Immunomodulatory effects of β-defensin 2 on tumor-associated macrophages induced antitumor function in breast cancer

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Research Article

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Abstract

Background: TAMs express cytokines and chemokines that can suppress antitumor immunity and promote tumor progression. The immunomodulatory and antitumor function of β-defensin 2 is still unclear, despite the evidence of infection response. So, this study aims to investigate the association between β-defensin 2 and TAMs and determine the role in tumor-promoting attributes of TAMs reversal of phenotype in tumor regression.

Methods: Swiss albino mice and C127i breast cancer cell line were used in this study. C127i conditioned media was prepared and generated macrophage-derived TAM to study antitumor function. Flow cytometry was performed for phenotype identification of macrophages and TAMs. MTT was performed to estimate cytotoxicity and dose optimization of β-defensin 2. Oxidative stress was analyzed by H\textsubscript{2}O\textsubscript{2} and NO estimation, and qPCR was performed for iNOS, cytokines and chemokines quantification.

Results: PEC harvested macrophages were characterized by flow-cytometry using F4/80, CD11c antibodies with 98% pure population of macrophages and cultured in C127i conditioned media for 7 days. TAM markers were estimated, and it was found that 98 % expression of F4/80, CD-206, and CD-115 expression compared to macrophages. Purified 100ng/ml of β-defensin 2 was used to stimulate the TAMs 98% population was viable, which was confirmed by cell viability assay. ROS levels decreased (TAMs alone p<0.05, TAMs treated p<0.001) compared to control. IL-6, IL-10, IL-3, TNF-α, and TGF-β and CXCL-1, CXCL-5, CXCL-15, CCL-24, and CCL-5 decreased drastically compared to control.

Conclusion: This is the first report of β-defensin 2 on TAMs to elucidate the immunomodulatory and antitumor function. It was found that the cytokines and chemokines molecules, ROS expression, pliably changed, which facilitates tumor regression. β-defensin 2 is a new therapeutic target peptide to revert tumor-promoting function.

Background

The tumor microenvironment consists of immunosuppressive factors and tumor-promoting cells. It continuously recruits immune cells such as macrophages, NK cells, B cells, T cells, and MSCs into the tumor microenvironment. These cells may aid cancer progression by downregulating their normal antitumor function. TAMs are largest population of infiltrating inflammatory cells in malignant tumors which promote the growth. TAMs facilitate the immunosuppressive tumor microenvironment (TME) by releasing cytokines, chemokines, and growth factors and triggering the inhibitory immune checkpoint proteins released in T cells. These functions directly or indirectly promote tumor growth by increasing tumor cell survival, proliferation, invasiveness, and motility [1]. TAMs have been demonstrated to have comparable roles in recent investigations, as TAMs tend to be pro-tumoral, reacting to IL-4, IL-10, TGF-β, and IL-13, and stimulating tissue regeneration [2]. TAMs are often located in the invasive front of cancer cells, where most cancer cells undergo EMT [3]. TAMs secrete TGF-β, as described above, which plays an important role in promoting EMT [4, 5]. M2-polarized TAMs facilitate EMT in PC via the TLR4/IL10 signaling
pathway [6]. Many studies report the cross-talk of M2 and MSCs in breast cancer, where M2 and secretory factors have a significant impact by promoting the EMT profiling of TA-MSCs, which helps in the breast cancer progression.

Immunologically, Defensins are associated with the disturbance of the numbers and function of peri-and intrafollicular inflammatory cells, which is more abundant during the active stage of the disease [7]. Defensins are cysteine-rich, cationic proteins of small amino acids (18–55), which are expressed predominantly in innate immune cells, for example, epithelial cells and neutrophils [8]. Their multifaceted immunomodulatory functions and antimicrobial activities have been extensively studied, establishing their role as a core host-protective component against bacterial, fungal, and viral infections [9]. β-defensins are expressed in humans, while α-defensins and defensins have different functions and are not well studied. β-defensin exhibits potent chemotactic activity for various innate immune cells and stimulates other cells to secrete cytokines [10]. However, they can also inhibit the inflammatory response by the specific binding of microbe-associated molecular patterns. These patterns can also induce the expression of β-defensins in gingival epithelial cell breast cancer cell lines, although significant differences are observed between different species of bacteria. Together these results suggest a complex model of a host-defense-related function in maintaining bacterial homeostasis and response to pathogens [11].

Previously we reported that β-defensin showed macrophages' immunomodulatory function and anti-tumor property increases in the breast cancer tumor model (). There is a gap in the knowledge of the immunomodulatory and tumoricidal part of tumor-associated macrophages with crosstalk with defensin 2. So, this study aims to investigate the association between β-defensin 2 and TAMs and determine the role in tumor-promoting attributes of TAMs reversal of phenotype in tumor regression. We explored whether β-defensin 2 (BD2) inductions have any toxicity on TAMs, ROS generation, effects of β-defensins 2 on cellular viability, and altered nuclear morphology. We also studied IL-6, IL-10, IL-3, TNF-α, and TGF-β, and CC and CXC chemokines CXCL-1, CXCL-5, CXCL-15, and CCL-24 expression in TAMs.

**Methods**

**Reagents**

DMEM, RPMI-1640 media, and FBS were purchased from Gibco, Waltham, Massachusetts, USA. Antibiotics, LPS, TiSO₄, and H₂O₂ were procured from Sigma-Aldrich, St. Louis, Missouri, USA. DAPI and DABCO were purchased from SRL, India. Anti-mouse F4/80, CD-206, and CD-115 conjugated with alexafluor, PE-A, and PE-CY-7-A were purchased from Thermo-Scientific, Waltham, Massachusetts, USA, β-defensin 2 (Prospec, Hamada, Israel) and RNAiso plus (Takara, Japan) were purchased. cDNA synthesis kit and SYBR green supermix were obtained from BIO-RAD, USA.

**Mice and tumor model**
The animal care committee approved all the experiments of the AIIMS, New Delhi. The experiments were performed on Swiss Albino mice, which were bred and maintained under defined flora conditions at the AIIMS, New Delhi. All mice used were 8–12 weeks of age at the onset of the experiments. Mice were sacrificed by cervical dislocation, a method authorized by Institutional Animal Ethical Committee (file no-100/IAEC-1/2018), AIIMS, New Delhi, for sacrificing experimental animals, and observed until all muscle activity and breathing had ceased for at least 120s. No mice died before meeting the endpoints described.

**Tumor conditioned media preparation**

C127i was cultured in DMEM media containing 10% FBS for 48 hr at 37°C in a CO2 incubator. Conditioned media was obtained from the mouse breast cancer cell line (C127i) by plating 1 × 10^6 cells in culture plates in DMEM media. The cells were let to adhered o/n and washed the following day twice with PBS to remove the remaining serum. The cultured cells were incubated at 37°C for 24 hours. The tumor conditioned medium was harvested and centrifuged to remove suspended cells. The supernatant was collected and used directly.

**Isolation and characterization of TAMs from mice**

Macrophages were harvested from Swiss albino mice as previously described [12, 13]. Briefly, mice were killed by cervical dislocation, and macrophages were harvested by peritoneal lavage as peritoneal exudate cells (PECs) using chilled PBS. 5ml chilled PBS was injected into the peritoneum of mice. PECs were harvested by adherent purification in plastic petri dishes at CO2 incubator for 2 hr. Adherent cells were collected and seeded in a flat-bottom culture flask at a cell density of 1×10^6 in the culture medium with or without LPS and BD-2 and incubated for time periods of 24 hr in a CO2 incubator for treatment. Macrophages (1× 10^6) were plated in 2 ml RPMI media. Macrophages were treated in a ratio of media and tumor conditioned media (1:1). Medium was refreshed every other day, and cells were harvested on 7 days and characterized by flow cytometry.

**Flow cytometry assay**

Macrophages were harvested from Swiss albino mice as described above [11] and at a cell density of 1×10^6 in the culture medium with or without LPS and BD-2 for 24 hr in RPMI 1640 containing 10% FBS at 37°C in 5% CO2 in humidified CO2 incubator. Tumor associated macrophages were suspended in RPMI-1640 with 10% FBS, 0.1% NaN3 and incubated with anti-mouse CD14, an antibody conjugated with PE. Isotype is used as a control conjugated with FITC as per manufacturer's instructions. After washing, cells were suspended in 0.1% PBS containing 0.1% NaN3 and then analyzed with a flow cytometry (BD Biosciences, Mountain View, CA, USA).

**Nitric oxide (NO) measurement**

The level of NO produced was quantified by determining the accumulation of nitrite using the Griess reagent. The optical density of the samples was measured at 540 nm with an ELISA reader. Briefly, cell supernatant was centrifuged to remove debris and clear the supernatant was mixed with an equal volume
of Griess reagent. The mixture was incubated in the dark for 40 min. The absorbance was measured at 540 nm using a microplate reader. The concentration of nitrite was determined from the sodium nitrite standard curve [13].

**H₂O₂ measurement**

The production of H₂O₂ was quantified by TISO₄. Add 50µl of titanium sulfate reagent per 100µl of sample. If peroxide is present in the sample, it will react with the titanium ions to give pertitanic acid, which will give the yellow color solution. The mixture with the reagent should be stable for several hours. Read the absorbance at 407 nm using the ELISA plate reader [13].

**Gene expression analysis**

Total RNA was extracted from the tumor associated MΦ harvested from control and experimental mice with RNAiso Plus reagent as instructed by the manufacturer (Takara, Japan). High-quality RNA (as estimated by absorbance ratio A260/280P1.8) from different groups were resolved on 1% agarose gel and stained with ethidium bromide to check the integrity of 18S and 28S rRNA using a UV transilluminator.

The total RNAs were used for cDNA synthesis using a kit, where total RNA (1µg/20µl reaction) was implied and further processes were followed as directed by the manufacturer. For real-time PCR, iTaq Universal SYBR Green Supermix was used. Reaction mix preparation and thermal cycling protocol was followed as directed by manufacturer guidelines. The primers used for the reaction are listed in Table 1 & 2.
### Table 1
Cytokine sequences used in this study

<table>
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<tr>
<th>Genes</th>
<th>Sequences</th>
<th>Tm (°C)</th>
</tr>
</thead>
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<tr>
<td>IL-6</td>
<td>Forward</td>
<td>ACA AAG CCA GAG TCC TTC AGA 56.0</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGG TCC TTA GCC ACT CCT TC 56.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>ACC CTC ACA CTC ACA AAC CA 56.4</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GGC AGA GAG GAG GTT GAC TT 56.6</td>
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<tr>
<td>IL-3</td>
<td>Forward</td>
<td>GCCTGCCTACATCTGCGAAT 54</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTTAGGAGAGACGGAGCCA 56</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward</td>
<td>GCTGCCTGCTTTTACTGACT 54</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGGCACTCATTCTTACCAGG 56</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Forward</td>
<td>GCTGAGCACCTTTTTGCTCC 54</td>
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<td></td>
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<td>GATGGCATTTTCGGAGGGGA 54</td>
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<td>iNOS</td>
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<td>CTATGGCCGCTTTTGATGTGC 59.97</td>
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<td></td>
<td>Reverse</td>
<td>CAACCTTGGTTGTAAGGGCG 59.97</td>
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<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CGT CCC GTA GAC AAA ATG GT 54.9</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>TTG ATG GCA ACA ATC TCC AC 53.3</td>
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</table>

### Table 2
Chemokine sequences used in this study

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<th>Genes</th>
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</thead>
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<td>Reverse</td>
<td>CGAGACCAGGAAACAGGG 56</td>
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<tr>
<td>CXCL-5</td>
<td>Forward</td>
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<td></td>
<td>Reverse</td>
<td>TCTTCTCTGGTTGGCACAC 54</td>
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<tr>
<td>CXCL-15</td>
<td>Forward</td>
<td>TTGGAGCCAAGGCAAGAACA 52</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AATGGAGAGGCATCCGTTTC 54</td>
</tr>
<tr>
<td>CCL-24</td>
<td>Forward</td>
<td>GGTCCTGCTCATGCTTCTGG 56</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGTAGCAGGAGGTTGAGTTG 56</td>
</tr>
<tr>
<td>CCL-5</td>
<td>Forward</td>
<td>TGCTCAAATCTTGCAGTCTG 52</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCAAGCAATGACGGGAGGC 54</td>
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</table>
Statistical analysis

Data were expressed as the mean ± SE. p value < 0.05, 0.01, 0.001 was considered significant. All graphs were generated using the Graph Pad Prism software. The multigroup comparisons of means were carried out by one-way analysis of variance (ANOVA) test. Student t-test was used for comparison between groups.

Results

Isolation and characterization of macrophages and TAMS (Fig. 1A-B)

TAMs generated using tumor conditioned media from mouse breast cancer cell line c127i. Flow cytometry was performed for quantification and characterization of harvested cells (Fig. 1). Purity greater than 90% for macrophages and TAMs was confirmed via flow cytometric analysis according to surface marker expression such as F4/80, CD206 and CD115 conjugated with alexafluor, PE-A and PE-CY-7-A respectively.

Nitric oxide and H₂O₂ measurement (Fig. 2A-C)

The ROS contains NO⁻ and H₂O₂ which was estimated by Griess method and TiSO₄ method. The amount of NO production in the supernatant of TAMs after 24 h treatment with β-defensin 2 was determined with the Griess reagent. During this period, the NO level decreased remarkably in the β-defensin 2 group as compared to the TAMs group (Fig. 2A) It was observed that in other experiments were conducted to examine the effect of β-defensin 2 on the production of H₂O₂. Production of H₂O₂ was found to be decreased in the β-defensin 2 treated group as compared to the TAMs group (Fig. 2B). mRNA level of iNOS was upregulated in the β-defensin 2 treated group as compared to the TAMs group (Fig. 2C).

qRT-PCR for selected gene expression on TAMs (Figs. 3 & 4)

We treated TAMs with mouse beta defensin 2 (BD-2) in vitro, followed by RT-PCR analysis to assess mRNA levels of respective cytokines and chemokines. Gene expression of cytokines (IL-6, IL-10, IL-3, TNF-α and TGF-β) and chemokines (CXCL-1, CXCL-5, CXCL-15, CCL-24 and CCL-5) were evaluated (Figs. 3 and 4). The results showed that BD-2 significantly increased the expression of cytokines TNF-α, TGF-β, IL-6 and IL-10, while diminished the expression of IL-3 as compared to only TAMs group (Fig. 3A-E). The results found that BD-2 reduced the expression of chemokines such as CXCL-1, CXCL-5, CXCL-15, CCL-24 and CCL-5 as compared to only TAMs group (Fig. 4A-E) suggesting that BD-2 may stimulate their anti-tumor promoting capacity.

Discussion
In the previously published report on β-defensin 2 shows that macrophage's immunomodulatory and antitumor functions increase (Ref). Herewith we are deciphering the association between β-defensin 2 and TAMs and determining the role in tumor-promoting attributes of TAMs reversal of phenotype in tumor regression. The present study establishes a new paradigm whereby the alteration in oxidative stress, chemokine's and cytokines pattern of TAMs by the β-defensin 2 is an effective strategy as a therapeutic agent. It plays an essential role in tumor regression and antitumor function.

Macrophages differentiate into TAMs via their interaction with tumor and tumor-derived factors. TAMs express F4/80, CD206 and CD163 [25, 26]. TAMs consist of transcriptionally, phenotypically, and functionally diverse populations of macrophages [14–16]. TAMs are a major tumor-promoting component of the tumor microenvironment. TAMs promote tumor growth through a variety of approach such as secretion of growth factors, matrix degradation enzymes, and proangiogenic factors [17–19]. TAMs represent an intriguing cell phenotype in different tumor compartments [19–21]. Tumor cells releasing cytokines and chemokines attract macrophages (tumor promoting TAMs phenotype) and other inflammatory cells to the tumor stroma. [22]. TAMs express chemokines and cytokines that can promote tumor progression and suppress antitumor immunity. Tumor-derived factors in the TME can stimulate macrophages to produce a wide array of tumor-promoting molecules, such as chemokines, cytokines, and growth factors, to stimulate tumor cell proliferation, tumor angiogenesis and metastasis [23, 24].

β defensin-2 may play role in controlled ROS release during tumor progression. The expression level of NO$^-$ and H$_2$O$_2$ was found to be decreased in β defensin-2 treated group as compared to only TAMs group (Fig. 2). It is well documented that nitric oxide (NO$^-$) is a highly reactive free radical that involves in many inflammatory processes [27]. Depending on the source of the NO$^-$, tumor cells can either be stimulated to grow or be encouraged to die. Similarly, the function of iNOS in cancer varies depending on the kind of cell. Consistent with previous reports that TAMs promote tumor initiation, progression and metastasis, it is evident that BD-2 suppressed the tumorigenesis in TAMs. NO$^-$ is a main mediator of angiogenesis and neovascularization [28]. It also activates immune responses against pathogens [29] and increased expression of iNOS on macrophages represents a marker for M1 polarization [30]. Although ROS are typically thought to be detrimental to cells, they also play a crucial role in controlling gene expression and signal transduction pathways [31, 32]. Because TAMs produce a variety of factors to promote tumor growth and angiogenesis and mediates immunosuppression [33]. This is the first demonstration that β-defensin 2 is a key protein on immune defense which regulates ROS generation and decreased NO$^-$ and H$_2$O$_2$, pro-inflammatory cytokines and chemokines.

TAMs with different polarization phenotypes can produce a variety of cytokines, chemokines, and growth factors that help to regulate inflammation and immunity. Chemokines are critical secretors derived from TAMs that mediate cancer progress and metastasis. In our study, we have found that higher expression of CXCL1 in untreated group (TAMs) while lower expression of CXCL-1 in TAMS when treated with BD-2 (Fig. 3). CXCL1 secreted by breast cancer cell, recruits CD11b$^+$Gr1$^+$ myeloid cells into the tumor, therefore supporting the cancer metastasis and survival by activating calprotectin expression [34]. CXCL1, a
chemotaxis-stimulating factor, recruits a variety of stromal cells into tumor surroundings to involve in tumor growth, angiogenesis and metastasis [35].

Here we showed that the expression of TNF-α, TGF-β, IL-6 and IL-10 is found to be increased in BD-2 treated group as compared to only TAMs group (Fig. 4). TAMs stimulate the expression of IL-6 and IL-10 in cancer cells through TLR signaling, serve in maintaining the tumor's inflammatory microenvironment and promoting development and progression of cancer [36]. IL-6 is an inflammatory cytokine, considered as a tumor-promoting and antiapoptotic factor. IL-6 contributes to the induction of skin tumors, triggers malignancy in breast tumor mammospheres, and participates in suppression of antigen-specific anti-tumor immunity. IL-3 known as hematopoietic cytokine which is secreted by activated T cells and mast cells (MCs). IL-10 is a pleiotropic cytokine produced by a variety of immune cells (e.g. T1,2 cells, macrophages and CD8+ cells) that can inhibit a broad array of immune and inflammatory responses. TGF-β secreted by tumor cells can also induce M2-type polarization of macrophages. TNF-α is a pleiotropic cytokine that regulates a broad range of biological activities, including cell differentiation, proliferation and death, as well as inflammation, innate and adaptive immune responses, and tissue development [37]. It is produced by monocytes and macrophages. TNF-α secreted by TAM leads to the activation of NF-kB in tumor cells. Activation of NF-kB pathway in tumor cells prevents tumor cell death and enhances tumor cell invasion [37].

**Conclusions**

This is the first demonstration that β-defensin 2 is a key protein on immune defense. Our findings indicated that BD-2 reduced the oxidative stress and expression of chemokines (CXCL-1, 5, 15, CCL-5 and 24) while induced the expression of cytokines (IL-3, 6, 10, TNF-α and TGF-β). Our data clearly demonstrated that treatment of BD-2 for targeting TAMs may be a potentially effective method for cancer treatment.

**Abbreviations**

Tumor associated macrophages (TAMs), Tumor necrosis factor (TNF), peritoneal exudate cells (PECs), Nitric oxide (NO), Peritoneal exudate cells (PECs), Reaction oxygen species (ROS)

**Declarations**

**Ethics approval and consent to participate**

All animal experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) (file no- 100/IAEC-1/2018) of AIIMS, New Delhi. All methods and animal monitoring were carried out in accordance with relevant guidelines and regulations provided by the Committee for the Purpose of Control and Supervision of Experimentation of Animals (CPCSEA) (Reg. No. 10/GO/ReBiBtS/99/CPCSEA). Animal
studies are conducted in compliance with the ARRIVE guidelines. Human subject consent was not required in the study because we did not use human samples.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available by the corresponding author Dr. Pramod Kumar Gautam (gautam@aiims.edu) on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Dr. Sonam Agarwal, Anita Chauhan and Khushwant Singh performed wet lab experiments. Dr. Sonam Agarwal and Dr. Pramod Kumar Gautam wrote the manuscript. Rupinder Kaur and Marilyn Masih arranged the references of the manuscript. Dr. Pramod Kumar Gautam was the supervisor and designed the experimental plan. He analyzed all the data and corrected the manuscript. All authors read and approved the final manuscript.

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References


Figures
Figure 1

Characterization of tumor associated macrophages (TAMs) based on F4/80, CD-206 and CD-115 expression through Flow cytometry

Figure 2

Effects of BD 2 on the level of NO, H₂O₂ and iNOS in TAMs after 24 hr treatment. Results are expressed as mean ±SE. Statistical analysis: *p < 0.05, **p < 0.01, ***p < 0.001
Figure 3

Effects of BD 2 on the expression of cytokines in TAMs after 24 h treatment. Results are expressed as mean ±SE. Statistical analysis: *p < 0.05, **p < 0.01.
Figure 4

Effects of BD 2 on the expression of chemokines in TAMs after 24 h treatment. Results are expressed as mean ±SE. Statistical analysis: ***p < 0.001, **p < 0.01.