Cancer Therapy: Preclinical

Clinical Cancer Research

TLR2 Agonist PSK Activates Human NK Cells and Enhances the Antitumor Effect of HER2-Targeted Monoclonal Antibody Therapy

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Abstract

Purpose: The therapeutic effect of trastuzumab monoclonal antibody (mAb) therapy has been shown to be partially dependent on functional natural killer (NK) cells. Novel agents that enhance NK cell function could potentially improve the antitumor effect of trastuzumab. We recently identified polysaccharide krestin (PSK), a natural product extracted from medicinal mushroom *Trametes versicolor*, as a potent toll-like receptor 2 (TLR2) agonist. This study was undertaken to evaluate the effect of PSK on human NK cells and the potential of using PSK to enhance HER2-targeted mAb therapy.

Experimental Design: Human peripheral blood mononuclear cells were stimulated with PSK to evaluate the effect of PSK on NK cell activation, IFN-γ production, cytotoxicity, and trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). Whether the effect of PSK on NK cells is direct or indirect was also investigated. Then, *in vivo* experiment in neu transgenic (neu-T) mice was carried out to determine the potential of using PSK to augment the antitumor effect of HER2-targeted mAb therapy.

Results: PSK activated human NK cells to produce IFN-γ and to lyse K562 target cells. PSK also enhanced trastuzumab-mediated ADCC against SKBR3 and MDA-MB-231 breast cancer cells. Both direct and interleukin-12–dependent indirect effects seem to be involved in the effect of PSK on NK cells. Oral administration of PSK significantly potentiated the antitumor effect of anti-HER2/neu mAb therapy in neu-T mice.

Conclusion: These results showed that PSK activates human NK cells and potentiates trastuzumab-mediated ADCC. Concurrent treatment with PSK and trastuzumab may be a novel way to augment the antitumor effect of trastuzumab. *Clin Cancer Res; 17(21); 6742–53. ©2011 AACR.*

Introduction

Trastuzumab is a humanized anti-HER2 monoclonal antibody (mAb) and is the first HER2-targeted therapy approved by the Food and Drug Administration. Trastuzumab has significantly advanced the clinical management of patients with $HER2^+$ breast cancer by prolonging disease-free survival and overall survival in patients with early-stage breast cancer, and progression-free survival and overall survival in patients with metastatic breast cancer (1, 2). Trastuzumab inhibits tumor cell growth through multiple mechanisms including signaling blockade and downregulating the HER2/neu receptor. One of the major mechanisms is believed to be antibody-dependent cell-mediated cytotoxicity (ADCC), in which the tumor cells are coated with trastuzumab and then lysed by immune cells via binding of Fc gamma receptor (FcγR) to the Fc portion of the mAb (3). Increase in tumor-infiltrating natural killer (NK) cells after trastuzumab therapy has been found in human breast cancer biopsy samples (4, 5), and *FcγR* gene polymorphism can impact the clinical response to trastuzumab (6). NK cells constitutively express FcγRIIIA (CD16) and are the major effectors of ADCC (7). Therefore, the function of NK cells may impact the efficacy of ADCC and clinical response to trastuzumab (8). Unfortunately, NK cell function is frequently impaired in patients with cancer as compared with healthy donors (9–11), and lytic function of NK cells in patients with advanced disease (stages II, III, and IV) is even lower than in those with limited disease (stage I; ref. 12). Therefore, novel approaches that can enhance NK cell function and improve ADCC would potentially benefit many patients with cancer receiving mAb therapy.

The activation of NK cells is determined by the coordination of inhibitory and activating receptors on the surface

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Translational Relevance

Trastuzumab has become the standard of care for patients with HER2$^+$ breast cancer. Although the mechanism of the antitumor actions of trastuzumab is multifaceted, antibody-dependent cell-mediated cytotoxicity (ADCC) is believed to be one of the major mechanisms. The clinical response to trastuzumab therapy has been shown to be partially associated with the function of natural killer (NK) cells, which are the main mediators of ADCC. Immune response modifiers that can enhance NK cell function and ADCC could potentially improve the clinical response to trastuzumab. In this study, we showed that polysaccharide krestin (PSK), a mushroom extract and a toll-like receptor 2 (TLR2) agonist, can activate human NK cells to secrete IFN-γ and exert enhanced cytolytic activity in lysing K562 target cells and trastuzumab-coated breast cancer cells. Using a mouse model of HER2$^+$ breast cancer, we further showed that concurrent administration of PSK can augment the antitumor effect of anti-HER2/neu mAb therapy. These findings suggest the potential of using PSK, a natural product with potent TLR2 agonist activity, as an adjuvant therapy for patients with breast cancer to improve the therapeutic effect of trastuzumab.

of NK cells. The inhibitory receptors include killer Ig-like receptor and CD94 (NKG2A/B), which prevent NK cell activation upon encounter of normal MHC class I. The activating receptors include CD16 that is involved in ADCC, NKG2D that recognizes stress-induced ligand MICA/B and UL16 binding proteins (ULBP) on malignant cells, and natural cytotoxicity receptors (NKp30, NKp44, and NKp46) whose ligands remain unclear (13). Two subsets of NK cells have been identified in humans according to their phenotype (CD56 expression) and function (regulatory vs. effector cells; ref. 14). CD56^{bright}CD16$^{-/low}$ NK cells, which account for approximately 10% of NK cells in peripheral blood, are the major producer of IFN-γ. CD56^{dim}CD16$^+$ NK cells, which account for approximately 90% of NK cells, are cytotoxic effector cells in mediating ADCC (14). Both types of NK cells have been found to express toll-like receptors (TLR; ref. 15). TLR agonists, especially the agonists of TLR3 [copolymer of polyinosinic and polycytidylic acids, poly(I):poly(C)], TLR7/8 (imiquimod, resiquimod, and 3M-002), and TLR9 (CpG), have been shown to activate NK cells either directly or indirectly via stimulation of accessory cells (15–19). A phase II clinical trial that combines rituximab and 1018 ISS (CpG) has been conducted in patients with relapsed or refractory follicular lymphoma, and the results showed that biologically relevant increase in ADCC was observed in 35% of patients (20), showing the potential of using TLR agonists to improve mAb therapy.

The effect of TLR2 agonist on NK cell function and ADCC is relatively less well known, although there is some evidence that NK cells can be activated via TLR2 either directly or indirectly via dendritic cells (DC; refs. 21–23). A recent publication by Moreno and colleagues compared the effects of TLR-2, 3, 4, 5, 8, and 9 agonists on NK cells and ADCC and found that only TLR2 agonist peptidoglycan, TLR8 agonist CL075, and TLR9 agonist CpG oligonucleotide (ODN) A showed enhanced anti-MUC1 mAb-mediated ADCC (24), indicating that TLR2 ligation could be as potent as TLR8 or TLR9 ligation in augmenting NK cell function. We recently identified polysaccharide krestin (PSK), a mushroom extract from *Trametes versicolor*, as a selective and potent TLR2 agonist and revealed the potential of using a natural product to enhance NK cell function (25).

The major component of PSK is protein-bound polysaccharide with an approximate molecular weight of 90 to 100 kDa. PSK was approved as a prescription drug for the treatment of cancer in Japan in 1977 (26). Clinical trials in Japan have shown that oral intake of PSK significantly extended survival at 5 years or beyond in patients with different types of cancer, especially stomach and colorectal cancer (27–29). Using HEK293 cells transfected with different TLRs, we showed that PSK is a selective and potent TLR2 agonist (25). We further showed that the antitumor effect of PSK in a mouse model of breast cancer is dependent on both CD8 T cells and NK cells (25). Expanding from our previous findings in mice, the current study was undertaken to investigate the effect of PSK on human NK cells and trastuzumab-mediated ADCC and the potential of using this natural product with TLR2 agonist activity to augment the antitumor effect of trastuzumab.

Materials and Methods

Animals

A colony of neu transgenic (neu-T) mice [strain name, FVB/N-TgN (MMTVneu)-202Mul] was established in our animal facilities from breeding pairs obtained from the Jackson Laboratory and maintained as previously described (30). Mice were maintained under strict inbreeding conditions. All of the procedures were conducted in compliance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Human peripheral blood mononuclear cells and cell lines

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood or leukapheresis products by centrifugation through a Ficoll-hypaque gradient. Blood or leukapheresis samples were collected from healthy volunteer donors with informed consent using a protocol approved by the Institutional Review Board of University of Washington. NK cells were purified from PBMCs by magnetic negative selection, using Miltenyi NK cell Isolation kit II. NK-92, a cell line that has the characteristics of human NK cells (31), was obtained from American Type Culture Collection (ATCC) and maintained in Alpha Minimum Essential Medium without ribonucleosides and deoxyribonucleosides but with 2 mmol/L L-glutamine, 0.2 mmol/L inositol, 0.1 mmol/L 2-mercaptoethanol,

0.02 mmol/L folic acid, 100 U/mL interleukin (IL)-2, 12.5% FBS, and 12.5% horse serum. The breast cancer cell lines SKBR3 and MDA-MB-231 were obtained from ATCC and maintained in Dulbecco's Modified Eagle's Medium (Cellgro) supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere. The K562 leukemia cell line was also obtained from ATCC and maintained in RPMI (Cellgro) with 10% FBS (Gemini Bioproducts).

Antibodies and other reagents

The HER2-specific mAb trastuzumab (Herceptin) was manufactured by Genentech and purchased from the University of Washington Pharmacy. Fluorochrome-conjugated mAbs against CD3, CD56, CD25, CD69, and CD107a were from eBiosciences. Fluorochrome-conjugated mAbs against CD16 and IFN-γ was from Biolegend. Recombinant human IL-12 and anti-human IL-12 neutralizing antibody were purchased from Peprotech. PBS, penicillin–streptomycin, and L-glutamine were obtained from Invitrogen. PSK was purchased from Kureha Corporation. PSK was dissolved in PBS at a stock concentration of 10 mg/mL. Aliquots of 100 μL were stored at −80°C. The frozen aliquots were thawed immediately before use. Anti-rat neu mAb (clone 7.16.4) was produced from 7.16.4 hybridoma cells (kindly provided by Dr. Mark Green) by the University of California, San Francisco, Hybridoma and Monoclonal Antibody Core.

Measurement of human NK cell activation and production of IFN-γ by fluorescence-activated cell sorting

PBMCs or purified NK cells were cultured in RPMI in the presence of PSK (100 μg/mL) or control PBS for 24 or 48 hours. Brefeldin-A (BFA, 5 μg/mL; Sigma–Aldrich), a secretion inhibitor, was included for the last 6 hours of the incubation. At the end of activation period, the cells were first stained with fluorophore-conjugated antibodies to surface markers (anti-CD3, anti-CD56, anti-CD25, and anti-CD69). After subsequent fixation and permeabilization, the cells were stained with anti-IFN-γ-PE. In some experiments with PBMCs, the cells were coincubated with anti-IL-12 to determine whether the production of IFN-γ by NK cells is dependent on this cytokine. In experiments with purified NK cells, a suboptimal dose of IL-12 (1 ng/mL) or PSK plus IL-12 was also included. Samples were acquired on FACSCanto II. List mode file was analyzed using FlowJo (Treestar).

Measurement of CD107a degranulation in NK cells

The degranulation of NK cells was measured by the expression of CD107a, lysosome-associated membrane protein-1 (LAMP-1). In brief, PBMCs treated with PSK (100 μg/mL, 24 hours) or medium alone were incubated with K562 target cells at effector/target (E:T) ratio of 2:1 for 6 hours. Anti-CD107a-PE antibody was added directly to the cocultures. After 1-hour incubation, BFA was included to the culture and incubated for another 5 hours. Cells were then stained with CD3 and CD56 and analyzed on FACSCanto II.

Cytotoxicity assay

A nonradioactive, fluorometric cytotoxicity assay with calcein–acetoxymethyl (calcein AM; ref. 32) was used to measure the lysis of K562 and trastuzumab-mediated ADCC. PBMCs were stimulated with PSK (10 μg/mL) or control PBS for 48 hours before coincubation with target cells. The K562 tumor target cells were loaded with calcein AM (10 μg/mL; Invitrogen) for 1 hour and washed. Labeled target cells were mixed with PBMCs at different E:T ratios (100:1, 50:1, 25:1, and 12.5:1) and plated on 96-well culture plates. After 4-hour incubation at 37°C, the release of calcein into culture medium was measured by a Victor 3 fluorescent plate reader (PerkinElmer). The percentages of specific lysis were calculated according to the formula: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100, where experimental release represents the mean fluorescence for target cells incubated in the presence of effector cells, spontaneous release represents the mean fluorescence for target cells incubated without effector cells, and maximal release represents the mean fluorescence for target cells incubated with Triton X-100. The measurement of trastuzumab-mediated ADCC was done similarly as described earlier for the K562 lysis assay except that the target breast cancer cells SKBR3 and MDA-MB-231 were coated with trastuzumab (5 μg/mL) or control IgG1 for 30 minutes before labeling with calcein AM. The percentages of specific lysis were calculated as earlier. Triplicate wells were set up for each E:T ratio. Results were expressed at mean ± SD of triplicate wells at each E:T ratio.

Analysis of TLR2 mRNA expression using real-time reverse transcriptase PCR

To analyze TLR2 expression on purified $CD56^{bright}$ and $CD56^{dim}$ NK cells, NK cells were first enriched with PBMCs by magnetic negative selection using Miltenyi NK cell Isolation kit II. Then, the enriched NK cells were stained with anti-CD3, CD56, and CD16 to sort for $CD56^{bright}CD16^{-/low}$ and $CD56^{dim}CD16^{+}$ NK cells using BD FACSAria sorter. The sorted populations had more than 99% purity. $CD3^{+}$ T cells, $CD19^{+}$ B cells, and $CD11c^{+}$ DC were also sorted from PBMCs as controls. RNA was isolated from fluorescence-activated cell-sorted cells or whole PBMCs using RNAqeous4PCR kit (Ambion). cDNAs were prepared using Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was carried out using TaqMan primer and probe from Applied Biosystems in 384-well plates using an ABI 7900 (Applied Biosystems). Cycling conditions were similar as previously described (30). The expression of TLR2 mRNA was normalized to hypoxanthine ribosyltransferase using the ΔC_t method (30).

Measurement of cytokine and chemokine secretion from PSK-stimulated PBMCs or purified NK cells using Luminex analysis

PBMCs or MACS-purified NK cells (200,000 per well) were plated in 96-well round-bottom culture plates and treated with serial dilutions of PSK (25–400 μg/mL) for 24

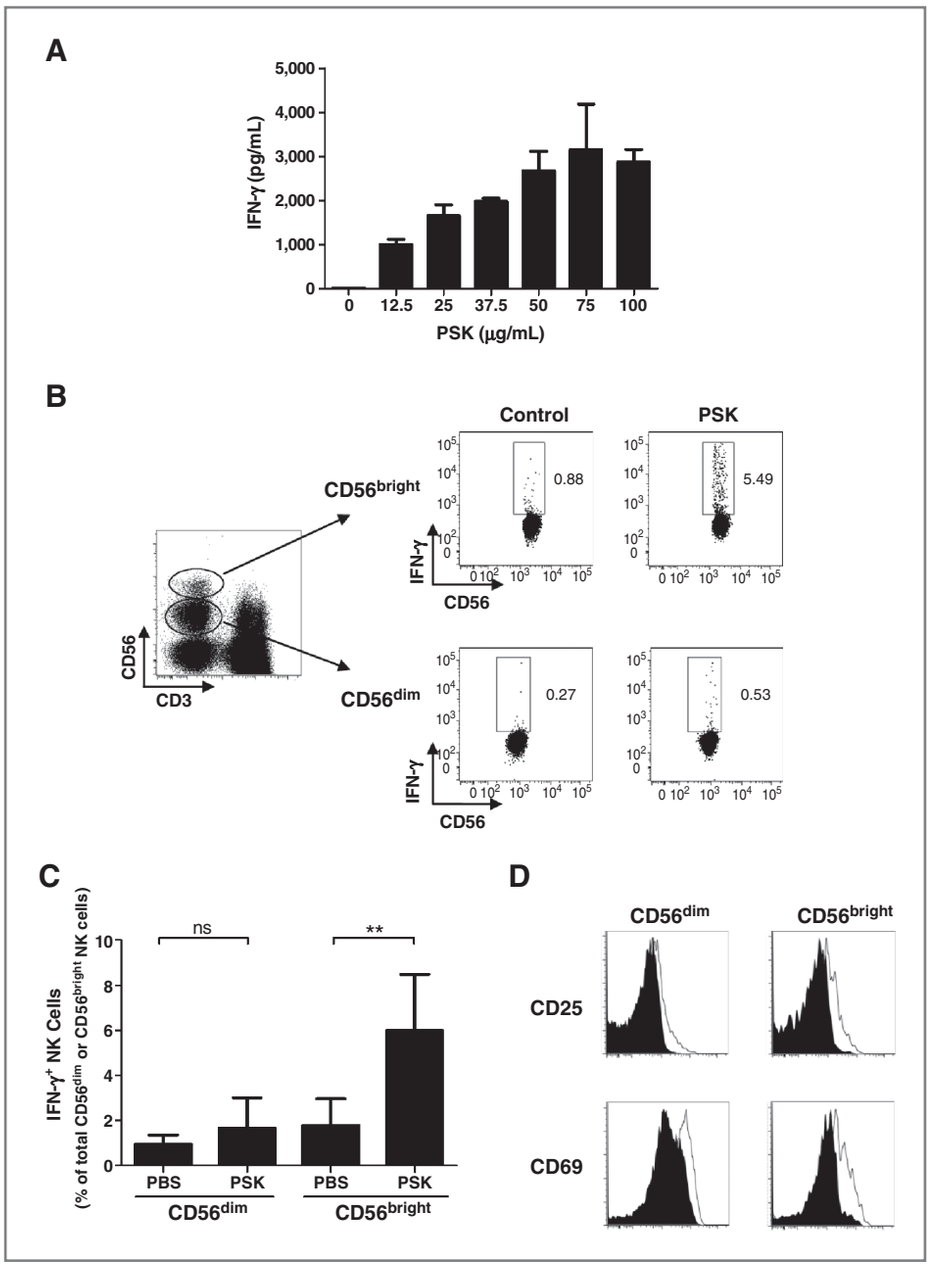

Figure 1. PSK stimulates IFN-γ production from CD56^{bright} NK cells. A, dose-dependent induction of IFN-γ secretion by PSK. Shown are IFN-γ concentrations (mean ± SD) in culture supernatant from duplicate culture wells of PBMCs stimulated with different concentrations of PSK for 24 hours. Similar results were obtained from 3 different donors. B, representative dot plots showing the gating of CD56^{dim} and CD56^{bright} NK cells and IFN-γ production in PBS control or PSK-stimulated CD56^{dim} or CD56^{bright} NK cells. C, summary graph showing the mean ± SD of the percentages of IFN-γ–positive cells among total CD56^{dim} and CD56^{bright} NK cells in PBMCs from 5 different donors. ns, not significant; **, $P < 0.01$ using 2-tailed Student *t* test. D, overlay histogram showing CD25 and CD69 expression on CD56^{dim} and CD56^{bright} NK cells. Filled histogram, NK cells from control PBS group; unfilled histogram, NK cells from PBMCs treated with PSK (100 μg/mL). Results are representative of 3 independent experiments.

or 48 hours. The supernatants were harvested and levels of various cytokines and chemokines [IL-12p40, IL-12p70, TNF-α, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, IL-1α, and IL-1β] were measured using a Luminex kit purchased from Millipore following the manufacturer's instruction.

Treatment of tumor-bearing mice with PSK and anti-HER2/neu mAb (7.16.4)

neu-T mice received subcutaneous implant of 1 million MMC cells, a cell line derived from a syngeneic spontaneous breast cancer in these mice (33). At 2 weeks after implantation (average tumor size = 50 mm^3), mice were randomly assigned to receive treatment with 7.16.4 alone (15 mg/kg, tail vein injection, 3 times per week), PSK alone (100 mg/kg, oral gavage, 3 times per week), 7.16.4 plus PSK, or PSK plus a control irrelevant IgG of the same isotype. Mice in the 7.16.4-alone group received oral gavage of PBS of the same volume. To determine the role of immune cells in the antitumor effect of PSK and 7.16.4, some mice received depletion of CD4, CD8 T cells, or NK cells using the monoclonal antibodies (clone GK1.5 for CD4, clone 2.43 for CD8, and clone PK136 for NK) at 1 week before and during PSK and 7.16.4 treatment, using similar

protocol as previously described (34). Tumors were measured every other day with Vernier calipers and tumor volume was calculated as the product of length × width × height × 0.5236. *In vivo* data are presented as mean ± SD of each treatment group.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism (GraphPad Software). Data were analyzed using the Student *t* test, ANOVA, or the Mann–Whitney *U* test when Guassian distribution cannot be assumed. A value of $P < 0.05$ was considered statistically significant.

Results

PSK stimulates human CD56^{bright} NK cells to produce IFN-γ

PSK induces IFN-γ secretion from PBMCs in a dose-dependent manner (Fig. 1A). Intracellular staining showed that IFN-γ is mainly produced by CD56^{bright} NK cells, although there is a slight induction of IFN-γ in CD56^{dim} NK cells (Fig. 1B). The percentages of cells that are positive for IFN-γ are 1.0 ± 0.2% in control CD56^{dim} cells and 1.6 ± 0.6% in PSK-treated CD56^{dim} NK cells ($P = 0.3$), and are 1.8 ± 0.5% in control CD56^{bright} cells and 5.9 ± 1.1% in PSK-stimulated CD56^{bright} NK cells ($P = 0.009$, Fig. 1C). To determine whether both CD56^{dim} and CD56^{bright} cells are activated, we measured the expression of activation markers, CD25 and CD69, on NK cells after PSK treatment. As shown in Fig. 1D, PSK upregulates the expression of CD25 and CD69 in both CD56^{dim} and CD56^{bright} NK cells.

PSK stimulates the cytolytic function of human NK cells and augments trastuzumab-mediated ADCC

Expression of CD107a in the presence of K562 cells, a MHCI-devoid leukemia cell line, has been used as a marker of NK cell cytotoxicity (35). Fluorescence-activated cell-sorting (FACS) analysis showed that NK cells in PSK-treated PBMCs have higher expression of CD107a (8.0 ± 0.2%) than that present in the control group (4.2 ± 1.0%, $P = 0.02$ between PBS and PSK, Fig. 2A and B). The specific lysis of K562, as measured by calcein AM release assay, was also significantly enhanced in PSK-stimulated PBMCs. As shown in Fig. 2C, the percentage of specific lysis was approximately 2-fold higher in PSK-stimulated PBMCs than in unstimulated PBMCs at different E:T ratios ($P < 0.0001$). We next measured the potential of PSK to augment trastuzumab-mediated ADCC against 2 breast cancer cell lines, SKBR3 and MDA-MB-231. As shown in Fig. 3A, SKBR3 expresses high levels of HER2 and MDA-MB-231 expresses low levels of HER2. Pretreatment of PBMCs with PSK (10 μg/mL, 72 hour) resulted in significantly enhanced ADCC against both cancer cell lines (Fig. 3B). Similar results were obtained using PBMCs from 5 different donors as summarized in Supplementary Table. Measurement of IFN-γ in ADCC supernatant showed that pretreatment with PSK results in significantly enhanced IFN-γ production in response to trastuzumab-coated cancer cells (Fig. 3C).

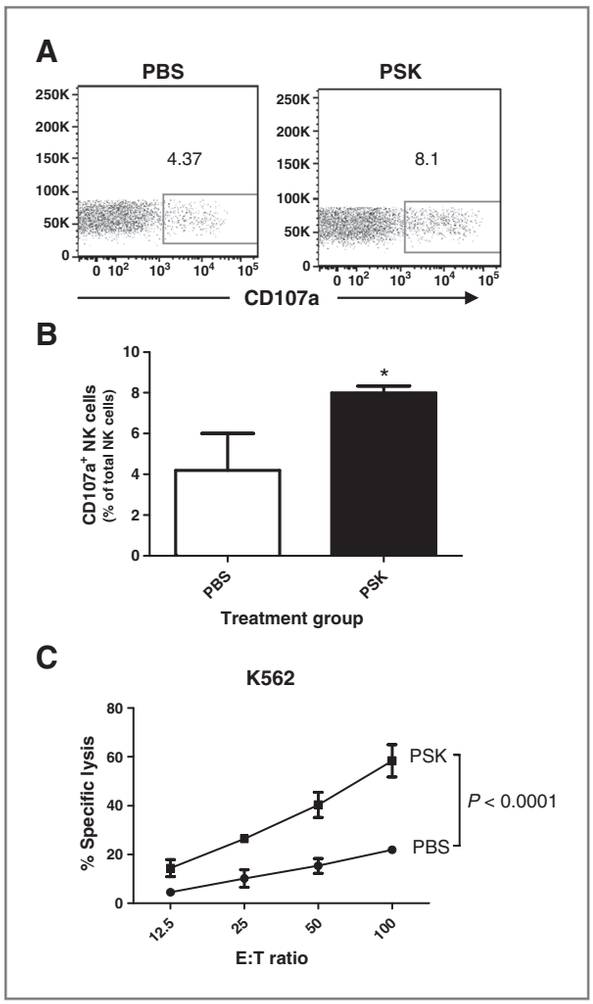

Figure 2. PSK stimulates CD107a mobilization and enhances the lysis of K562 tumor cells. A, representative dot plots showing expression of CD107a in NK cells in control and PSK-treated PBMCs. The PBMCs were stimulated with PSK (100 μg/mL) or control PBS for 24 hours. Then, the cells were mixed with target K562 cells at 2:1 ratio. Anti-CD107a was added to the culture and incubated for 6 hours. B, summary graph showing the percentage (mean ± SD) of CD107a-positive NK cells from 3 different donors and treated with or without PSK stimulation. *, $P < 0.05$ using 2-tailed Student *t* test. C, the lysis of K562 target tumor cells by PSK-stimulated or unstimulated PBMCs. Shown are the percentages of specific lysis (mean ± SD in triplicate wells) at the indicated E:T ratios. PBMCs were stimulated with PSK (or control PBS) for 48 hours before the initiation of cytolytic assay. Differences between PBS and PSK group at different E:T ratio were analyzed using 2-way ANOVA. Similar results were obtained from 3 independent experiments using PBMCs from 3 different donors.

PSK has both direct and IL-12–dependent indirect effects on NK cells

There are controversial reports as to whether NK cells are activated by TLR2 agonists directly or indirectly via accessory cells (21–23). To determine whether the effect of PSK on NK cells is direct or indirect, we first used IL-12 blockade by including anti-IL-12 antibody during PSK treatment of PBMCs. As shown in Fig. 4A–C, IL-12 blockade did not

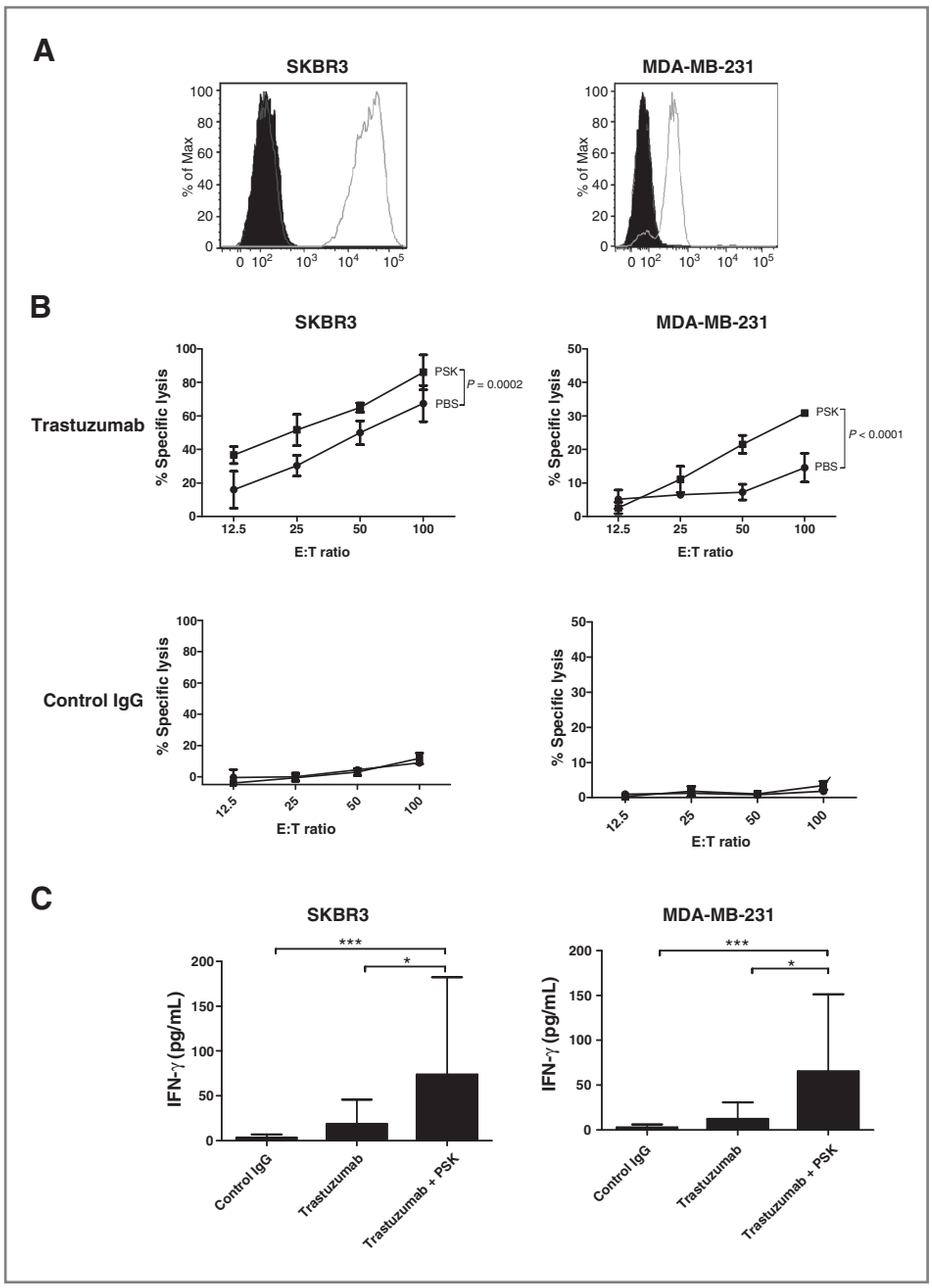

Figure 3. PSK enhances trastuzumab-mediated ADCC against SKBR3 and MDA-MB-231 breast cancer cells. A, expression of HER2 on SKBR3 and MDA-MB-231 cells. The cells were stained with PE-conjugated anti-human HER2 (empty histogram) or isotype control (filled histogram). B, percentages of specific lysis of trastuzumab- or control IgG–coated SKBR3 and MDA-MB-231 target cells. Shown are mean ± SD of triplicate wells at different E:T ratios. ■, PSK-stimulated effector PBMCs; ●, control PBS-treated effector cells. PBMCs were treated with PSK (10 μg/mL) in RPMI for 72 hours before the initiation of cytolytic assay. Differences between PBS and PSK groups at different E:T ratios were analyzed using ANOVA. Similar results were obtained using PBMCs from 5 different donors as summarized in Supplementary Table. C, IFN-γ levels (mean ± SD) in culture supernatant from the 4-hour ADCC incubation. Control IgG, tumor targets were coated with control irrelevant IgG and incubated with PBMCs with no prior PSK stimulation; trastuzumab, tumor cells were coated with trastuzumab and then incubated with PBMCs with no prior PSK stimulation; trastuzumab + PSK, tumor cells were coated with trastuzumab and incubated with PSK (10 μg/mL, 72 hour)-stimulated PBMCs. *, $P < 0.05$; ***, $P < 0.001$ by the Mann–Whitney *U* test. Results are representative of 3 independent experiments.

affect PSK-induced upregulation of CD25 on NK cells but significantly decreased PSK-induced IFN-γ production, as shown by decreased levels of IFN-γ^+ CD56^{bright} NK cells (Fig. 4B) and decreased levels of IFN-γ in culture supernatant from PBMCs (Fig. 4C). It is noted that there is residual amount of IFN-γ production even when IL-12 is blocked, suggesting that IL-12 independent induction of IFN-γ by PSK may also exist (Fig. 4B and C). In contrast, PSK-induced TNF-α production by PBMCs was not decreased in the presence of anti-IL-12 antibody (Supplementary Fig. S1). This could be due to that fact that monocytes and DCs can produce large amounts of TNF-α in addition to NK cells. It also suggests that TNF-α production by NK cells could be regulated differently than IFN-γ production by NK cells and is independent of IL-12. To confirm that PSK-stimulated IFN-γ production by NK cells is dependent on IL-12, we treated MACS-purified NK cells with PSK, a suboptimal dose of IL-12 (1 ng/mL, as determined by dose titration experiment shown in Supplementary Fig. S2), or PSK plus IL-12. Results showed that PSK by itself significantly induced the expression of CD25 on purified NK cells (Fig. 4D), but the effect on IFN-γ production is moderate (not significant by intracellular staining but significant by ELISA measurement, Fig. 4E and F). In the presence of IL-12, PSK

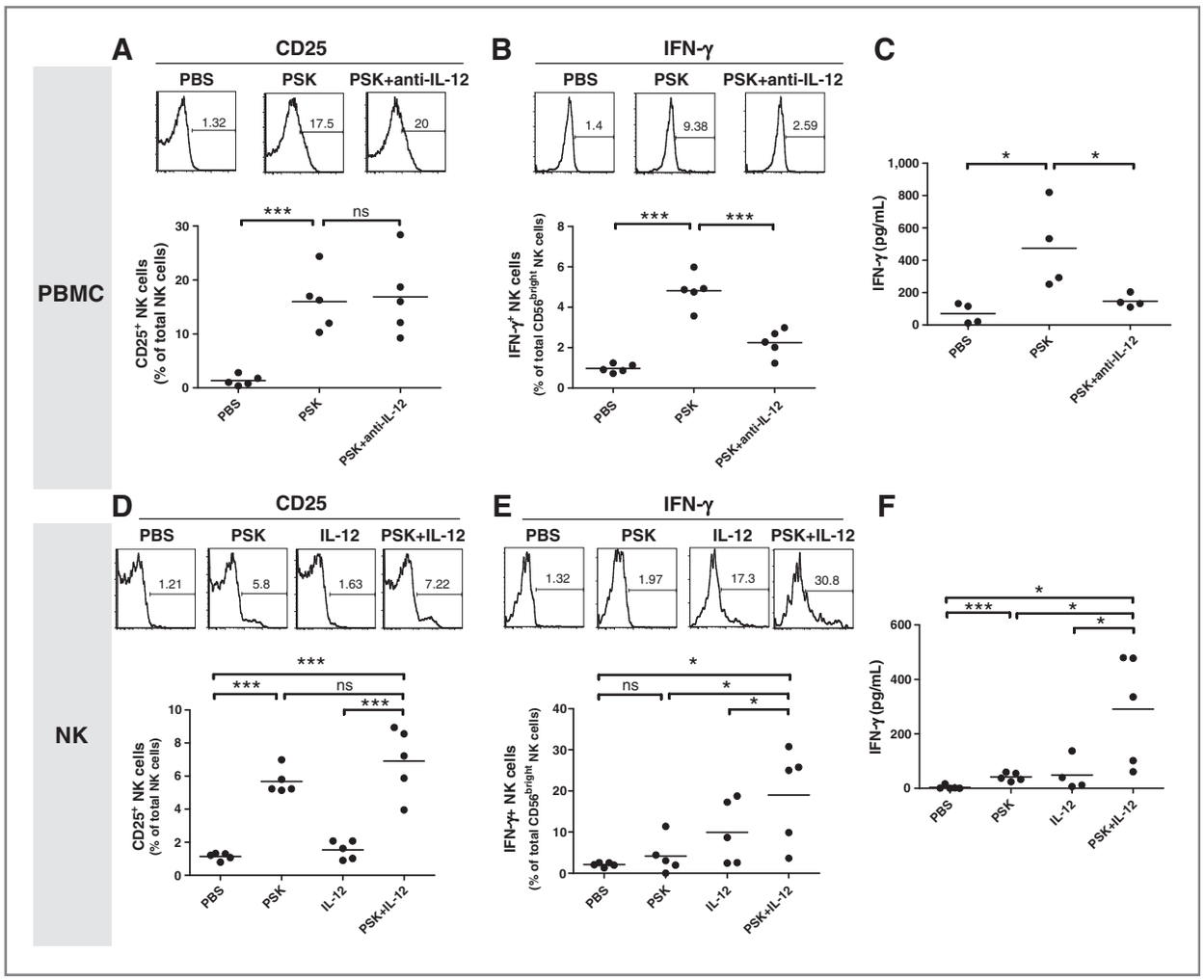

Figure 4. PSK has both direct and IL-12–dependent effects on NK cells. A–C, results generated using PBMCs; D–F, results generated using purified NK cells. A, expression of CD25 on NK cells in PBMC treated with PBS, PSK (100 μg/mL), or PSK plus anti-IL-12 Ab (10 μg/mL) for 24 hours. B, percentage of IFN-γ–positive NK cells among total CD56^{bright} NK cells in PBMC treated with PBS, PSK, or PSK plus anti-IL-12 Ab. C, the level of IFN-γ in culture supernatant from PBMCs treated with PBS, PSK, or PSK plus anti-IL-12 Ab. D, CD25 expression in purified NK cells stimulated with PBS, PSK (100 μg/mL), IL-12 (1 ng/mL), or PSK + IL-12 for 24 hours. E, percentage of IFN-γ–positive NK cells among total CD56^{bright} NK cells in purified NK cells stimulated with PBS, PSK, IL-12, or PSK + IL-12. F, the level of IFN-γ in culture supernatant of purified NK cells stimulated with PBS, PSK, IL-12, or PSK + IL-12. The FACS graphs show response from a representative donor. The summary graphs show data from 4 to 5 different donors analyzed in independent experiments. Each dot represents an individual donor. The horizontal line represents the group average. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ between 2 treatment groups using the Student *t* test.

resulted in enhanced IFN-γ production, which is significantly higher than either PSK or IL-12 treatment alone (Fig. 4E and F). These data suggest that PSK-induced IFN-γ production but not CD25 upregulation is dependent on IL-12. Experiments using NK-92 cells yielded results consistent with those from purified NK cells showing that the production of IFN-γ in response to PSK is dependent on IL-12 (Supplementary Fig. S3). We also measured the expression of TLR2 on NK cells by FACS analysis and real-time PCR. FACS analysis showed that TLR2 is detectable in CD56^{bright} NK cells, but the expression level is much lower than that on B cells or DCs (Supplementary Fig. S4A). Reverse transcriptase-PCR analysis using fluorescence-activated cell-sorted cells confirmed that CD56^{bright} NK cells express more TLR2 mRNA than CD56^{dim} NK cells and T cells, although there is inconsistency between mRNA and FACS data on the relative expression levels of TLR2 on CD56^{bright} NK cells as compared with that on B cell or DCs (Supplementary Fig. S4B). TLR2 expression was not induced upon PSK treatment (data not shown).

PSK stimulates the production of IL-12 and other proinflammatory cytokines and chemokines from PBMCs

Culture supernatant from PSK-treated PBMC was collected for Luminex analysis of IL-12 and other cytokines

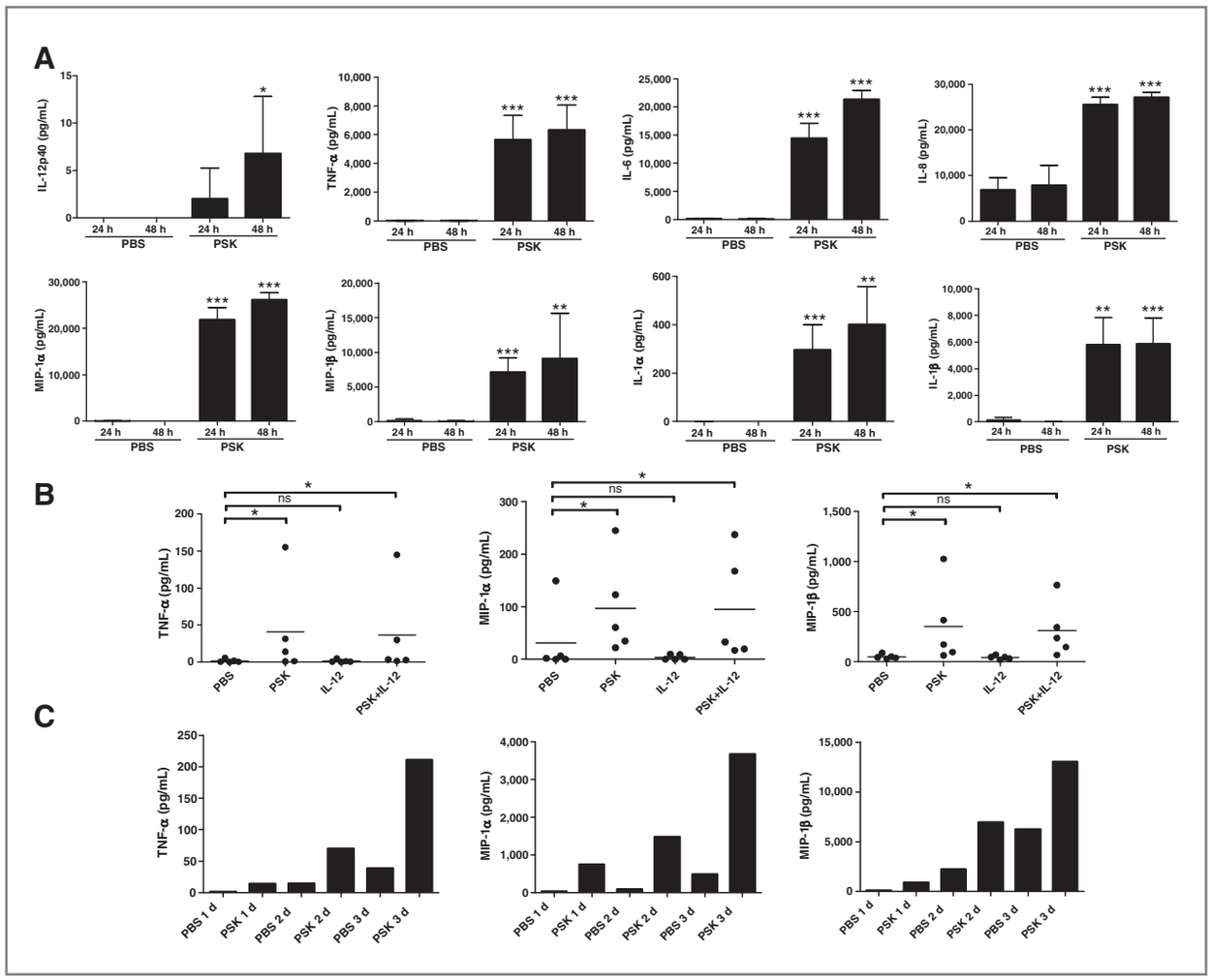

Figure 5. PSK induces the secretion of proinflammatory cytokines and chemokines by PBMCs and NK cells. A, the levels of IL-12p40, TNF-α, IL-6, IL-8, MIP-1α, MIP-1β, IL-1α, and IL-1β in culture supernatant from PBMCs treated with PSK (100 μg/mL) or control PBS for 24 or 48 hours, as determined in Luminex analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ between PSK and PBS group at the same time point using 2-tailed Student t test. Shown are mean ± SD of results from 3 independent donors. B, shown are levels of TNF-α, MIP-1α, and MIP-1β in culture supernatant from purified NK cells treated with PSK (100 μg/mL), IL-12 (1 ng/mL), or PSK + IL-12. Each data point represents response from an individual donor ($N = 5$). C, time course of TNF-α, MIP-1α, and MIP-1β induction by PSK in one donor.

and chemokines. As shown in Fig. 5A, PSK significantly induced the production of IL-12p40. PSK also significantly induced other proinflammatory cytokines and chemokines (TNF-α, IL-6, IL-8, MIP-1α, MIP-1β, IL-1α, and IL-1β) from PBMCs. The level of cytokine/chemokine induction is similar to our previous observation on the effect of PSK on mouse splenocytes (25). Interestingly, this panel of cytokines and chemokines (except IL-12) was also induced in the cytolytic assay when PSK-stimulated PBMCs were coincubated with K562 (Supplementary Fig. S5), indicating that PSK stimulates the cytokine-secreting and cytolytic activity of NK cells simultaneously.

Luminex analysis was also conducted to measure the potential secretion of cytokine/chemokines by PSK-treated purified NK cells. As shown in Fig. 5B, PSK induced the production of TNF-α, MIP-1α, and MIP-1β by NK cells. Interestingly, the pattern of induction for these cytokine/chemokines seems to be different from that for IFN-γ (Fig. 4F) and seems to be independent of IL-12 (Fig. 5B). The time course of cytokine/chemokine induction in 1 of the 5 donors tested in Fig. 5B was shown in Fig. 5C.

Combination of PSK and anti-HER2/neu mAb (7.16.4) has enhanced antitumor effect in a mouse model of breast cancer

To evaluate the potential synergistic antitumor effect between PSK and anti-HER2 mAb therapy, we treated neu-T mice bearing HER2/neu$^+$ breast tumors with 7.16.4, an anti-ErbB2 mAb, alone or in combination with oral PSK. The mechanisms of action of 7.16.4 remain

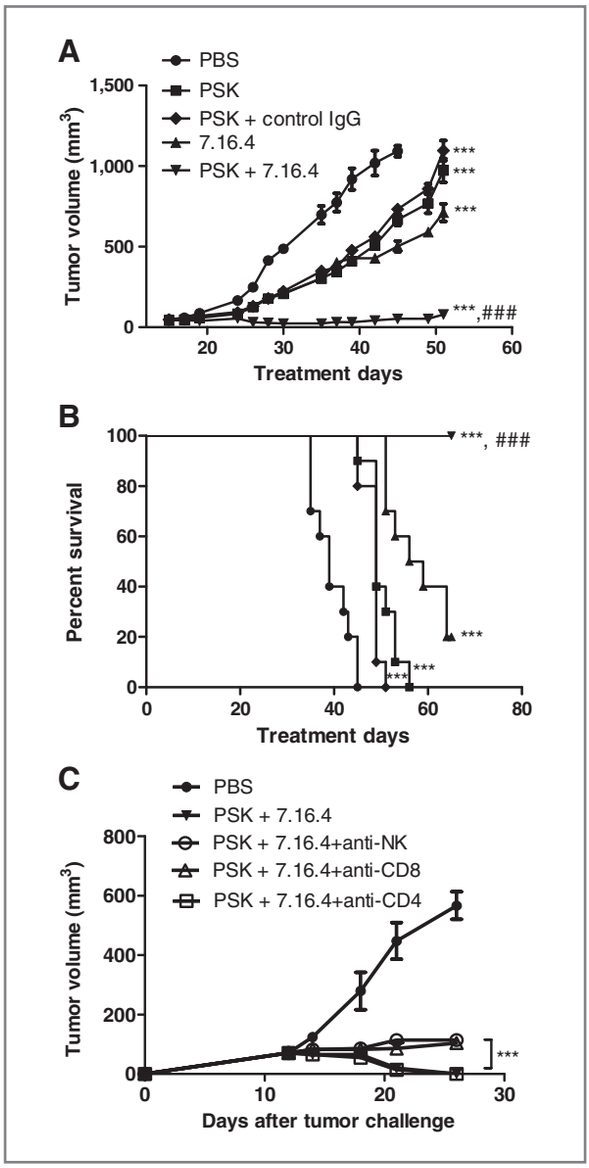

Figure 6. PSK enhances the antitumor effect of 7.16.4 in neu transgenic mice, and the antitumor effect of combinatorial PSK and 7.16.4 is partially dependent on NK cells and CD8 T cells. Tumor growth curve (A) and overall survival (B) in mice receiving PBS (●), PSK (■), control IgG + PSK (◆), 7.16.4 (▲), 7.16.4 plus PSK (▼). Each data point in A represents mean ± SD in the treatment group, $n = 10$ per group. Similar results were obtained from 2 independent experiments. ***, $P < 0.001$ from control PBS group using ANOVA. ###, $P < 0.001$ between 7.16.4 and PSK + 7.16.4 using ANOVA. C, tumor growth curves in mice receiving PBS (●), PSK + 7.16.4 (▼), PSK + 7.16.4 with NK cell depletion (○), PSK + 7.16.4 with CD8 T cell depletion (Δ), or PSK + 7.16.4 with CD4 T cell depletion (□). The treatment with PSK + 7.16.4 mAb or control PBS was initiated on day 12. The depletion antibodies were administered 3 times a week before PSK treatment and then 2 times a week during PSK treatment. Each data point represents the average tumor volume in each group (mean ± SD, $n = 5$ per group). The difference between no-depletion group (PSK + 7.16.4) and depletion groups was calculated using 2-tailed Student t test. ***, $P < 0.001$ between CD8 T cell or NK cell depletion group and no-depletion group (PSK + 7.16.4). There is no difference between CD4 T cell depletion group and no-depletion group.

unclear, and both direct inhibition of tumor cell growth (36) and immune cell-mediated antitumor effect (37) have been reported. As shown in Fig. 6A, 7.16.4 mAb by itself inhibits tumor growth by 58% ± 2%. PSK by itself inhibits tumor growth by 50% ± 3%. The combination of the two treatments inhibits tumor growth by 96% ± 2% ($P < 0.0001$ compared with either treatment alone), showing the potential of PSK to augment the antitumor effect of trastuzumab. The overall survival was also significantly improved in the group of mice that received both PSK and 7.16.4 mAb ($P = 0.0003$ between 7.16.4 alone and 7.16.4 plus PSK, Fig. 6B). Selective depletion of CD4, CD8 T cells, or NK cells during 7.16.4 plus PSK treatment showed that the antitumor effect is partially dependent on CD8 T cells and NK cells but not on CD4 T cells (Fig. 6C).

Discussion

Enhancing NK cell function is important to improve the clinical response to trastuzumab and other mAb therapy. In this study, we have shown that *in vitro* treatment with TLR2 agonist PSK can activate human NK cells and augment trastuzumab-mediated ADCC. In a mouse model of $HER2^+$ breast cancer, orally administered PSK augments the antitumor effect of anti-HER2/neu mAb therapy. These findings indicate the potential of using a natural product as an adjuvant to improve the clinical response to trastuzumab.

NK cells are the major mediator of ADCC, and the function of NK cells has been shown to impact the treatment outcomes of trastuzumab, rituximab, and cetuximab (8). NK cell function is frequently impaired in patients with cancer and improving NK cell function via cytokines or TLR agonists has shown promise to augment ADCC (38–42). For example, IL-2 *ex vivo* treatment of NK cells was shown to restore the impairment of trastuzumab-mediated ADCC in the patients with gastric cancer (11). IL-12 has also been shown to augment trastuzumab-mediated ADCC and enhance the antitumor actions of trastuzumab via NK IFN-γ secretion (41, 42). Multiple TLR agonists, especially the agonists of TLR9 and 7/8, have shown potential to augment NK cell function (24, 40, 43, 44). For example, CpG ODN has been reported to increase IFN-γ production by NK cells and enhance trastuzumab-mediated lysis of breast cancer cells (17). TLR7 and 8 agonists have also been shown to induce IFN-γ production by NK cells (15, 18). Our study shows the potential of using a natural product with TLR2 agonist activity to augment NK cell function. The concentration of PSK used in our study (10–100 μg/mL) has been reported to be achievable in the blood of patients with cancer who received standard oral administration of the drug (3 g daily; ref. 45).

Whether TLR2-mediated activation of NK cells is direct or indirect remains controversial in the literature (21–23). Our results showed that TLR2 is expressed on NK cells, although the level is significantly lower than that on

B cells or DCs. Our finding that $CD56^{bright}$ NK cells expresses more TLR2 than $CD56^{dim}$ cells is consistent with the results by Gorski and colleagues showing that $CD56^{bright}$ NK cells express more TLR2 mRNA than $CD56^{dim}$ NK cells (15). Similar to our observation that PSK induces CD25 expression on NK cells independent of IL-12, Gorski and colleagues observed upregulation of CD69 in both $CD56^{dim}$ and $CD56^{bright}$ NK cells when purified NK cells were stimulated with the TLR2 agonist MALP2 (15). It is interesting that although $CD56^{bright}$ cells, the major producer of IFN-γ, express more TLR2 than $CD56^{dim}$ NK cells, they still seem to require the help from IL-12–producing DCs to produce IFN-γ. This might be explained by the concept that $CD56^{bright}$ NK cells generally need 2 signals to produce IFN-γ, and one of these almost always includes IL-12 (46). Previously, we have reported the effect of PSK on DC maturation and induction of both IL-12p40 and p70 using mouse bone marrow–derived DCs. In the current study, we found significant induction of IL-12p40 in PSK-treated human PBMCs. IL-12p70 was detected in some PSK-treated PBMCs and not in untreated samples. However, the levels were very low and did not reach statistical significance (data not shown).

In addition to IFN-γ and IL-12, PSK induced the secretion of other proinflammatory cytokines and chemokines (TNF-α, MIP-1α, and MIP-1β) by PBMCs and purified NK cells, which could potentially promote chemotaxis. A previous study by Roda and colleagues has shown that IL-8, MIP-1α, and RANTES secreted by IL-21–stimulated NK cells in the presence of mAb-coated tumor cells resulted in enhanced migration of T cells (38, 39). Although we did not evaluate the potential chemotactic effect of culture supernatant from PSK-stimulated NK cells, our previous study in mouse using selective depletion during PSK treatment showed that the antitumor effect of PSK is dependent on both NK cells and CD8 T cells (25). Selective depletion of CD4, CD8 T cells, or NK cells during PSK and 7.16.4 combination therapy also showed the involvement of both NK and CD8 T cells (Fig. 6C). This indicates that chemokines released by NK cells could have led to recruitment of T cells that contributed to the antitumor effect of PSK. It is noted that NK or CD8 T cell depletion only partially abrogated the antitumor effects of PSK and 7.16.4 therapy. This could be explained by the potential of 7.16.4 to directly inhibit tumor cell growth, as suggested in publication (36). It is also noted that in about 50% of the mice that received both PSK and 7.16.4 mAb, the tumor will relapse at a later date, indicating that long-term immunologic memory has not been established (data not shown). Whether the tumor-free mice can reject a second tumor challenge remains to be tested.

In summary, our study indicates the potential of using PSK, a natural product with potent TLR2 agonist activity, to augment the function of NK cells and enhance ADCC. The major advantage of PSK as compared with other TLR agonists that are currently evaluated in clinical trials, such as CpG, imiquimod, or poly(I):poly(C), is its known safety profile. PSK is a mushroom extract that has been widely used in Asian countries for its immune potentiating and antitumor effects. A meta-analysis of data from 3 randomized clinical trials in Japan in 1,094 patients with colorectal cancer showed that PSK significantly increased both overall survival and disease-free survival of patients with curatively resected colorectal cancer (29). The antitumor effect of PSK has also been shown in other types of cancer, including stomach cancer (27, 47) and lung cancer (48). To our knowledge, our study represents the first report on the potential of PSK to augment trastuzumab-mediated ADCC and the synergistic antitumor effect between PSK and HER2-targeted mAb therapy in a preclinical model. This provides rationale for future clinical trials testing the adjuvant effect of PSK when administered concurrently with trastuzumab. Although the study reported in this article used only PBMCs from normal healthy donors, a recently finished clinical study in patients with breast cancer conducted by Standish and colleagues (manuscript in preparation) also showed a trend toward increased NK cell cytolytic activity after oral administration of Turkey tail mushroom extract, the same species of mushroom from which PSK is extracted. Whether PSK can enhance the therapeutic effect of trastuzumab and a HER2-targeted vaccine in patients with breast cancer will be tested in our group. Because our previous studies have shown that the potential of PSK to stimulate NK cells is dependent on TLR2 (25), we speculate that the clinical response to combination therapy with PSK and trastuzumab may also be dependent on TLR2 and may be impacted by functional *TLR2* gene polymorphism that have been reported (49). Hopefully, these questions can be addressed in clinical trials in the future. NK cell function impacts the clinical response not only to trastuzumab but also to other mAb therapies, such as rituximab for lymphoma and cetuximab for head and neck cancer (8). Thus, results from the current study could potentially be expanded to other types of cancer. Our study highlights the potential of combining complementary and alternative medicine therapy to mainstream cancer therapy for enhanced therapeutic effect.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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