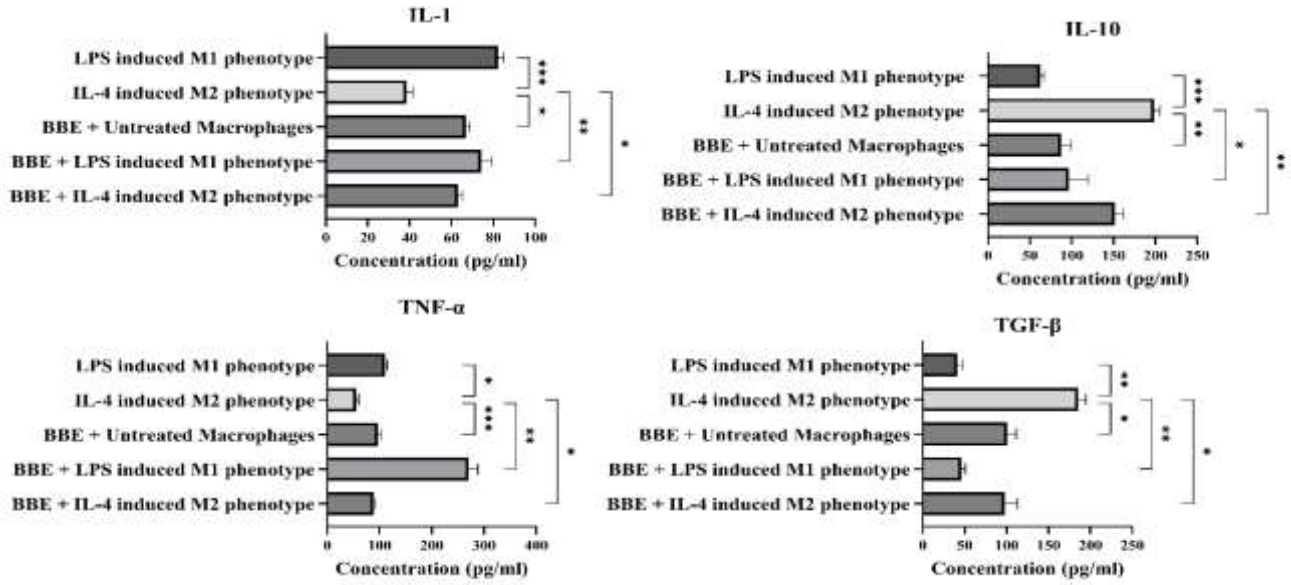

Fig. 5. The Burn blister exudate (BBE) treated groups demonstrated elevated secretion of M1 cytokines (IL-1, TNF-α) and reduced levels of M2 cytokines (IL-10, TGF-β) compared to the IL-4 induced M2 phenotype. Cytokine concentrations are presented in pg/ml. Data presented are mean ± SD (n=3). Statistical significance: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.

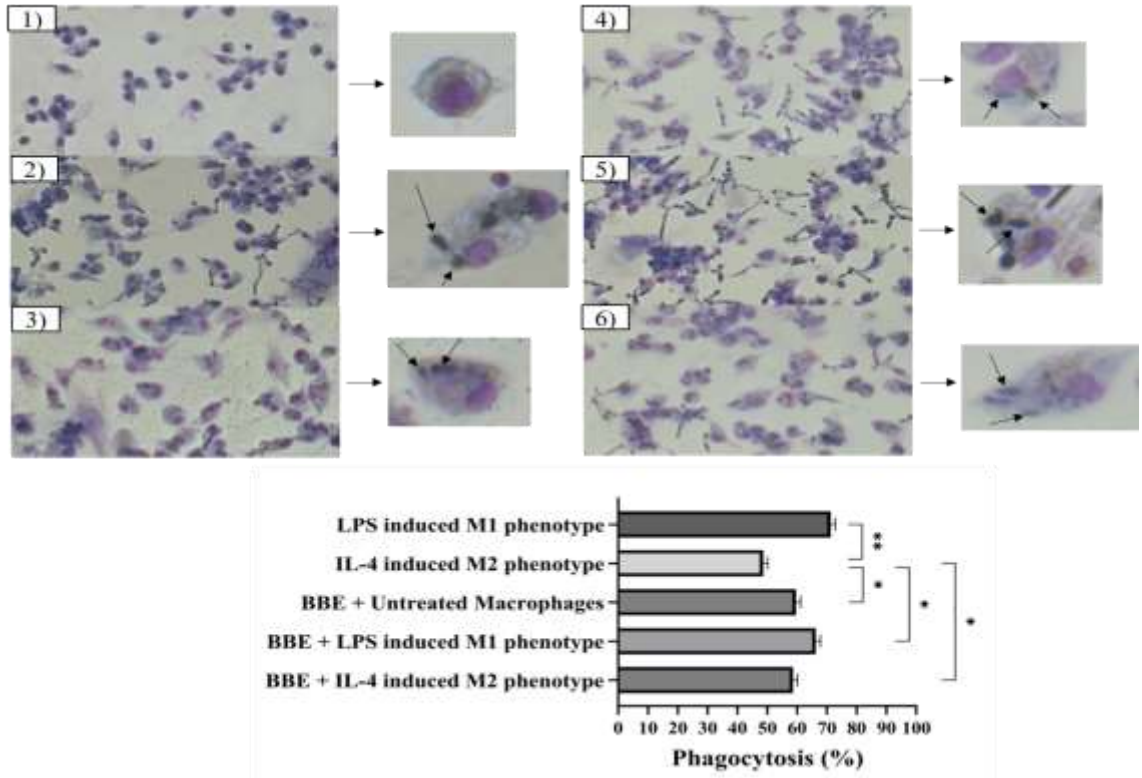

Fig. 6. Burn blister exudate (BBE) was found to promote the polarization of macrophages toward M1-like phenotype, enhancing their phagocytic activity. a), yeast phagocytosis by various macrophage groups was visually assessed, with arrows indicating yeast particles engulfed by the macrophages. b) Macrophages treated with BBE exhibited a higher phagocytic potential compared to those with an IL-4-induced M2 phenotype. Data presented are mean ± SD (n=3). *p< 0.05 and **p< 0.01.(1) Control Macrophages, 2) LPS induced M1 phenotype, 3) IL-4 induced M2 phenotype, 4) BBE + Untreated macrophage, 5) BBE + LPS induced M1 phenotype,6) BBE + IL-4 induced M2 phenotype).

Burn blister exudate treatment groups' conditioned media Suppressed the migration of CT-26 cells

We performed a scratch wound healing assay to determine whether BBE-induced macrophages can inhibit CT-26 tumor cell migration as a potential antitumor mechanism. The results indicated that conditioned media obtained from BBE-treatment groups impeded wound healing at both 24 and 48 hours after wound formation compared conditioned media from IL-4-induced M2 macrophages treated CT-26 cells (Fig. 7). These findings suggest that the conditioned media derived from macrophages exposed to BBE inhibits the migratory capacity of CT-26 cells. Collectively, these results support the notion that BBE may promote macrophage polarization toward an M1-like phenotype.

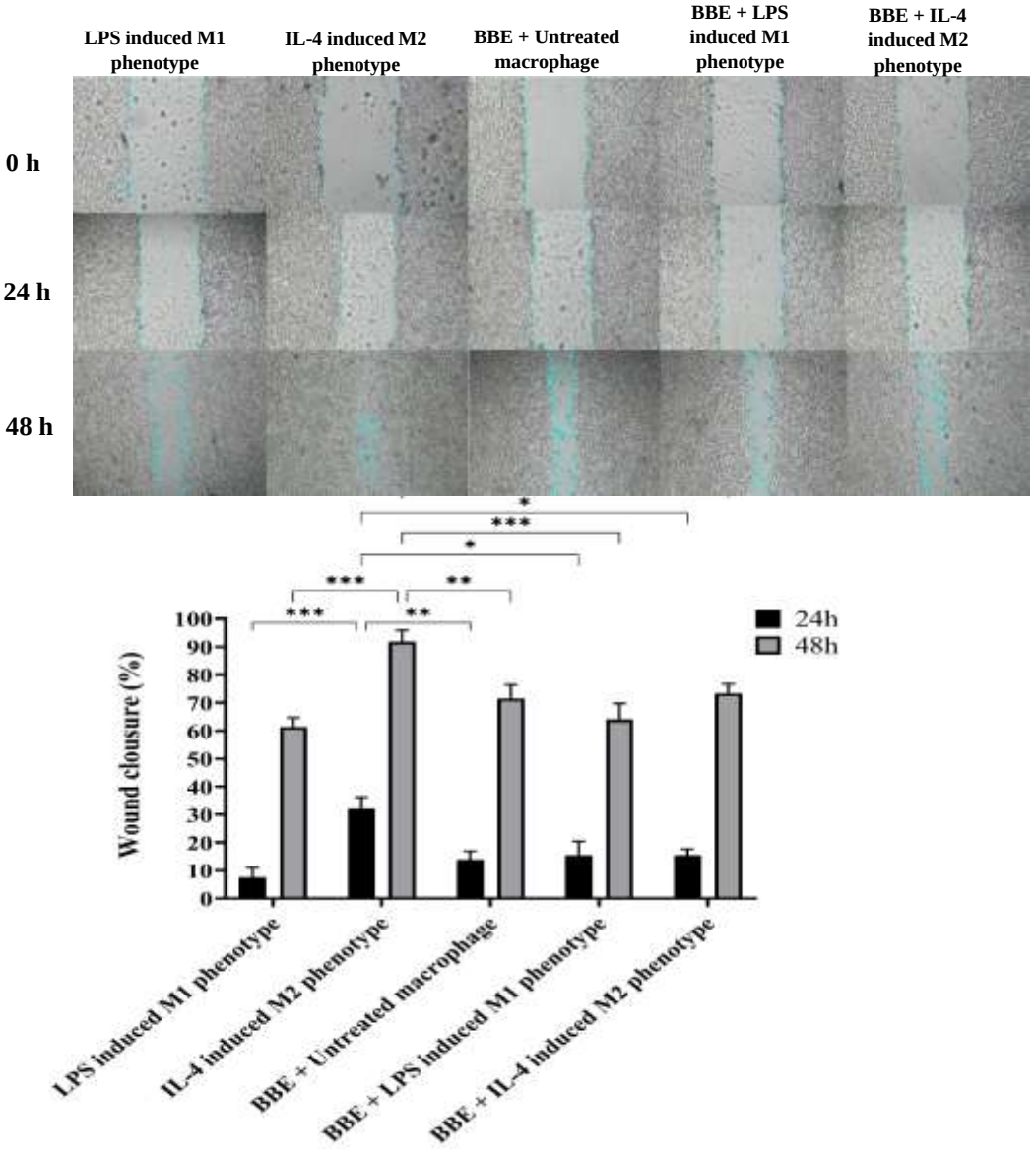

Fig. 7. a) Scratch assay of CT-26 cells exposed to conditioned media of macrophages from various treatment groups. b) The results demonstrated a reduction in the migration and percentages of wound closure of the CT-26 cells treated with the conditioned media of burn blister exudate (BBE) treatment groups. Data presented are mean ± SD (n=3). *p< 0.05, **p< 0.01, ***p< 0.001.

Burn blister exudate treatment groups' conditioned media reduced the proliferation of CT-26 cells

Upon conducting an MTT assay, we observed that using conditioned media from BBE-treated groups macrophages decreased CT-26 cell proliferation after 72 hours of treatment compared to conditioned media from the IL-4-induced M2 phenotype. After treatment with conditioned media from different groups for 24 hours, CT-26 cells did not display substantial differences in proliferative rates. It should be noted that only the conditioned media from the LPS-induced M1 phenotype significantly reduced CT-26 colon cancer cell proliferation after 48 hours (Fig. 8). These findings suggest that BBE may induce macrophages to develop an anti-tumor M1-like phenotype, thereby suppressing the growth and proliferation of CT-26 tumor cells over time.

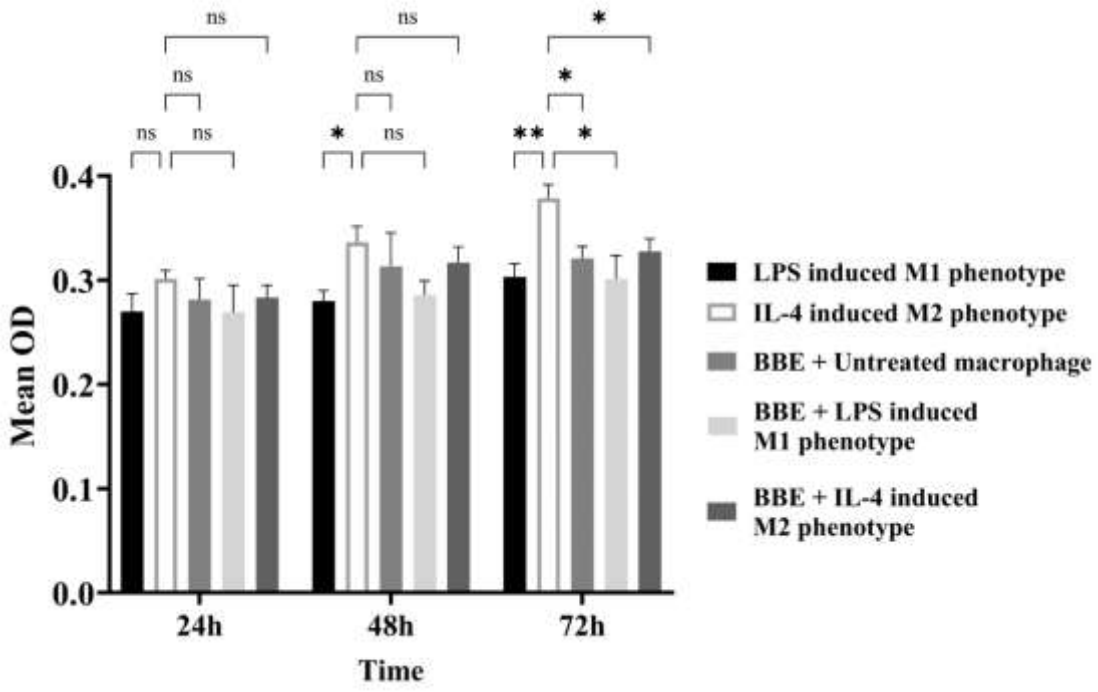

Fig. 8. As a result of an MTT analysis that compared the response of CT-26 cells to conditioned media from various BBE treatment groups with the IL-4-induced M2 phenotype. BBE-conditioned media groups exhibited a reduced proliferative activity compared to IL-4 conditioned media groups treated CT-26 cells, indicating their potential to promote a tumor-suppressing M1-like phenotype and restrict malignant cell proliferation. Data presented are mean ± SD (n=3). *p< 0.05, **p< 0.01 and ns = nonsignificant.

Discussion

Several cell phenotypes and signaling pathways contribute to the idea that tumors behave similarly to wounds that do not heal (26-28). These similarities include immune cell infiltration, neovascularization, and cellular plasticity. Macrophages are among the highly plastic cells found in both the tumor microenvironment (TME) and wound microenvironment (WME) (32, 33). The purpose of this study was to determine whether BBE can polarize macrophages to a phenotype similar to tumor-associated macrophages (TAMs) by examining the effects of BBE as burn WME (16, 18) on macrophage polarization and evaluating the in vitro antitumor activity of these BBE-induced

macrophages against CT-26 colon cancer cells. In the present study, human BBE samples were collected within 48 hours of blister formation. The findings indicated that both untreated macrophages and IL-4-induced M2 macrophage treated with BBE exhibited an M1-like gene expression profile, with upregulation of M1-associated markers and increased secretion of M1-related cytokines (CD86, iNOS, IRF5, TNF-α and IL-1). Conversely, the expression of M2- associated markers was downregulated, and the secretion of M2-related cytokines was decreased (CD206, Arg-1, FIZZ-1, TGF-β and IL-10).

The LPS-induced M1 phenotype group treated with BBE maintained an M1-like phonotype, in terms of gene expression profile and cytokine secretion. Consequently, we propose that early-stage BBE is pro-inflammatory in nature and promotes M1-like macrophage polarization. In addition, our yeast phagocytosis assay showed that BBE enhances the phagocytic activity of both untreated macrophages and IL-4-induced M2 macrophages. Additionally, our findings indicate that conditioned medium from untreated and IL-4-induced M2 phenotype macrophages treated with BBE for 24 or 48 hours inhibits CT-26 colon cancer cell migration in vitro, suggesting a potential antitumor effect. Further, our proliferation assay demonstrated that conditioned medium from both untreated and IL-4-induced M2 macrophages treated with BBE significantly reduced CT-26 colon cancer cell proliferation. These results suggest that BBE may polarize macrophages toward an M1-like phenotype, thereby contributing to tumor growth inhibition.

In a study by Kim et al, the increase in IL-10 levels in BBE was shown to promote healing and inflammation alleviation (34). This finding supports our observation that BBE is characterized by an inflammatory state in its early stages, which then shifts to an anti-inflammatory state with time. Mosser et al. and Fan et al. indicated that M1 macrophages play a crucial role in suppressing tumor cell survival by initially acting in the tumor microenvironment (35, 36). According to Yang et al. and Schultze et al. M1-like TAMs secrete co-stimulatory molecules like CD86 and pro-inflammatory factors, such as IL-1β and TNF-α. Also, they release IFN-γ and iNOS (37, 38). These observations are consistent with our findings. Untreated and IL-4-induced M2 phenotype macrophages treated with BBE exhibited upregulated expression levels of CD86 and iNOS, increased concentrations of IL-1 and TNF- α cytokines, and downregulation of M2 markers, indicating that BBE can polarize macrophages toward an M1-like phenotypes.

On the other hand, it has been reported by Huang et al. that M2-like macrophages can promote the migration of cancer cells (39), and studies by Sadeghi et al. and Chen et al. suggest that if the macrophages switch to the M1 phenotype, they can limit tumor cell migration (40, 41). Based on our findings, IL-4 induced M2 phenotype macrophages treated with BBE repolarized toward an M1-like phenotype and demonstrated antitumor activity by reducing the migration and proliferation of CT-26 colon cancer cells in vitro. While IL-4 induced M2 macrophages are known to promote cancer cell proliferation and growth, some studies by Xiao et al, Wang et al., and Lv et al. have suggested that M1-like macrophages, in contrast to our findings, can also promote the growth and invasion of cancer cells in vitro (42-44).

This may be explained, at least in part, by the findings of Nakao et al., who indicated that this effect could result from inflammatory cytokines that directly or indirectly promote vaso-proliferation (45). Overall, this study found that untreated and IL-4-induced M2 macrophages treated with early-stage second-degree BBE were polarized toward an M1-like phenotype and inhibited the migration and proliferation of CT-26 colon cancer cells. Understanding how macrophages respond to external stimuli and polarize into different activation states is essential to determining how they function. As a result of the current study, we demonstrate that untreated and IL-4-induced M2 macrophages treated with BBE

possess M1-like properties like those of macrophages in the early stages of TME.

Therefore, by reshaping the microenvironment toward a more inflammatory state, similar to the early stages of burn injuries, we may prevent cancer development by stimulating M2-like macrophages to polarize toward an M1-like phenotype. Through studying macrophage polarization in disease, we can gain a deeper understanding of how macrophage plasticity works, and we'll be and leverage this knowledge to our advantage. The shift from the M2 phenotype that promotes tumor growth to the anti-tumor M1 phenotype is crucial for developing more effective cancer treatments.

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Ethics

This study was conducted under the supervision of the Biomedical Research Ethics Committee of Ahvaz Jundishpur University of Medical Sciences, Ahvaz, Iran (Approval ID: IR.AJUMS.ABHC.REC.1402.401).

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors contribution

Amir Mohamad Amiri: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. Ali Asadirad: Formal analysis, Supervision, Writing - Review & Editing. Alireza Rafati Navaei: Resources, Writing - Review & Editing. Ali Khodadadi: Conceptualization, Methodology, Investigation, Funding acquisition, Project administration, Writing - Review & Editing.

References

1. Li MO, Wolf N, Raulet DH, Akkari L, Pittet MJ, Rodriguez PC, et al. Innate immune cells in the tumor microenvironment. Cancer Cell. 2021;39(6):725-9.

2. Smyth T, Payton A, Hickman E, Rager JE, Jaspers I. Leveraging a Comprehensive Unbiased RNAseq Database Uncovers New Human Monocyte-Derived Macrophage Subtypes Within Commonly Employed in Vitro Polarization Methods. Sci Rep. 2024;14:26753.

3. Zhang W, Zheng X, Yu Y, Zheng L, Lan J, Wu Y, et al. Renal Cell Carcinoma-Derived Exosomes Deliver lncARSR to Induce Macrophage Polarization and Promote Tumor Progression via STAT3 Pathway. Int J Biol Sci. 2022;18(8):3209-3222.

4. Oishi S, Takano R, Tamura S, Tani S, Iwaizumi M, Hamaya Y, et al. M2 Polarization of Murine Peritoneal Macrophages Induces Regulatory Cytokine Production and Suppresses T-cell Proliferation. Immunology. 2016;149(3):320-8.

5. Wang YC, He F, Feng F, Liu XW, Dong GY, Qin HY, et al. Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. Cancer Res. 2010;70(12):4840-9.

6. Amin Mohedin J, Rezaiemanesh A, Asadi S, Haddadi M, Abdul Ahmed B, Gorgin Karaji A, Salari F. Resolvin D1 (Rvd1) Attenuates *In Vitro* LPS-Stimulated Inflammation Through Downregulation of miR-155, miR -146, miR -148 and Krupple Like Factor 5. Rep Biochem Mol Biol. 2024;12(4):566-574.

7. Zhao P, Cai Z, Zhang X, Liu M, Xie F, Liu Z, et al. Hydrogen Attenuates Inflammation by Inducing Early M2 Macrophage Polarization in

Skin Wound Healing. Pharmaceuticals (Basel). 2023;16(6):885.
8. de Visser KE, Joyce JA. The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth. Cancer Cell. 2023;41(3):374-403.
9. Christofides A, Strauss L, Yeo A, Cao C, Charest A, Boussiotis VA. The complex role of tumor-infiltrating macrophages. Nat Immunol. 2022;23(8):1148-56.
10. Malika N, Zhansaya A, Kasym M, Kanat T, Yerlan R, Kanatbek M. Analysis of Antibody Induction by Macrophages Treated *Ex Vivo* with Human Proteins in Mice. Rep Biochem Mol Biol. 2023;11(4):694-701.
11. Jeschke MG, van Baar ME, Choudhry MA, Chung KK, Gibran NS, Logsetty S. Burn injury. Nat Rev Dis Primers. 2020;6(1):11.
12. Li B, Xu K, Liu X. Curative Effect Observation of Applying Silver-Zinc Bacteriostatic Cream to Patients with Second-Degree Burn under Targeted Nursing Intervention and Its Effect on Wound Healing Rate. J Healthc Eng. 2021;2021:7591959.
13. Xue Y, Yang F, Li J, Zuo X, Pan B, Li M, et al. Synthesis of an Effective Flame-Retardant Hydrogel for Skin Protection Using Xanthan Gum and Resorcinol Bis(diphenyl phosphate)-Coated Starch. Biomacromolecules. 2021;22(11):4535-4543.
14. Paquet P, Piérard GE. Soluble fractions of tumor necrosis factor-alpha, interleukin-6 and of their receptors in toxic epidermal necrolysis: a comparison with second-degree burns. Int J Mol Med. 1998;1(2):459-62.
15. Gäddnäs F, Sutinen M, Koskela M, Tervahartiala T, Sorsa T, Salo T, et al. Matrix-Metalloproteinase -2, -8 and -9 in Serum and Skin Blister Fluid in Patients With Severe Sepsis. Crit Care. 2010;14(2):R49.
16. Widgerow AD, King K, Tocco-Tussardi I, Banyard DA, Chiang R, Awad A, et al. The burn wound exudate-an under-utilized resource. Burns. 2015;41(1):11-7.
17. Gupta S, Chittoria RK, Chavan V, Aggarwal A, Reddy LC, Mohan PB, et al. Role of Burn Blister Fluid in Wound Healing. J Cutan Aesthet Surg. 2021;14(3):370-373.
18. Ono I, Gunji H, Zhang JZ, Maruyama K, Kaneko F. A study of cytokines in burn blister fluid related to wound healing. Burns. 1995;21(5):352-5.
19. Lestari T, Syukur S, Revilla G, Sukma Rita R, Rustini R. The Burn Wound Healing Process: A Review. Int J Prog Sci Technol. 2023;40(1):77.
20. Willis ML, Mahung C, Wallet SM, Barnett A, Cairns BA, Coleman LG Jr, Maile R. Plasma extracellular vesicles released after severe burn injury modulate macrophage phenotype and function. J Leukoc Biol. 2022;111(1):33-49.
21. Zhang G, Li J, Wang D, Lou H, Zhang C, Liu W. The mechanisms related to fibroblasts in burn surface. Skin Res Technol. 2023;29(8):e13431.
22. Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB, Beelen RH. Macrophages in skin injury and repair. Immunobiology. 2011;216(7):753-62.
23. Daley JM, Brancato SK, Thomay AA, Reichner JS, Albina JE. The phenotype of murine wound macrophages. J Leukoc Biol. 2010;87(1):59-67.
24. Lateef Z, Stuart G, Jones N, Mercer A, Fleming S, Wise L. The Cutaneous Inflammatory Response to Thermal Burn Injury in a Murine Model. Int J Mol Sci. 2019;20(3):538.
25. Patel U, Rajasingh S, Samanta S, Cao T, Dawn B, Rajasingh J. Macrophage polarization in response to epigenetic modifiers during infection and inflammation. Drug Discov Today. 2017;22(1):186-193.
26. Dvorak HF. Tumors: Wounds That Do Not Heal-A Historical Perspective with a Focus on the Fundamental Roles of Increased Vascular Permeability and Clotting. Semin Thromb Hemost. 2019;45(6):576-92.
27. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315(26):1650-9.
28. Dvorak HF. Tumors: wounds that do not heal-redux. Cancer Immunol Res. 2015;3(1):1-11.

29. Shaw J, Dibble C. Best evidence topic report. Management of burns blisters. Emerg Med J. 2006;23(8):648-9.
30. Reagan BJ, Staiano-Coico L, LaBruna A, Mathwich M, Finkelstein J, Yurt RW, et al. The effects of burn blister fluid on cultured keratinocytes. J Trauma. 1996;40(3):361-7.
31. Suarez-Arnedo A, Torres Figueroa F, Clavijo C, Arbeláez P, Cruz JC, Muñoz-Camargo C. An image J plugin for the high throughput image analysis of in vitro scratch wound healing assays. PLoS One. 2020;15(7):e0232565.
32. Bied M, Ho WW, Ginhoux F, Blériot C. Roles of macrophages in tumor development: a spatiotemporal perspective. Cell Mol Immunol. 2023;20(9):983-992.
33. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 2018;233(9):6425-6440.
34. Kim HS, Kim JH, Yim H, Kim D. Changes in the levels of interleukins 6, 8, and 10, tumor necrosis factor alpha, and granulocyte-colony stimulating factor in Korean burn patients: relation to burn size and postburn time. Ann Lab Med. 2012;32(5):339-44.
35. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008;8(12):958-69.
36. Fan Z, Yu P, Wang Y, Wang Y, Fu ML, Liu W, et al. NK-cell activation by LIGHT triggers tumor-specific CD8+ T-cell immunity to reject established tumors. Blood. 2006;107(4):1342-51.
37. Yang Q, Guo N, Zhou Y, Chen J, Wei Q, Han M. The role of tumor-associated macrophages (TAMs) in tumor progression and relevant advance in targeted therapy. Acta Pharm Sin B. 2020;10(11):2156-70.
38. Schultze JL, Schmidt SV. Molecular features of macrophage activation. Semin Immunol. 2015;27(6):416-23.
39. Huang W, Jiang M, Lin Y, Qi Y, Li B. Crosstalk between cancer cells and macrophages promotes OSCC cell migration and invasion through a CXCL1/EGF positive feedback loop. Discov Oncol. 2024;15(1):145.
40. Chen M, Lai R, Lin X, Chen W, Wu H, Zheng Q. Downregulation of triggering receptor expressed on myeloid cells 1 inhibits invasion and migration of liver cancer cells by mediating macrophage polarization. Oncol Rep. 2021;45(4):37.
41. Sadeghi M, Amari A, Asadirad A, Nemati M, Khodadadi A. F1 fraction isolated from Mesobuthus eupeus scorpion venom induces macrophage polarization toward M1 phenotype and exerts anti-tumoral effects on the CT26 tumor cell line. Int Immunopharmacol. 2024;132:111960.
42. Xiao M, Zhang J, Chen W, Chen W. M1-like tumor-associated macrophages activated by exosome-transferred THBS1 promote malignant migration in oral squamous cell carcinoma. J Exp Clin Cancer Res. 2018;37(1):143.
43. Wang H, Wang X, Li X, Fan Y, Li G, Guo C, et al. CD68(+) HLA-DR (+) M1-like macrophages promote motility of HCC cells via NF-κB/FAK pathway. Cancer Lett. 2014;345(1):91-9.
44. Lv C, Li S, Zhao J, Yang P, Yang C. M1 Macrophages Enhance Survival and Invasion of Oral Squamous Cell Carcinoma by Inducing GDF15-Mediated ErbB2 Phosphorylation. ACS Omega. 2022;7(13):11405-14.
45. Nakao S, Kuwano T, Tsutsumi-Miyahara C, Ueda S, Kimura YN, Hamano S, et al. Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1 beta-induced neovascularization and tumor growth. J Clin Invest. 2005;115(11):2979-91.