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Correlation Between NKG2DL Expression and Antitumor Effect of Protein-bound Polysaccharide-K in Tumor-bearing Mouse Models

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Abstract. *Background/Aim: We investigated the relationship between the expression of natural killer group 2, member D ligands (NKG2DLs) and the antitumor effects of protein-bound polysaccharide-K (PSK). Materials and Methods: PSK was administered to evaluate its effectiveness against tumor growth. The expression of Rae-1 and H60 were analyzed in multiple cell lines. Results: PSK showed the highest antitumor effects in mice implanted with cells expressing neither Rae-1 nor H60. PSK had little antitumor effect in mice implanted with cells expressing both Rae-1 and H60. A correlation between the expression of NKG2DLs and the antitumor effect of PSK was observed. After PSK administration, INF-γ production in CD8$^+$ T cells increased in mice with cells expressing neither Rae-1 nor H60, but did not change in mice implanted with cells expressing both Rae-1 and H60. Conclusion: We demonstrated that the expression of NKG2DLs affects tumor immunity and the efficacy of immunotherapy in tumor-bearing mouse model.*

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Natural killer group 2 member D (NKG2D) is an active receptor expressed on NK cells, CD8$^+$ T cells, and γδ T cells. Tumor cells expressing NKG2D ligands (NKG2DLs) are more likely to be targeted by NK cells because of the function of NK cell activation and the interaction between NKG2DL and NKG2D. However, the correlation between tumor cells expressing NKG2DL and T cells expressing NKG2D in the mechanism of T cell activation remains unclear. Although NKG2DLs interact actively with NKG2D expressed on NK cells, immune effector cells chronically exposed to NKG2DLs inhibit the activity of NK cells expressing NKG2D (1). In other words, NKG2DLs have paradoxical immunological characteristics, exerting both immunostimulatory and immunosuppressive effects on NK cell activation. We observed that NKG2D-expressing tumor infiltrating cells were not NK cells but CD8$^+$ T cells in surgically resected tissues (Oncology letters in press). Therefore, the focus of this study was to determine the role of NKG2DL/NKG2D interaction in T cell activation or regulation and its potential to affect the efficacy of immunotherapy.

The NKG2DLs are stress-induced proteins. UL16 binding protein 1 to 6 (ULBP1-6) (2) and MHC class I chain-related proteins A and B (MICA/B) (3) are the major NKG2DLs for human NKG2D, while Rae-1, H-60, and Mult-1 are the major NKG2DLs for murine NKG2D (4, 5). NKG2DLs are widely expressed on tumor cells and function as activating factors of NK cells, $CD8^+$ T cells, and γδ T cells that express NKG2D. An exception is Mult-1; this NKG2DL is expressed on normal tissues, unlike the Rae-1 family proteins and H60. Instead, the Rae-1 family and H60 are induced by viral infection or intracellular stress (such as DNA injury), and are widely expressed on tumor cells (6). However, immune effector cells are suppressed by NKG2DLs in some instances (7, 8).

To identify the tumor-derived immunosuppressive or immunostimulatory factors involved in the NKG2DLs/NKG2D interaction, we used protein-bound polysaccharide-K (PSK), also known as Krestin, as a therapeutic immunoactivator (9, 10). Protein-bound PSK is extracted from *Coriolus versicolor* (CM101) (11) and has been used clinically in combination therapy for gastrointestinal cancer and small cell lung carcinoma. First, we attempted to determine the relationship between the antitumor effect of PSK in a tumor-bearing mouse model and the expression profile of NKG2DLs on a tumor. Next, we investigated whether NKG2DL expression on tumor cells affects T cell activation by examining the expression of NKG2D on and IFN-γ production in $CD8^+$ T cells in mice bearing NKG2DL-expressing and NKG2DL-nonexpressing tumors.

Materials and Methods

Mice and tumor cell lines. Female syngeneic BALB/c mice (8–10 weeks of age) were purchased from CLEA Japan Inc. (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility of Yamaguchi University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University. The following cell lines were used: Meth-A, a methylcholanthrene-induced fibrosarcoma cell line of BALB/c origin (12); colon tumor 26 (CT26), a murine colon adenocarcinoma cell line derived from BALB/c mice (13); 4T1 breast tumor cell line, spontaneous tumor developed in BALB/c mice (14); S180, a murine sarcoma cell line (15); K-BALB, a sarcoma cell line of BALB/c origin (16); ISOS-1, an angiosarcoma cell line from severe-combined-immunodeficiency (SCID) mice (17); RAG, a renal adenocarcinoma cell line from BALB/c mice; and CMT93 (18), a polyploidy carcinoma cell line from C57BL/icrf mice (19). All tumor cell lines were purchased from the American Type Culture Collection (Rockville, Maryland). Cells were maintained in DMEM-F12 (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Tokyo, Japan) at 37˚C in 5% CO_2.

Antitumor effect of PSK in mice. Tumor cells were implanted subcutaneously into BALB/c mice at 1×10^6 cells per mouse, and PSK was administered intraperitoneally three times a week from day 1 after implantation (n=7-9, Figure 1A). Mice were monitored and euthanized if tumors became necrotic or exceeded the predetermined maximum size of 6,000 mm^3. Tumor diameters were measured using calipers and tumor volume was calculated using the following formula: $A \times B^2 \times 0.5$, where A is the largest diameter and B is the smallest diameter. Tumor control mice were implanted with tumor cells at 1×10^6 cells per mouse and administered saline three times a week. Normal control mice were not implanted with tumor cells or administered PSK.

Analysis of cell surface antigens by flow cytometry. Flow cytometric analysis was performed to examine the expression of Rae-1 and H60 on the cell surface of Meth-A, S180, 4T1, CT26, K-BALB, and ISOS-1 cells. The expression of MHC class I molecules; H-$2D^d$, H-$2K^d$ and H-$2L^d$/H-$2D^b$, on Meth-A, 4T1, and K-BALB cells was also analyzed. The levels of NKG2D expression on $CD49b^+$ NK cells and $CD8^+$ T cells were measured in Meth-A-bearing and in K-BALB-bearing mice. Blood was collected and red blood cells were lysed by the addition of BD Pharm Lyse (BD Biosciences, San Jose, CA, USA). The mononuclear cells harvested were stained for CD8a and CD49b as T cell and NK cell surface markers, respectively, and also for NKG2D. After washing, the cells were resuspended in phosphate-buffered saline (PBS) and analyzed by flow cytometry. For cell surface staining, the following antibodies were used: PE-conjugated anti-Rae-1 antibody (Ab) (R&D Systems, Minneapolis, MN, USA), PE-conjugated anti-H60 Ab (R&D Systems Minneapolis, MN, USA), PE-conjugated anti-H-2Dd Ab (BioLegend, San Diego, CA, USA), PE-conjugated anti-H-2Kd Ab (BioLegend, San Diego, CA, USA), PE-conjugated anit-H-2Ld/H-2Db Ab (BioLegend, San Diego, CA, USA), PE-conjugated isotype-control Ab (R&D Systems), FITC-conjugated anti-CD4 Ab (BD Biosciences, San Jose, CA, USA), PerCP-Cy5.5-conjugated anti-CD8a Ab (BD Biosciences, San Jose, CA, USA), APC-conjugated anti-CD49b Ab (BD Biosciences, San Jose, CA, USA), and PE-conjugated anti-NKG2D Ab (BioLegend, San Diego, CA, USA). Cells were analyzed using a FACSCalibur system (BD Biosciences, San Jose, CA, USA).

Analysis of intracellular cytokines. Meth-A and K-BALB cells were implanted subcutaneously at 1×10^6 per mouse. After 4 weeks, spleen cells were collected. Spleen cells (2×10^6) were incubated with brefeldin A for 5 h at 37˚C. For stimulation, phorbol 12-myristate 13-acetate (PMA; 5 ng/mL) and A23187 (250 ng/mL) was added. Cells were washed and incubated on ice with Fcg III/II receptor blocker [anti-mouse CD16/32 (eBioscience, San Diego, CA, USA)]. After reacting with PE-conjugated anti-CD49b, PerCP-Cy5.5-conjugated anti-CD4, and APC-conjugated anti-CD8a Abs, cells were fixed and permeabilized in a fixation/permeabilization solution (BD Biosciences, San Jose, CA, USA). Finally, FITC-conjugated anti-IFN-g Ab was added and cells were analyzed using a FACSCalibur system (BD Biosciences, San Jose, CA, USA). Data were analyzed by FlowJo software (Treestar Inc., San Carlos, CA, USA).

Statistical analysis. Results are expressed as means±standard error (SE). All data were analyzed using GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA). One- and two-way analyses of variances (ANOVA) were used for data analysis of more than two groups, and a Bonferroni *post-hoc* test was used to determine significant differences between treatment groups. Two-group analysis was performed using a Mann-Whitney *U*-test. *p*-Values ≤0.05 were considered statistically significant.

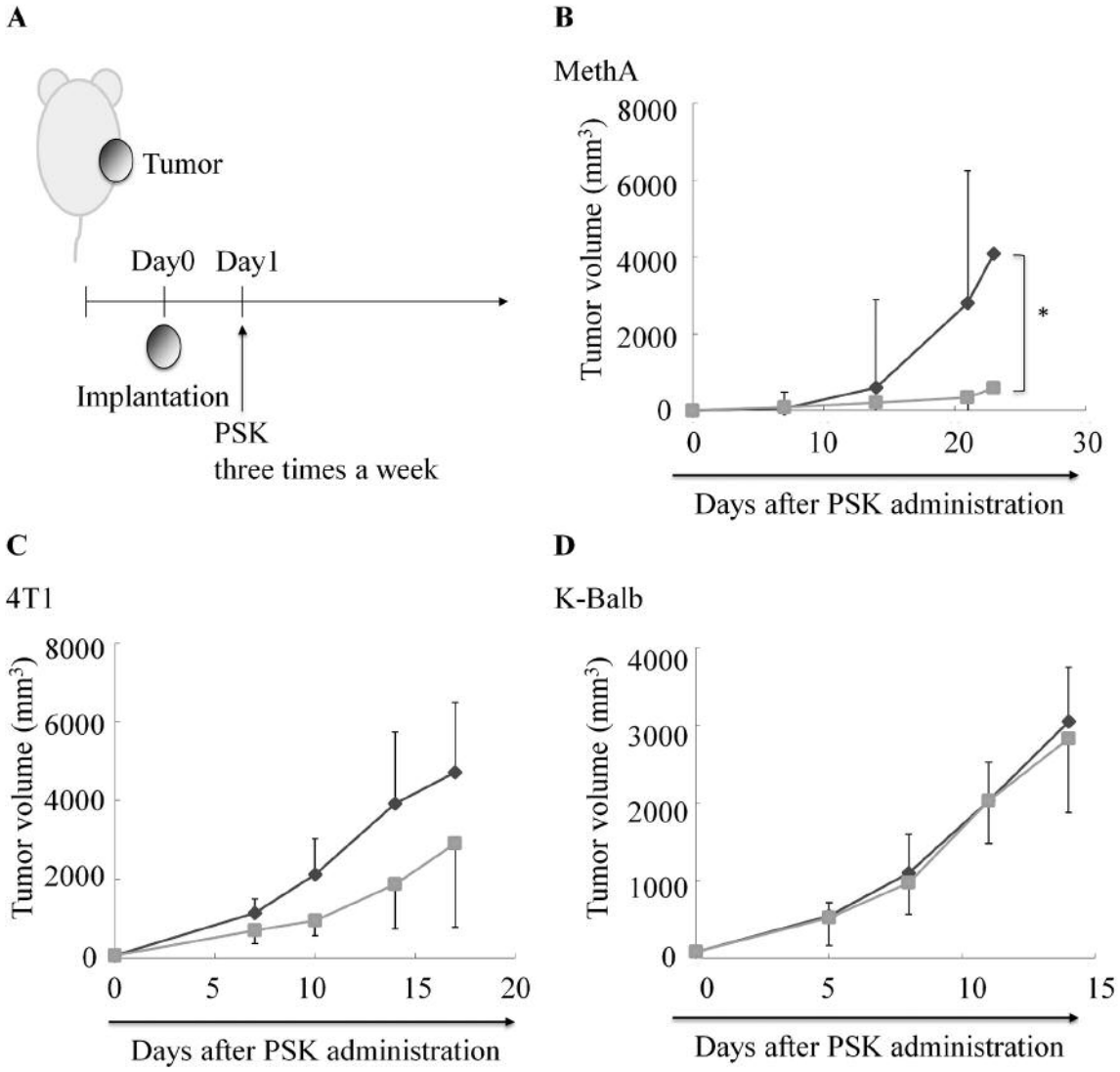

Figure 1. *Effect of PSK on tumor growth. (A) Tumor cells were implanted subcutaneously into mice at 1×10^6 cells per mouse, and polysaccharide-K (PSK) was administered intraperitoneally three times per week from day 1 after implantation. (B-D) Effect of PSK on tumor growth in mice. Gray line: mice treated with PSK, black line: tumor control mice treated with saline. The antitumor effect of PSK was in decreasing order of Meth-A > 4T1 > K-BALB (*p=0.0001).*

Results

Effect of PSK on tumor growth. PSK showed the highest antitumor effect in mice implanted with Meth-A, and the difference in tumor volume between the PSK-treated group and the tumor control group was significant (Figure 1B, n=9, p=0.0001). Similar result was obtained in S180-implanted mice (data not shown). An apparent weak antitumor effect was observed in mice implanted with 4T1, although the tumor volume was not significantly different from control mice (Figure 1C, n=7). Similar result was obtained in CT26-implanted mice (data not shown). PSK had little or no effect in mice implanted with K-BALB (Figure 1D, n=8). Similar result was obtained in ISOS-1-implanted mice (data not shown). Mice were not successfully implanted with RAG and CMT93 cell lines (data not shown).

Expression of NKG2DLs in cell lines. Meth-A and S180 did not express Rae-1 family members or H60 (Figure 2A). 4T1 and CT26 expressed H60 only (Figure 2B). K-BALB and ISOS-1 expressed both Rae-1 family members and H60 (Figure 2C). RAG and CMT expressed Rae-1 but not H60 (Figure 2D). Thus, both representative NKG2DLs were not expressed on Meth-A that showed high response to PSK

treatment, while only one NKG2DL was expressed on 4T1 that showed mild response, and both NKG2DLs were expressed on K-BALB that showed no response (Table I).

Expression of MHC Class I. H-2D^d, H-2K^d, and H-2L^d were highly expressed in Meth-A, 4T1, and K-BALB cell lines (Figure 3).

NKG2D expression and IFN-g production in CD49b$^+$ NK cells and CD8$^+$ T cells. NKG2D expression levels on CD49b$^+$ NK cells tended to be lower in mice implanted with Meth-A or K-BALB cells than in mice without tumor, although the differences were not significant. Furthermore, the level of expression was similar in mice implanted with Meth-A and in those implanted with K-BALB cells (Figure 4A, n=4). However, the level of NKG2D expression on CD8$^+$ T cells was significantly higher in mice implanted with Meth-A cells compared to mice without tumor (Figure 4B, n=4, $p<0.0001$). Mice without tumors expressed almost no NKG2D on CD8$^+$ T cells, indicating that the presence of tumor induced NKG2D expression (Figure 4B). Meth-A-implanted mice showed significantly higher NKG2D expression on CD8$^+$ T cells than K-BALB-implanted mice (Figure 4B, n=4, $p=0.0004$). IFN-g levels in CD8$^+$ T cells were similar in mice implanted with Meth-A and K-BALB, and mice without tumor (Figure 4C, n=4).

Effect of PSK on NKG2D expression and intracellular IFN-g production in CD8$^+$ T cells. Level of NKG2D expression on CD8$^+$ T cells in mice implanted with Meth-A cells and treated with PSK was significantly higher than that in mice implanted with Meth-A cells but not treated with PSK (Figure 4D, n=5, $p=0.0159$). Mice implanted with K-BALB showed a similar (but not significantly different) tendency of NKG2D expression as mice implanted with Meth-A, but there was no significant difference between with and without PSK treatment (Figure 4E, n=4). IFN-g level in mice implanted with Meth-A and treated with PSK was significantly higher than that in mice implanted with Meth-A cells but not treated with PSK (Figure 4F, n=5, $p=0.0079$). In mice implanted with K-BALB, INF-γ levels were similar with and without PSK treatment (Figure 4G, n=4).

Effect of PSK in mice implanted with both K-BALB and Meth-A cells. In mice implanted with K-BALB 7 days (day -7) prior to implantation with Meth-A (day 0) and treated with PSK three times a week from day 1 (Figure 5A), the antitumor effect of PSK was completely lost, resulting in growth of Meth-A cells (Figure 5B). On the other hand, in mice implanted with Meth-A alone and treated with PSK, tumor growth was significantly suppressed compared to control mice implanted with Meth-A, but not treated with PSK (Figures 5C-D, $p=0.0004$, n=8).

Table I. *Relationship between NKG2DL expression and effect of PSK on tumor growth.*

Tumor cell	NKG2DL expression		Antitumor effect of PSK
	Rae-1 family	H60	
Meth-A	Not expressed	Not expressed	Strong
S180	Not expressed	Not expressed	-
4T1	Not Expressed	Expressed	Weak
CT26	Not Expressed	Expressed	-
K-BALB	Expressed	Expressed	None
ISOS-1	Expressed	Expressed	-

PSK-responsive tumors did not express Rae-1 and H60, but PSK-nonresponsive tumor expressed both. Moderately responsive tumors expressed only H60.

Discussion

Mice implanted with Meth-A cells showed the greatest response to PSK, while mice implanted with 4T1 cells showed weak response. Mice implanted with K-BALB cells showed little or no response to PSK. Differences in the response to PSK among tumor cells appeared to correlate with the expression patterns of representative NKG2DLs; Rae-1 family and H60, on tumor cells. Specifically, both Rae-1 and H60 were not expressed on Meth-A cells, while H60 but not Rae-1 family was expressed on 4T1 cells, and both Rae-1 and H60 were expressed on K-BALB cells. We also confirmed the expression of MHC class I molecules on the tumor cells. MHC class I molecules were widely expressed in all three cell lines, suggesting no relationship between MHC class I molecule expression and PSK response.

To date, few studies have reported a relationship between NKG2DL expression and antitumor effects of drugs. One study showed that fluorouracil (5-FU) treatment upregulated the expression of Rae-1 and H60 in MC38 cells, and that the cytolytic activity induced by 5-FU partially depended on NKG2D-Rae-1 or H60 signaling (20). Another study showed that NKG2D and CD16 expressed on NK cells were associated with lower antitumor effect compared with NKG2D and CD16 expressed on NK cells from mice vaccinated with a recombinant vaccinia virus expressing interleukin-2 (rvv-IL-2) (13). In that study, tumors derived from SCC VII/SF cell line, which expressed Rae-1 but not H60, expressed high levels of transforming growth factor-b1 (TGF-b1) and TGF-b1 expression was down-regulated by vaccination with rvv-IL-2. Co-culture of NK cells with tumor cells expressing Rae-1 but not H60 resulted in the downregulation of NKG2D and CD16 expression. This mechanism might be mediated by TGF-b produced by the cancer cells (21). Finally, a study showed that blockade of

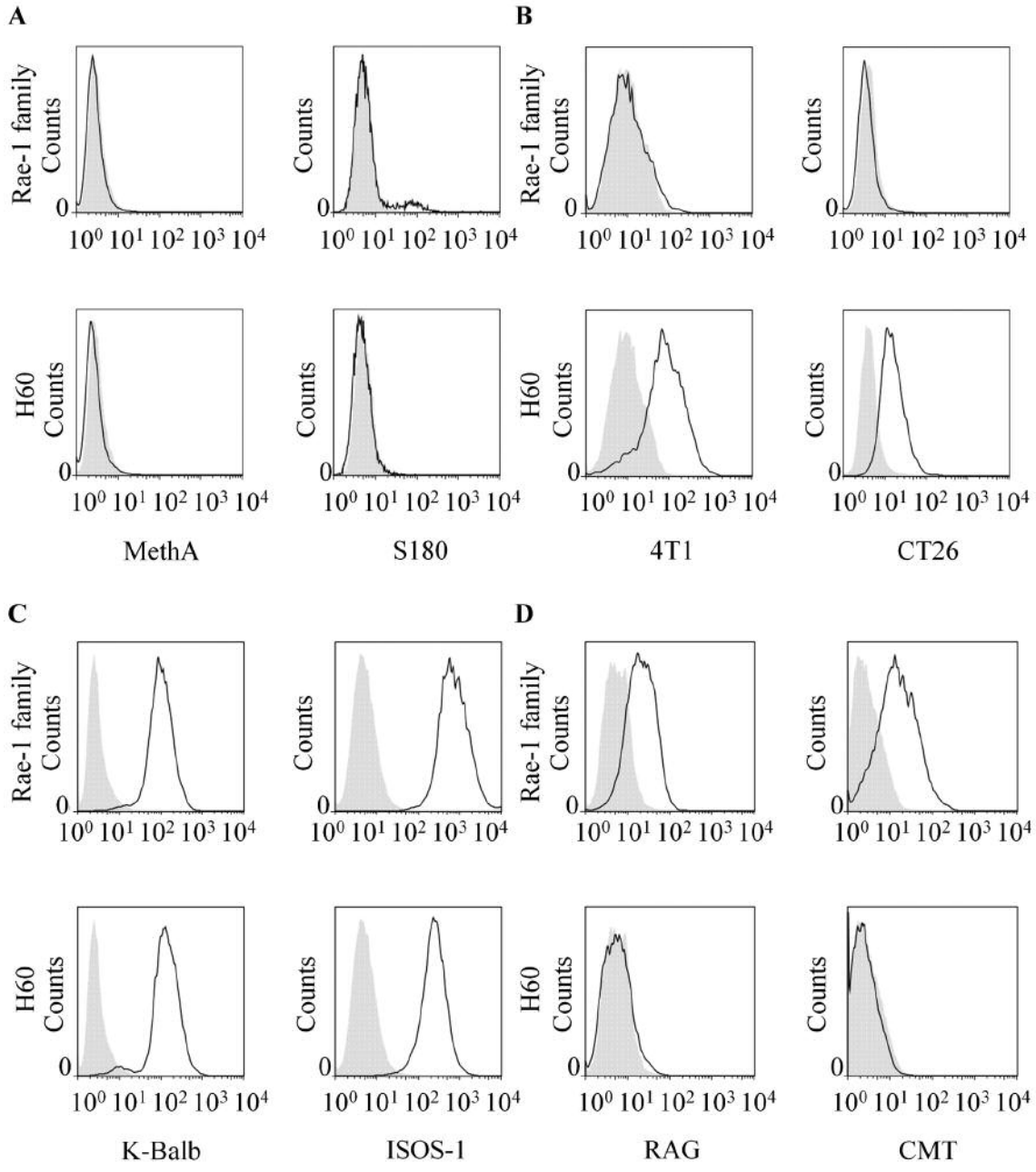

Figure 2. *Expression of NKG2DLs on cancer cell lines. (A) Expression of NKG2DLs; Rae-1 family and H60, on Meth-A and S180 tumor cells. (B) Expression of Rae-1 family and H60 on 4T1 and CT26 tumor cells. (C) Expression of Rae-1 family and H60 on K-BALB and ISOS-1 tumor cells. (D) Expression of Rae-1 family and H60 on RAG and CMT tumor cells.*

NKG2D signaling prevented murine $CD4^+$ T cell-mediated colitis, suggesting that the NKG2D signaling pathway is critically involved in $CD4^+$ T cell-mediated disease progression and thus represents a novel therapeutic target for inflammatory bowel diseases (22). These studies evaluated the interaction between NKG2DLs and NKG2D to determine their relationship with NK cells or with $CD4^+$ T cells and tumors. Although the function of NKG2D in NK cells has been the focus of several studies to date, the mechanism by which $CD8^+$ T cell activation induced by NKG2DLs/ NKG2D interaction exerts antitumor effects has not been fully elucidated. The results in the present study suggest that interaction between NKG2D expressed on $CD8^+$ T cells and NKG2DLs expressed on tumors has a

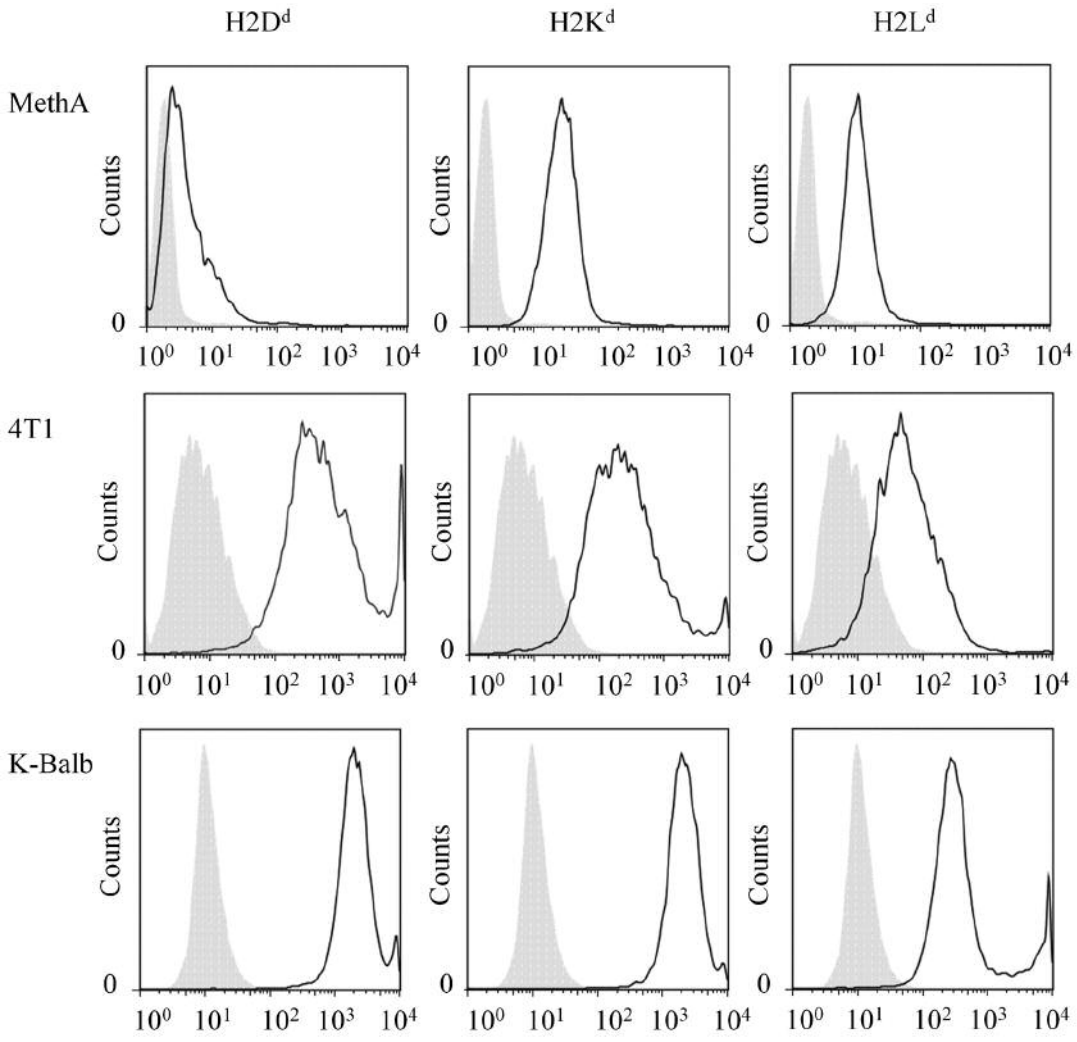

Figure 3. *Expression of MHC Class I molecules in Meth-A, 4T1, and K-BALB cells. Upper row indicates expression of MHC Class I molecules in Meth-A. Middle and lower row indicate expression of MHC Class I molecules in 4T1 and K-BALB respectively. All cell lines express MHC Class I molecules.*

significant effect on tumor immunity. To examine T cell immune activation by NKG2DLs/NKG2D interaction in the tumor micro-environment, we used PSK to stimulate the immune system, instead of using direct T cell stimulators such as anti-4-1BB stimulating antibody (23). The purpose of stimulating the immune system was to determine whether difference in NKG2DL expression on tumor affects the activity of NKG2D expressed on T cells in a stimulated immune system *in vivo*. In mice implanted with NKG2DL-non-expressing Meth-A, PSK treatment resulted in a significant increase in NKG2D expression on $CD8^+$ T cells accompanied by a significant increase in intracellular INF-γ in $CD8^+$ T cells. On the other hand, in mice implanted with NKG2DL-expressing K-BALB, PSK treatment resulted in no significant increase of NKG2D expression on $CD8^+$ T cells and no increase in intracellular INF-γ. These findings demonstrated that $CD8^+$ T cell activation by PSK was dependent on the expression of NKG2DLs on tumor cell lines, indicating that the expression of NKG2DLs had an immunosuppressive effect and contributed to tumor evasion of the immune system. The differences in NKG2DL expression pattern among tumor cells may therefore predict response to PSK therapy. This finding may also have implications for other immunotherapies.

We demonstrated that by implanting the PSK-non-responsive K-BALB tumor seven days prior to implantation of the PSK-responsive Meth-A tumor, the antitumor effect of PSK against Meth-A was lost. These findings suggest that the difference in response to PSK of the two tumors was due to not only the aggressiveness and susceptibility to immune cells of the individual tumor, but also the systemic immune system altered by the tumor cells.

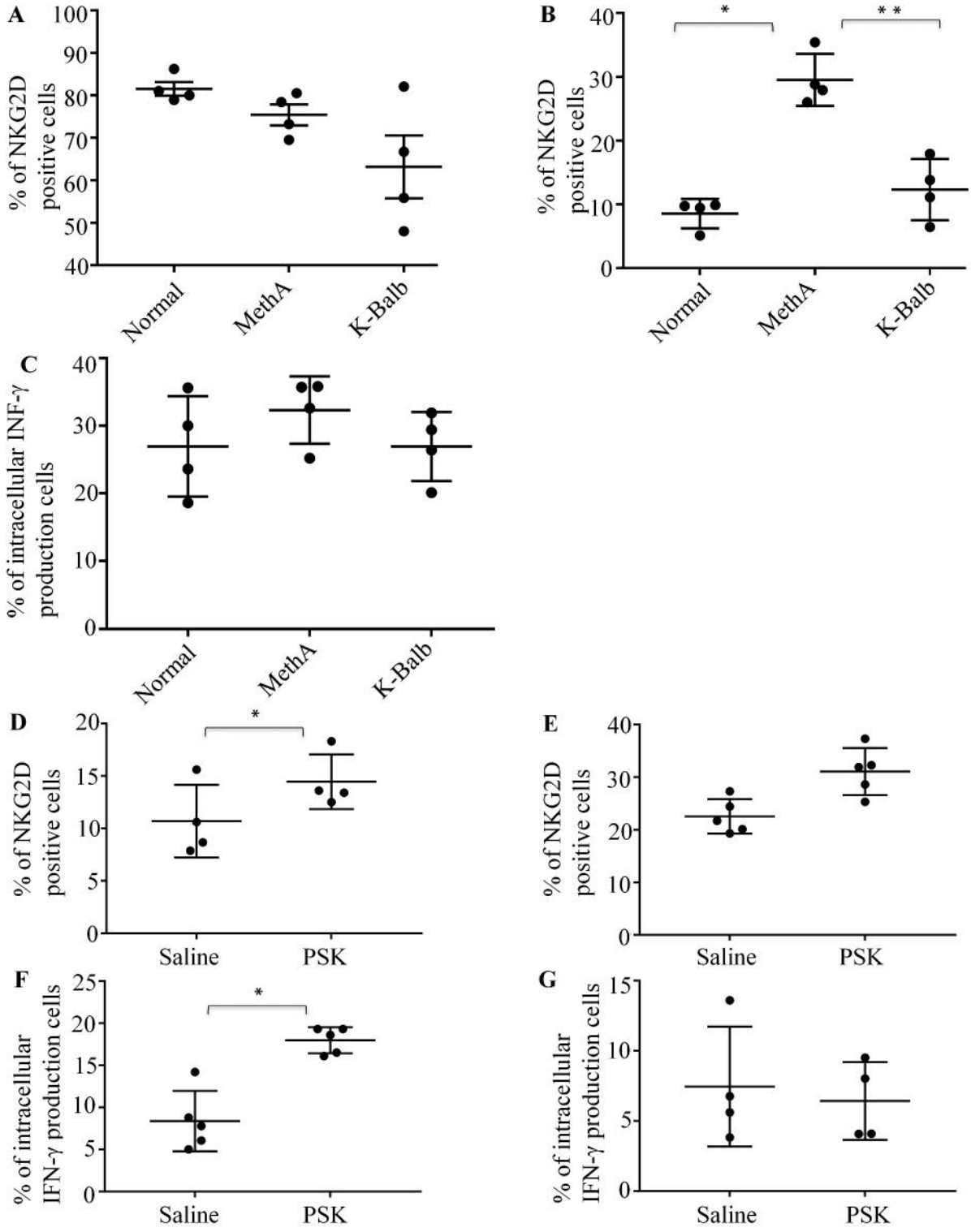

Figure 4. *NKG2D expression and IFN-g production in immune cells. (A) NKG2D expression on CD49b+ NK cells. (B) NKG2D expression on CD8+ T cells (*p<0.0001, **p<0.0004). (C) Percentage of IFN-γ production in CD8+ T cells of mice implanted with Meth-A or K-BALB cell lines. (D) Expression of NKG2D on CD8+ T cells in Meth-A-implanted mice with/without PSK treatment (*p=0.0159). (E) Expression of NKG2D on CD8+ T cells in K-BALB-implanted mice with/without PSK treatment. (F) IFN-γ production in CD8+ T cells in Meth-A-implanted mice with/without PSK treatment. (*p=0.0079). (G) IFN-γ production in CD8+ T cells in K-BALB-implanted mice with/without PSK treatment.*

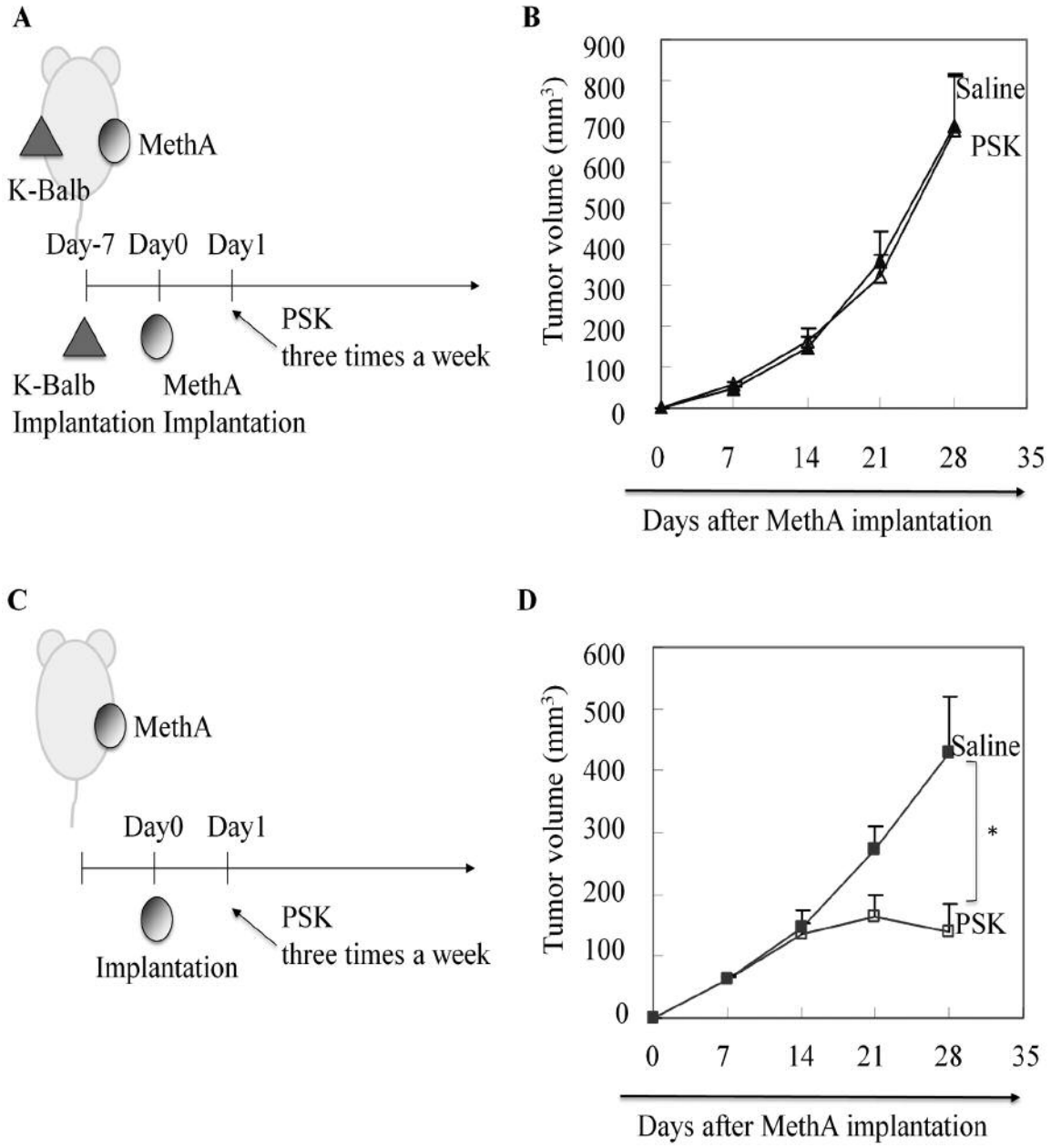

Figure 5. *Effect of PSK in mice implanted with K-BALB and Meth-A cells. (A) Mice were implanted with K-BALB cells 7 days (day -7) before implantation with Meth-A cells (1×10^6 cells per mouse) (day 0) and treated with PSK three times a week from day 1. (B) Antitumor effect of PSK was completely lost, resulting in growth of both tumors. The curve indicates growth of tumor derived from Meth-A cells. (C) Mice were implanted with Meth-A cells alone at 1×10^6 cells per mouse on day 0, and then treated with PSK on day 1 and three times a week thereafter. (D) Effect of PSK on mouse xenograft tumor growth (*p=0.0004).*

In conclusion, we demonstrated that the expression pattern of NKG2DLs affects tumor immunity and the efficacy of immunotherapy in a tumor-bearing mouse model. In particular, the expression of H60 and Rae-1 family members may be an immunosuppressive factor. Further analysis is required to gain a better understanding of the mechanism of interaction between NKG2D expressed on immune effector cells and H60/Rae-1 expression on tumors. Although NKG2DL expression may represent a marker predicting response to immunotherapy, further studies in humans are required to validate this finding in the clinical setting.

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