Bitter Melon Enhances Natural Killer–Mediated Toxicity against Head and Neck Cancer Cells

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Abstract

Natural killer (NK) cells are one of the major components of innate immunity, with the ability to mediate antitumor activity. Understanding the role of NK-cell–mediated tumor killing in controlling of solid tumor growth is still in the developmental stage. We have shown recently that bitter melon extract (BME) modulates the regulatory T cell (Treg) population in head and neck squamous cell carcinoma (HNSCC). However, the role of BME in NK-cell modulation against HNSCC remains unknown. In this study, we investigated whether BME can enhance the NK-cell killing activity against HNSCC cells. Our results indi-cated that treatment of human NK-cell line (NK3.3) with BME enhances ability to kill HNSCC cells. BME increases granzyme B accumulation and translocation/accumulation of CD107a/LAMP1 in NK3.3 cells exposed to BME. Furthermore, an increase in cell surface expression of CD16 and NKp30 in BME-treated NK3.3 cells was observed when cocultured with HNSCC cells. Collectively, our results demonstrated for the first time that BME augments NK-cell–mediated HNSCC killing activity, implicating an immunomodulatory role of BME. *Cancer Prev Res; 10(6); 337–44. ©2017 AACR.*

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and one of the leading causes of cancer-related mortality worldwide. Improvement has been made with respect to chemotherapeutic drugs, radiation, and surgical tech-niques for HNSCC. However, recurrence of the disease and drug resistance still causes a significant amount of death. Thus, there is a great need for additional effective therapies to increase cure rates and reduce morbidity.

Natural killer (NK) cells are cytotoxic lymphoid cells of the innate immune system. Unlike T cells, these cells are able to respond quickly to "invaders" without a "priming" period (1). They recognize target cells with low cell surface expression of MHC class I (MHC-I). Cancer cells having characteristics of reduced MHC-I expression thus can be recognized by NK cells, which consequently release cytokines and cytolytic gran-ules containing perforin and granzyme B to kill tumor cells. Because of the variability in the expression of MHC-I on the surface of different tumor cells, NK-cell responses vary (2, 3). NK-cell–based therapies include different strategies such as increasing NK-cell lytic function or enhancing their homing to tumor sites, cytokine-mediated activation of endogenous NK cells, antibody-dependent cell-mediated cytotoxicity (ADCC)-promoting therapeutic antibodies, NK-cell infusions, anti-KIR antibody, bispecific antibody, or combined therapies (4–7).

Preclinical and clinical studies suggest that NK-cell–mediated immunotherapy would be efficient in treating minimal resid-ual disease (8).

Natural products play a leading role in the discovery and the development of various drugs for the treatment of human diseases including cancer. Bitter melon (*Momordica charantia*) is extensively cultivated in Asia, Africa, and South America, and is widely used in folk medicines to treat diabetes (9). In our previous studies, we demonstrated that BME is effective in killing various types of solid tumors including head and neck cancer (10–13). We found that BME inhibits HNSCC cell proliferation through modulation of c-Met signaling in *in vitro* as well as *in vivo* using a xenograft model (12). We recently observed that BME treatment reduces the regulatory T cell (Treg) activity in a HNSCC syngeneic mouse model (14). NK cells display rapid and potent killing of hematologic cancers (15). However, the effect of BME on NK-cell cytotoxicity remains unknown in solid tumors including HNSCC. In this study, we demonstrated for the first time that pretreatment of NK cells with BME enhances their killing activity against HNSCC cells. We also observed that BME-mediated increase in NK-cell killing activity is associated with translocation of CD107a/LAMP1, increased accumulation of granzyme B, and increase of CD16 (FcγRIIIa) and NKp30 cell surface expression.

Materials and Methods

BME preparation

BME was prepared from the Chinese variety of young bitter melons (raw and green) as discussed previously (12, 14). Briefly, BME was extracted using a household juicer and centrifuged at $560 \times g$ at 4°C for 30 minutes, freeze dried at −45°C for 72 hours, and stored at −80°C. We next prepared BME by suspending 1 gm of freeze-dried powder in 10 mL of water, mixed overnight, and separated the aqueous portion by centrifugation for 30 minutes. BME was aliquoted and stored at −80°C. We generally prepare a

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big batch and tested each batch for cytotoxicity using 3–4 previously tested cancer cell lines.

Cell lines and cytotoxicity assay

We used two HNSCC cell lines in this study. Cal27 cell line (tongue origin) was purchased from ATCC and was maintained in DMEM (Sigma) supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma). JHU-29 (tongue) cell line was procured from the Johns Hopkins University, and was maintained in RPMI1640 medium (Sigma) supplemented with 10% FBS and 1% penicillin/streptomycin. The human NK-cell line (NK3.3) was cultured in RPMI1640 medium supplemented with 10% FBS, 1% glutamine, 1% penicillin–streptomycin, and 200 IU/mL recombinant IL2 (rIL2; R & D Systems) (16). We added IL-2 overnight to the NK 3.3 cells, then removed residual IL2 by washing, exposed with BME (1% v/v) for additional 20 hours before incubating with cancer cells. HNSCC cells were cocultured with BME-treated NK3.3 cells at different tumor cell/target: effector cell (T:E; 1:10) ratios for 24 hours. Cytotoxicity was measured by using a multiTox-fluor multiplex cytotoxicity assay kit (Promega) following the manufacturer's protocol, and readings were taken using a Bio-Tek plate reader.

Western blot analysis

Cell lysates were analyzed by SDS-PAGE and transferred onto 0.45 μm nitrocellulose membrane (Bio-Rad). Membranes were blocked using 5% low fat dry milk and probed with the specific antibodies. Proteins were detected using ECL Western blotting substrate (Thermo Scientific) and autoradiography. The protein loading was normalized using antibody to β-actin. The following antibodies were used in this study: granzyme B, pSTAT3, STAT3, and LAMP1 (Cell Signaling Technology) and β-actin (Santa Cruz Biotechnology).

Flow cytometry

NK cells were treated with 2% BME or left untreated as a control for 16 hours, washed extensively, and then cocultured with adherent HNSCC cells for another 24 hours. NK3.3 cells were separated from the HNSCC cells, washed with buffer (0.5% BSA in $1\times$ phosphate buffer, pH 7.4) and stained with anti-CD45 (FITC), anti-CD56 (APCA700), anti-CD107a (PE), anti-CD16 (ECD), anti-NKp30 (PE), anti-CD314/NKG2D (APC), anti-CD161 (A750), anti-CD158e (BV421), or anti-NKp46 (PECy7) antibody for surface expression. Brilliant stain buffer (BD Biosciences) was used for the dilution of the stains. Next, cells were washed with staining buffer, fixed with 4% formalhyde, and analyzed using an LSRII flow cytometer (BD Biosciences). Data were evaluated using FlowJo software. The antibodies were purchased from Beckman Coulter, Miltenyi Biotec, or Biolegend.

Statistical analysis

Results were expressed as the mean $\pm$ SD, and statistical analyses were performed using two-tailed paired or unpaired Student t test in GraphPad Prism 6 (GraphPad). A P value of <0.05 was considered statistically significant.

Results

BME enhances NK-cell-mediated cytotoxicity

We initially examined whether BME has an effect on NK3.3 cell growth. For this, NK cells were exposed to BME for 24 hours and cell viability were assessed. We did not observe an effect of BME treatment on NK-cell growth or viability (Fig. 1A). We next examined whether BME treatment of NK3.3 cells enhanced tumor cell killing activity. For this, control or BME-treated NK3.3 cells were cocultured with HNSCC (Cal27 or JHU-29) cells at different T:E ratios for 24 hours. Our results suggested that BME-treated NK cells showed enhanced cytotoxicity as compared with untreated NK cells (Fig. 1B and C). BME treatment alone, without prior IL2 stimulation of NK cells, does not significantly induce NK-cell activity.

BME treatment enhances NK-cell cytotoxicity by increasing granzyme B expression

NK cells, upon encounter with potential target cells, form an immunologic synapse. This event provides the activation signals to NK cells to augment granzyme B expression and release (17). One of the defining properties of NK cells is the expression and regulated secretion of granzyme B. To gain further insights into the effects of BME on NK-cell–mediated cytotoxicity, we examined the effect of BME on the expression of granzyme B. We observed higher granzyme B expression in BME-treated NK3.3 cells as compared with untreated cells. BME-treated NK3.3 cells, when cocultured with HNSCC tumor cells, also displayed a significantly higher expression of granzyme B as compared with untreated NK3.3 cells (Fig. 2A). We did not observe a significant upregulation of perforin in BME-treated NK cells when exposed to HNSCC cells.

Recent studies suggested that STAT3 activation impairs tumor immune surveillance and allows the tumor to escape immune control (18). STAT3 activation in NK cells indeed suppressed cytotoxicity in mouse model (19). We therefore examined the status of activated STAT3. Our results demonstrated that phospho-STAT3 activation is significantly lower in BME-treated NK3.3 cells as compared with control NK3.3 cells when cocultured with Cal27 cells (Fig. 2B). We also observed inhibition of phospho-STAT3 in BME treated NK3.3 cells alone. The modulation of STAT3 has been shown to inversely correlate with expression of granzyme B (19). Together, our results suggested that tumor–NK-cell interaction is a prerequisite for BME treatment to maximally enhance granzyme B expression.

BME treatment of NK cells enhances CD107a/LAMP1 surface expression and accumulation

NK-cell cytotoxicity is a multistep complex process that involves adhesion to target cells, synapse formation, and signal transduction leading to granule polarization and exocytosis. CD107a/LAMP1 participates in the degranulation of NK-cell lytic granules, mobilizing perforin and granzyme B toward the immunologic synapse. CD107a/LAMP1 also plays a crucial role in the protection from degranulation-associated suicide of NK cells (20). Therefore, it is likely that BME might affect the degranulation process by modulating the surface expression of CD107a on NK cells. Our results demonstrated that BME-treated NK3.3 cells, when cocultured with HNSCC cells, display higher surface CD107a expression than control NK3.3 cells (Fig. 3A). In addition, we examined the expression of total CD107a/LAMP1 by Western blot analysis. Our data indicates an accumulation of CD107a/LAMP1 in BME-treated NK3.3 cells cocultured with Cal27 or JHU-29 cells (Fig. 3B). Interestingly, NK3.3 cells treated only with BME displayed a similar level of CD107a/LAMP1 expression to that of untreated NK3.3 cells.
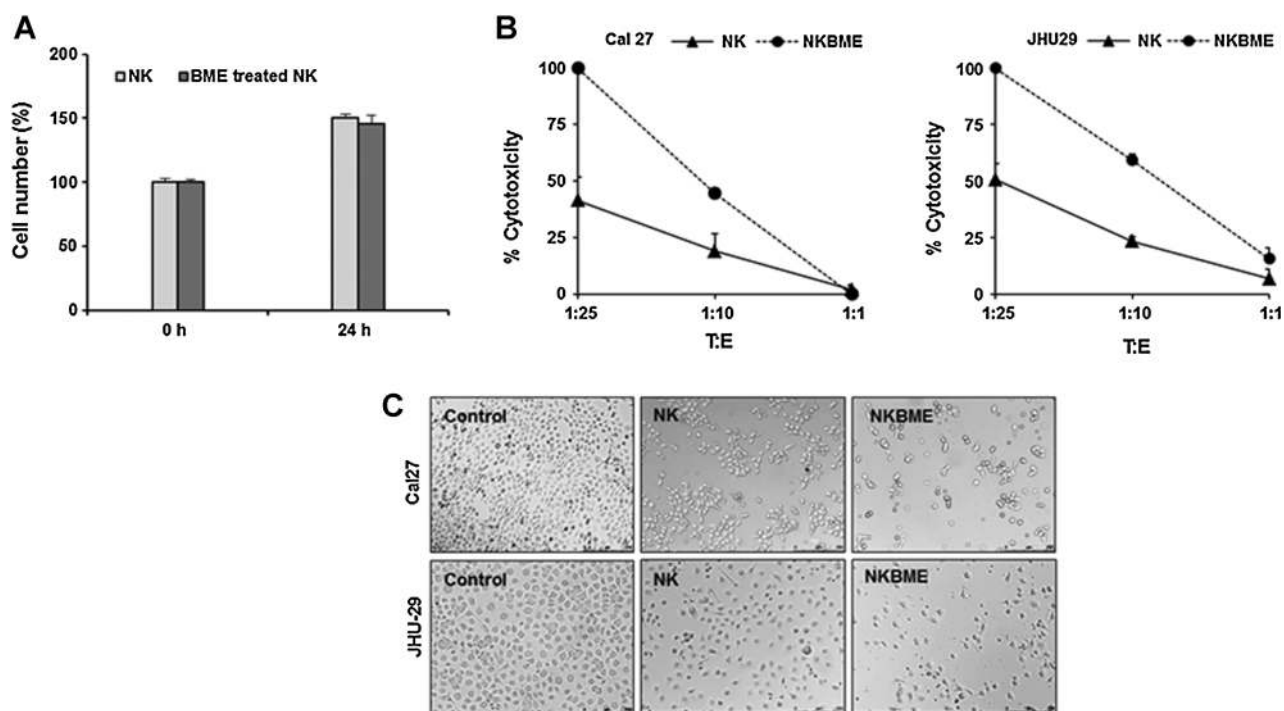
Figure 1.
BME treatment increases cytotoxic activity of NK3.3 cells on HNSCC cells. **A,** NK3.3 cells were treated with BME for 24 hours. Cell number and viability was measured by Trypan blue dye exclusion method. Results shown are an average of three independent experiments. Cell viability at 0-hour time point was arbitrarily set to 100%. **B,** Cal27 or JHU-29 cells were cocultured with different T:E ratios of control or BME-treated NK cells, and cytotoxicity was measured. Data are represented as mean ± SD. *, $P < 0.05$. **C,** Representative microscopic images (10×) showing untreated HNSCC cells (control), HNSCC cells exposed to untreated NK (NK) or BME-treated NK cells (NKBME) with T:E ratio 1:10. Photographs were taken after 24 hours of coculturing BME-treated NK cells with target cells.

BME treatment of NK cells enhances CD16 and NKp30 expression

CD16, a member of the IgG superfamily, is expressed on a subset of NK cells. It is involved in ADCC and in mediating direct NK-cell cytotoxicity (21). Engagement of CD16 by its ligand, the Fc region of IgG, triggers degranulation of lytic granules from NK cells (22). We observed an increase in CD16 surface expression in BME-treated NK3.3 cells cocultured with HNSCC cells (Fig. 4A). CD16 signaling is mediated by transmembrane adaptor proteins that possess immunoreceptor tyrosine–based activation motifs (ITAM). The natural cytotoxicity receptor (NCR) NKp30 is also associated with ITAM-containing adaptor proteins (22). We observed an increase in NKp30 surface expression in BME-treated NK3.3 cells cocultured with tumor cells as compared with untreated NK cells (Fig. 4B). We did not find modulation of other NK receptors (NKG2D, CD161, or NKp46) on BME-treated NK3.3 cells when cocultured with HNSCC cells (Fig. 4C).

Discussion

BME displayed anticancer activities in various cancer models (10–13). In addition to its direct inhibitory effect on tumor growth and survival, BME also has demonstrated immunomodulatory activity in a syngeneic mouse model of HNSCC by inhibiting Treg activity (14). However, the effect of BME on NK-cell activation and subsequently killing of HNSCC cells was unknown. The tumor microenvironment is not only a passive recipient of immune cells but an active contributor to the establishment of immunosuppressive conditions (23). In the tumor microenvironment, NK cells display a modified phenotype with reduced cytotoxic activity as well as reduced immune surveillance. A direct correlation between high intratumoral levels of NK cells and increased survival has been shown in several types of cancers (24). Studies suggested that various chemotherapeutic drugs augment IL2-activated NK-cell lysis of tumor cells (25).

In this study, we demonstrated that BME treatment augments NK-cell–mediated tumor cell toxicity to induce tumor cell death via activation of NK-cell receptor–dependent pathways. The mechanistic study suggested that BME-treated NK cells, when cocultured with HNSCC cells, accumulate and translocate CD107a/LAMP1 to the cell surface. We further observed upregulation of CD16 and NKp30 receptors on BME-treated NK cells when cocultured with HNSCC cells.

NK cells contain various proteins including granzyme and perforin, which are secreted from NK cells upon contact with target cells, and ultimately cause death of target cells. CD107a/LAMP-1 is present in the membranes of cytolytic granules. This protein is also expressed on the surface of NK cells upon degranulation and is considered a discrete marker for the
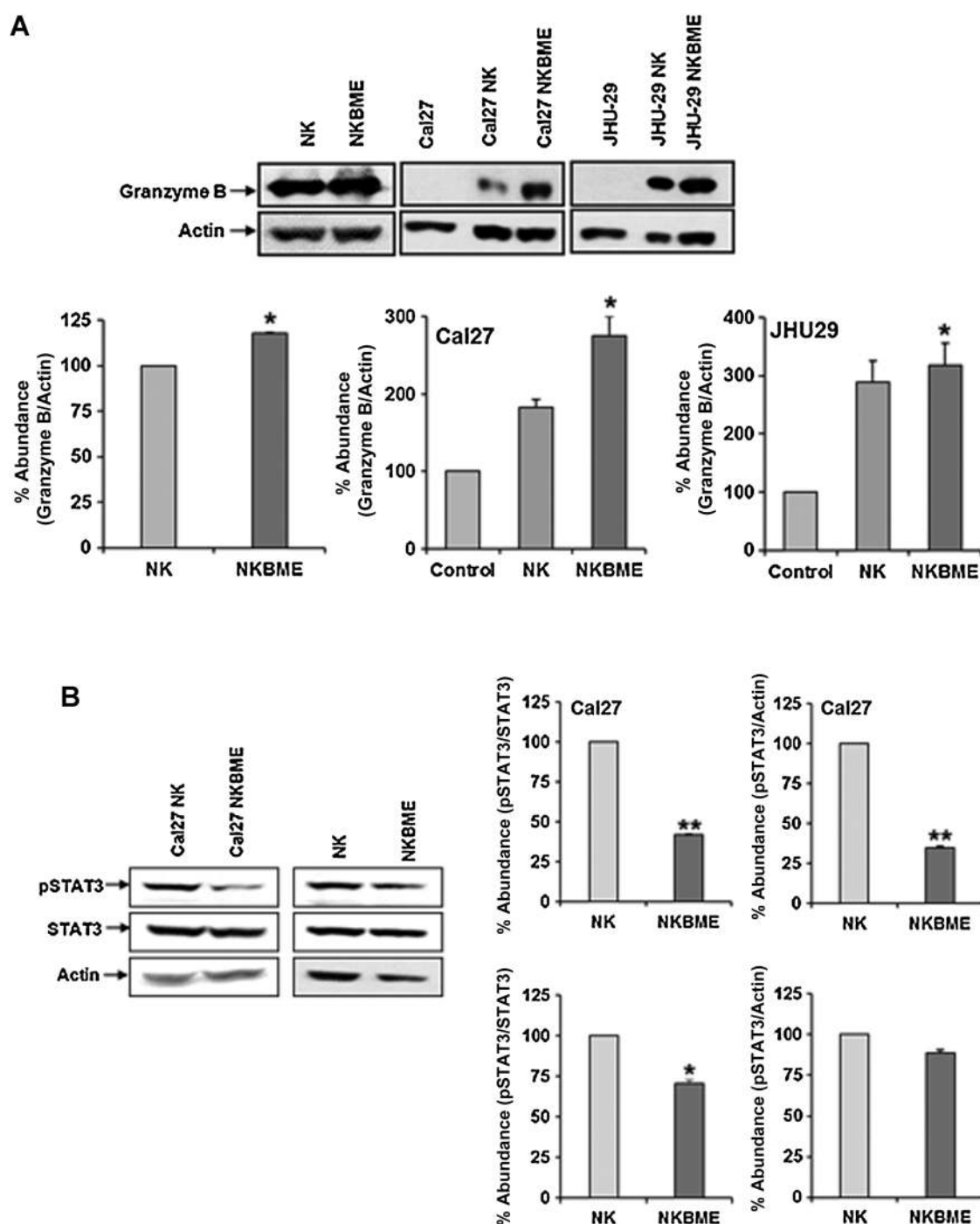
Figure 2.
BME treatment increases granzyme B expression in NK 3.3 cells. **A,** Western blot analysis of granzyme B expression in control or BME-treated NK3.3 cells when cocultured with Cal27 and JHU-29 cells. **B,** Phospho-STAT3/STAT3 expression in control or BME-treated NK3.3 cells cocultured with Cal27 was examined by Western blot using specific antibody. Similarly, lysates from NK cell were untreated or BME-treated were analyzed for phospho-STAT3/STAT3 expression. Blots were reprobed with an antibody to actin for comparison of protein loading in each lane. Densitometric analyses of was performed using ImageJ software. Data are represented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$.

NK-cell–mediated killing of target cells (26). CD107a plays a vital role in the survival of NK cells against degranulation mediated self-destruction (20). Silencing of CD107a/LAMP1

inhibits cytotoxic activity of NK cells (27). We observed the enhanced expression of CD107a on the surface of NK cells when they were exposed to BME and cocultured with HNSCC
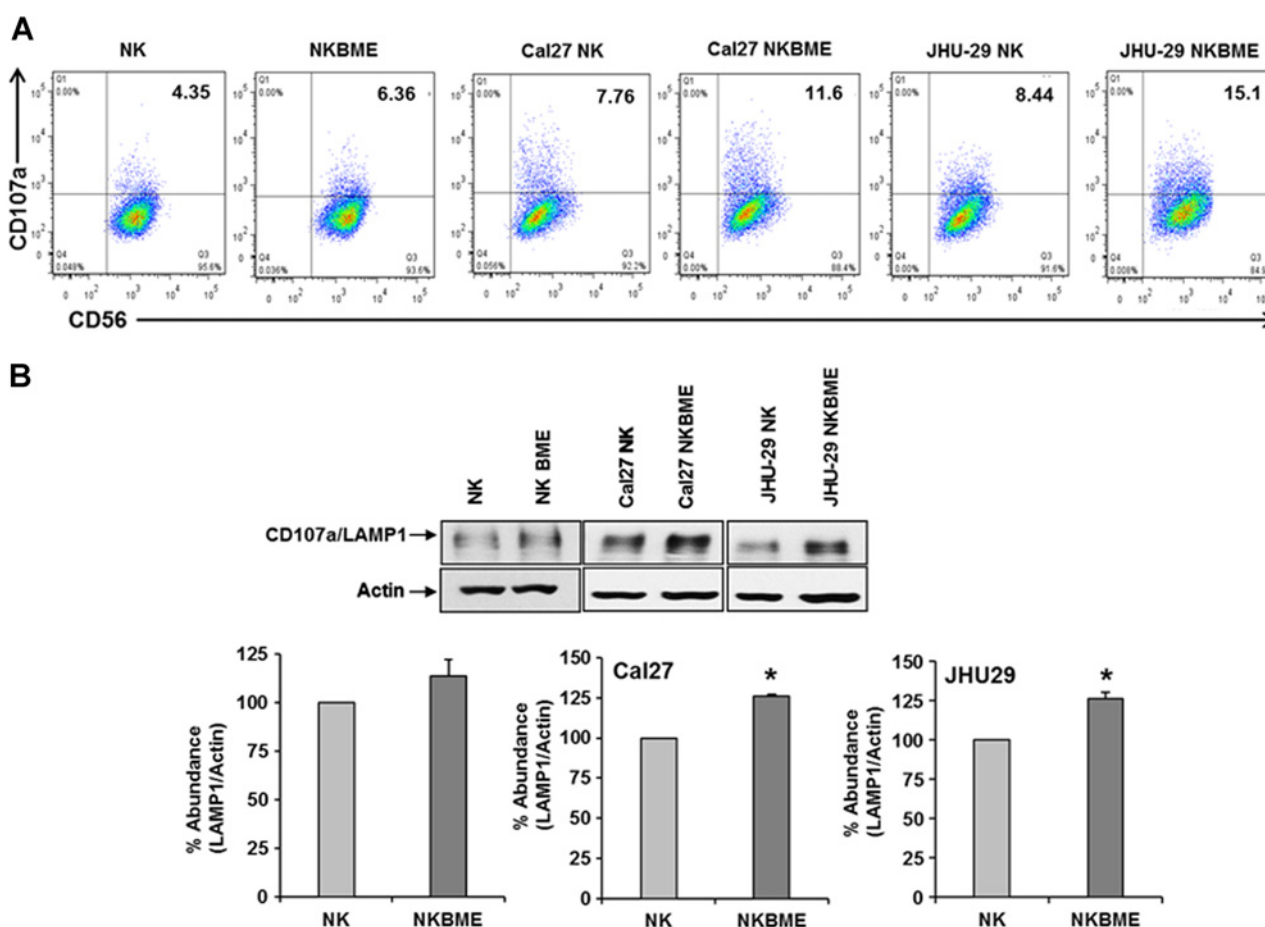
Figure 3.
BME treatment increases CD107a/LAMP1 expression on NK 3.3 cells. **A,** FACS analysis of the cell surface expression of CD107a/LAMP-1 on NK cells. Dot plot analysis of the surface expression of CD107a on CD56-positive NK3.3 cells and BME exposed NK3.3 cells with or without coculture with HNSCC cells. **B,** Western blot data showing CD107a/LAMP1 expression in NK3.3 cells. Blots were reprobed with an antibody to actin for comparison of protein loading in each lane. Densitometric analyses were performed using ImageJ software. Data are represented as mean ± SD. *, $P < 0.05$.

cells. This phenomenon is closely related to the effect of BME on degranulation and the expression of granzyme B on BME-treated NK cells. Furthermore, STAT3 activation was inversely correlated with granzyme B expression. It is likely reflecting the ability of BME to induce the killing of HNSCC cells through the enhancement of NK cytolytic activity. The function of NK cells is dependent on the balance between engagement of both activation and inhibitory receptors with the target cell. CD16 is a member of IgG superfamily, involved in ADCC and also mediating direct NK-cell cytotoxicity (21). We observed that BME treatment increases CD16 expression on NK cells when cocultured with HNSCC cells. In addition, we observed that treatment with BME also increases the expression of NKp30, a natural cytotoxic receptor. NKp30 plays an important role in mediating tumor immunosurveillance in several clinical settings. One of its ligands, B7H6, is expressed on many types of tumor cells but absent on normal tissues. Interaction of NKp30 with B7H6 has been shown to enhance degranulation of NK cells (28). Other activation receptors on NK cells, including NKG2D and NKp46, did not indicate a modulation after BME treatment.

On the basis of our *in vitro* data, we examined granzyme B and CD107a/LAMP1 expression in tumor tissues from HNSCC syngeneic mouse as described recently (14). We performed Western blot analysis and qRT-PCR to examine the status of granzyme B in tumor following BME treatment. In addition, we examined LAMP1 expression by Western blots. Interestingly, we did not observe a significant modulation of granzyme B or CD107a/LAMP1 expression in tumors isolated from BME-treated mice as compared with the control group in the HNSCC syngeneic mouse model (data not shown). The role of NK cells in antitumor responses *in vivo* may be difficult to interpret due to the difference between human and mouse NK cells (29). Human NK cells can be subclassified based upon the level of expression of CD56. CD56 dim–positive subsets of NK cells present in the circulation have high level of cytotoxicity while CD56 bright–positive cells have less cytotoxicity. These subsets of NK cells are not present in mice. BME treatment in a HNSCC syngeneic mouse model does not appear to alter NK-cell activity. One possible reason for these results might be the difference in expression of NKp30 by mouse and human NK cells. NKp30 expression is increased in human NK3.3 cells after
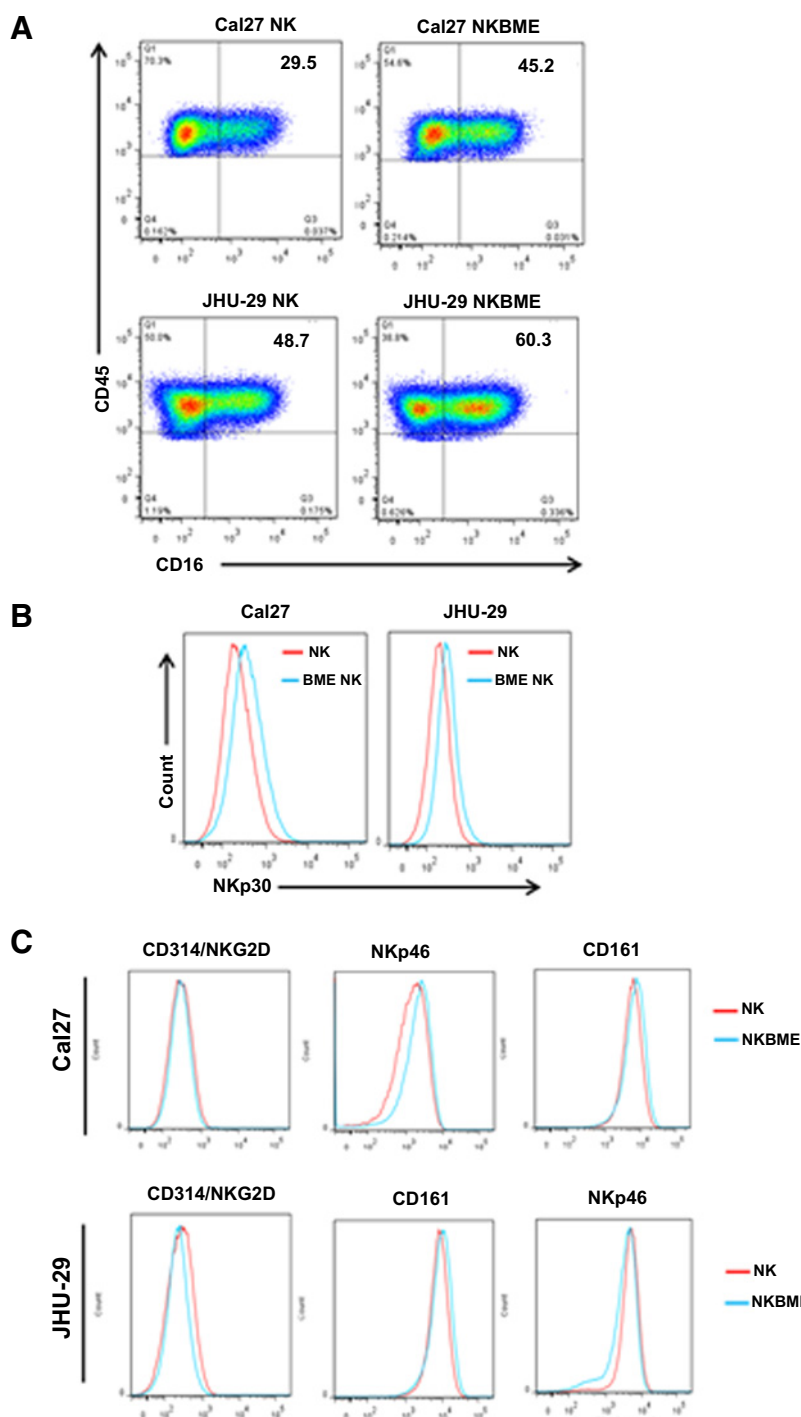
Figure 4.
BME treatment increases CD16 and NKp30 surface expression on NK3.3 cells. **A,** FACS analysis showing the populations of CD16$^+$ NK3.3 cells exposed with BME or left untreated when cocultured with Cal27 or JHU-29 cells. **B,** FACS analysis showing expression of NKp30 on NK3.3 and BME-treated NK3.3 cells cocultured with Cal27 and JHU-29 cells. Data are represented as mean $\pm$ SD. *, $P < 0.05$. **C,** Overlay of histograms showing expression of CD314/NKG2D, NKp46, and CD161 by FACS analysis on NK3.3 cells exposed with BME as described above.

BME treatment. NKp30 (NCR3 or 1C7), a natural killer (NK)-cell activation receptor is a functional gene in human (30) but a pseudogene in mouse (31). Further work is necessary to tease out the in-depth mechanism for BME-mediated NK-cell killing against HNSCC. Together our data demonstrated that BME modulates human NK-cell cytotoxic activity against HNSCC by modulating LAMP1, Granzyme BCD16, and NKP30 expression. Our findings also suggested that besides direct antitumor action, enhancement of effector functions of the immune system may also contribute to the therapeutic effects of BME in HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R.B. Ray
Development of methodology: S. Bhattacharya, N. Muhammd, R. Steele, R.B. Ray

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