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BCc1, a Chelation-Based Nanomedicine, modulates splenic cytokine networks toward a profile associated with antitumor immunity in 4T1 breast tumor–bearing mice

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Abstract

Background and Aim: Breast cancer remains a major cause of cancer mortality in women, and tumor-driven cytokine dysregulation promotes immune escape. This study aimed to define the splenic immunomodulatory activity of BCc1 nanomedicine in 4T1 breast tumor-bearing BALB/c mice by measuring interleukin-12 (IL-12), transforming growth factor-β1 (TGF-β1), and tumor necrosis factor-α (TNF-α), and the (TGF-β1)/(IL-12) balance.

Methods: Seventy female mice with palpable tumors received a 24-day regimen of intraperitoneal or oral BCc1, cyclophosphamide, or phosphate-buffered saline (PBS). Splenic cytokines were quantified by ELISA, and the (TGF-β1)/(IL-12) ratio was calculated as an index of cytokine polarization.

Results: BCc1 reshaped splenic cytokine patterns in a dose- and route-dependent manner. High-dose intraperitoneal BCc1 produced the strongest effects, markedly reducing $\mathrm{TGF}-\beta1$ and $\mathrm{TNF}-\alpha$, inducing controlled modulation of $\mathrm{IL}-12$, and substantially lowering the $^{\mathrm{TGF}-\beta1}/_{\mathrm{IL}-12}$ ratio. These changes indicate reduced immunoregulatory dominance and a shift toward an antitumor-permissive cytokine milieu. For several cytokine endpoints, high-dose BCc1 showed effects comparable to or greater than cyclophosphamide. Oral BCc1 elicited milder dose-dependent responses, with stronger immunomodulation at the higher oral dose.

Conclusion: BCc1 therefore mediates route- and dose-dependent splenic cytokine remodeling, supporting further evaluation as a multifunctional immunomodulatory nanotherapeutic; cellular phenotyping and functional antitumor assays are required to determine whether these cytokine shifts translate into immune-mediated tumor control

Keywords: Chelation therapy, Cytokine remodeling, Immunomodulatory nanomedicine, Tumor immune escape, Splenic microenvironment

1. Introduction

Despite substantial progress in early detection and therapeutic management, breast cancer continues to impose a substantial burden on female cancer mortality globally (1). While conventional modalities including chemotherapy, targeted therapy, and radiotherapy have improved survival outcomes, their clinical efficacy is frequently compromised by

systemic toxicity, off-target effects, and treatment-associated morbidity (2). These limitations underscore the urgent need for innovative therapeutic approaches that harmonize potent antitumor efficacy with enhanced safety profiles (3, 4).

In oncology, nanomedicine has become an increasingly influential strategy, owing to its capacity to improve delivery precision and provide versatile, multifunctional therapeutic platforms (5). Beyond simple drug encapsulation, engineered nanoparticles can be designed to interact with multiple biological pathways simultaneously, enabling direct antitumor activity alongside the strategic modulation of the tumor–immune interface (6-8). Within this landscape, chelating nanoparticles have emerged as a sophisticated frontier, offering a specialized approach to modulating metal-dependent signaling pathways and cellular oxidative states (9). A prominent example of this technology is BCc1, a novel, self-assembled nano-complex engineered to modulate metal ion homeostasis specifically through the redistribution of iron-thereby influencing critical iron-dependent signaling pathways involved in tumor proliferation and innate immune activation (10-12).

By targeting the metabolic requirements of malignant cells, BCc1 has demonstrated significant antitumor efficacy through the disruption of redox processes essential for tumor survival (13, 14). Notably, BCc1 functions by sequestering bioavailable iron, which mitigates oxidative stress and disrupts the signaling cascades required for cancer cell persistence (9). The immunological activity of BCc1 may not be limited to its cytotoxic properties, as iron-dependent pathways are critically involved in shaping myeloid cell responses and cytokine production. To date, the precise immunological mechanisms through which BCc1 influences systemic innate immune responses—particularly regarding splenic immune regulation remain incompletely defined.

The spleen serves as a critical secondary lymphoid organ, acting as a central hub for cytokine production, immune cell priming, and systemic immune surveillance (14). Because the spleen functions as a major reservoir for myeloid cells and a systemic regulator of cytokine homeostasis, alterations in its cytokine networks can exert profound downstream effects on antitumor immunity (15, 16). Consequently, the splenic compartment represents a vital, yet underexplored, site for evaluating the systemic impact of immunomodulatory nanotherapeutics.

Here, we examined the immunoregulatory impact of BCc1 nanomedicine on the splenic cytokine milieu in a 4T1 breast tumor–bearing mouse model, specifically assessing splenic concentrations of IL-12, TGF-β1, and TNF-α.

By examining how BCc1 modulates the equilibrium between immunostimulatory and immunosuppressive signaling, this study seeks to elucidate its role as an active immunomodulatory agent rather than a passive nanocarrier. Specifically, we hypothesize that BCc1 promotes a cytokine milieu conducive to a Th1-skewed antitumor immune response, characterized by enhanced IL-12 and TNF-α production and the attenuation of TGF-β1-mediated immunosuppressive signaling. The findings presented herein aim to provide mechanistic insight into the immunological basis of BCc1 activity and to support its further development as a multifunctional nanotherapeutic platform for immunologically informed cancer treatment strategies.

2. Methods

2.1 Expansion of 4T1 Murine Mammary Carcinoma Cells for Tumor Antigen Generation

The 4T1 murine mammary carcinoma cell line (ATCC CRL-2539) was obtained from the National Cell Bank, Pasteur Institute of Iran, and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 $\mu g/ml$ streptomycin, and 2 mM L-glutamine. Cultures were kept at 37 °C in a humidified atmosphere containing 5% CO_2 and routinely passaged at 80–90% confluency to preserve optimal viability and growth characteristics. Cell viability was assessed by trypan blue exclusion before downstream experimental use. Expanded 4T1 cells were subsequently used for in vivo tumor induction and for preparing tumor-associated antigens for ex vivo stimulation of splenic immune cells.

2.2 Generation of Tumor Antigen Lysate from 4T1 Cells

To generate tumor-associated antigen preparations for downstream immune assays, harvested 4T1 murine mammary carcinoma cells were adjusted to 1x10^7 cells/ml in ice-cold phosphate-buffered saline (PBS) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) to minimize proteolytic degradation. The cells underwent five cycles of rapid freezing at −70 °C followed by thawing at 37 °C, after which they were subjected to ultrasonic disruption (60 Hz, 0.5 amplitude) for ten consecutive cycles to ensure complete lysis.

The resulting homogenate was clarified by centrifugation at approximately 21,900 × g for 10 min at 4 °C. The supernatant was carefully collected, dialyzed against chilled PBS to remove low-molecular-weight impurities, and sterilized via passage through a 0.2 µm membrane filter.

The protein content of the antigen preparation was estimated using the Coomassie Brilliant Blue dye-binding method described by Bradford. The samples

were subsequently divided into single-use aliquots and preserved at −70 °C until their use in downstream experiments evaluating systemic immunomodulatory responses, with particular emphasis on the splenic immune microenvironment.

2.3 Tumor Modeling

2.3.1 Animals and Housing

Seventy BALB/c females aged 6–8 weeks, with an average body weight of 20 ± 0.5 g, were procured from the Pasteur Institute, Karaj, Iran. Before experimental manipulation, mice were allowed to acclimate for one week in individually ventilated cages, with unrestricted access to standard laboratory chow and water. Housing was provided under controlled conditions at 20–22 °C with a 12-h light/12-h dark cycle. The BALB/c female strain was chosen because it offers an immunocompetent syngeneic host for the 4T1 mammary carcinoma model and supports investigation of tumor development and cytokine-mediated immune regulation within a physiologically relevant female endocrine setting.

Ethical clearance for this animal study was granted by the Institutional Animal Ethics Committee of Islamic Azad University, Tehran Medical Sciences, Tehran, Iran, under authorization number IR.IAU.PS.REC.1400.341. Experimental procedures involving animals adhered to ARRIVE 2.0 reporting standards and the 3Rs framework. Mice were examined daily for health and welfare status, and humane endpoints were implemented whenever required.

2.3.2 Tumor Cell Preparation and Initial Implantation

Identity-authenticated 4T1 breast carcinoma cells (BALB/c-derived; ATCC, catalogue no. CRL-2539) were expanded in vitro and suspended in sterile phosphate-buffered saline (PBS) to a final concentration of 1x10^6 cells/ml. To establish donor tumors, 200 µL of this suspension was injected subcutaneously into the perimammary region of five mice. Neoplastic progression was closely monitored to ensure the maximum tumor diameter never exceeded the predefined humane endpoint of 20 mm. Upon reaching this size, tumors were surgically excised under aseptic conditions and sectioned into fragments of approximately 2–3 mm. These tissue fragments were preserved in chilled PBS supplemented with penicillin–streptomycin until subsequent use.

2.3.3 Breast Tumor Model Establishment

For surgical procedures, each mouse was administered 100 µL of a freshly prepared anesthetic mixture (xylazine: ketamine, 1:2 ratio) via intraperitoneal injection. The formulation was prepared for each batch of five animals by combining 40 µL xylazine, 80 µL ketamine, and 380 µL sterile physiological saline. The right flank was shaved, and tumor fragments were implanted subcutaneously. The incision was sutured, and animals were housed individually in a temperature-controlled environment (25 °C) during recovery. Experimental treatments were initiated 48 h post-transplantation. No significant acute mortality occurred during the treatment period, and survival mice remained comparable to those of the control groups.

2.4 Treatment Protocols

2.4.1 Control Treatment (PBS)

Animals assigned to the control group received PBS as the vehicle control. The isotonic buffer solution, with a pH range of 7.2–7.4, was prepared by dissolving commercially supplied PBS tablets in distilled water in accordance with the supplier's recommended protocol (Table 1). When required for procedures involving cultured cells, the prepared buffer underwent liquid-cycle autoclaving at 121 °C and 15 psi for 20 min to obtain a sterile preparation, followed by refrigeration at 4 °C until needed. Before administration, the solution was allowed to reach room temperature to ensure complete redissolution of any precipitated material.

PBS was administered at a volume of 0.1 mL per mouse using the same route and schedule applied to treatment groups.

Table 1. PBS Buffer Preparation

Salt	Concentration (mmol/L)	Concentration (g/L)
Nacl	137	**8.01**
Kcl	2.7	**0.20**
$Na_2HPO_4 \cdot 2H_2O$	10	**1.78**
KH_2PO_4	2	**0.27**
PH (adjusted with HCl or NaOH)	7.4	**7.4**

2.4.2 Cyclophosphamide Treatment

Cyclophosphamide, an alkylating chemotherapeutic agent, was used as a reference treatment (17). The drug was freshly prepared in sterile PBS immediately before administration. Cyclophosphamide was administered according to the experimental design at

the specified dose and schedule to evaluate its immunomodulatory and antitumor effects. All treatments were performed under sterile conditions, and dosing volumes were adjusted to mouse body weight to ensure consistency across experimental groups.

2.4.3 BCc1 Nanomedicine Treatment

After tumor induction using 4T1 mammary carcinoma cell inoculation, mice were allowed to recover for 48 h and were subsequently distributed through random allocation across seven study arms, with 10 animals included in each arm. Randomization was performed manually to ensure unbiased allocation across all treatment modalities. Animals were individually identified via picric acid staining. The experimental design was structured as follows: (i) two negative control groups receiving the vehicle via intraperitoneal (i.p.) injection and oral gavage, respectively; (ii) one positive control group treated with cyclophosphamide; and (iii) four treatment groups receiving various formulations and dosage levels of BCc1 nanomedicine (Table 2).

The BCc1 nanomedicine, kindly provided by Sodour Ahrar Shargh Company, was administered daily for 24 consecutive days at a fixed time. Depending on the specific group protocol, administration was conducted either via i.p. injection or oral gavage. Each treatment was administered according to the predetermined dosing schedules defined in the experimental protocol.

Table 2. Treatment Groups and Administration Routes

Group	Number of Mice	Injection Site	Treatment	Dose/Concentration
A	10	Right hind paw	Intraperitoneal (i.p.)	**Tumor + PBS**
B	10	Left hind paw	Oral gavage	**Tumor + PBS**
C	10	Right forelimb	Intraperitoneal (i.p.)	**Tumor + BCc1 0.1 mg/kg**
D	10	Left forelimb	Oral gavage	**Tumor + BCc1 10 mg/kg**
E	10	Forehead	Intraperitoneal (i.p.)	**Tumor + BCc1 0.4 mg/kg**
F	10	Abdomen	Oral gavage	**Tumor + BCc1 40 mg/kg**
G	10	Both forelimbs	Intraperitoneal (i.p.)	**Tumor + Cyclophosphamide 20 mg/ kg**

2.4.4 Routes of Administration

2.4.4.1 Intraperitoneal Injection

Intraperitoneal injections were performed in the lower right abdominal quadrant, lateral to the midline, using a shallow insertion angle (~10°) to minimize the risk of visceral injury. To ensure correct needle placement and avoid inadvertent intravascular or organ puncture, gentle aspiration was performed prior to injection (18, 19).

2.4.4.2 Oral Gavage

For oral administration, mice were gently restrained, and a gavage needle was introduced parallel to the palate and advanced along the esophagus. Proper placement was confirmed by unobstructed respiration and absence of distress. The head was maintained in a slightly elevated position to reduce the risk of tracheal insertion (20).

2.4.5 Dose Volume

Based on standard dosing guidelines for murine models, the administration volume was set at 0.1 mL per mouse for both intraperitoneal and oral routes (21). This volume was maintained consistently across all treatment groups to ensure comparability.

2.5 Isolation and Culture of Murine Splenocytes

2.5.1 Spleen Harvesting

At the predetermined study endpoint, or earlier upon fulfillment of prespecified humane endpoints, mice were deeply anesthetized before humane euthanasia by gradual-fill CO_2 exposure. Cervical dislocation was subsequently used to verify death. The protocol adhered to AVMA recommendations on animal euthanasia, CCAC standards, as well as EU Directive 2010/63/EU.

Immediately following euthanasia, animals were briefly immersed in 70% ethanol for surface decontamination and transferred to a laminar-flow biosafety cabinet. Under sterile conditions, a small incision was made in the left abdominal flank to access the peritoneal cavity, and the spleen was carefully excised to avoid mechanical disruption. Upon excision, spleen tissues were promptly transferred to aseptic Petri plates containing 5 mL of cold splenic rinse medium formulated with PBS, 5–10% FBS, and penicillin–streptomycin. The samples were processed immediately for subsequent immunological analyses.

2.5.2 Preparation of Splenic Single-Cell Suspension

Following excision, spleen specimens were placed in sterile 6-cm Petri dishes containing splenic washing solution and mechanically dissociated by gentle pressure with a sterile plunger tip until a homogeneous cellular suspension was obtained. The resulting preparation was transferred into an aseptic 15-mL conical tube, carefully homogenized through repeated pipetting, and processed with a refrigerated spin at 447

× g and 4 °C for 5 min to pellet the cells. The overlying liquid was subsequently aspirated with care and discarded.

2.5.3 Red Blood Cell Lysis

The cell pellet was gently resuspended by tapping, and erythrocytes were lysed by incubation with 5 mL of lysis buffer (5–10 min, room temperature). To restore isotonic conditions and maintain leukocyte viability, 5 mL of splenic washing buffer (0.9% saline, 5% FBS, L-glutamine, and penicillin–streptomycin) was added. The suspension was centrifuged (447 × g, 4 °C, 5 min), and the supernatant was carefully aspirated, ensuring the integrity of the cell pellet.

2.5.4 Removal of Residual Splenic Tissue

The remaining cell fraction was gently dispersed in 5–6 mL of splenic washing solution and kept at 4 °C for 30 min to allow residual splenic tissue fragments to sediment. The leukocyte-rich upper phase was then recovered carefully without disrupting the settled material, transferred to a sterile tube, and processed by refrigerated centrifugation at 447 × g and 4 °C for 5 min, producing a purified splenocyte pellet.

2.5.5 Cell Counting and Viability Assessment

Cell concentration and viability were determined using the trypan blue exclusion assay. Briefly, 10 µL of cell suspension was mixed with an equal volume of 0.4% trypan blue solution. A 10-µL aliquot was loaded onto a hemocytometer, and cells were visualized via bright-field microscopy (×10 and ×40 objectives). Viable cells, characterized by dye exclusion, remained unstained, whereas cells with compromised membrane integrity appeared blue. Cell enumeration was performed across 16 large hemocytometer squares, with final concentrations calculated using standard equations adjusted for the dilution factor.

2.5.6 Preparation of Cells for Culture

Following cell counting, splenocyte suspensions underwent refrigerated centrifugation at 447 × g and 4 °C for 5 min to obtain a compact cell pellet. The overlying medium was aspirated carefully, and the recovered cells were resuspended in complete RPMI-1640 culture medium. The resulting suspension was mixed gently to ensure uniformity and brought to a final concentration of $4x10^6\ cells/ml$.

2.5.7 Splenocyte Culture and Tumor Antigen Stimulation

Each well of a 24-well culture plate received an aliquot of splenocyte suspension containing $4x10^6\ cells$. For antigen-specific restimulation, 100 µL of the 4T1 tumor-antigen preparation was added to the designated wells, yielding a final antigen concentration of $10\ \mu g/ml$. The plates were gently agitated to facilitate homogeneous distribution of the antigen within the cell cultures. Splenocytes were maintained for 72 h in a humidified atmosphere at 37 °C. At the end of the culture period, conditioned supernatants were collected and stored at −70 °C until cytokine measurement by ELISA.

2.6 Quantification of Splenic Cytokines by Sandwich ELISA

Splenocyte-derived levels of $IL-12$, $TGF-\beta1$, and $TNF-\alpha$ were quantified in culture supernatants using a capture–detection ELISA format. For ex vivo cytokine profiling, isolated splenocytes were stimulated with lysates generated from 4T1 tumor cells. Briefly, splenocyte suspensions were distributed into multiwell culture vessels containing 24 wells and stimulated with 4T1-derived tumor lysate. Following 72 h of incubation, the cultures were centrifuged to remove cells and debris, and the resulting cell-free supernatants were collected and stored at −70 °C until cytokine analysis.

Mouse cytokine concentrations were quantified using commercially available ELISA kits specific to the target analytes, with all assay steps conducted according to the suppliers' instructions. For assay-plate preparation, polystyrene 96-well microplates were coated with cytokine-specific capture antibodies against $IL-12$, $TGF-\beta1$, or $TNF-\alpha$ and incubated at 4 °C until the following day. After PBS washing, remaining unoccupied binding surfaces were blocked at ambient temperature for 60 min with a PBS-based buffer supplemented with 1% BSA to reduce nonspecific binding.

Samples and recombinant cytokine standards were added in triplicate at 100 µL per well and incubated at ambient temperature for 120 min. After washing, the corresponding biotinylated detection antibodies for each cytokine were added and allowed to react for 60 min. Streptavidin–horseradish peroxidase conjugate, diluted 1:1000, was then introduced, followed by an additional 60-min incubation under ambient laboratory conditions.

Following multiple wash cycles, TMB substrate was added, and color development proceeded for 20–30 min in the absence of direct light. The enzymatic reaction was stopped with 2 N sulfuric acid, and absorbance was measured at 450 nm using a microplate spectrophotometer. Cytokine concentrations were calculated from standard curves generated using serial dilutions of recombinant cytokines and expressed as pg/ml.

2.7 Statistical Analysis

Statistical evaluation was performed using GraphPad

Prism software, version 6.01, developed by GraphPad Software Inc. (La Jolla, CA, USA). The analyzed endpoints comprised splenic IL − 12 and TNF − α concentrations, along with the $TGF-\beta1/IL-12$ ratio. Intergroup differences were examined using a single-factor analysis of variance, followed by Tukey-adjusted pairwise comparisons when applicable. Data are presented as means with standard deviations, and statistical significance was defined at an alpha level of 0.05.

3. Results

3.1 BCc1 Treatment Differentially Modulates Splenic IL − 12 Production

Quantitative assessment of IL − 12 protein levels in splenic suspensions revealed a treatment-dependent effect (Figure 1). Parenteral administration of BCc1 significantly reduced IL − 12levels compared with PBS-treated controls. Specifically, BCc1 treatment at 0.1 mg/kg lowered IL-12 concentrations by 19.65% (P = 0.0003), while the 0.4 mg/kg dose produced a more pronounced decrease of 32.66% (P < 0.0001). The extent of IL-12 suppression was similar to that achieved with cyclophosphamide, which reduced IL-12 levels by 34.72% (P < 0.0001). Direct comparison of the 0.1 and 0.4 mg/kg parenteral BCc1 regimens yielded no evidence of additional dose-dependent suppression (P = 0.2492), consistent with a possible plateau in the response at higher exposure levels.

Oral administration produced a milder immunomodulatory response. Treatment with 10 mg/kg BCc1 did not significantly alter IL − 12levels compared with oral PBS controls (5.21% decrease; P = 0.8478), whereas 40 mg/kg BCc1 reduced IL − 12levels by 17.72% (P = 0.0037). The comparison of oral BCc1 dosing regimens showed no reliable separation in IL − 12response (P = 0.1320); nevertheless, the higher oral dose was associated with a stronger reduction in IL − 12production.

Cross-route analysis showed that cyclophosphamide produced significantly greater IL − 12 suppression than the 10 mg/kg oral BCc1 regimen, corresponding to an 18.26% lower level (P = 0.0051). In contrast, IL − 12 concentrations in mice given 40 mg/kg BCc1 orally closely matched those measured after cyclophosphamide treatment (P = 0.8910).

Overall, these results show that the effect of BCc1 on splenic IL − 12 production varies according to both dose and delivery route, with parenteral delivery generating immunomodulatory responses similar to those induced by cyclophosphamide, whereas oral dosing produced comparatively modest cytokine changes.

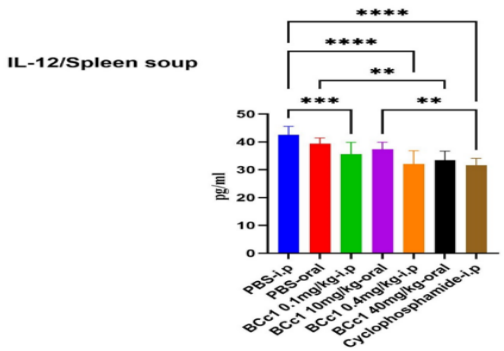

Figure 1. BCc1-mediated modulation of IL − 12 levels in splenic suspensions.

Splenic IL − 12 protein levels were quantified by ELISA following treatment with BCc1 Nanomedicine or cyclophosphamide. Data are presented as mean ± standard deviation for each experimental group. Statistical significance was determined relative to the corresponding PBS control. *P < 0.05.

3.2 BCc1 Suppresses Splenic TGF − β1 in a Dose- and Route-Dependent Manner

Quantification of splenic TGF − β1 revealed a strong inhibitory effect of BCc1 on this immunoregulatory cytokine (Figure 2). Compared with PBS controls, parenteral delivery of BCc1 markedly lowered TGF − β1 levels. At 0.1 mg/kg, BCc1 produced a pronounced reduction in TGF − β1 (P < 0.0001), and this effect became stronger at 0.4 mg/kg, with significantly greater suppression than both PBS treatment (P < 0.0001) and the lower BCc1 dose (P < 0.0001). Cyclophosphamide injection likewise decreased TGF − β1 levels significantly (P < 0.0001). However, animals receiving 0.4 mg/kg BCc1 had significantly lower TGF − β1 concentrations than cyclophosphamide-treated mice (P = 0.0005), whereas the response to 0.1 mg/kg BCc1 was not significantly different from that induced by cyclophosphamide (P = 0.0755). These results suggest that parenteral BCc1 at the higher dose exerts stronger suppression of splenic TGF − β1 production than cyclophosphamide.

Oral delivery of BCc1 resulted in a weaker, yet dose-related, response. The 10 mg/kg oral dose did not significantly change TGF − β1 levels relative to oral PBS controls (P = 0.2400), whereas 40 mg/kg BCc1 substantially reduced TGF − β1 expression (P = 0.0008). A clear significant separation was detected across the two oral doses (P < 0.0001), supporting a dose-linked effect after oral treatment.

Comparisons across delivery routes showed that TGF − β1 levels in cyclophosphamide-treated mice were similar to those measured in mice given 10 mg/kg BCc1 orally (P = 0.1358), but remained significantly higher than levels detected after 40

mg/kg oral BCc1 treatment (P = 0.0022). Taken together, the results identify BCc1 as a potent suppressor of splenic TGF − β1, with the strongest immunomodulatory effect achieved at the higher dose, particularly when delivered parenterally.

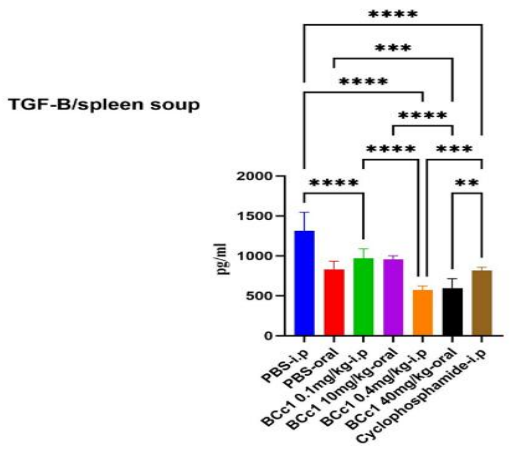

Figure 2. BCc1-mediated suppression of TGF − β1levels in splenic suspensions.
Splenic TGF − β1 protein concentrations were quantified by ELISA following treatment with BCc1 Nanomedicine or cyclophosphamide. Data are presented as mean ± standard deviation. Columns represent individual treatment groups. Statistical significance was determined relative to the corresponding PBS control or between indicated groups. *P < 0.05.

3.3 BCc1 Shifts the $^{TGF-\beta1}/_{IL-12}$ Cytokine Balance Toward an Immunostimulatory Profile

To assess the overall immunomodulatory impact of BCc1, splenic cytokine balance was evaluated by calculating the $^{TGF-\beta1}/_{IL-12}$ ratio, representing the relationship between an immunosuppressive mediator and an immunostimulatory cytokine (Figure 3). Parenteral delivery reshaped this balance in a dose-sensitive pattern. BCc1 at 0.1 mg/kg caused only a limited, statistically non-significant decline in the $^{TGF-\beta1}/_{IL-12}$ ratio compared with PBS controls, corresponding to a 13.04% decrease (P = 0.4347). By contrast, BCc1 at 0.4 mg/kg markedly suppressed this ratio, producing a 70.62% reduction from the PBS baseline (P < 0.0001).

Cyclophosphamide produced a smaller and statistically non-significant decrease in the $^{TGF-\beta1}/_{IL-12}$ ratio, equivalent to a 20.19% reduction (P = 0.0742). Pairwise analysis showed that the higher parenteral BCc1 dose lowered the cytokine ratio more effectively than the lower BCc1 dose, with a 50.92% reduction (P < 0.0001). The same high-dose BCc1 regimen also exceeded cyclophosphamide in reducing the ratio, showing a 41.95% decrease (P = 0.0014). In contrast, responses to BCc1 at 0.1 mg/kg and cyclophosphamide were statistically indistinguishable (P = 0.9707). These results emphasize that parenteral delivery of high-dose BCc1 can shift splenic cytokine balance away from immunoregulatory predominance.

Oral delivery generated a less pronounced effect on the $^{TGF-\beta1}/_{IL-12}$ ratio. Neither BCc1 at 10 mg/kg nor BCc1 at 40 mg/kg produced a significant change compared with oral PBS controls, with P values of 0.1806 and 0.6031. However, the 40 mg/kg oral regimen reduced the ratio more strongly than the 10 mg/kg oral regimen, corresponding to a 42.35% decrease, consistent with a dose-linked response after oral dosing.

Across-route analysis showed that cyclophosphamide and oral BCc1 at 10 mg/kg yielded comparable cytokine ratios, with a non-significant 1.05% increase. In contrast, oral BCc1 at 40 mg/kg produced a lower ratio than cyclophosphamide, corresponding to a 43.84% decrease. Overall, these data support high-dose BCc1, especially through parenteral delivery, as a strong modulator of the $^{TGF-\beta1}/_{IL-12}$axis, directing the splenic cytokine environment toward a state more compatible with antitumor immune permissiveness.

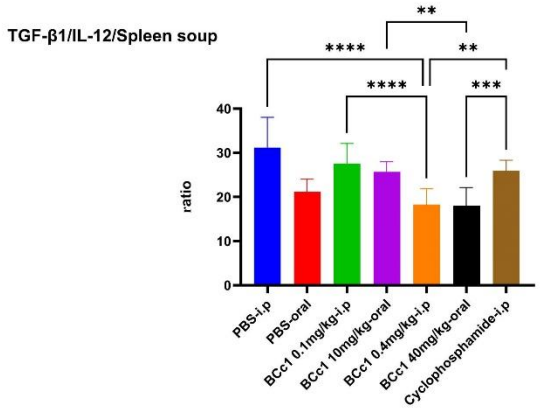

Figure 3. BCc1-induced modulation of the $^{TGF-\beta1}/_{IL-12}$ cytokine ratio in splenic suspensions**.**
The ratio of TGF − β1 to IL − 12 in splenic suspensions was quantified by ELISA following treatment with BCc1 Nanomedicine or cyclophosphamide. Data are presented as mean ± standard deviation. Columns correspond to experimental treatment groups. Statistical significance was assessed relative to the corresponding PBS control or between indicated groups. *P < 0.05.

3.4 BCc1 Attenuates Splenic TNF − α Production in a Dose- and Route-Dependent Manner

ELISA-based quantification revealed route- and dose-related modulation of splenic TNF − α by BCc1

(Figure 4). After parenteral dosing, BCc1 at 0.1 mg/kg induced only a modest reduction in TNF − α concentrations, which did not reach statistical significance versus PBS controls (14.65% decrease; P = 0.2659). By contrast, parenteral BCc1 at 0.4 mg/kg markedly suppressed TNF − α production, producing a statistically robust 43.62% decrease (P < 0.0001). Cyclophosphamide likewise lowered TNF − α levels in comparison with PBS controls, corresponding to a 27.95% reduction (P = 0.0041).

Head-to-head evaluation of the injectable BCc1 regimens showed lower TNF − α levels under the 0.4 mg/kg regimen than under the 0.1 mg/kg regimen, with a 25.27% reduction (P = 0.0381). This pattern supports a dose-linked response after parenteral delivery. TNF − α concentrations after BCc1 exposure were similar to those measured after cyclophosphamide, with no meaningful statistical separation between cyclophosphamide and BCc1 at either 0.1 mg/kg (P = 0.6679) or 0.4 mg/kg (P = 0.7280).

Oral delivery of BCc1 also reduced splenic TNF − α production. BCc1 at 10 mg/kg lowered TNF − α levels versus oral PBS controls (P = 0.0212), whereas the 40 mg/kg oral regimen generated a stronger suppressive effect (P < 0.0001). In addition, TNF − α concentrations were lower after oral BCc1 at 40 mg/kg than after oral BCc1 at 10 mg/kg (P = 0.0117), further supporting dose-linked activity through the oral route. Across-route comparisons showed that cyclophosphamide produced TNF − α levels similar to those achieved with oral BCc1 at 10 mg/kg (P = 0.1257) and oral BCc1 at 40 mg/kg (P = 0.9699). Overall, the data indicate that BCc1 suppresses splenic TNF − α production effectively, with higher-dose regimens delivered either parenterally or orally producing immunomodulatory effects comparable to cyclophosphamide.

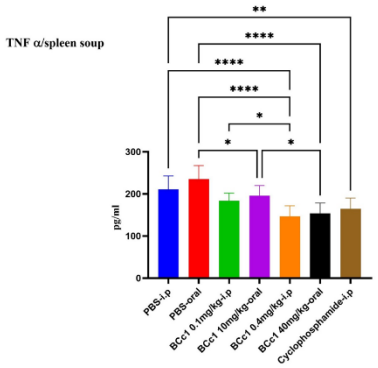

Figure 4. BCc1-mediated regulation of TNF − α levels in splenic suspensions

Splenic TNF − α protein concentrations were quantified by ELISA following treatment with BCc1 Nanomedicine or cyclophosphamide. Data are presented as mean ± standard deviation. Columns represent individual treatment groups. Statistical significance was determined relative to the corresponding PBS control or between indicated groups. *P < 0.05.

4. Discussion

Collectively, the cytokine profiling presented in this study indicates that BCc1 does not act solely as a cytotoxic nanomedicine, but also modulates cytokine networks within the splenic immune microenvironment of 4T1 breast tumor–bearing mice. BCc1 reshaped the splenic cytokine profile in a regimen-dependent manner, as reflected by changes in IL − 12 , TNF − α, and TGF − β1 levels and, most importantly, by alterations in the $^{TGF-\beta1}/_{IL-12}$ balance across different doses and routes of administration. The most prominent effects were observed after higher-dose parenteral administration, particularly for suppression of TGF − β1and reduction of the $^{TGF-\beta1}/_{IL-12}$ ratio. These findings suggest that BCc1 recalibrates tumor-associated splenic cytokine imbalance toward a profile associated with reduced immunoregulatory dominance and greater antitumor immune permissiveness. Accordingly, BCc1 may be considered an active immunomodulatory nanotherapeutic candidate rather than a passive delivery platform.

The findings should be interpreted in view of two methodological constraints. First, the experimental treatments were evaluated mainly against PBS-treated tumor-bearing mice, whereas a naïve tumor-free control group was not incorporated. Second, although earlier investigations have documented marked anticancer activity of BCc1 in both in vitro and in vivo settings, including direct antineoplastic effects, the cytokine-oriented design of this work did not include a concurrent assessment of direct cytotoxicity or 4T1 cell viability in BCc1-treated cultures, such as an MTT-based assay (10). Accordingly, the current findings should be interpreted primarily as evidence of in vivo splenic cytokine-network immunomodulation rather than as a quantitative measure of the direct cytotoxic potency of BCc1 against 4T1 tumor cells.

Therefore, although the observed reduction in splenic TGF − β1 and modulation of the $^{TGF-\beta1}/_{IL-12}$ axis indicate attenuation of tumor-associated immunoregulatory cytokine activity, the current dataset does not determine whether BCc1 restores cytokine patterns toward physiological steady-state levels or induces a distinct immune state beyond the naïve baseline. Incorporating age- and sex-matched naïve controls in future studies would provide an essential reference for defining the directionality, magnitude, and biological boundaries of BCc1-

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induced immune remodeling. Such comparisons would help clarify whether BCc1 acts predominantly as a homeostasis-restoring immunomodulator in the tumor-bearing host or as an immune-state–shifting nanotherapeutic capable of inducing cytokine configurations distinct from those observed under non-tumor-bearing conditions.

Nanoparticle-based therapeutic systems are increasingly recognized in oncology for their capacity to engage multiple biological pathways, an attribute of particular relevance in metastatic breast cancer, where immune evasion and treatment resistance remain major therapeutic barriers(22). The structural and chemical versatility of nanomaterials, including tunable surface properties and chelating functionalities, enables interactions with diverse molecular and cellular networks, thereby extending their biological effects beyond single-pathway modulation (23-25).

BCc1, developed through advanced chelation chemistry, has previously been shown to influence iron homeostasis through redistribution of metal ions, thereby affecting iron-dependent pathways implicated in tumor progression and immune regulation (9, 13). However, the immunological basis of its antitumor activity has remained incompletely defined (10, 26). The present findings extend this understanding by demonstrating that BCc1 modulates cytokine networks at the level of the spleen, a central secondary lymphoid organ involved in systemic innate and adaptive immune regulation (27).

The spleen integrates circulating inflammatory and tumor-associated signals through pattern-recognition and cytokine-responsive pathways expressed largely by myeloid and lymphoid immune populations (28). Within this compartment, splenic monocytes, macrophages, dendritic cells, and lymphocyte subsets can either support antitumor immunity or contribute to immunoregulatory states, depending on the balance of cytokine cues. $TGF-\beta1$is particularly relevant in this context because it promotes immune tolerance, suppresses cytotoxic lymphocyte activity, and supports regulatory T-cell differentiation (29). Thus, modulation of splenic $TGF-\beta1$by BCc1 may have systemic immunological implications, especially in a tumor-bearing host in which $TGF-\beta1$-driven pathways contribute to immune escape.

$IL-12$ functions as a central immunoregulatory mediator at the interface of innate and adaptive immune pathways and plays a pivotal role in directing type 1 immune polarization (30-32). However, $IL-12$ activity is tightly regulated within the cytokine network, and excessive or sustained $IL-12$ signaling has been associated with systemic toxicity in clinical settings despite robust antitumor activity in preclinical models (33, 34). In the present study, BCc1 induced a modest, dose- and route-dependent reduction in splenic $IL-12$ levels. Rather than indicating broad immune suppression, this pattern suggests that BCc1 does not indiscriminately amplify pro-inflammatory signaling. Instead, BCc1 may operate within a constrained immunoregulatory window, limiting excessive inflammatory activation while modulating cytokine balance in the tumor-bearing host.

In parallel, BCc1 markedly reduced splenic $TGF-\beta1$, with the strongest effect observed after high-dose parenteral administration. This finding is immunologically relevant because $TGF-\beta1$can antagonize type 1 immune polarization and has been reported to interfere with $IL-12$ -associated signaling pathways, including $^{JAK}/_{STAT}$ -dependent downstream responses (35-37).

Notably, high-dose parenteral BCc1 reduced $TGF-\beta1$more effectively than cyclophosphamide, supporting the capacity of BCc1 to modulate immunoregulatory cytokine pathways. This route-dependent pattern further underscores the importance of pharmacokinetic behavior and lymphoid tissue accessibility in shaping systemic cytokine modulation (38).

The apparent divergence between reduced $IL-12$ levels and a cytokine profile associated with greater antitumor immune permissiveness can be interpreted by considering cytokine network balance rather than absolute abundance of individual mediators. In this regard, the $^{TGF-\beta1}/_{IL-12}$ ratio emerged as an integrative indicator of the splenic cytokine state. BCc1, particularly at the higher parenteral dose, substantially reduced this ratio, suggesting a shift away from dominant $TGF-\beta1$-associated immunoregulation. This interpretation is consistent with the broader concept that attenuation of suppressive cytokine pathways may be as important as, or more important than, isolated enhancement of pro-inflammatory cytokines in supporting antitumor immune conditions (35, 39).

$TNF-\alpha$ modulation further supports the concept of cytokine network recalibration rather than nonspecific immune activation. Although $TNF-\alpha$ can contribute to tumor cell killing and inflammatory immune activation, chronic or excessive $TNF-\alpha$ production has also been associated with tumor progression, systemic inflammation, and immune dysfunction (40-42). The attenuation of splenic $TNF-\alpha$ observed after BCc1 treatment, particularly at higher doses, may therefore reflect fine-tuning of inflammatory tone within the tumor-bearing host. Importantly, high-dose BCc1 administered by either the parenteral or oral route produced $TNF-\alpha$ -lowering effects comparable to cyclophosphamide, suggesting that BCc1 can modulate inflammatory cytokine output without

necessarily inducing broad pro-inflammatory amplification.

Notably, the immunological effects of BCc1 were context- and compartment-dependent. In our previous tumor-focused analysis, oral administration exerted stronger local effects within the tumor microenvironment (43) , whereas in the present study, parenteral delivery more effectively modulated systemic splenic cytokine networks. These differences likely reflect distinct biodistribution patterns, route-dependent exposure profiles, and tissue-specific immune architectures rather than inconsistencies in the biological activity of BCc1. The approximately 100-fold higher oral dose required to elicit significant and partially overlapping splenic cytokine-level effects relative to intraperitoneal administration likely reflects route-dependent differences in effective systemic exposure rather than differences in the intrinsic immunomodulatory activity of BCc1. Limited gastrointestinal absorption, presystemic hepatic metabolism, mucosal barrier constraints, and differential lymphatic uptake may reduce the fraction of orally administered BCc1 reaching systemic and splenic immune compartments, whereas intraperitoneal delivery may provide more efficient access to peritoneal immune cells and lymphatic drainage pathways involved in systemic immune regulation.

Consistent with this interpretation, lower-dose oral administration did not produce broad splenic cytokine changes, whereas higher oral exposure was required to induce significant modulation of selected cytokine parameters, particularly $TGF-\beta 1$and $TNF-\alpha$. These findings indicate that dose and route of administration are critical determinants of BCc1-induced systemic immune effects. However, because the present study assessed soluble cytokine profiles rather than immune cell phenotypes or effector functions, the data should be interpreted as evidence of cytokine-level immune modulation rather than direct proof of enhanced innate antitumor immune function. Future studies incorporating cellular phenotyping, NK-cell cytotoxicity assays, macrophage polarization analysis, myeloid activation profiling, and tumor-growth or survival endpoints will be necessary to determine whether BCc1-induced cytokine remodeling translates into functional antitumor immunity.

In conclusion, BCc1 induces a systems-level reconfiguration of the splenic cytokine network in 4T1 breast tumor–bearing mice, characterized primarily by suppression of $TGF-\beta 1$-associated immunoregulatory cytokine activity and controlled modulation of $IL-12$ - and $TNF-\alpha$ -related inflammatory pathways. Rather than acting as a direct cytokine enhancer, BCc1 appears to recalibrate tumor-associated cytokine imbalance toward a more immunologically permissive state. However, whether this represents restoration toward naïve physiological homeostasis or induction of a distinct immune state remains to be determined. Taken together, our results warrant expanded preclinical validation of BCc1 within multifunctional nanotherapeutic development, while emphasizing the need to refine dose selection and administration route to maximize immunological and therapeutic benefit without compromising safety.

A further point that should be considered when interpreting the present findings is that naïve mice without tumor burden were not included as an independent reference group. Therefore, the cytokine alterations observed after BCc1 treatment can only be interpreted in comparison with PBS-treated tumor-bearing animals, and not against the normal immunological baseline of healthy mice. Future experiments including naïve non-tumor-bearing controls would make it possible to clarify whether BCc1 reverses tumor-associated cytokine disturbances toward physiological immune balance or, alternatively, promotes an immune cytokine pattern that differs from the naïve steady state. Such a comparison would strengthen the interpretation of BCc1-driven immune remodeling and help define its biological significance more precisely.

5. Conclusion

Collectively, these findings demonstrate that BCc1 nanomedicine reshapes the splenic cytokine milieu in 4T1 breast tumor–bearing mice. This immunomodulatory activity is evidenced by modulated IL-12 and TNF-α production, altered TGF-β1 levels, and a distinct shift in the (TGF-β1)/(IL-12) balance, with effects contingent upon both dosage and administration route. Notably, the most pronounced responses were observed following high-dose parenteral regimens. These data suggest that BCc1 recalibrates the splenic cytokine environment relative to PBS-treated controls, favoring a profile consistent with attenuated immunoregulatory dominance and enhanced antitumor-associated immune modulation. However, in the absence of naïve non-tumor-bearing controls and functional innate immune endpoints, these observations should be interpreted as cytokine-level immune modulation rather than definitive evidence of restored physiological homeostasis or augmented innate antitumor function. Future investigations incorporating naïve control groups and comprehensive functional immune assays are warranted to determine whether this BCc1-induced cytokine modulation reflects immune normalization and translates into robust antitumor immune activity.

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This trial received the ethical approval of the Faculty of Pharmacy and Pharmaceutical Sciences - Islamic Azad University of Medical Sciences, Tehran, Iran; reg. No. IR.IAU.PS.REC.1400.341.

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Conflict of interest

The authors declare no conflict of interest.

AI Using Declaration

The authors declare that during the manuscript revision process on May 14, 2026, the AI-assisted tool GPT-5.5 was employed solely for linguistic editing, grammar correction, paraphrasing, and improving the readability of the text. All scientific content and the original intellectual contributions were produced entirely by the authors, who retain full responsibility for the originality, integrity, and accuracy of this work.

Author's contributions

MEA, MM, and MH designed this research. FMA was involved in most of the experiments and data collection, and has written the first version of the manuscript. SK synthesized the nano medicine. MHN contributed to the conception and design of the BCc1. SF, MH, and MM edited the manuscript. AS, MH, and MM performed the statistical analysis. All authors read and approved the final manuscript.

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