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Original Article

Granzyme B-producing B cells: a bidirectional relationship with breast cancer cells and implications for immunotherapy

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Abstract: Granzyme B (GrB)-producing B cells have dual roles in tumor immunity, either killing tumor cells or suppressing antitumor responses by eliminating effector T cells. In this study, we aimed to investigate how breast cancer cells influence GrB-producing B cells from tumor-draining lymph nodes and whether B cell activation enhances their cytotoxic potential. Mononuclear cells were isolated from 14 fresh axillary lymph node samples by density gradient centrifugation using Ficoll-Hypaque. Lymphocytes were co-cultured with breast tumor cell lines (MCF-7 and MDA-231) in the presence of recombinant interleukin-21 (rIL-21) and anti-B cell receptor (BCR). B cell granzyme B production was measured by flow cytometry, while tumor cell (MCF-7) apoptosis was assessed using calcein AM release assays. Direct co-culture of lymphocytes with MCF-7 or MDA-MB-231 significantly reduced the frequency of GrB-producing B cells (P=0.001 and P=0.031, respectively), while tumor supernatants alone had no effect. When B cells were pre-stimulated with IL-21 and anti-BCR for 24 hours before direct co-culture, GrB expression was maintained at baseline levels (no significant difference vs. control). Additionally, B cells activated with IL-21 and anti-BCR caused significant apoptosis in MCF-7 cells (38±8.9%, P=0.023). In conclusion, breast cancer cells suppress GrB^+ B cell responses via direct contact, but this suppression is reversible through B cell activation. Importantly, pre-activated B cells exhibit direct cytotoxic activity against tumor cells, highlighting their potential as an effector population for breast cancer immunotherapy.

Keywords: Granzyme B, B cells, tumor-draining lymph node, breast cancer

Introduction

B cells play various functions in immune responses, ranging from producing antibodies to presenting antigens and providing costimulation. In the tumor microenvironment, they can either promote or inhibit tumor progression depending on their functional state [1]. Regulatory B cells (Bregs) suppress antitumor immunity through suppressive cytokines such as interleukin (IL)-10, IL-35, and transforming growth factor-beta (TGF-β) and checkpoint molecules such programmed cell death protein-1 (PD-1), programmed death-ligand 1 (PD-L1) and T-cell immunoglobulin and mucin domain-containing protein-1 (TIM-1) [2]. In contrast, effector B cells can contribute to tumor clearance through antibody production and cytotoxic mechanisms, including the expression of granzyme B (GrB), perforin, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [3].

IL-21 is a pleiotropic cytokine primarily secreted by T follicular helper (TFH) cells, T helper (Th) 17 cells, and natural killer T (NKT) cells. It plays a pivotal role in cancer immunity by modulating the functions of various immune cells, including

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B cells [4, 5]. IL-21 supports B cell proliferation and differentiation, particularly promoting their maturation into plasma cells. It also facilitates germinal center formation and antibody class switching [6-8]. In addition, IL-21 enhances cytotoxic effector functions in $CD8^+$ T cells, NK cells, and NKT cells by upregulating granzyme B and/or perforin expression [9, 10]. Importantly, in the absence of CD40 signaling, IL-21 in combination with B cell receptor (BCR) stimulation can also induce B cells to adopt a regulatory-cytotoxic phenotype characterized by granzyme B production [11, 12].

GrB-producing B cells have been implicated in both immune suppression and tumor cell killing. They may dampen T cell responses by degrading the CD3ζ chain [13], but can also directly induce apoptosis in tumor cells [12, 14, 15]. It has been shown that the frequency of GrB-producing B cells was lower in involved lymph nodes (LNs) compared with uninvolved LNs in breast cancer and it was suggested that the tumor cells could suppress the production of GrB in B cells [16]. However, the ability of breast cancer cells to modulate this pathway and affect B cell-mediated cytotoxicity remains poorly understood.

This study aims to investigate whether breast cancer cell lines suppress GrB production in B cells derived from tumor-draining lymph nodes (TDLNs), and whether B cell activation with IL-21 and anti-BCR can restore their cytotoxic potential. We hypothesize that tumor cells inhibit GrB expression in B cells through direct contact, but that this suppression is reversible through ex vivo stimulation.

Materials and methods

Patients and sample collection

Fresh uninvolved axillary LNs were obtained from 11 female breast cancer patients (mean age: 57±12.6 years) for GrB expression analysis. Tumor types included invasive ductal carcinoma (IDC, n=7), IDC with medullary features (n=2), and unreported (n=2). Cancer stages were: stage I (n=3), stage II (n=5), and unknown (n=3). For cytotoxicity assays, LNs were collected from 3 stage II patients (mean age: 44±2.6 years). None had received chemotherapy or radiotherapy. Written informed consent was obtained from all participants. The study was approved by the Ethics Committees of Shiraz and Shahid Sadoughi Universities of Medical Sciences in accordance with the Declaration of Helsinki. A portion of each LN was stored in Roswell Park Memorial Institute medium 1640 (RPMI 1640) +10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (All from Gibco, Life Technologies, USA) for experiments; the remainder was submitted for routine histopathology.

Preparing conditioned media (CM)

MCF-7 and MDA-MB-231 cell lines (Pasteur Institute, Tehran, Iran) were cultured in RPMI 1640+10% FBS +1% Penicillin/Streptomycin. After two passages and reaching > 90% confluency, cells were trypsinized, centrifuged, and resuspended in serum-free RPMI at a concentration of 4.5×10^5 cells/mL. After 48 hours, CM was collected, centrifuged (800×g, 10 min), and stored at -70°C.

Lymphocyte-tumor cell co-culture and stimulation

Mononuclear cells were isolated from LNs using Ficoll-Hypaque gradient centrifugation [17]. Co-cultures with tumor cells (MCF-7 or MDA-MB-231) were set at a 10:1 lymphocyte:tumor cell ratio in 24-well plates for 24 or 48 hours. In some experiments, lymphocytes were pre-activated with recombinant IL-21 (25 ng/mL, R&D systems, USA) and anti-BCR (2.8 µg/mL, Jackson ImmunoResearch, USA) for 24 hours. Brefeldin A (1 µL/mL, BD bioscience, USA) was added during the final 6 hours.

CM experiments were performed by mixing CM and fresh medium at 1:1 (50%) and 3:1 (75%) ratios, followed by lymphocyte stimulation as described above. Controls included unstimulated (negative) and IL-21/anti-BCR-stimulated (positive) cells without tumor exposure.

Flow cytometry for GrB expression

Cells were washed and stained with anti-CD19 antibody (clone HIB19, BioLegend, USA), fixed with 1% paraformaldehyde (Sigma, Germany), permeabilized (BioLegend perm/wash buffer), and stained intracellularly with anti-GrB antibody (clone GB11, BD Biosciences). Data were

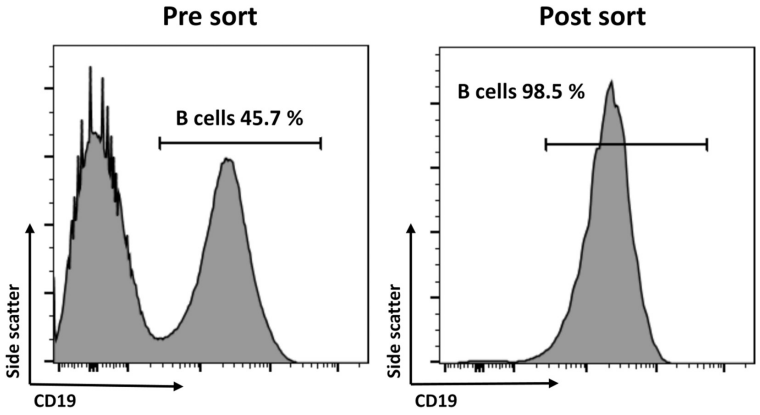

Figure 1. Isolation of B cells from breast tumor-draining lymph nodes (TDLNs) using fluorescence-activated cell sorting (FACS). Mononuclear cells from TDLNs were surface-stained with anti-CD19 antibody, and $CD19^+$ B cells were isolated by positive selection using FACS. Representative histograms depict the percentage of $CD19^+$ cells before and after sorting.

acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo v10.0.7 (USA). $CD19^+$ lymphocytes were gated and GrB^+ populations quantified.

Cytotoxicity assay

$CD19^+$ B cells were isolated by flow sorting (FACSAria™ III; purity ≥ 98%, **Figure 1**). Cells were pre-activated with IL-21 (50 ng/mL) and/or anti-BCR (6.6 μg/mL) for 24 hours, then co-cultured with Calcein (BD bioscience)-labeled MCF-7 cells (effector:target ratio 17:1) for 36 hours. During co-culture, fresh activation media (IL-21: 12.5 ng/mL, anti-BCR: 1.7 μg/mL) was added. Supernatants were collected, and Calcein release was measured using a microplate reader (Omega Polar Star reader, USA). To determine the maximum release we treated MCF-7 cells with 2% Triton X100, while to assess the spontaneous release only fresh medium was added. Apoptosis was calculated as: Apoptosis (%) = (Experimental release - spontaneous release)/(Maximum release - spontaneous release) × 100.

Statistical analysis

Non-parametric Kruskal-wallis H test with Dunn's post hoc test was used to compare GrB^+ B cell frequencies and apoptosis levels. All data analysis and graphical representations were generated using GraphPad Prism 6 (GraphPad Software, Inc., USA).

Results

Granzyme B production in B cells is induced by IL-21/anti-BCR and suppressed by tumor cells

Mononuclear cells from eight uninvolved axillary lymph nodes of breast cancer patients were stimulated with recombinant IL-21 and anti-BCR for 24 hours. This led to a significant increase in the frequency of GrB^+ B cells compared to unstimulated controls (16.8±2.4% vs. 1±0.1%, P=0.0002; **Figure 2A**, **2B**).

Direct co-culture of lymphocytes with MCF-7 or MDA-MB-231 breast cancer cell lines significantly decreased GrB expression in B cells. The frequency of GrB^+ B cells was reduced to 3.6±1.3% with MCF-7 (P=0.001) and 5.2±2% with MDA-231 (P=0.031) compared to the IL-21/anti-BCR-stimulated control (**Figure 3A**, **3B**). No significant difference was observed between the effects of the two cell lines (P > 0.05).

Reduced GrB expression results from suppressed production, not secretion

To confirm that the observed decrease in GrB was due to reduced intracellular production rather than secretion, Brefeldin A (1 μL/mL) was added at the start of the 24-hour co-culture. GrB expression remained significantly lower in B cells co-cultured with MCF-7 (1.1±0.5%) or MDA-231 (1.4±0.4%) compared to control B cells (16.1±7%), even with Brefeldin A present (**Figure 4A**).

To rule out the cytotoxic effect of prolonged Brefeldin A exposure, it was added only during the final 10 hours of the culture. Results were consistent: GrB expression remained significantly reduced in the presence of either tumor cell line (**Figure 4B**), supporting that suppression results from impaired production rather than release of GrB.

Tumor-conditioned media has limited suppressive effect on GrB production

To assess whether soluble factors from tumor cells influenced GrB expression, lymphocytes

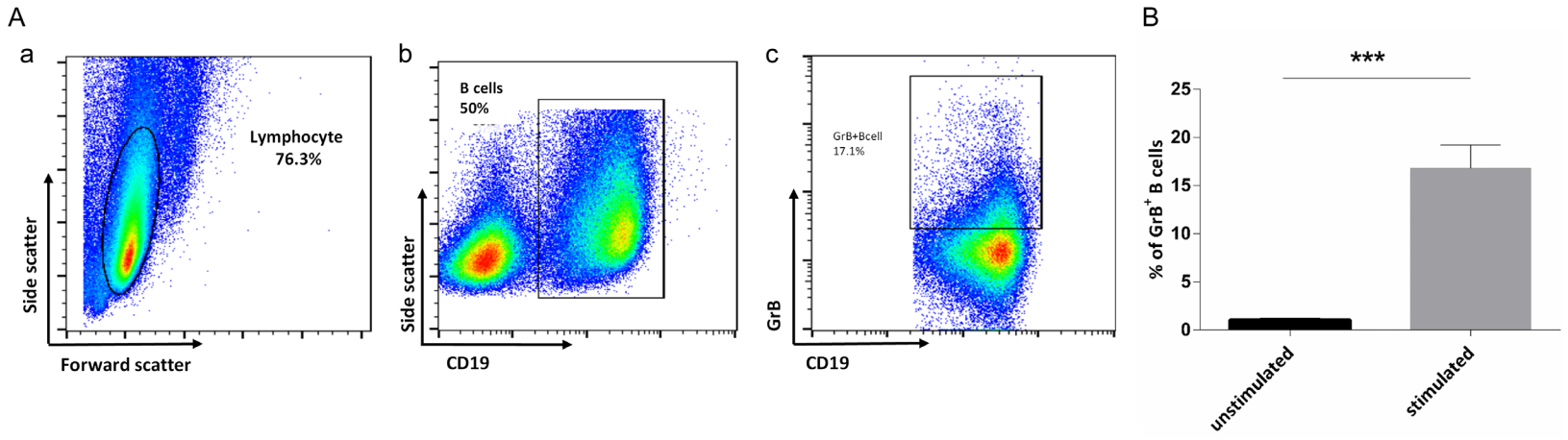

Figure 2. Assessment of granzyme B (GrB) expression in B cells from breast tumor-draining lymph nodes. A. Flow cytometry gating strategy: a. lymphocytes were selected based on forward and side scatter; b. $CD19^+$ cells were gated as B cells; c. GrB expression was assessed within the $CD19^+$ population. B. Comparison of GrB^+ B cell frequency in stimulated vs. unstimulated conditions. Cells were cultured for 24 hours with recombinant IL-21 (rIL-21) and anti-BCR antibodies, or left unstimulated as controls. Data are presented as mean ± SEM. ***P < 0.001. GrB, granzyme B; rIL-21, recombinant interleukin-21; BCR, B cell receptor; SEM, standard error of the mean.

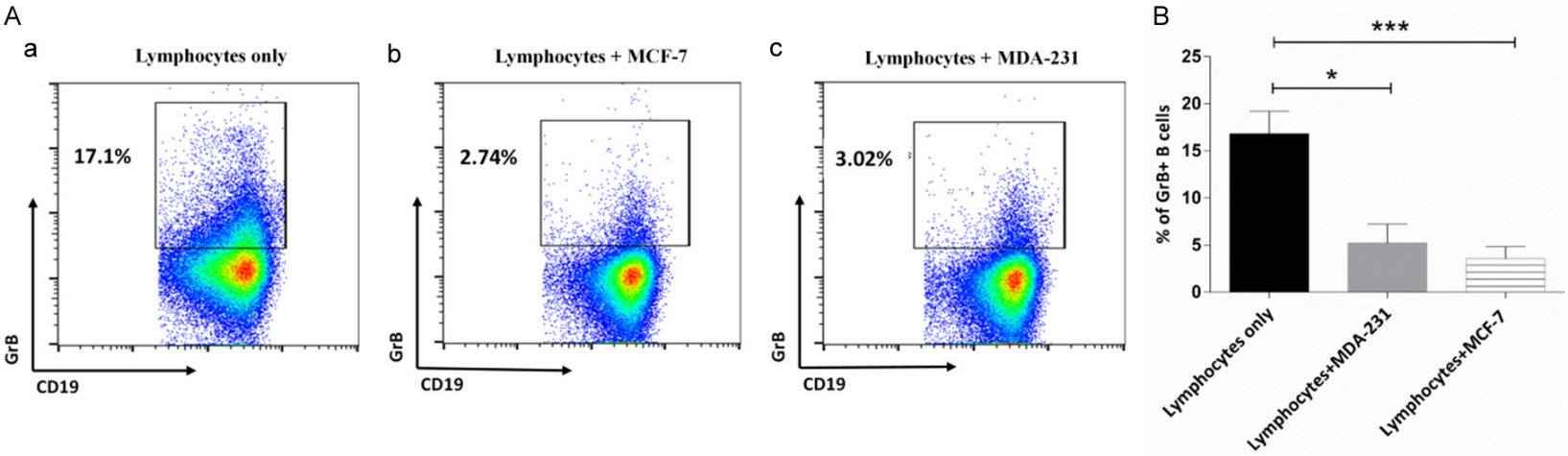

Figure 3. Effect of breast cancer cell lines on granzyme B (GrB) expression in B cells from breast tumor-draining lymph nodes. A. Flow cytometry analysis of GrB expression in B cells co-cultured with or without tumor cells. a. Lymphocytes cultured without tumor cells served as positive control. b. Lymphocytes co-cultured with MCF-7 cells. c. Lymphocytes co-cultured with MDA-MB-231 cells. In all conditions, cells were stimulated with recombinant IL-21 (rIL-21) and anti-BCR antibodies

for 24 hours. B cells were identified by gating on $CD19^+$ lymphocytes as shown in **Figure 2A**, and GrB^+ cells were quantified within this population. B. Quantitative comparison of GrB^+ B cell frequency across the different culture conditions. Data are presented as mean ± SEM. *P < 0.05, ***P < 0.001. GrB, granzyme B; rIL-21, recombinant interleukin-21; BCR, B cell receptor; SEM, standard error of the mean.

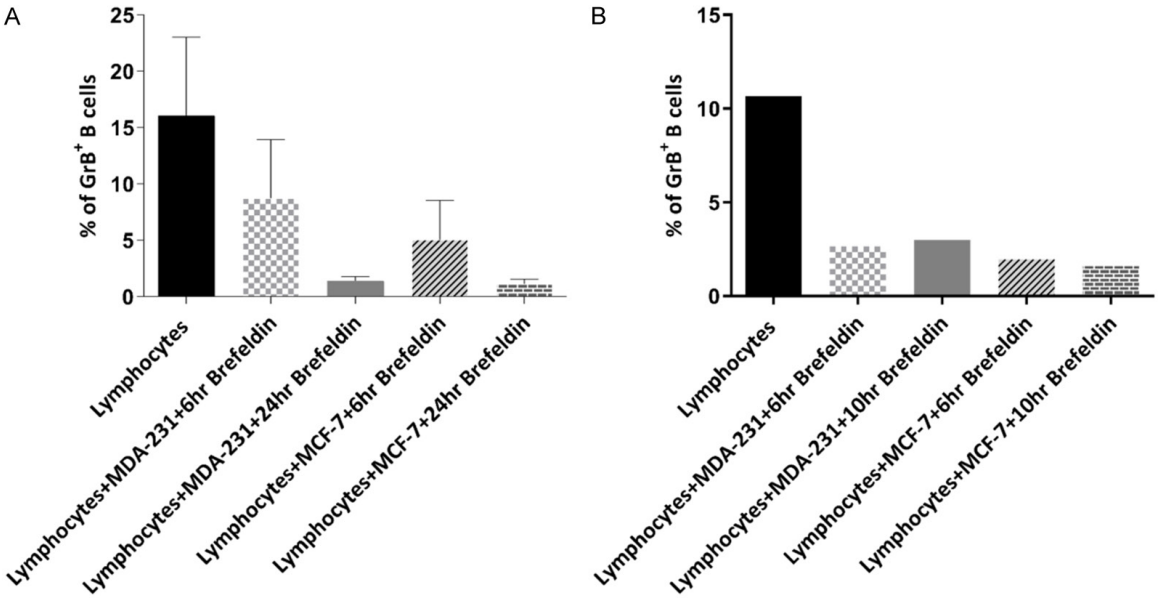

Figure 4. Effect of Brefeldin A treatment duration on GrB expression in B cells co-cultured with breast cancer cell lines. A. Comparison of the frequency of GrB^+ B cells co-cultured with MCF-7 or MDA-MB-231 breast cancer cell lines, with Brefeldin A added either for the full 24 hours or during the final 6 hours of culture. All cells were stimulated with recombinant IL-21 (rIL-21) and anti-BCR antibodies for 24 hours in the presence or absence of tumor cells. B. Comparison of GrB^+ B cell frequencies when Brefeldin A was added either during the final 6 or final 10 hours of co-culture, based on two independent experiments. Stimulation conditions were identical to part A. Data are presented as mean ± SEM. GrB, granzyme B; rIL-21, recombinant interleukin-21; BCR, B cell receptor; SEM, standard error of the mean.

were cultured with conditioned media (CM) from MCF-7 and MDA-231 cells at concentrations of 50% and 75%, in the presence of IL-21 and anti-BCR (**Figure 5A**). MCF-7 CM (both 50% and 75%) had no significant effect on GrB expression compared to control-stimulated cells (**Figure 5B**). Similarly, MDA-231 CM at 50% showed no significant suppression. However, 75% MDA-231 CM resulted in a modest, non-significant reduction in GrB^+ B cells (from 16.8±2.4% to 8.5±1.6%, P=0.082; **Figure 5C**).

Pre-activation of B cells reverses tumor-mediated suppression of GrB expression

To evaluate whether pre-activation could counteract the suppressive effects of tumor cells, B cells were stimulated with IL-21 and anti-BCR for 24 hours prior to co-culture with tumor cells. Pre-activated B cells maintained higher levels of GrB expression even in the presence of MCF-7 or MDA-231 compared to non-pre-activated B cells (**Figure 6A**). These results support the use of pre-activation to preserve B cell cytotoxic function in the tumor microenvironment.

Activated B cells exhibit cytotoxicity against MCF-7 breast cancer cells

$CD19^+$ B cells were isolated from TDLNs using FACS (purity ≥ 98%, **Figure 1**), pre-activated with IL-21 and anti-BCR for 24 hours, and co-cultured with Calcein-labeled MCF-7 cells at a 17:1 ratio for 36 hours. Activated B cells induced apoptosis in 38.0±8.9% of MCF-7 cells (P=0.023 vs. unstimulated B cells; **Figure 6B**, **6C**). B cells stimulated with either IL-21 or anti-BCR alone, or unstimulated B cells, did not show significant cytotoxicity. Control wells containing IL-21 and anti-BCR alone (no B cells) resulted in only 0.6% tumor cell apoptosis, confirming the effect was B cell-mediated.

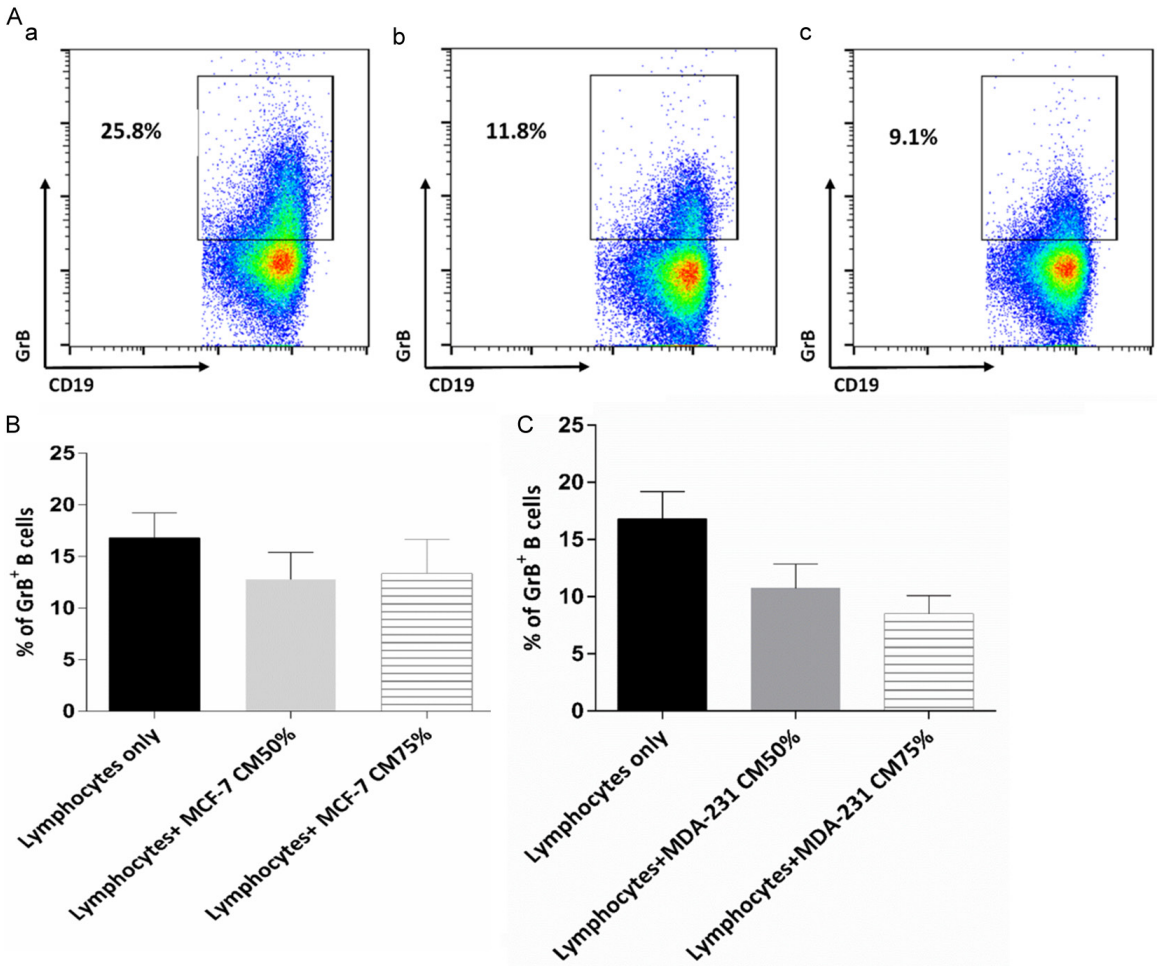

Figure 5. Effect of breast cancer cell line conditioned media on GrB expression in B cells derived from breast tumor-draining lymph nodes. A. Representative gating strategy for GrB expression in $CD19^+$ B cells cultured for 24 hours in different concentrations of MDA-MB-231 conditioned media (CM). a. Cells cultured in complete media (positive control). b. Cells cultured in 50% MDA-MB-231 CM. c. Cells cultured in 75% MDA-MB-231 CM. All conditions were stimulated with recombinant IL-21 (rIL-21) and anti-BCR. B. Comparison of the frequency of GrB^+ B cells in the presence or absence of MCF-7 conditioned media (50% and 75%). C. Comparison of the frequency of GrB^+ B cells in the presence or absence of MDA-MB-231 conditioned media (50% and 75%). Data are presented as mean ± SEM. GrB, granzyme B; rIL-21, recombinant interleukin-21; BCR, B cell receptor; SEM, standard error of the mean.

Discussion

Although immunotherapy has traditionally focused on T cells and NK cells, recent studies have highlighted the potential role of B cells in modulating antitumor responses [18]. GrB-producing B cells represent a unique cytotoxic subset capable of killing target cells independently of antibodies [12, 19]. Our findings build on prior data showing that metastatic breast cancer lymph nodes have a reduced frequency of GrB^+ B cells compared to non-metastatic nodes [16]. This study extends those observations by demonstrating that tumor cells directly inhibit GrB expression in B cells via contact-dependent mechanisms. This suppression did not occur in the presence of tumor-conditioned media, even at high concentrations, suggesting involvement of membrane-bound inhibitory signals rather than soluble factors.

B cell GrB expression requires dual stimulation through IL-21 and B cell receptor (BCR) pathways [16]. The observed inhibition could stem from interference with one or both of these signaling axes. While IL-21 receptor expression appears unaltered in metastatic versus non-metastatic LNs [20], it remains unclear whether downstream IL-21R or BCR signaling is functionally impaired. Tumor-induced expression of

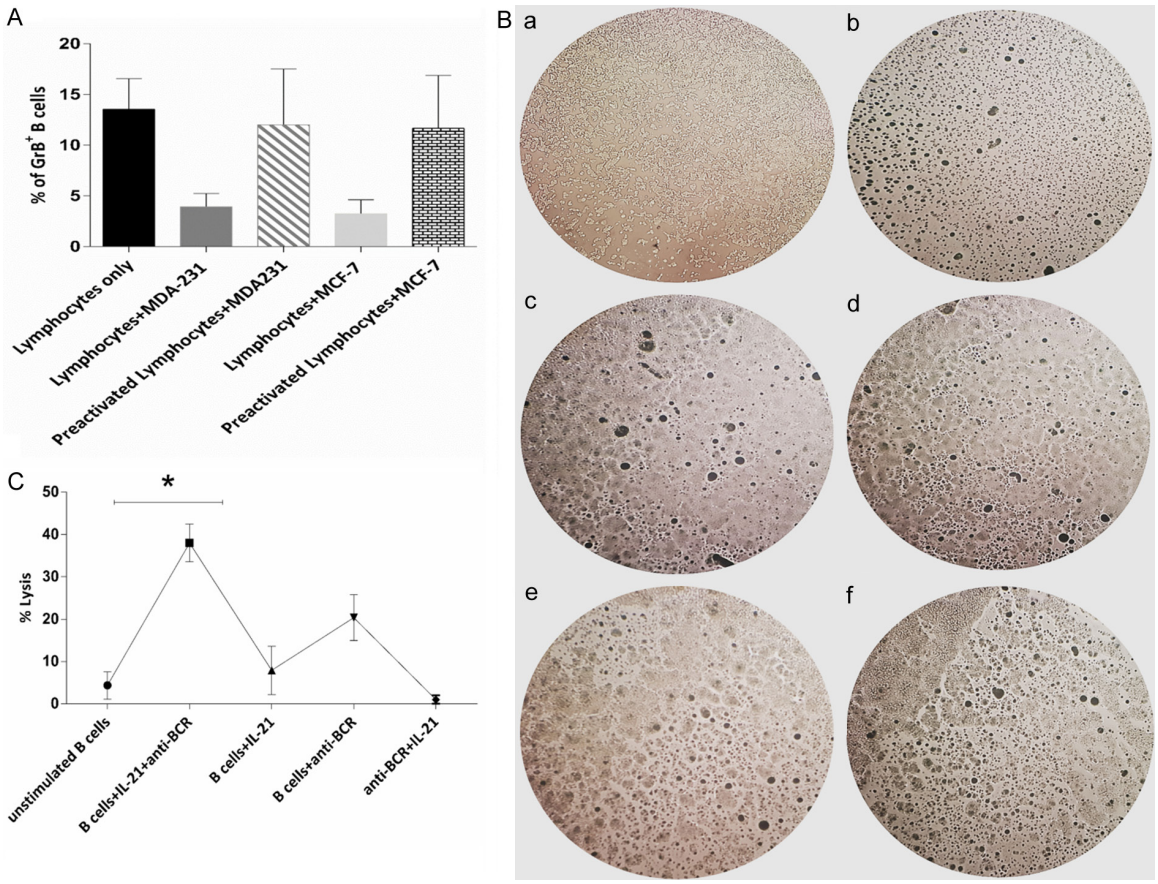

Figure 6. Effect of B cell pre-stimulation on GrB expression and cytotoxicity against MCF-7 cells. A. Comparison of GrB expression in B cells co-cultured with tumor cell lines (MCF-7 and MDA-MB-231), with or without 24-hour pre-stimulation. B cells were stimulated with recombinant IL-21 (rIL-21) and anti-BCR either during or prior to co-culture. GrB^+ B cells were quantified after 24 hours. Data are shown as mean ± SEM. B. Morphological assessment of Calcein-labeled MCF-7 cells following co-culture with B cells under different stimulation conditions. a. MCF-7 cells alone (negative control for spontaneous release). b. MCF-7 cells treated with Triton X-100 (positive control for maximum release). c. MCF-7 cells co-cultured with unstimulated B cells. d. MCF-7 cells co-cultured with B cells stimulated with rIL-21. e. MCF-7 cells co-cultured with B cells stimulated with anti-BCR. f. MCF-7 cells co-cultured with B cells stimulated with both rIL-21 and anti-BCR. C. Quantification of apoptosis in MCF-7 cells following co-culture with stimulated or unstimulated B cells. B cells were isolated from breast tumor-draining lymph nodes, stimulated for 24 hours, and then co-cultured with Calcein-labeled MCF-7 cells. Unstimulated B cells served as control. Data are presented as mean ± SEM. *P < 0.05. GrB, granzyme B; rIL-21, recombinant interleukin-21; BCR, B cell receptor; SEM, standard error of the mean.

inhibitory B cell receptors such as CD22 or FcRL4 may also contribute to this phenomenon. These mechanisms warrant further investigation. Additionally, the use of Brefeldin A to block protein secretion confirmed that decreased GrB levels were due to reduced production, not enhanced release, supporting a true suppressive effect on B cell function.

Interestingly, although MDA-MB-231 conditioned media at 75% concentration showed a slight non-significant reduction in GrB production, MCF-7 media had no effect, whereas direct co-culture with both cell lines led to suppression. These differences underscore the importance of tumor subtype in shaping immune interactions, possibly reflecting variations in surface ligands or tumor immunogenicity.

A key finding was that pre-activating B cells with IL-21 and anti-BCR for 24 hours prior to tumor exposure preserved GrB expression and overcame tumor-induced suppression. These results suggest that once B cells commit to a cytotoxic program, they become less susceptible to tumor interference, or that tumors require

extended time to exert their inhibitory effects. Activated B cells exhibited significant cytotoxicity against MCF-7 cells, while unstimulated or partially stimulated B cells (anti-BCR or IL-21 alone) did not. These data strongly suggest that GrB plays a functional role in tumor cell killing. While our study did not directly visualize GrB transfer into tumor cells, prior work has shown that IL-21-activated B cells can induce apoptosis in HeLa cells via GrB-dependent mechanisms [12].

Several limitations should be acknowledged. First, the sample size was limited, particularly for cytotoxicity assays. Second, all experiments were conducted in vitro; in vivo confirmation using animal models is essential to validate the therapeutic relevance of GrB^+ B cells. Third, GrB was the only cytotoxic molecule evaluated; future studies should also examine perforin, FasL, and other effector molecules. Lastly, the precise tumor-derived ligands mediating B cell suppression were not identified.

Future research should aim to define the tumor ligands and B cell receptors involved in this suppressive interaction. In vivo models could test the therapeutic efficacy of adoptively transferred GrB^+ B cells, particularly in combination with checkpoint inhibitors. Further, characterizing GrB^+ B cells in different breast cancer subtypes and disease stages may offer insights into their prognostic and therapeutic potential.

Conclusion

This study reveals that breast cancer cells can suppress the cytotoxic potential of B cells via direct contact, impairing GrB production. However, B cell pre-activation with IL-21 and anti-BCR restores this function and enables effective tumor cell killing. These findings support the emerging concept that cytotoxic B cells, particularly those primed for GrB expression, may be harnessed as novel effectors in breast cancer immunotherapy.

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Disclosure of conflict of interest

None.

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