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Original Research

NKp44-based chimeric antigen receptor effectively redirects primary T cells against synovial sarcoma

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ABSTRACT

Background: T-cell receptor-engineered T-cell therapies have achieved promising response rates against synovial sarcoma in clinical trials, but their applicability is limited owing to the HLA matching requirement. Chimeric antigen receptor (CAR) can redirect primary T cells to tumor-associated antigens without requiring HLA matching. However, various obstacles, including the paucity of targetable antigens, must be addressed for synovial sarcoma. Ligands for natural killer (NK) cell-activating receptors are highly expressed by tumor cells.

Methods: The surface expression of ligands for NK cell-activating receptors in synovial sarcoma cell lines was analyzed. We analyzed RNA sequencing data deposited in a public database to evaluate NKp44-ligand expression. Primary T cells retrovirally transduced with CAR targeting NKp44 ligands were evaluated for their functions in synovial sarcoma cells. Alterations induced by various stimuli, including a histone deacetylase inhibitor, a hypomethylating agent, inflammatory cytokines, and ionizing radiation, in the expression levels of NKp44 ligands were investigated.

Results: : Ligands for NKp44 and NKp30 were expressed in all cell lines. NKG2D ligands were barely expressed in a single cell line. None of the cell lines expressed NKp46 ligand. Primary synovial sarcoma cells expressed the mRNA of the truncated isoform of *MLL5*, a known cellular ligand for NKp44. NKp44-based CAR T cells specifically recognize synovial sarcoma cells, secrete interferon-γ, and exert suppressive effects on tumor cell growth. No stimulus altered the expression of NKp44 ligands.

Conclusion: NKp44-based CAR T cells can redirect primary human T cells to synovial sarcoma cells. CAR-based cell therapies may be an option for treating synovial sarcomas.

Introduction

Synovial sarcoma is a malignant solid tumor derived from different types of soft tissue that can occur throughout the body. Synovial sarcoma is characterized by t(X;18) translocation and the presence of an SS18 (also known as SYT)-SSX1/2/4 fusion gene. Adolescents and young adults are more likely to be affected. Resectable primary lesions can be treated surgically. However, patients who present with metastasis show poor clinical outcomes, with a five-year overall survival rate of less than 10% [1,2]. Therefore, the development of novel therapies is urgently required.

Cancer immunotherapy is a candidate in future treatment strategies

Abbreviations: 5-AZA, azacytidine; CAR, chimeric antigen receptor; GEM, gemcitabine; HER2, human epidermal growth factor receptor-2; NKG2D, natural killer group 2 member D; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

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for refractory and relapsed solid tumors, including synovial sarcoma. Immune checkpoint inhibition using Programmed Cell Death Protein 1 blocking antibodies for refractory and relapsed cancers has been a vital area of cancer research. For example, pembrolizumab usage has been investigated in advanced soft tissue and bone sarcomas, including synovial sarcoma. However, it showed only modest response rates against synovial sarcoma (response observed in only one of eight synovial sarcoma patients) [3]. In contrast, T-cell-based cancer immunotherapy has shown promising results. A recent study observed an antitumor response in six out of 12 patients who were infused with gene-transduced autologous T cells expressing a T-cell receptor (TCR) with an enhanced affinity for the cancer-testis antigen New York esophageal squamous cell carcinoma-1 (NY-ESO-1) [4]. However, HLA-A2-restriction of the T-cell receptor gene caused strict patient selection, and only 15 patients were enroled in the trial despite an initial recruitment of 120 candidates. Recently, promising results of a phase 2 trial of melanoma-associated antigen A4-specific TCR gene-transduced autologous T-cell therapy for advanced synovial sarcoma or myxoid/round cell liposarcoma have been reported. Among the 330 candidates in the trial, 176 were HLA-A*02 positive and 106 were melanoma associated antigen-A4 positive. Ultimately, 59 patients were enroled in the study [5].

Adoptive immunotherapy using genetically modified autologous T cells to express the chimeric antigen receptor (CAR) has been investigated for refractory and relapsed solid tumors, including soft tissue and bone sarcomas. CAR comprises an extracellular antigen-binding domain, which is usually derived from a single-chain antibody fragment, hinge, transmembrane domain, and intracellular signaling/activation domain(s); therefore, it lacks HLA restriction and can be universally utilized for all subjects requiring treatment. CD19-targeted CAR T-cell therapy has shown significant clinical efficacy against hematological malignancies [6]. Several experimental and clinical studies on human epidermal growth factor receptor-2 (HER2)-targeted CAR T-cell therapy for HER2-positive cancers have been performed, including ours [7–10]. Importantly, a successful case of a patient with relapsed rhabdomyosarcoma who maintained long-term tumor control with multiple infusions of HER2 CAR T cells has been reported [9].

Although great success resulting in regulatory approval has been observed for CD19-targeted or B-cell maturation antigen-targeted CAR T-cell therapies for hematological malignancies, treatment failure has also been observed in approximately half of all patients. One of the crucial causes of treatment failure in CD19-targeted CAR T-cell therapy is CD19 antigen loss [11]. A similar phenomenon has been observed in CAR T-cell therapy trials for solid tumors [12,13]. Since antigen heterogeneity is more prevalent in solid tumors than in hematological malignancies, single-target CAR T-cell therapy may not be expected to have the same dramatic effect as observed in hematological malignancies; targeting more than two antigens might be a way to solve this problem [14]. Therefore, more antigens that can be simultaneously targeted on the cell surface of solid tumors are required in addition to currently preferred antigens such as HER2.

Natural killer (NK) cells can detect and rapidly respond to tumor cells while sparing normal cells using various activating and inhibitory receptors on their surface. Activating receptors include natural killer group 2 member D (NKG2D), DNAX accessory molecule-1, CD16, and natural cytotoxicity receptors (NCR) [15]. NKp44 (also known as NCR2) is an NCR that binds to several ligands on the surface of tumor cells or virally infected cells, including a peculiar isoform of mixed-lineage leukemia protein-5 (MLL5), proliferating cell nuclear antigen, heparan sulfate proteoglycans, nidogen-1, and platelet-derived growth factor-DD [16]. Recently, we developed a novel CAR that targets NKp44 ligands and reported that T cells transduced with this CAR showed antitumor effects against various pediatric solid tumors, including some sarcomas [17]. Ligands for NKp44 are only expressed in malignant tumor cells, and not in normal tissues [16]. This property makes it a useful target for CAR T-cell therapies. In the present study, we investigated the effects of NKp44-based CAR T cells on synovial sarcoma cells.

Materials and methods

Plasmids and cell lines

The MSCV-IRES-GFP vector, pEQ-PAM3(-E), and pRDF plasmids were obtained from St. Jude Vector Development and Production Shared Resource. Various NKp44-based CAR genes with structural and functional diversity have been developed in our laboratory [17]. The CD19-targeted 4-1BB co-stimulated CAR gene has been reported previously [18].

Three synovial sarcoma cell lines with disease-specific SYT-SSX1 or SYT-SSX2 fusion mRNA, proven by reverse transcriptase polymerase chain reaction, were used for this study. SYO-1 was kindly provided by Dr. Akira Kawai (Department of Orthopedic Surgery, Okayama University, Okayama, Japan) [19], and HS-SY-II by Dr. Hiroshi Sonobe (Department of Pathology, Kochi Medical School, Kochi, Japan) [20]. Yamato-SS was obtained from RIKEN BRC Cell Bank (Tsukuba, Japan) [21]. HEK293T and HeLa cells were obtained from the American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) was used for cell culturing.

Detection of NK-cell receptor ligands on the surface of synovial sarcoma cells and human primary T cells

Expression of NKp44, NKp30, and NKp46 ligands was detected using recombinant human NKp44 Fc chimera protein, recombinant human NKp30 Fc chimera protein, or recombinant human NKp46 Fc chimera protein (R&D Systems, MN, USA) and stained with PE-conjugated F(ab')2 fragment goat-anti-human IgG secondary antibodies (Jackson ImmunoResearch, PA, USA). Recombinant human IgG1 Fc protein (R&D Systems) was used as a control. Prior to surface staining, the adherent cells were detached from the bottom of the culture flasks using phosphate-buffered saline containing 0.5 mM ethylenediaminetetraacetic acid. Expression of NKG2D ligands was detected using PE-conjugated MICA/B monoclonal antibody (Biolegend, CA, USA), human ULBP-1 PE-conjugated antibody (R&D systems), human ULBP-2 PE-conjugated antibody (R&D Systems), and human ULBP3 antibody (R&D Systems), followed by staining with PE-conjugated F(ab')2 fragment goat anti-mouse IgG secondary antibodies (Jackson ImmunoResearch). Purified mouse IgG2a, κ isotype antibody (BioLegend) was used as the control. Antibody staining was detected using a BD FACSCalibur flow cytometer (Becton, Dickinson and Company, NJ, USA).

Analysis of the expression of a NKp44 ligand using data from a public database

It has been reported that a truncated isoform of *MLL5* [*KMT2E*] called *NKp44L*, which has a special exon 21, is a cellular ligand of NKp44 [22]. Unlike a normal MLL5 protein, NKp44L is not present in the nucleus but is expressed on the cell surface. To investigate the mRNA expression levels of NKp44L in primary synovial sarcoma relative to the adjacent normal tissues, we analyzed three datasets, including GSE144190 with 10 pairs of primary tumor tissues and adjacent normal tissues, and GSE108028 with 19 primary tumor samples (GSM2887701–2887719) [23]. Data on the three synovial sarcoma cell lines (Yamato-SS, HS-SY-II, SYO-1 [n=2]; GSM2887616–2887619, 2887622–2887623) [23] were included in the analysis and served as a positive control. All reads in these RNA-seq datasets were mapped to the mRNA sequences of normal *MLL5* (NM_018682) and a truncated isoform of *MLL5* (JQ809698) [22] using the Minimap2 software [24]. Reads with blast-like alignment identities greater than or equal to 0.95 were counted. The expression of different regions was considered as read coverages that overlapped special exon 21 (36 bp) in JQ809698 or normal exons 21–25 (2861 bp) in NM_018682. Read coverages normalized by total reads were used to compare the expressions of

special exon 21 among different tissue types (Supplementary Fig. 1). Read coverages per base were used for comparing the expression of special exon 21 in JQ809698 and normal exons 21–25 in NM_018682.

Transduction of T cells with NKp44-based CAR gene

Recombinant retroviruses containing the NKp44-based CAR gene were produced by transient transfection of the three plasmids into HEK293T cells using FuGENE HD reagent (Promega, WI, USA), and the titers of the supernatant, which contains recombinant retrovirus, were measured using HeLa cells. Peripheral blood samples were obtained from healthy volunteers. This study has been carried out in accordance with the Declaration of Helsinki. It was approved by the ethical committee of the Niigata University School of Medicine, Niigata, Japan (approval #2015-2686), and written informed consent was obtained from all volunteers. CD3-positive T cells were isolated using RosetteSep™ human T cell enrichment cocktail (STEMCELL Technology, Vancouver, Canada) and Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) and stimulated with Dynabeads™ human T-activator CD3/CD28 (Thermo Fisher Scientific, MA, USA) in RPMI-1640 containing 10% fetal bovine serum and 200 IU/mL of recombinant human interleukin-2 (rhIL-2; PeproTech, NJ, USA). After 48 to 72 h of incubation, T cells were transduced via exposure to the retroviral supernatant in the presence of RetroNectin® (Takara, Otsu, Japan), and the transduced T cells were maintained by adding 200 IU/mL rhIL-2 to the complete medium every 2–3 days. The surface expression of NKp44-based CARs on transduced T cells was detected using the CD336 (NKp44)-PE monoclonal antibody (Beckman Coulter, CA, USA).

Assessment of specific recognition and cytotoxicity by NKp44-based CAR T cells

The cytokine secretion assay was performed using a cytometric bead array (Becton, Dickinson and Company) according to the manufacturer's instructions. Briefly, 1×10^5 synovial sarcoma cells were seeded in a 96-well flat-bottom plate and left to adhere for 12–24 h. NKp44-based CAR T cells were added at an effector to target ratio (E:T) of 1:1 in 200 μL of a medium containing 10% FBS and 200 IU/mL of rhIL-2. Cells were co-cultured for 24 h, and IFN-γ levels in the supernatant were measured.

Colorimetric cytotoxicity assay was performed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In a 96-well flat-bottom plate, 2×10^5 synovial sarcoma cells were seeded and left to adhere for 12–24 h. NKp44-based CAR T cells were added at E:T ratios of 4:1, 2:1, and 1:1. After seven days, the viable residual tumor cells were stained with a water-soluble tetrazolium dye produced by the tetrazolium salt WST-8 and quantitatively analyzed by measuring the absorbance at 450 nm using a microplate reader (Synergy HTX; BioTek Instruments, VT, USA).

Real time cell analysis (RTCA) was performed using iCELLigence (ACEA Biosciences, CA, USA). In an iCELLigence™ E-plate (ACEA Biosciences, CA, USA), 1×10^4 synovial sarcoma cells were seeded and allowed to adhere overnight. The next day, NKp44-based CAR T cells were added at E:T ratios of 2:1 and 1:1, and the cellular impedance of cells attached to the plate was measured continuously for 7–10 days.

Alterations in surface expression of NKp44 ligands

To investigate the alterations in the surface expression of NKp44 ligands induced by various stimuli, including the histone deacetylase inhibitor sodium valproate (VPA; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), hypomethylating agent 5-azacytidine (5-AZA; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), IFN-γ (Peprotech), and tumor necrosis factor (TNF)-α (R&D Systems), we cultured synovial sarcoma cells (seeded and grown overnight) or human primary CD3-positive T cells for 48–72 h in the presence or absence of an indicated agent. CD3-positive T cells were isolated from three healthy adults using RosetteSep™ human T cell enrichment cocktail and Ficoll-Paque™ Plus and then activated using the Dynabeads™ human T-activator CD3/CD28 in RPMI-1640 containing 10% fetal bovine serum and 200 IU/mL of rhIL-2. To investigate alterations induced by the anticancer agent gemcitabine (GEM; Adipogen, CA, USA), we exposed synovial sarcoma cells to GEM for 2 h, washed them twice with complete medium thereafter, and cultured them for 48–72 h. Ionizing radiation using a ^{137}Cs irradiator (PS-3000SB; PONY INDUSTRY CO. LTD., Osaka, Japan) was also employed as a stimulus. Surface expression of NKp44 ligands was detected as mentioned above and expressed and compared as a ratio calculated using the following formula: Expression ratio = surface binding level (median fluorescent intensity) of recombinant NKp44 Fc chimera protein/recombinant human IgG1 Fc chimera protein.

Statistical analysis

Statistical analysis was conducted using EZR software (version 4.0.0) [25]. Cytokine production, antitumor effect, and alteration of surface expression of NKp44 ligands were analyzed using Student's t-test. The cytokine secretion assay, colorimetric cytotoxicity assay, and real-time cell analysis were performed in three technical replicates. Alterations in the surface expression of NKp44 ligands, induced by various stimuli, were examined in three independent experiments. The results are presented as mean ± standard deviation (SD). Statistical significance was set at $P < 0.05$.

Results

Expression of ligands for NK-cell activating receptors on synovial sarcoma cells

To examine whether synovial sarcoma cells can be targeted by NK receptor-based CAR T cells, including NKp44-based CAR, NKp30-based CAR, and NKG2D-based CAR, we first examined the expression of ligands on the surface of three types of synovial sarcoma cells (SYO-1, HS-SY-II, and Yamato-SS). SYO-1 expressed NKp44 and NKp30 ligands; HS-SY-II expressed ligands NKp44, NKp30, MICA/B, and ULBP3; Yamato-SS expressed ligands for NKp44, NKp30, and ULBP3. Both ligands for NKp44 and NKp30 were expressed in all three cell lines, and the levels of ligands for NKp44 were shown to be higher than those for NKp30. Hence, we considered that ligands for NKp44 might be vital targets for CAR T cell therapy in synovial sarcoma (Fig. 1A). Next, we investigated the expression of NKp44 ligands in primary tumors obtained from patients with synovial sarcoma. We analyzed public-database-derived RNA sequencing data of primary synovial sarcoma, normal tissues adjacent to the tumors, and the three tumor cell lines investigated in the current study, which were shown to have high surface expression of NKp44 ligands, to detect the truncated isoform of *MLL5*, which has been shown to be a cellular ligand of NKp44 that stimulates effector functions of primary human NK cells. We found that the tumor samples, as well as the cell lines, had significantly higher numbers of "normalized reads" of an *MLL5* truncated isoform than the normal tissues do, indicating the expression of NKp44 ligand in primary synovial sarcoma tissues as well as in cell lines (Yamato-SS, HS-SY-II, and SYO-1). Notably, the number of normalized reads of primary tumors was not statistically different from those of the cell lines (Fig. 1B).

Specific recognition of and cytotoxicity against synovial sarcoma by NKp44-based CAR T cells

Fig. 2A illustrates the NKp44-based CAR gene construct used in this study, which is composed of an extracellular antigen-binding domain (immunoglobulin-like), CD8α hinge and transmembrane domains, and 4-1BB and intracellular signaling domains. T cells transduced with the

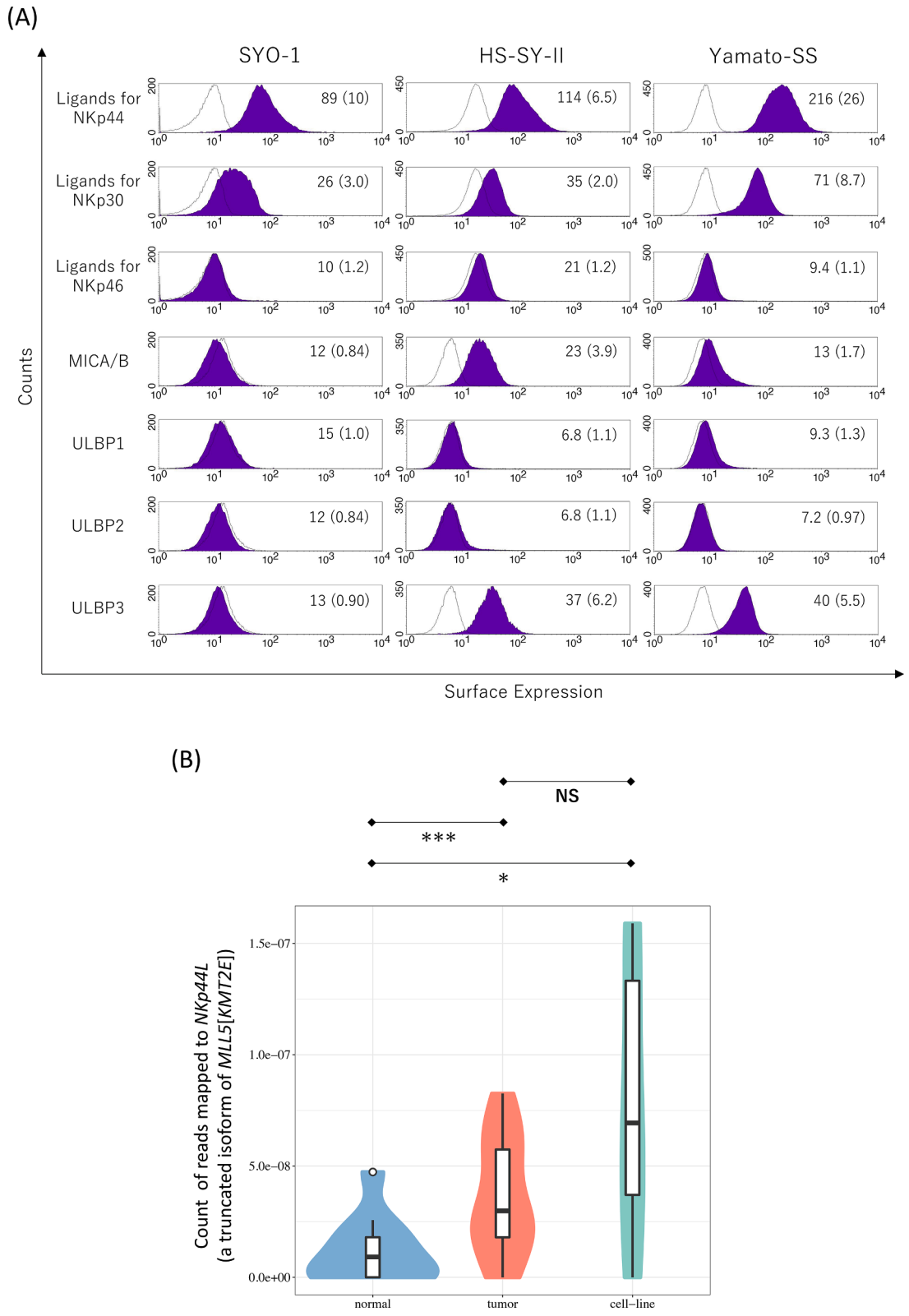

Fig. 1. Expression of ligands for natural killer (NK) cell activating receptors on synovial sarcoma cells. (A) Surface expression of NK cell activating receptor ligands. Each histogram shows the signal intensity of phycoerythrin (PE) channels when the cells were stained with NKp44, NKp46, NKp30 recombinant human Fc chimera proteins, or PE-anti ULBP1-3 antibodies (filled area). Recombinant human IgG1 Fc protein or PE mouse IgG2a, κ isotype control antibody was used as a control (open area). The mean fluorescence intensity of the filled histogram and the ratio of the intensities of the filled and open histograms in parentheses are presented at the top-right corner of each histogram. The experiments were performed at least three times, and representative data are shown. (B) Comparison of RNA expression levels in primary tumor samples, adjacent normal tissue samples, and the cell lines Yamato-SS, HS-SY-II, and SYO-1. The Y-axis in the box plot shows the count of reads mapped to special exon 21 of an *MLL5* truncated isoform that was normalized by all reads in each sample. $^{*}P < 0.05$, $^{***}P < 0.001$. NS: not significant.

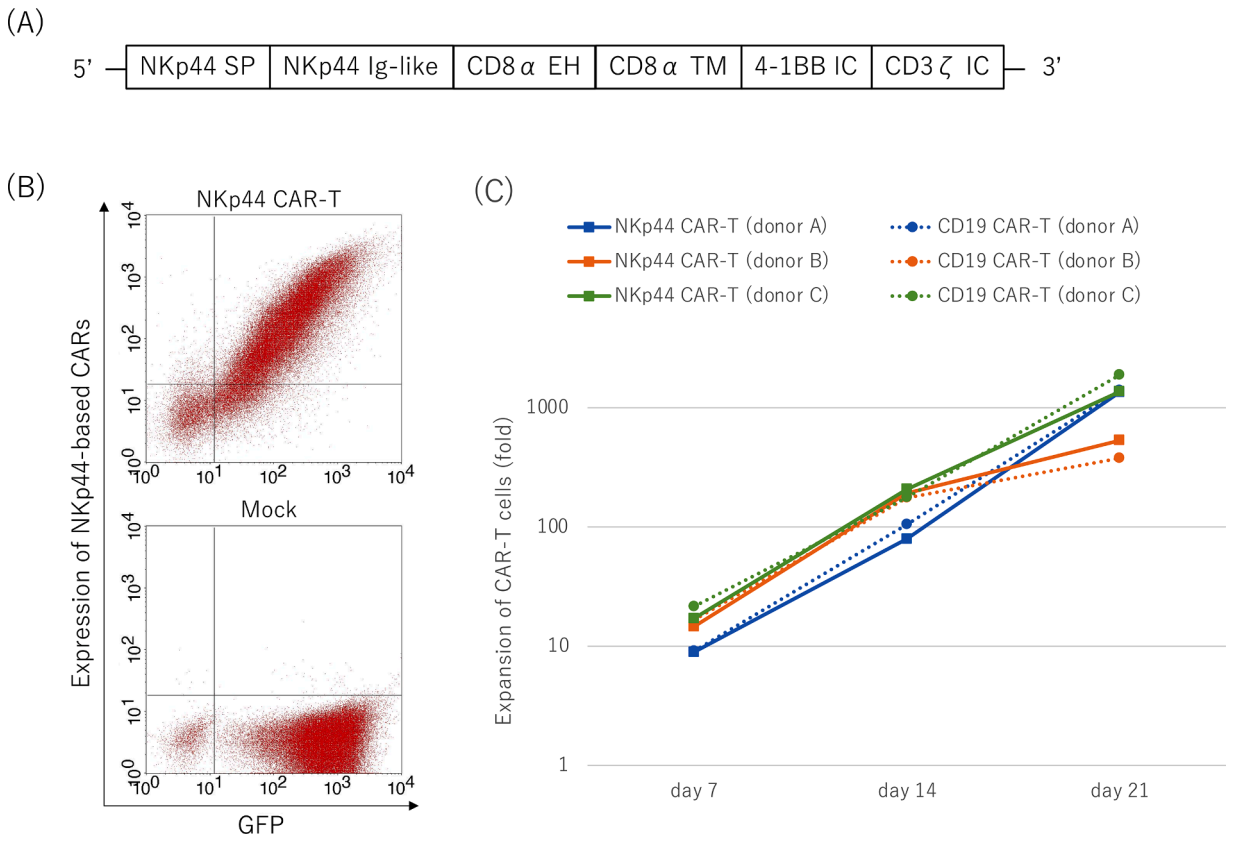

Fig. 2. Gene construct and T-cell surface expression of NKp44-based chimeric antigen receptor (CAR). (A) Gene construction of NKp44-based chimeric antigen receptor. SP: signal peptide domain, Ig-like: immunoglobulin-like domain, EH: extracellular hinge domain, TM: transmembrane domain, IC: intracellular domain. (B) Expression of NKp44-based CARs on the surface of transduced T cells. The horizontal axis represents the level of green fluorescent protein (GFP). The vertical axis represents the surface expression levels of NKp44-based CAR, as demonstrated by the anti-phycoerythrin (PE)-anti NKp44 antibody. (C) Expansion of CAR T cells after gene transduction. The horizontal axis represents the number of days after gene transduction and the vertical axis represents the expansion folds of cell culture.

NKp44-based CAR gene showed high surface expression of the NKp44-based CAR protein, as demonstrated by the PE anti-human NKp44 antibody (Fig. 2B). Fig. 2C illustrates the comparable expansion folds of NKp44-based CAR T cells compared to CD19-targeted CAR T cells. In the final products used for subsequent functional assays, the average CD3-positive cell percentage was 86.4 ± 12.3%, CD8-positive cells 50.0 ± 10.3%, and CD4-positive cells were 46.7 ± 9.0%.

A cytokine secretion assay was performed to analyze whether NKp44-based CAR T cells respond specifically to synovial sarcoma cells. The NKp44-based CAR T cells released significantly higher levels of IFN-γ into the culture medium than T cells transduced with the empty vector (mock) after 24 h exposure to synovial sarcoma cells ($P < 0.001$) (Fig. 3).

To investigate the NKp44-specific cytotoxic activity of CAR T cells, the antitumor effect of NKp44-based CAR T cells was measured quantitatively using a cell proliferation assay (WST-8 assay) and RTCA. In the WST-8 assay, NKp44-based CAR T cells from three healthy T-cell donors showed significantly higher antitumor effects against SYO-1, HS-SY-II, and Yamato-SS cells in a dose-dependent manner than mock-transduced control T cells at almost all E:T ratios (Fig. 4). RTCA showed that NKp44-based CAR T cells exerted significantly higher growth-suppressive effects on all three cell lines at E:T ratios of 2:1 and 1:1 compared to mock-transduced T cells ($P < 0.001$) (Fig. 5).

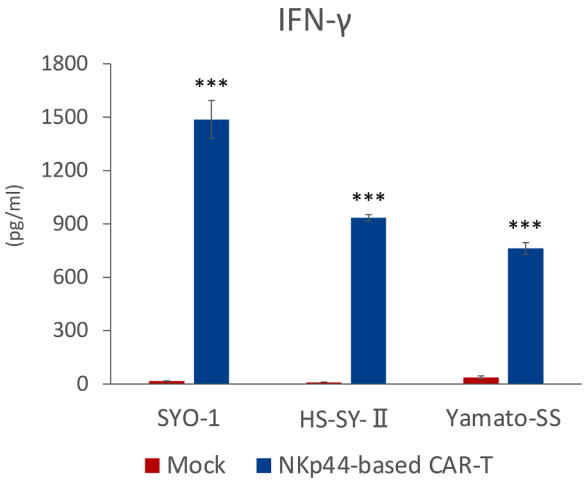

Fig. 3. Specific recognition and secretion of interferon gamma (IFN-γ) by chimeric antigen receptor (CAR) T cells. Secretion of IFN-γ by NKp44-based CAR-T cells was assessed using a cytometric bead array after 24 h exposure to synovial sarcoma cells. NKp44-based CAR T cells (blue bars) produced significantly higher levels of IFN-γ than T cells transduced with the empty vector (mock; red bars). Data are presented as the mean ± standard deviation of triplicate experiments. The data are representative results from experiments using at least two healthy donors. ***$P < 0.001$.

Ligand induction

The expression of cell surface ligands may be altered by various stimuli or stressors. For example, NKG2D ligands are upregulated by chemotherapy, radiation, and inflammation [26,27]. We investigated whether VPA, inflammatory cytokines (IFN-γ plus TNF-α), 5-AZA, GEM,

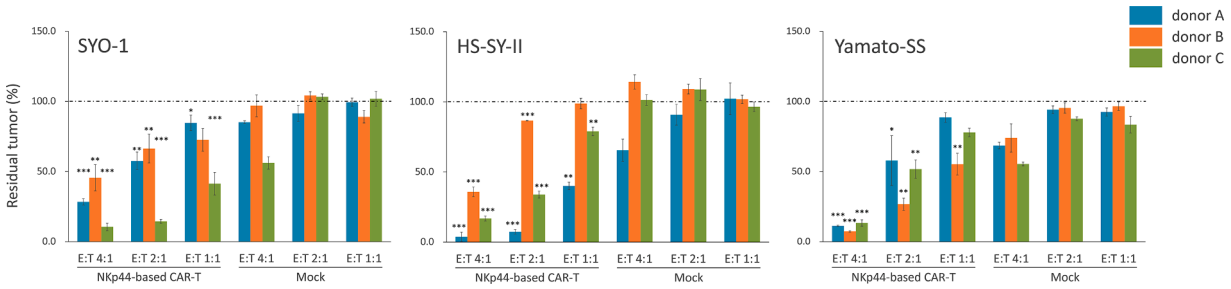

Fig. 4. Cytotoxicity of NKp44-based chimeric antigen receptor (CAR) T cells against synovial sarcoma. NKp44-based CAR T cells from three healthy T-cell donors were co-cultured for 7 days with synovial sarcoma cells at E:T ratios ranging from 1:1 to 4:1. NKp44-based CAR T cells show significantly higher antitumor effect than mock-transduced T cells. Some inter-individual variation is noticeable. The y-axis represents the percentage of residual tumors (compared with tumor cells without effector cells). Data are presented as the mean ± standard deviation of triplicate experiments. The data are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

and ionizing radiation (two gray) could induce the expression of NKp44 ligands. Since various stimuli or stress may increase the autofluorescence of cells, we evaluated the ratios of the surface binding of recombinant NKp44 Fc chimeric protein /recombinant human IgG1 Fc protein. No agents caused statistically significant alterations in NKp44 ligands (Fig. 6). To investigate whether such stimuli, including VPA, IFN-γ plus TNF-α, or 5-AZA, could induce NKp44 ligands on the normal tissue, we used primary lymphocytes as a model. No statistically significant changes in the expression levels of NKp44 ligands were observed (Supplementary Fig. 2).

Discussion

In this study, we investigated the expression of NK cell-related receptor ligands on the surface of synovial sarcoma cells. Among the various activation ligands, NKp44 ligands were found to be most prevalent in synovial sarcoma cells and are therefore suitable for use in CAR T cell therapy. In addition, we showed that primary synovial sarcoma cells expressed the mRNA of an *MLL5* truncated isoform, a known cellular ligand for NKp44. We then examined the response of NKp44-based CAR T cells to synovial sarcoma cells. NKp44-based CAR T cells produced significantly higher IFN-γ levels than the control cells and showed superior antitumor effects against all three synovial sarcoma cell lines. Finally, we investigated whether NKp44 ligands could be upregulated by various stimuli, including a histone deacetylase inhibitor (sodium valproate), inflammatory cytokine combination (IFN-γ plus TNF-α), demethylating agent (azacytidine), anticancer agent (gemcitabine), and ionizing radiation, and found that there were no changes in surface expression after stimulation.

The success of CAR T-cell therapy against hematologic malignancies has yet to be replicated in solid tumors. A significant reason for this might be the heterogeneity of solid tumors, such that CAR T cells targeting a single antigen cannot eradicate a tumor cell that does not express that antigen [28]. To overcome this problem, strategies for simultaneously targeting multiple antigens have been developed. Hedge et al. created a CAR gene incorporating two antigen-binding sites in a serial sequence (tandem CAR) and reported that T cells transduced with the tandem CAR gene showed stronger antitumor effects than those expressing the two CARs separately [29]. We believe that HER2 [10] and NKp44 ligands are suitable for combinatorial targeting strategies for the treatment of synovial sarcoma using CAR T cells.

The efficacy of CAR T-cell therapy also depends on the expression levels of ligands on the surface of tumor cells. NKG2D ligands are induced by various inflammatory stimuli or stresses [26,27]. The upregulation of ligands can enhance the effectiveness of CAR T cells. Simultaneously, it can also enhance the on-target off-tumor effects on normal tissues, which may lead to multiple organ injuries. In this study, the surface expression of NKp44 ligands did not increase (or decrease) in tumor cells after exposure to VPA, inflammatory cytokines (IFN-γ and TNF-α), 5-AZA, GEM, or ionizing radiation. The surface expression of NKp44 ligands also did not change in normal lymphocytes after exposure to VPA, inflammatory cytokines (IFN-γ and TNF-α), or 5-AZA. The lack of an increase in ligands by stimuli suggests that there is less risk of on-target off-tumor effects in normal tissues, which may be an advantage of NKp44-based CAR over NKG2D-based CAR. However, our study only used primary lymphocytes. Therefore, it is important to further investigate, preferably using a mouse model, whether the stimuli used in the current study and other stimuli such as irradiation and anticancer agents have any impact on NKp44 ligand expression in the normal tissues of various organs or whether infusions of high doses of the NKp44-based CAR-T cells could injure the normal organs.

However, this study had several limitations. First, we assessed the efficacy of NKp44-based CAR T cells against synovial sarcoma cells using *in vitro* experiments only. However, Eisenberg et al. reported that NKp44-based CAR T cells incorporating the CD28 costimulatory domain showed antitumor effects against various cancer cells in mouse xenograft models [30]. Subsequently, we reported that T cells transduced with NKp44-based CAR construct incorporating 4-1BB costimulatory domain, used in this study, exhibited more powerful effects than those transduced with the CD28-costimulated construct against various hematological and solid tumor cells lines *in vitro* [17]. Therefore, our NKp44-based 4-1BB co-stimulated CAR T cells might also be expected to exert a potent antitumor effect in preclinical mouse models. Secondly, to the best of our knowledge, NKp44 ligand expression has not been investigated in primary tumor samples using immunohistochemistry because of the lack of suitable monoclonal antibodies. Although NKp44 ligands can be detected by flow cytometry using the NKp44-ectodomain fused to the human Fc region of immunoglobulin, it is difficult to perform it on primary samples because of the need to isolate single cells from a fresh tumor sample without using an enzyme treatment that might cause damage to the surface proteins. *In situ* RNA hybridization may be useful for estimating the prevalence of NKp44 ligand expression in human tissues.

In conclusion, we have reported that NKp44-based CAR-redirected human primary T cells are active against synovial sarcoma cells, suggesting that this treatment strategy could be an option for the treatment of refractory and relapsed synovial sarcoma.

Ethics approval statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethical committee of Niigata University School of Medicine (approval #2015-2686).

Informed consent statement

Informed consent was obtained from all volunteers who participated in this study.

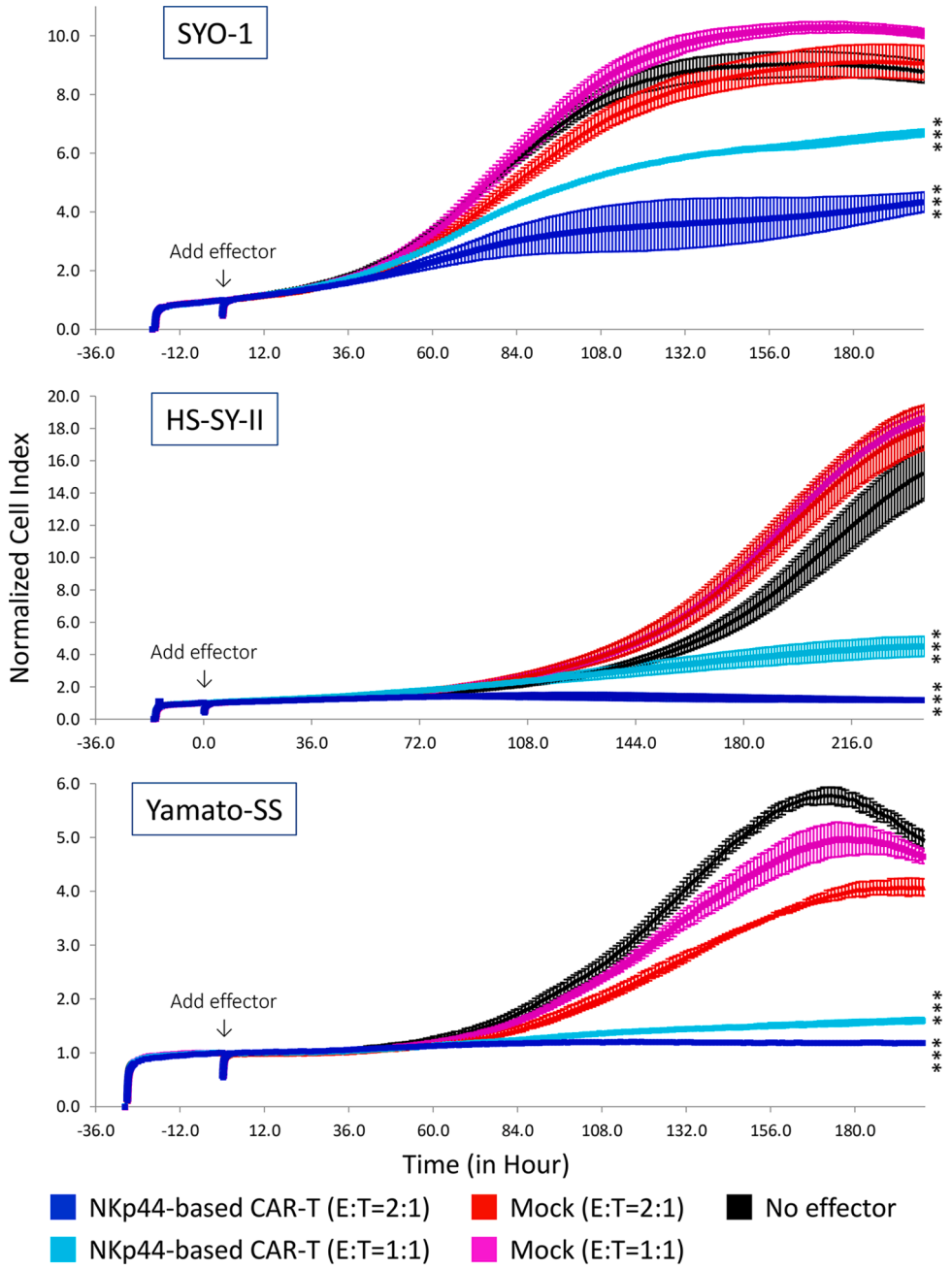

Fig. 5. Quantitative evaluation of the antitumor effect using real-time cell analysis. NKp44-based chimeric antigen receptor (CAR) T cells and mock-transduced T cells were co-cultured with synovial sarcoma cells at effector (E): target (T) ratios of 1:1 and 2:1, respectively. NKp44-based CAR T cells exerted significantly higher suppressive effects on tumor cell growth than mock-transduced T cells in a dose-dependent manner. ***$P < 0.001$.

Consent for publication

Not applicable.

Availability of data and materials

The data presented in this study are available upon request from the corresponding author.

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Supplementary Fig. 1. All reads in the three RNA-seq datasets were mapped to the mRNA sequences of normal *MLL5* (NM_018682) and of the truncated isoform of *MLL5* (JQ809698) using the Minimap2 software.

Supplementary Fig. 2. Alterations in the surface expression of NKp44 ligands in normal lymphocytes. Each bar shows the ratio of the median fluorescence intensity of NKp44 recombinant human Fc chimeric protein to that of the recombinant human IgG1 Fc protein. Sodium valproate, 5-azacytidine, and interferon-γ plus tumor necrosis factor-α, were employed as stimuli. The experiments were performed under the same conditions as those for synovial sarcoma cells. No agents caused statistically significant alterations in NKp44 ligands. Data are presented as mean ± standard deviation of three independent experiments. NS: not significant.

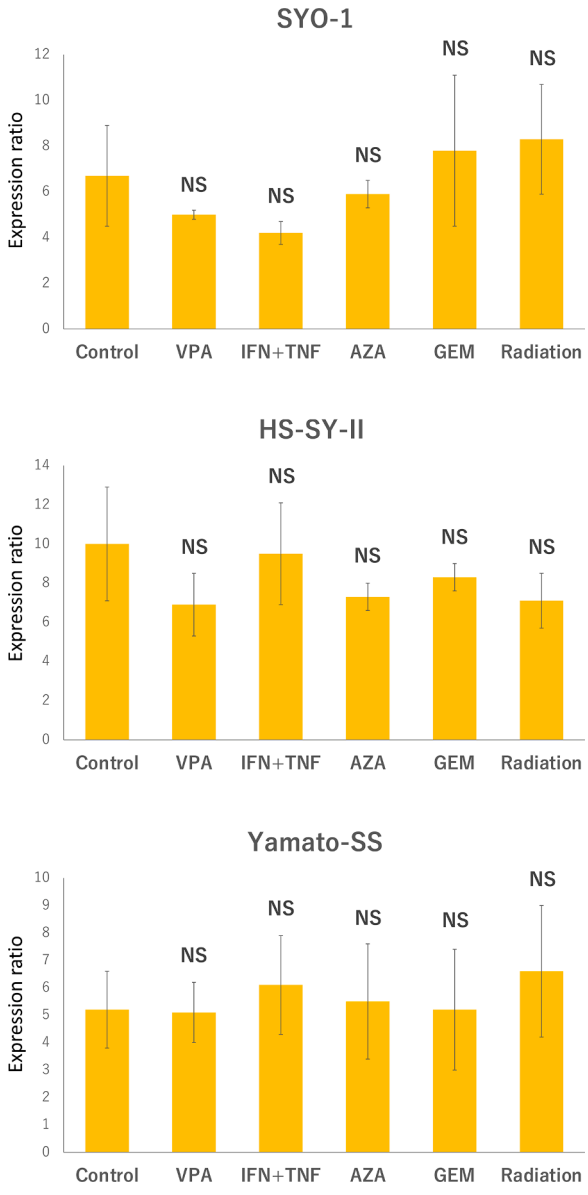

Fig. 6. Alterations of surface expression of NKp44 ligands. Each bar shows the ratio of the median fluorescence intensity of NKp44 recombinant human Fc chimeric protein/recombinant human IgG1 Fc protein. Sodium valproate (VPA: 200 μg/mL, 24–72 h), 5-azacytidine (5-AZA: 5 μm, 24–72 h), interferon-γ (IFN-γ: 1000 U/mL, 24–72 h) in combination with tumor necrosis factor-α (TNF-α: 400 U/mL, 24–72 h), gemcitabine (GEM: 30 ng/mL, 2 h), or ionizing radiation (Radiation: two gray) were employed as stimuli. No agents caused statistically significant alterations in NKp44 ligands. Data are presented as mean ± standard deviation of three independent experiments. NS: not significant compared to the control.

CRediT authorship contribution statement

Yudai Murayama: Investigation, Formal analysis, Writing – original draft. **Yasushi Kasahara:** Resources. **Nobuhiro Kubo:** Investigation. **Chansu Shin:** Resources. **Masaru Imamura:** Funding acquisition. **Naoki Oike:** Resources. **Takashi Ariizumi:** Resources. **Akihiko Saitoh:** Resources. **Minori Baba:** Investigation. **Tomohiro Miyazaki:** Investigation. **Yuko Suzuki:** Investigation. **Yiwei Ling:** Formal analysis. **Shujiro Okuda:** Formal analysis. **Keichiro Mihara:** Resources. **Akira Ogose:** Funding acquisition. **Hiroyuki Kawashima:** Resources. **Chihaya Imai:** Project administration, Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

Chihaya Imai reports patent royalties from Juno Therapeutics, patent royalty, research fund, and advisory fee from CURED Inc. Yuko Suzuki has been an employee of CURED Inc. since February 2022. The other authors have no conflicts of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101521.

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