Soluble NKG2D ligands impair CD8+ T cell antitumor function dependent of NKG2D downregulation in neuroblastoma

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Abstract. T cell-based immunotherapy has achieved remarkable beneficial clinical outcomes. Tumor-derived NKG2D ligands (NKG2DL) allow tumors to escape immunologic surveillance. However, the mechanism underlying NKG2DL-mediated immune escape in neuroblastoma (NB) remains incompletely understood. In the present study, first soluble NKG2DL, soluble major histocompatibility complex (MHC) class-I-related chain A and soluble UL-16 binding proteins expression levels were determined in both the serum from patients with NB and in NB cell line culture supernatants. NB cell-derived sNKG2DL was initially cleaved by ADAM10 and ADAM17. Furthermore, sNKG2DL expression levels were positively correlated with the immunosuppressive microenvironment and poor prognosis. Tumor-derived sNKG2DL induced degradation of NKG2D on $CD8^+$ T cells and impaired $CD8^+$ T cell proliferation, IFN-γ production, and CD107a translocation. More importantly, blockage of sNKG2DL increased the antitumor activity of $CD8^+$ T cells. Thus, the results showed that NB-induced immunosuppression was achieved through tumor-derived sMICA and sULBP-2, and blockage of the tumor-derived sNKG2DLs with sNKG2DL neutralizing antibodies was a novel strategy to recover T-cell function and enhance antitumor immunotherapy.

Introduction

Neuroblastoma (NB) is the most common type of pediatric extracranial neurogenic tumor. The most common location for the primary development of NB is in the adrenal glands. NB can also develop anywhere along the sympathetic nervous system from the neck to the pelvis. The frequencies in different locations are: neck (1%), chest (19%), abdomen (30% nonadrenal), or pelvis (1%). The current standard therapy for high-risk NB consists of 5-7 cycles of high-dose chemotherapy, surgery, autologous stem cell transplantation, radiation therapy, and monoclonal antibody immunotherapy with anti-GD2 antibodies (1,2). These treatment options are fairly toxic and patients still have low survival rates, with NB still accounting for 15% of all childhood cancer deaths (3). NB can result in central nervous system (CNS) metastasis, which makes treatment more difficult. Tumor microenvironment hypoxia and blood-brain barrier (BBB) functioning drive resistance to several cancer therapies (4). Novel transcriptome analysis (5), advanced 2D and 3D *in vitro* models (6), and drug design approaches (7) are thus needed for the improved management of NB. Activating receptor (transmembrane protein) NKG2D belongs to the NKG2 family of C-type lectin-like receptors (CLRs) (8,9). It is typically expressed on natural killer (NK) cells, all activated $CD8^+$ T cells, and certain activated $CD4^+$ T cells (10,11). As the primary activating receptor of NK cells, activating NKG2D can induce cytotoxicity, cytokine secretion, and proliferation of NK cells (12).

T cell surface NKG2D is an effective co-stimulator of T cell receptor (TCR)-mediated effector function and can upregulate antigen-specific T cell-mediated cytotoxic effects against cells or tissues expressing stress-induced NKG2D ligand (NKG2DL), particularly in the presence of suboptimal TCR (13). The NKG2DLs include major histocompatibility complex (MHC) class-I-related chain (MIC) A/B, and the UL-16 binding proteins 1-6 (ULBP1-6) (14), which is typically expressed on 'stressed' cells such as infected or malignant cells. The majority of NKG2DLs are membrane-bound glycoproteins, whereas certain ULBPs are GPI-anchored, which can be shed under certain circumstances (15-17). It has been found that NB can downregulate NKG2DL expression to prevent the recognition and elimination mediated by NK cells (18). Additionally, NKG2DLs released by NB cells can impair NK or T cell-mediated antitumor immunity by promoting the internalization of NKG2D receptors in NK or T cells (19).

Adoptive transfer cell therapy with chimeric antigen receptor (CAR)-T cells is a relatively novel and promising approach for the management of NB (20). First-generation

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CAR-T cells have been used in two clinical studies in patients with NB (21,22). CAR-NK cells also have been assessed, and they were shown to achieve increased killing of tumor cell lines *in vitro* (23). Indeed, the efficacy of CAR-T/NK-cell therapy in solid tumors depends on the local infiltration efficiency of CAR-engineered cells, the tumor immunosuppressive microenvironment, and the long-term viability of these CAR cells. Considering the T-cell immune suppressive conditions induced by NKG2DL release, the clinical efficacy of CAR-engineered cells in NB tumors may likely be inhibited.

In the present study, it was shown that high levels of soluble MICA and ULBP2 (NKG2DLs that activate the NK cell receptor NKG2D) in the sera of patients with NB was closely associated with poorer outcome and T cell exhaustion. Additionally, these tumor-derived NKG2DLs were cleaved by a disintegrin and metalloproteinases (ADAM)10 and ADAM 17. Consequently, sNKG2DLs impaired the proliferation, IFN-γ production, and cytotoxicity of $CD8^+$ T cells through NKG2D receptor. The blockade of these tumor-derived sNKG2DLs increased $CD8^+$ T cells antitumor activity, highlighting a novel potential strategy for improving T cell-mediated immunotherapy.

Materials and methods

Patient samples. Peripheral blood from patients with NB was collected from 35 stage IV NB patients pre and post-surgery between January 2021 and December 2021. Of the 35 patients, 24 were male and 11 were female, and the mean age at diagnosis was 26.11±9.13 months (range, 9-43 months). Gross total resection was achieved in 27 cases and subtotal resection in 8 cases. Peripheral blood was equally obtained from 10 healthy control patients (range, 22-32 months; mean age, 25.4±3.23 months) with benign pediatric surgical disease (oblique inguinal hernia) at the Children's Hospital of Fudan University (Shanghai, China) between January 2021 and December 2021.

Mononuclear cells were isolated using a Lymphocyte Separation Medium 1077 (PromoCell GmbH) from peripheral blood. Fresh or cryopreserved mononuclear cells were used for immunophenotype analysis. Plasma was used for the analysis of soluble NKG2D ligands.

The use of human material was approved (approval no. 2020238) by the Local Ethics Committee of Fudan University (Shanghai, China).

Cell culture. Flow cytometry was used to analyze the cell purity of isolated subpopulations, and the T cell purity was >95%. T cells were re-suspended in complete medium at a density of $1x10^6$ cells/ml. For immune function analysis, T cells were cultured with anti-CD28 (2 μg/ml) and IL-2 (10 U/ml) in 24 well plates (pre-coated with 5 μg/ml anti-CD3). Before detection and treatment, T cells were harvested, washed twice with PBS, and counted.

NB cell lines, including SH-SY5Y, SK-N-BE (2), and SK-N-SH, were purchased from the Cell Bank of the Chinese Academy of Science. SH-SY-5Y cell lines were authenticated by short tandem repeat (STR) testing provided by the Cell Bank of the Chinese Academy of Science. The other two cell lines, CHLA-15 and LA-N-5 cells were a gift from Professor Mujie Ye of Fudan University. All NB cells were cultured under the same conditions: DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and maintained in a 37˚C humidified incubator supplied with 5% CO_2.

Flow cytometry. NB tumor cells ($1x10^6$) were stained with unconjugated antibodies (1:100) according to the manufacturer's instructions: MICA/B, ULBP1-3 (cat. nos. ab54413, ab256515, ab88645 and ab280087, respectively; all from Abcam), ADAM10, ADAM17 (cat. nos. ab252234 and ab57484; both from Abcam) or with the appropriate isotype-matched control IgG (cat. no. ab37355; Abcam).

T cells were stained for the surface markers CD3, CD8, CD4, CD25, CD45RO, CD69, CCR7, NKG2D, CD107a, PD-1, and TIM-3 (cat. nos. 11-0038-42, 12-0088-42, 25-0049-42, 17-0257-42, 17-0457-42, 12-0699-42, 46-1979-42, 12-5878-42, 53-1079-42, 46-9969-42 and 17-3109-42; all from eBioscience; Thermo Fisher Scientific, Inc.), and the intracellular markers Foxp3, IFN-γ, and Ki67 (cat. nos. 12-4776-42, 12-7319-42 and 11-5698-82; all from eBioscience; Thermo Fisher Scientific, Inc.) with fluorescence-conjugated antibodies according to the manufacturer's instructions (eBioscience; Thermo Fisher Scientific, Inc.). For flow cytometric analysis, cells were counted, preincubated with an Fc blocker (cat. no. 14-9161-73; eBioscience; Thermo Fisher Scientific, Inc.) for blocking non-specific Fc receptors, then incubated with conjugated antibodies (1:100) in the dark at 4˚C for 30 min for surface marker analysis. For the intracellular cytokine analysis, cells were pre-stimulated with Cell Stimulation Cocktail (cat. no. 00-4975-93; eBioscience; Thermo Fisher Scientific, Inc.) *in vitro*. After stimulation for 5 h at 37˚C, an Intracellular Fixation and Permeabilization Buffer Set was used for intracellular staining and flow cytometry analysis (eBioscience; Thermo Fisher Scientific, Inc.). For cell markers, $CD3^+CD4^+CD25^+Foxp3^+$ was used to characterize regulatory T cells (Tregs), $CD3^+CD4^+$ was used to characterize $CD4^+$ T cells, and $CD3^+CD8^+$ was used to characterize $CD8^+$ T cells. The cells were examined by flow cytometry (BD FACSCanto™ II; BD Biosciences) and the data were analyzed using FlowJo software (version 10.0; FlowJo LLC).

ELISA. NB tumor cells ($1x10^6$/ml) in 2-ml complete medium were cultured in a six-well culture plate for 48 h as aforementioned. Then, tumor cell-free supernatant (Tumor sup) was obtained by centrifugation at 12,000 g at 4˚C for 10 min. Soluble MICA and ULBP1-3 levels were determined by ELISA (EHMICA; cat. nos. EH476RB, EH477RB, and EH478RB; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Inhibition or activation of ADAMs. Tumor cell lines were treated for 24 h with the ADAM10 inhibitor GI254023X (0, 10, 20, 30, or 40 μM), ADAM17 inhibitor TAPI-1 (0, 15, 30, or 60 μM), or both. The ADAM promotor PMA (0, 2.5, 5, or 10 ng/ml) was added for 40 min before collecting supernatants and tumor cell lines. GI254023X (cat. no. A4436), TAPI-1 (cat. no. B4686), and PMA (cat. no. N2060) were purchased from APeXBIO Technology LLC. Soluble NKG2DLs (sMICA, sULBP2, and sULBP3) levels were measured by ELISA in

the supernatants. The expression levels of NKG2DLs on tumor cells (MICA/B, ULBP2, and ULBP3) were assessed by flow cytometry.

T-cell function assays. T cells were resuspended in complete medium with a density of $1x10^6$ cells/ml and were cultured with anti-CD28 (2 μg/ml) and IL-2 (10 U/ml) in a 24-well plate pre-coated with 5 μg/ml anti-CD3. Subsequently, T cells were co-cultured with or without 100 μl serial diluted tumor sup for 24 h. For neutralizing sNKG2DL, single and combined soluble NKG2DL neutralizing antibodies (1, 2, or 5 μg/ml anti-MICA/B, ULBP1, ULBP2, and/or ULBP3) were added to T cells and to the tumor cell supernatant co-culture system. Then, these T cells were harvested and prepared for flow cytometric analysis. Neutralized antibodies (anti-MICA/B, anti-ULBP1, anti-ULBP2, and anti-ULBP3) were purchased from R&D Systems, Inc. (cat. nos. MAB13001, MAB1380, MAB1298 and MAB1517, respectively).

Statistical analysis. Normally distributed data are presented as the mean ± SD, whereas for non-normally distributed data, the median and interquartile range (IQR) are used. Clinically relevant immune parameters were analyzed using a Mann-Whitney non-parametric test. NKG2D/NKG2DL expression levels on the cell surface, sNKG2DL levels in the tumor sup and in the patient's plasma, immune parameters of T cells were analyzed using a one-way ANOVA followed by Tukey's post hoc test. GraphPad Prism version 6.0 (Dotmatics) was used for all analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Tumor-derived sNKG2DLs are initially cleaved by ADAM10 and ADAM17. To investigate the role of NKG2DLs, first ELISA kits were used to detect the levels of sMICA, and sULBP2 in serum samples from 35 patients with NB and 10 healthy donors. sMICA levels in the serum from NB patients were 269.2±88.3 pg/ml compared with 3.94±0.73 pg/ml in healthy donors (Fig. 1A). sULBP2 was 42.61±13.63 compared with 4.69±0.87 in healthy donors (Fig. 1A). There was a positive association between sMICA with sULBP2, indicating that immune escape in NB involves multiple soluble NKG2D ligands (Fig. S1). Based on the Mann-Whitney U test, there was a statistically significant difference between patients with NB and healthy donors in the concentrations of sMICA and sULBP-2 ($P<0.0001$; Fig. 1A). Consistently, the expression of MICA/B and ULBP 1-3 was detectable in NB cell lines, SH-SY-5Y, CHLA-15, SK-N-BE (2), LA-N-5, and SK-N-SH using flow cytometry (Fig. 1B). Additionally, the presence of sMICA and sULBP 1-3 in culture supernatants from the NB cell lines was assessed using ELISA. After 48 h of culture, there were high levels of sMICA, sULBP-2, and sULBP-3 detected in the supernatants of SH-SY-5Y, SK-N-BE (2), and LA-N-5 (Fig. 1C). sULBP-1 levels were lower in all cell lines (Fig. 1C). Additionally, the surface expression of ADAM10 and ADAM17 was assessed in all NB cells using FACS analysis (Fig. 1D).

To evaluate whether ADAM10 and ADAM17 expression was associated with sNKG2DL release, SH-SY-5Y NB cells were treated with selective ADAM10 or ADAM17 promoters and inhibitors.

SH-SY-5Y cells were cocultured with the ADAM10 inhibitor GI-X (10-40 μM), ADAM 17 inhibitor TAPI-1 (15-60 μM), or the solvent alone (DMSO) for 24 h. Then, the supernatant was collected and sMICA, sULBP2, and sULBP3 levels were assessed using ELISA. For enhancing ADAM analysis, the ADAM promotor PMA was added and cells were co-cultured for 40 min before the supernatant and tumor cells were collected for MICA/B, ULPB2, and ULBP3 expression analysis on the tumor cell surface using FACS. PMA enhanced the enzymatic effects of ADAM10 and ADAM17, thus inducing the releasing of sNKG2DLs into the supernatant and shielding NKG2DLs on the SH-SY-5Y cell surface (Fig. 1E and G). The combined ADAM10 inhibitor and ADAM 17 inhibitor could inhibit the shedding of NKG2DLs and increase its cell surface expression in NB cells in a concentration-dependent manner (Fig. 1F and G). Neither ADAM10 inhibitors GI-X nor ADAM 17 inhibitors TAPI-1 could produce similar effects (Fig. 1F). Thus, the production of sNKG2DL is a characteristic of primary NB and NB cell lines, and these tumor-derived sNKG2DLs were cleaved by ADAM10 and ADAM17 allowing its release into the supernatant.

sNKG2DLs levels are negatively correlated with patient prognosis and the low-exhausted phenotype of T cells. Serum samples from patients pre- and post-surgery, and the levels of both sMICA and sULBP-2 levels were determined. Compared with the pre-operative and subtotal resection (STR) groups, sMICA and sULBP-2 levels were significantly lower in the gross total resection (GTR) group (Fig. 2A), indicating that MICA/B and ULBP-2 expression levels may be negatively associated with patient prognosis. Next, given that both MICA/B and ULBP-2 were immune-related molecules, whether the decreased expression of sULBP2 and sMICA affected the immune pattern of patients was assessed. Peripheral blood mononuclear cells were isolated from patients after standard treatment and stained with different immunophenotypic markers. There was no difference in the percentages in Tregs, B cells, NK cell, and NKT lymphocyte subpopulations percentages between the GTR and STR groups (data not shown). However, there was a significant decrease in the percentage in $CD3^+$ T cells in the STR group compared with that in the GTR group (Fig. 2B). On further analysis of IFN-γ^+CD4^+ and IFN-γ^+CD8^+ T cell subpopulations, significantly increased percentages of PD-1^+, and Tim-3^+ T cell subpopulations were found in the STR group compared with the GTR group (Fig. 2C). These results imply that downregulation of sULBP2 and sMICA, and the upregulated frequency of T cells with a low-exhausted phenotype were associated with each other.

sNKG2DLs impair $CD8^+$ T cell function and memory formation. In humans, NKG2D was reported to be expressed on several types of lymphocytes, including activated NK, NKT, and $CD8^+$T cells (15). To evaluate whether soluble NKG2DLs separately affected $CD8^+$ T cells and whether this was associated with the NKG2D receptor, NKG2D expression was analyzed using FACS. After culturing cells with different concentrations of tumor cell supernatants for 12 h,

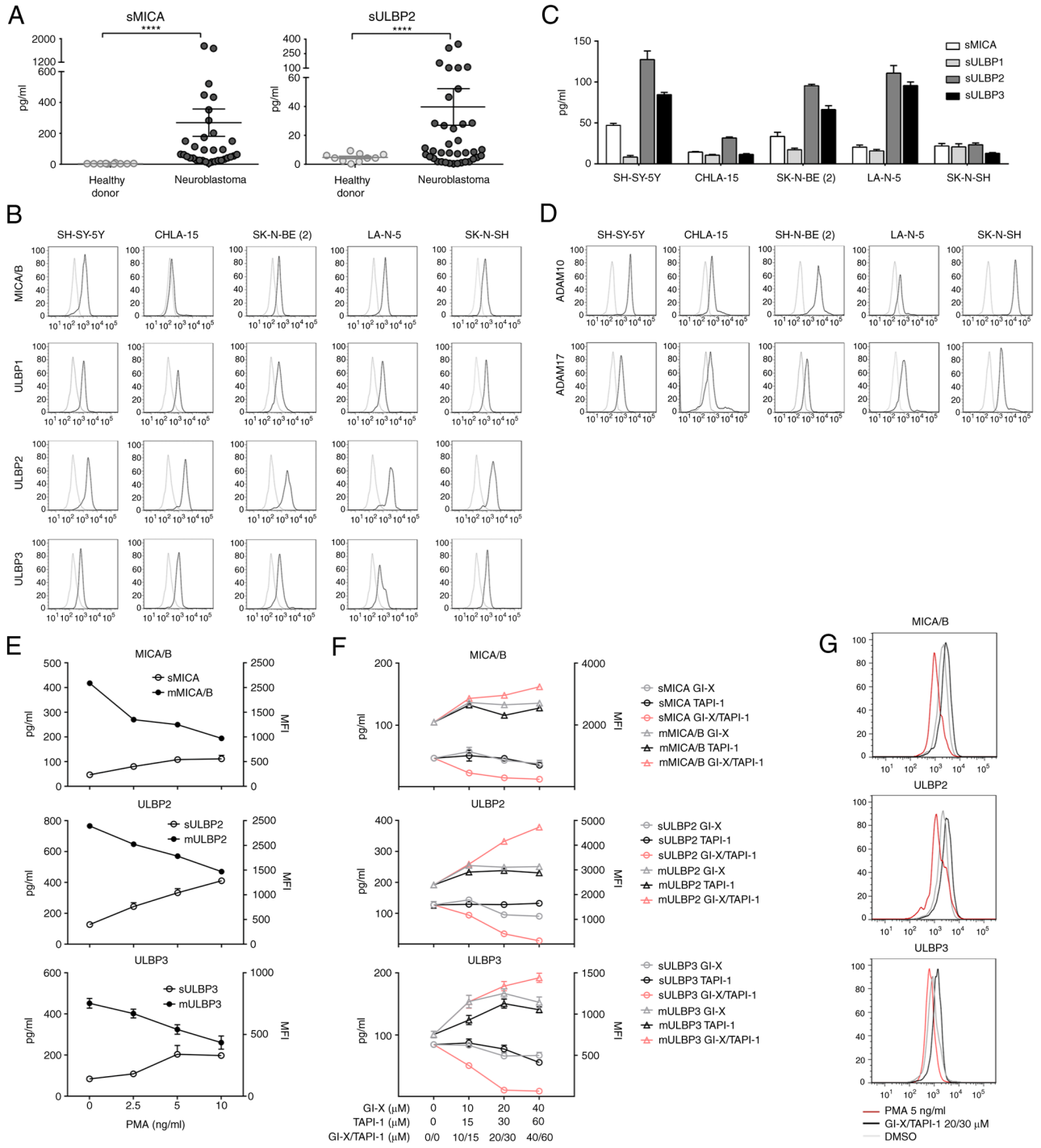

Figure 1. Tumor-derived sNKG2DLs are shed by ADAM10 and ADAM17. (A) Detection of sMICA and sULBP-2 levels by ELISA in serum samples from 35 patients with NB and 10 healthy controls. (B) MICA/B and ULBP1-3 expression in the NB cell lines were assessed using flow cytometry. (C) After 2 days of culture, sMICA and sULBP1-3 production in the culture supernatant was assessed using ELISA. (D) Cell surface expression of ADAM10 and ADAM17 in NB SH-SY-5Y cells was analyzed by flow cytometry. (E-G) SH-SY-5Y cells were pretreated with the ADAM10/ADAM17 promotor PMA, ADAM10 inhibitor GI-X, or ADAM17 inhibitor TAPI-1 for 24 h. Cell surface MFI of MICA/B and ULBP2 was detected by flow cytometry. The levels of sMICA and sULBP2 in the culture supernatant were analyzed by ELISA. Data are presented as the mean ± SEM. P-values were determined using a Mann-Whitney U test in (A). ****P<0.001. s, soluble; ADAM, a disintegrin and metalloproteinase; NB, neuroblastoma; MFI, mean fluorescence intensity.

NKG2D receptor downregulation was observed in the CD8$^+$ T cells (Fig. 3A), and the degree of NKG2D downregulation was associated with the supernatant concentration (Fig. 3A). To determine whether NKG2D downregulation affected CD8$^+$ T cell function, human T cell function was assessed after culturing with the different concentrations of supernatants from the NB cells for 12 h. When separately assessing CD8$^+$ and CD4$^+$ T lymphocyte presence, it was observed that sNKG2DL selectively downregulated CD8$^+$ T cell function, including proliferation, IFN-γ production and cytotoxicity (Fig. 3B). The percentage of IFN-γ$^+$, Ki67$^+$, and CD107a$^+$ in CD8$^+$ T cells was significantly decreased, particularly in the cells treated with the higher concentrations of tumor culture supernatant (Fig. 3B).

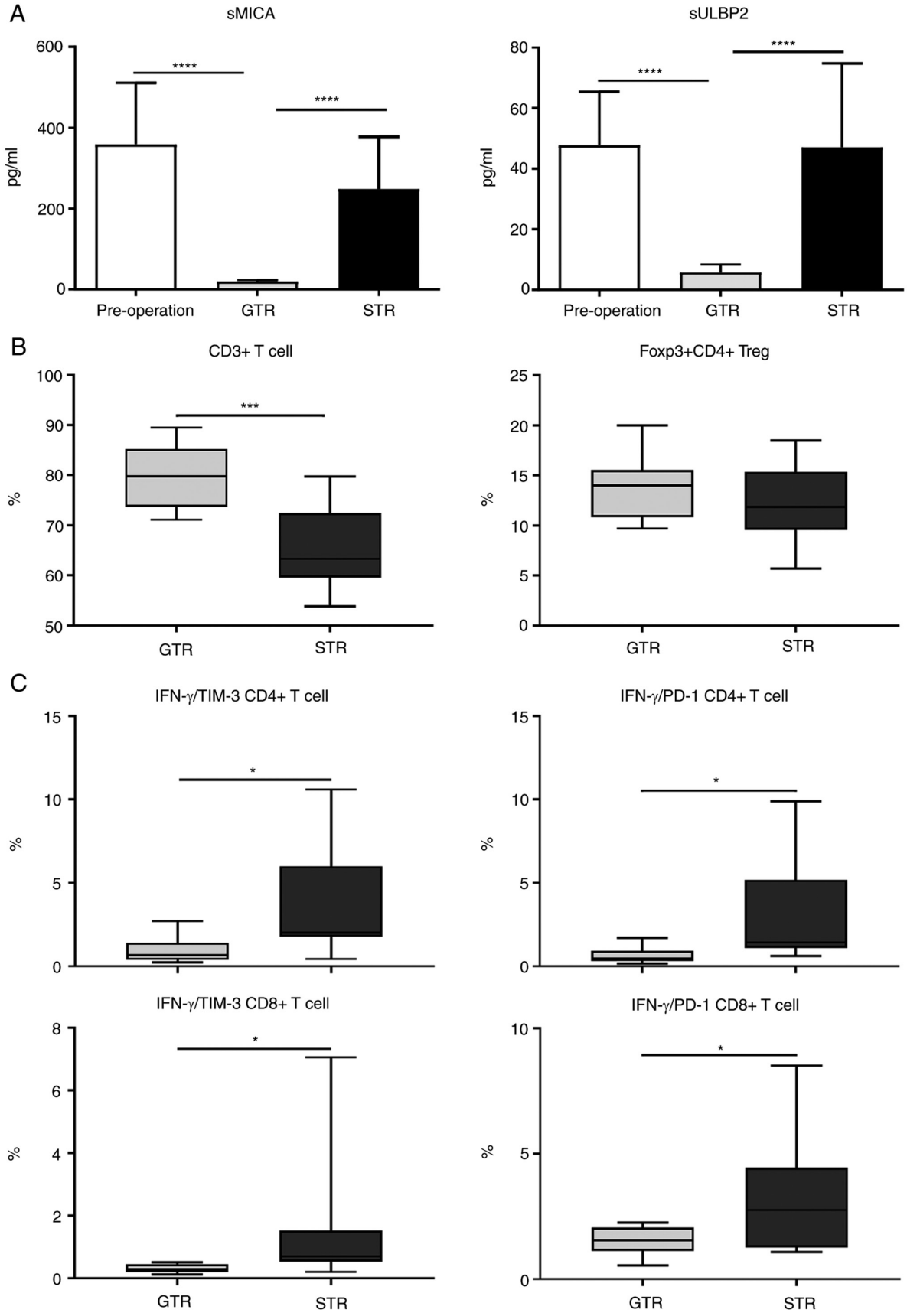

Figure 2. The levels of sNKG2DLs are negatively correlated with patient prognosis and the low-exhausted phenotype of T cells. (A) sMICA and sULBP-2 levels in serum samples from patients prior to operation, and in the GTR and STR patients after surgery were measured by ELISA. (B) The percentage of T cells and Tregs derived from PBMCs of the GTR and STR patients were determined using flow cytometry. (C) The expression of inhibitory receptors PD-1 and TIM-3 in IFN-γ^+CD4$^+$ or CD8$^+$ T cells derived from the PBMCs of the GTR and STR patients were determined using flow cytometry. Data are presented as the mean ± SEM. P-values were determined by one-way ANOVA in (A) and a Mann-Whitney U test in (B and C). *P<0.05, ***P<0.001 and ****P<0.0001. s, soluble; MICA, major histocompatibility complex (MHC) class-I-related chain A; ULBP, UL-16 binding protein; GTR, gross total resection; STR, subtotal resection; Tregs, regulatory T cells; PBMCs, peripheral blood mononuclear cells.

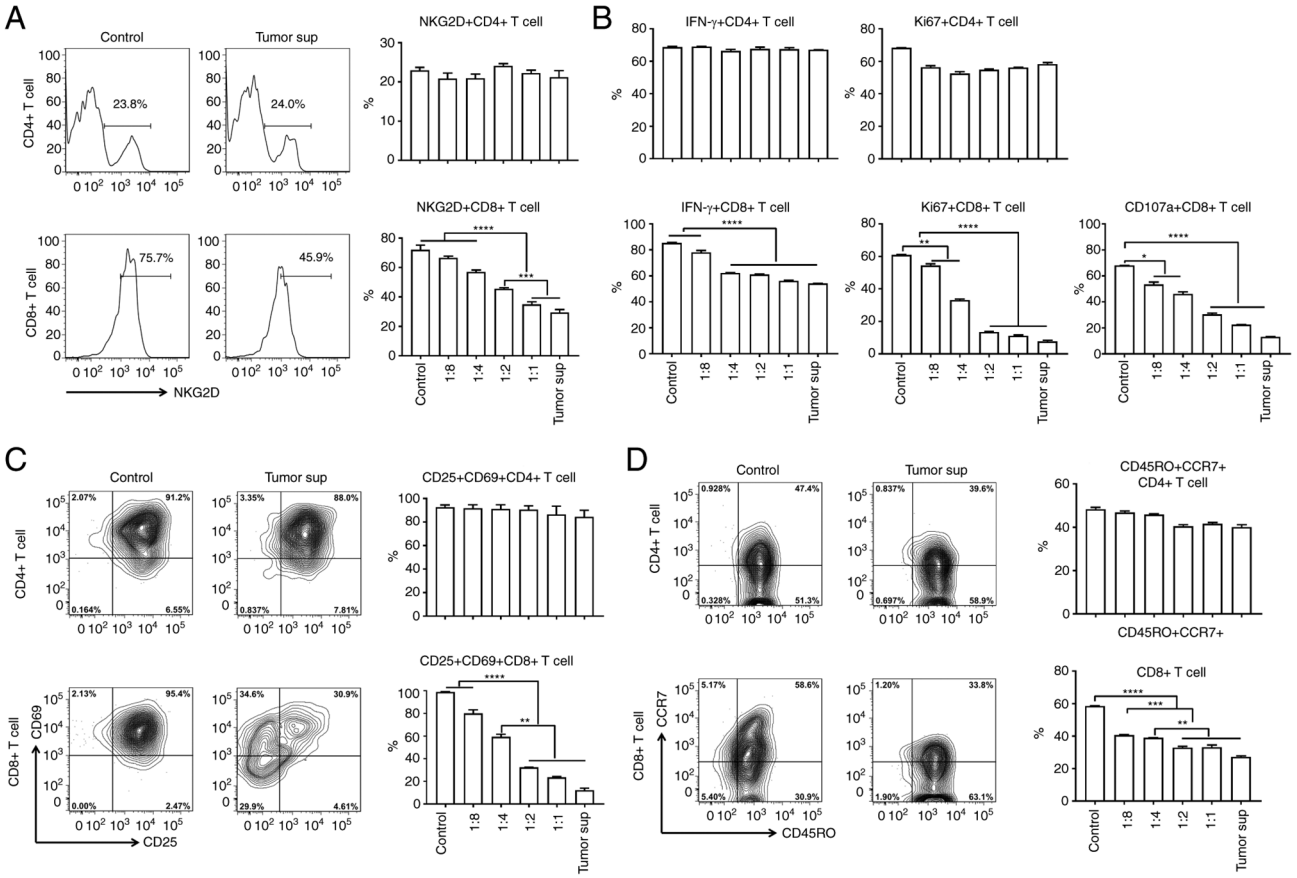

Figure 3. sNKG2DLs impair CD8$^+$ T cell function and memory formation. (A) Human T cells derived from healthy donors were activated and treated with different concentrations of the tumor cell culture supernatant for 12 h. NKG2D expression was detected using FACS. (B) Intracellular IFN-γ production, Ki67 expression and CD107a translocation of CD8$^+$ T cells were detected by flow cytometry. (C and D) Surface markers (CD25, CD69, CD45RO, and CCR7) of CD8$^+$ T cells were also analyzed by FACS. Data are presented as the mean ± SEM. P-values were determined using a one-way ANOVA in (A-D). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. s, soluble.

NKG2D has been shown to act as a co-stimulatory receptor in CD8$^+$ T cells, and was demonstrated to be involved in the function of CD8$^+$ T cell memory formation (11). Thus, whether T cells treated with sNKG2DL exhibited reduced effector functions and memory formation was assessed. Cytofluorimetric analysis of the expression of the early (CD69$^+$) and late activation markers (CD25$^+$) on T cells was performed in T cells treated with sNKG2DL. The cultured T cell memory profiling was defined based on CD45RO and CCR7 expression. The percentage of activated CD8$^+$ T cells (CD69$^+$CD25$^+$) was significantly decreased in cells treated with the high concentration of tumor cell supernatants (Fig. 3C), indicating that there was an inverse correlation between tumor cell supernatant concentration and CD8$^+$ T cell activation. A similar trend was observed in memory formation of CD8$^+$ T cells. The difference in the concentration of the tumor cell supernatants induced a decrease in the percentage of CD45RO$^+$CCR7$^+$ memory CD8$^+$ T cells (Fig. 3D). Activation and memory profiling of CD4$^+$ T cells was not affected. Taken together, sNKG2DLs impaired the antitumor effects and memory formation of CD8$^+$ T cells, including cell activation, proliferation, cytokine production and cytotoxicity.

Blockage of tumor-derived sNKG2DLs results in increased antitumor function of T cells. It was found that the combination of anti-MICA/B and ULPB2-3 increased NKG2D receptor expression on CD8$^+$ T cells, suggesting a cooperative therapeutic effect of targeting all the different types of sNKG2DLs (Fig. 4A and B). Furthermore, to investigate whether targeting sNKG2DLs could result in the recovery of CD8$^+$T cell function, a single and combined sNKG2DL neutralizing antibody was added to the T cells treated with sNKG2DL. Therapy with the single anti-ULBP2 antibody significantly increased the percentage of IFN-γ$^+$, Ki67$^+$, and CD107a$^+$ in CD8$^+$ T cells, whereas the use of the anti-MICA/B or anti-ULBP3 antibodies alone did not have a notable impact (Fig. 4C and D). Combined therapy with all the sNKG2DL neutralizing antibodies resulted in a significant increase in the antitumor function of CD8$^+$ T cells compared with the monotherapy (Fig. 4C and D). Therefore, blockage of the tumor-derived sNKG2DLs resulted in the recovery of CD8$^+$ T function and this may serve as a novel tumor immunotherapy approach.

Discussion

Immune cell-based antitumor therapy has achieved incredible clinical outcomes, and tumor-derived NKG2DLs are a key factor involved in immunosuppression to facilitate tumor cell escape from antitumor immunity. However, the underlying mechanisms require further elucidation. In the present study,

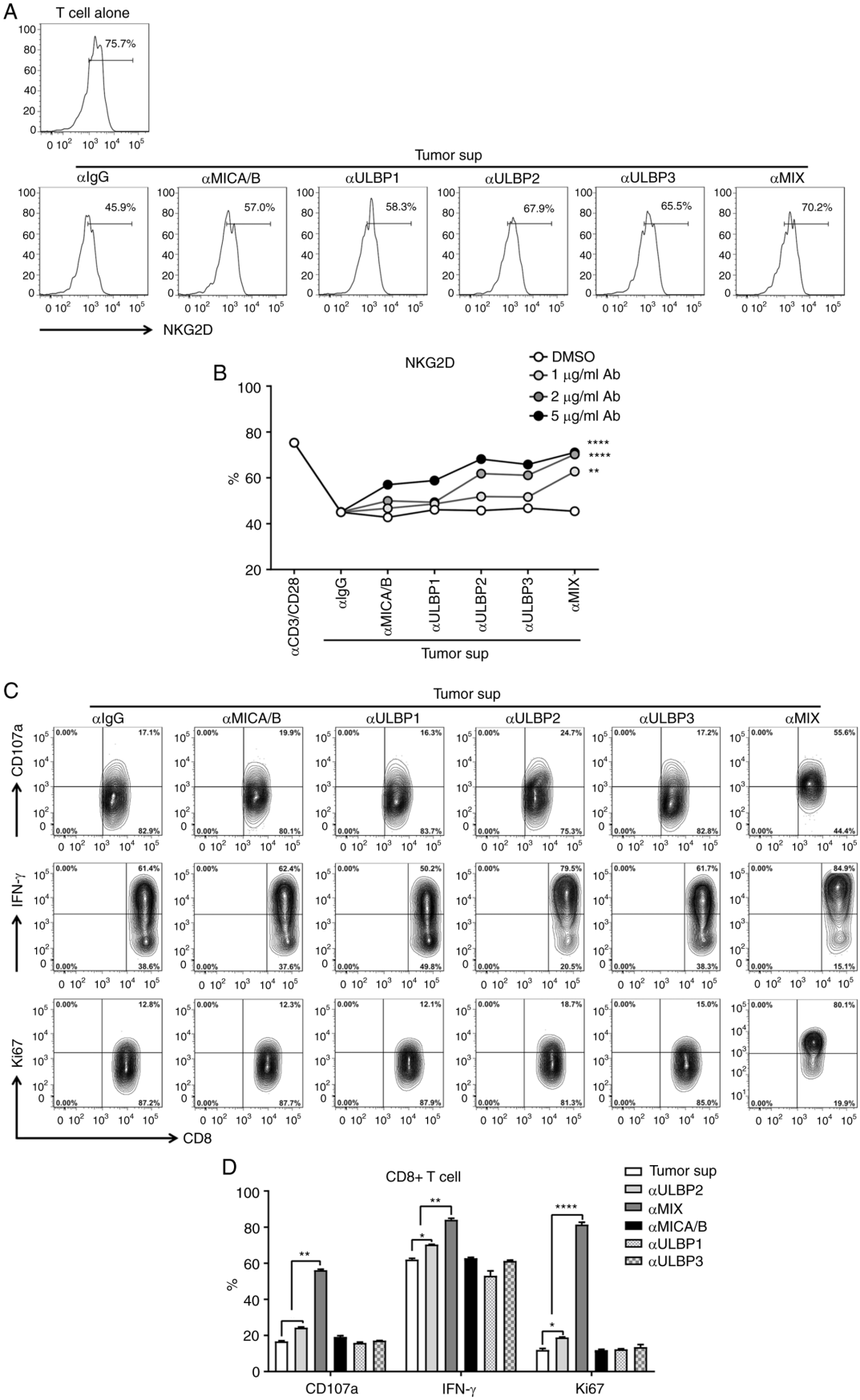

Figure 4. The blockage of tumor-derived sNKG2DLs results in the recovery of the antitumor function of T cells. (A and B) Human T cells derived from healthy donors were activated and treated with tumor cell culture supernatant for 12 h. Then, these T cells were treated with neutralizing antibodies against a single or several sNKG2DLs (anti-MICA/B and/or ULBP1~3). After 2 h of treatment, cell-surface expression of NKG2D was analyzed on $CD8^+$ T cells by FACS. (C and D) After an additional 10 h of treatment, intracellular IFN-γ production, Ki67 expression, and CD107a translocation of $CD8^+$ T cells were detected by flow cytometry. Data are presented as the mean ± SEM. P-values were determined using a one-way ANOVA in (B and D). $^*P<0.05$, $^{**}P<0.01$ and $^{****}P<0.0001$. s, soluble; MICA, major histocompatibility complex (MHC) class-I-related chain A; ULBP, UL-16 binding protein.

using both clinical data and *in vitro* analysis, it was found that the immunosuppressive effects of NKG2D and its soluble ligands on $CD8^+$ T cells were achieved by impairing $CD8^+$ T cell activation, proliferation, IFN-γ production, cytotoxicity and memory formation.

There were several mechanisms in NB immune escape of immune surveillance mediated by NK cells, including increasing the expression of ligands for inhibitory receptors and downregulating the expression of ligands for activation of NK cell receptors. The ligands for inhibitory receptors include PD1L for PD1 (24) and human leukocyte antigen-E (HLA-E) for NKG2A (25). Ligands that reduced activation of NK cell receptors include PVR and Nectin-2 for DNAM1 (26), CD137L (4-1BBL) for 4-1BB (27), and soluble MICA/B and ULBPs for NKG2D. These soluble molecules not only passively block NKG2D but also downregulate NKG2D on the surface of NK cells by inducing receptor internalization. These effects eventually lead to a decline in NK cell function (28). Diefenbach *et al* (29) indicated that NKG2D served a costimulatory function in $CD8^+$ T cells. It has also been revealed that the effect of NKG2D has also been contested with regard to $CD8^+$ T cells, and the impact of sNKG2DL on antitumor function in $CD8^+$ subtype T cells has not been elucidated. Additionally, the relevance of NKG2DLs in NB and NKG2D on T cell function have not been determined. A previous study reported that MICA/B in sera of most patients with NB and the downregulated NKG2D expression on the surface of CD8, after which the reduced cytotoxicity of IL-2-activated NK cells against $MICA^+$ target cells was shown (30). Based on analysis of the sera of patients with NB in the present study, it was found that MICA/B and ULBP-2 were the major sNKG2DLs present in patients with NB, characterized by the high concentration of sMICA and sULBP2 in the patient's plasma. Cell surface expression of MICA/B and ULBP1, 2 and 3 for activation of the NK cell receptor NKG2D, was observed in the majority of NB tumor cell lines, whereas high levels of sMICA, sULBP-2, and sULBP-3 were detected in the supernatants of SH-Y5Y, SK-N-BE (2), and LA-N-5 cells accompanied by low levels of sULBP-1 was observed in all the NB cell lines. sMICA has been used as a prognostic marker in certain solid tumors, showing a positive association between expression and a poor prognosis (31). In the present study, a similar phenomenon was observed in that sMICA and sULBP2 were associated with an immunosuppressive microenvironment in patients with NB, and this was indicative of a poor clinical prognosis.

NKG2D ligands are cleaved by several proteases for shedding, including different members of the metalloprotease family and ADAM family (32). Amongst the members of the ADAM family, ADAM10 and ADAM17 are involved in the migration and differentiation of tumor cells through cleavage of Notch (33). While direct ADAMs knockout may be reminiscent of the restoration of microRNA (miR). In hepatocellular carcinoma, ADAM17 knockout restored miR122 both *in vitro* and *in vivo*. In gastric cancer, modulating the miR-140/ADAM10 axis promoted GC cell proliferation and invasion. The net effect of MicroRNA restoration represented an overall response from all target genes and produced extensive impact. Previous studies indicated that knockout of ADAM10 and ADAM17 decreased tumor growth and invasiveness, and altered the behavior of the tumorigenic cells (34,35). The relevant functions of ADAM10 and ADAM17 in NKG2DLs cleavage remain undefined (36,37). In glioblastoma initiating cells, ADAM10 and ADAM17 have been found to be involved in the process of ULBP-2 cleavage, and the ADAM inhibitor significantly increased the secretion of IFN-γ by NK cells (38). In Hodgkin's lymphoma cells, ADAM10 showed higher specificity for the shedding of MICA/B and ULBP-3 over ADAM17 (39). Inhibition of ADAM10 and ADAM17 with small-molecule inhibitors is a common experimental method used in this type of research and without altered the tumorigenic behavior of the tumor cells. ADAM10 and ADAM17 small-molecule inhibitors also could be used to develop specific therapeutic applicability (40,41). Thus, in the present study, by inhibiting ADAM10 and ADAM17 with small-molecule inhibitors, it was shown that both ADAM10 and ADAM17 contributed to the cleavage of MICA/B, ULBP2, and ULBP3 from NB cells.

It has been reported that sNKG2DL suppressed NK cell antitumor function by disturbing its peripheral maintenance and activation (42). In preclinical models, it has been shown that NK cell function is enhanced by preventing sNKG2DL release (43). sNKG2DL was shown to induce immune-suppressive effects through other mechanisms, including destabilization of CD3ζ in TCR/CD3 pathways (44), and increasing the percentage of MDSCs and tumor-associated macrophages (45). Consistently, in the present study it was revealed that sMICA and sULBP-2 selectively downregulated NKG2D expression on the surface of $CD8^+$ T cells and impaired the proliferation, IFN-γ production, and cytotoxicity of these T cells. In a mouse model, it was identified that NKG2D malfunction critically impaired the effector response of tumor-specific memory T cells to NKG2DL-expressing tumors (46). With regard to the NKG2D receptor, it has been reported to serve as a costimulatory molecule responsible for memory formation in $CD8^+$ T cells (47), and in the current study it was reported that sNKG2DL significantly downregulated the percentage of activated $CD8^+$ T cells ($CD69^+CD25^+$) and $CD45RO^+$ $CCR7^+$ memory $CD8^+$ T cells.

In humans, NKG2D is abundantly expressed on the surface of NK cells, activated $CD8^+$ T cells, certain activated $CD4^+$ T cells, and other subtypes of T cells, including NKT and Tregs (10). In the present study, >75% of $CD4^+$ T cells did not express NKG2D *in vitro* after anti-CD3 and anti-CD28 treatment. Thus, there was no change in cytokine secretion (IFN-γ), proliferation (Ki67), activation (CD69 and CD25), and memory phenotypes (CCR7 and CD45RO) of $CD4^+$T cells after treatment with the tumor cell culture supernatant containing sNKG2DL *in vitro*. However, in the $CD4^+$ T cells derived from patients with NB, it was revealed that the high levels of sNKG2DLs (sMICA and sULBP2) were accompanied by a decrease in the frequency of $CD4^+$ T cells with a high-exhausted phenotype. On account of the low NKG2D expression on $CD4^+$ T cells, it is hypothesized that sNKG2DLs may mediate a suppressive immune environment, which changed the phenotype of $CD4^+$ T cells but did not directly impact $CD4^+$ T cells.

Adoptive transfer therapies applied in high-risk NB patients in addition to current standard treatments, include NK cells and CAR-T cells directed against NB-associated antigens (such as GD2), and CAR-NK cells (20,48,49).

The immunosuppressive effects of sNKG2DL are achieved by acting on the NKG2D receptor to directly or indirectly suppress the antitumor effects of NK and $CD8^+$ T cells, thus reducing the effectiveness of immunotherapy based on these types of cells (50). Immunotherapy strategies targeting NKG2DL include interventions that result in the upregulation of NKG2DL expression on tumor cell surfaces, reduced tumor cell shedding, and the formation of sNKG2DL or antibodies that neutralize already produced sNKG2DL (51). Kloess *et al* (52) found that soluble NKG2D ligands drastically reduce the cytotoxicity of activated dNK cells in pediatric patients suffering from NB and demonstrated a strategy that used overexpression of NKG2D to maintain the antitumor function of dNK cells. However, they suggested that the excess of NKG2D leads to clearance of sMICA and preserves the cytotoxicity of dNK cells via non-occupied NKG2D. The aforementioned study did not assess the cytotoxicity of $CD8^+$ T cells or use neutralizing antibodies of soluble NKG2D ligands. The results of the present study showed that the clearance of sNKG2DL rescued $CD8^+$ T cell function and enhanced the sensitivity to the antitumor effects in the NB. The antitumor effect of $CD8^+$ T cells potentiated by clearing sNKG2DL plays an important role in cellular immunotherapy, and it may serve as an adjunct to CAR T therapy to enhance the therapeutic effect.

NB metastasis to the CNS is rare and often occurs in recurrent or progressive cases (53). Even with aggressive treatment strategies, the treatment outcomes of patients with NB with CNS metastasis remain poor. An increasing number of studies have confirmed that a therapeutic strategy in which the tumor hypoxic microenvironment is targeted may be a potential treatment for high-risk NB. Hypoxia has been also shown to affect CNS metastatic spread of NB by affecting the BBB integrity (54). In the lymphatic system, the BBB aquaporins (AQPs) play an important role. Treatment targeting AQP4 may allow an increase in the entry of immune molecules and cells into the CNS (55).

Inhibitors of ADAM10 and 17 in the present study indicated that small-molecule drugs may serve as a novel therapeutic option for NB; however, the development of therapeutic methods for clinical needs is still an urgent problem that remains to be solved. Computer-aided drug design tools (56) and High-Throughput Screening (57) platforms are required to develop new drugs. AQPs are required for MMP-dependent migration of cancer cells. Hypoxia-dependent upregulation of AQPs has been demonstrated, and targeting AQP trafficking as a potential drug therapeutic target has been suggested (58). Regarding AQP0-5 relocation, novel drug targets have been discovered by identifying the downstream proteins involved in the underlying molecular mechanism (59). Recent studies on neurodegenerative diseases described that the use of brain organoid systems generated from human pluripotent stem cells demonstrated considerable potential in recapitulating key features of diseases' pathophysiology. Organoid systems are defined as self-organized and self-patterning three-dimensional (3D) structures that share certain similarities with complex organs. Additionally, the use of small-molecule drugs in this system was found to regulate key disease markers phosphorylation. Thus, it was identified that stem cell-derived 3D *in vitro* systems can potentially serve as drug treatment platforms against neurodegenerative diseases (60). An *in vitro* Microvessel-on-a-Chip open microfluidic model, designed and implemented by using human brain microvascular endothelial cells, was validated permeability of fluorescent dextran and a human monoclonal antibody (61). These advanced systems not only provide the possibility for *in vitro* research involving the treatment of NB with small-molecule drugs and monoclonal antibodies, but also are amenable for advanced imaging which are enable real-time monitoring of cancer cell spread and invasion. The high-throughput screening and computer-aided drug design will be applied in future research, and inability to test the effects of NB-derived sNKG2DL on these systems is a limitation of the present study.

In conclusion, it was found that high levels of soluble MICA and ULBP2 in the sera of patients with NB was closely associated with poorer outcome and T cell exhaustion. These tumor-derived NKG2DLs were cleaved by both ADAM10 and ADAM17 in NB. NB-derived sNKG2DL induced degradation of NKG2D on $CD8^+$ T cells and impaired the proliferation, IFN-γ production and cytotoxicity of $CD8^+$ T cells. Blockage of sNKG2DL may highlight a novel strategy to recover T-cell function and enhance antitumor immunotherapy.

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Availability of data and materials

The datasets used and/or analyzed during current study are available from the corresponding author upon reasonable request.

Authors' contributions

KD and FL designed the study. YZ collected the data and performed experiments. YZ and FL analyzed and interpreted the data. YZ and FL wrote the main manuscript text. YZ, FL and KD confirm the authenticity of all the raw data. DK and FL were involved in critical reviewing of the manuscript. All authors reviewed, read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no .2020238) by the Institute Research Ethics Committee at the Children's Hospital of Fudan University (Shanghai, China). Informed consent was acquired from every patient's legal guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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