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Targeting multiple types of tumors using NKG2D-coated iron oxide nanoparticles

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Abstract

Iron oxide nanoparticles (IONPs) hold great potential for cancer therapy. Actively targeting IONPs to tumor cells can further increase therapeutic efficacy and decrease off-target side effects. To target tumor cells, a natural killer (NK) cell activating receptor, NKG2D, was utilized to develop pan-tumor targeting IONPs. NKG2D ligands are expressed on many tumor types and its ligands are not found on most normal tissues under steady state conditions. The data showed that mouse and human fragment crystallizable (Fc) -fusion NKG2D (Fc-NKG2D) coated IONPs (NKG2D/NPs) can target multiple NKG2D ligand positive tumor types *in vitro* in a dose dependent manner by magnetic cell sorting. Tumor targeting effect was robust even under a very low tumor cell to normal cell ratio and targeting efficiency correlated with NKG2D ligand expression level on tumor cells. Furthermore, the magnetic separation platform utilized to test NKG2D/NP specificity has the potential to be developed into high throughput screening strategies to identify ideal fusion proteins or antibodies for targeting IONPs. In conclusion, NKG2D/NPs can be used to target multiple tumor types and magnetic separation platform can facilitate the proof-of-concept phase of tumor targeting IONP development.

Keywords

nanoparticles; ovarian cancer; lymphoma; NKG2D; MICA; Rae-1

Introduction

Cancer is a major disease burden to society and will continue to be a major threat to the ageing population. Lessons learned from successful cancer therapy strategies, such as Gleevec for chronic myeloid leukemia (CML) and Rituximab for B cell malignancies, highlight the importance of specificity in cancer therapy. However, many of the major therapeutic agents, such as chemotherapies, are not tumor-specific. More than 40 years ago, scientists already attempted to enhance the specificity of chemotherapies by increasing their delivery to tumor sites using nano-scale liposomes [1]. So far, FDA had approved several formats of nano-scale liposomes for chemotherapy delivery [2, 3]. Due to the limitation of nanotechnology, these first generation nanoparticles did not actively target tumor cells,

instead they utilized a less effective passive targeting phenomenon known as the enhanced permeability and retention effect [4]. Eventually, advances in material engineering and nanotechnology have led to the recent clinical translation of a few active tumor targeting nanoparticles for drug delivery [5]. Compared to the slower advance of nanoparticle targeting strategies, the format of nanoparticles has expanded robustly. During the past 40 years, nanoparticles in the format of liposomes, polymer particles, gold, and iron oxide particles have been developed. Among these particles, iron oxide nanoparticles (IONPs) are functionally versatile and have been designed as vehicles to deliver drugs [6–9], to enhance MRI imaging contrast [10–12], and to deliver heat to kill tumors [13–16]. Although functionally versatile, there are limited numbers of pre-clinical studies that use active targeting of IONP to tumor cells. Furthermore, most of these studies targeted tumor-associated antigens, such as human epidermal growth factor receptor 2 (HER-2) [6, 8], α -folate receptor [17], and epidermal growth factor receptor (EGFR) [18] that are not highly tumor-specific. New strategies to target IONP to tumor cells with greater specificity are needed.

The aim of this study was to create IONPs which target tumor cells with specificity and that can potentially target multiple tumor types. This aim was accomplished by utilizing a natural killer (NK) cell activating receptor, NKG2D. NKG2D recognizes stress-induced ligands expressed on many types of malignancies, and its ligands are not found on most normal tissues, although they can be transiently induced under certain conditions [19, 20]. In this study, it is shown that NKG2D/NP efficiently targets multiple NKG2D ligand positive tumor types *in vitro*. The targeting of tumor cells was very robust even under a very low tumor cell to normal cell ratio. Furthermore, the magnetic separation platform used to test NKG2D/NP specificity can potentially be used to screen the capability of any given fragment crystallizable (Fc) fusion protein or antibody conjugated IONPs to target tumor cells.

Materials and methods

Cell line and cell culture

293F cell line was obtained from Life Technologies (Carlsbad, CA). P815/MICA, RMA, RMA/RG, ID8 cell lines were described previously [21–23]. 293F cell line was cultured in Gibco® FreeStyle 293™ Expression Medium (Life Technologies). ID8 and Bosc23 cell lines were cultured in DMEM with a high glucose concentration (4.5g/L) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA), 10mM HEPES, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 100U/mL penicillin, 100ug/mL streptomycin, and 50uM 2-ME. RMA, RMA/RG, P815, P815/MICA, K562, and RPMI8866 cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactive FBS, 10mM HEPES, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 100U/mL penicillin, 100ug/mL streptomycin, and 50uM 2-ME.

Construction of Fc-NKG2D

A murine NKG2D based Fc-NKG2D (Fc-msNKG2D) was constructed by using standard molecular biology techniques to fuse the cDNA of mouse Dap10 signal peptide [aa 1–17]

with mouse IgG2a Fc (CH2-CH3) [aa 96–330], followed by mouse NKG2D extracellular (EC) domain [aa 90–232]. A human NKG2D based Fc-NKG2D (Fc-huNKG2D) was constructed by fusing cDNA of mouse Dap10 signal peptide [aa 1–17] with human IgG1 Fc (Hinge-CH2-CH3) [aa 99–330], followed by human NKG2D EC [aa 78–216]. Both Fc-NKG2D constructs were further cloned into a CMV promoter based expression vector.

Production and purification of Fc-NKG2D proteins

Fc-msNKG2D was produced from transiently transfected Bosc23 cells. Transfection of Bosc23 cells was done with Lipofectamine 2000 (Life technologies) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were grown in Gibco® FreeStyle 293™ Expression Medium and cell-free conditioned medium was harvested after 5 days. Fc-huNKG2D was produced by transiently transfected 293F cells. Transfection of 293F cells was done by mixing suspension growing 293F cells with Fc-NKG2D plasmids and 40Kd polyethylenimine (PEI) (Polysciences Inc, Warrington, PA) at a final concentration of 2×10^7 cells/mL, 12.5ug/mL plasmid DNA, and 25ug/mL PEI. The mixture was shaken at 120 rpm on an orbital shaker at 37°C for 3 h. The cells were diluted with FreeStyle 293™ Expression Medium at a 1:9 ratio to reach a final cell concentration of 2×10^6 cells/mL. Cells were cultured for 6 days and cell-free conditioned medium was harvested. For purification of Fc-NKG2D, cell-free culture conditioned medium was loaded directly onto PBS equilibrated HiTrap rProteinA HP columns (GE Healthcare, Waukesha, WI). After the supernatants were run through the columns, the columns were washed with 10 column volumes of PBS. Elution was done with 10 column volumes of Glycine buffer (PH=2.7). Eluted fractions containing Fc-NKG2D were collected and buffer was exchanged to PBS by Amicon ultrafiltration column (30Kd MW cutoff) (EMD Millipore, Billerica, MA) following the manufacturer's protocol. The final protein solution was analyzed by SDS-PAGE followed by SYPRO orange (Life Technologies) staining. Protein concentration was quantified by comparing protein staining intensity to ovalbumin standard curve using Image J software (NIH, Bethesda, MD).

Production of Fc-NKG2D conjugated nanoparticles (NKG2D/NP)

Protein A conjugated Bionized NanoFerrite-starch coated 100nm iron oxide nanoparticles (product no. 10-20-102) were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). These particles have an average diameter of 100nm, and the surface was conjugated with 1.5 to 2μg of protein A per mg of nanoparticles. These particles are estimated to have around 40 protein A molecules on each particle. For conjugating Fc-NKG2D proteins to the iron-oxide nanoparticles, a Fc-NKG2D to protein A molar ratio of 2:1 was used. 1mg of nanoparticles were washed with PBS by magnetic separation using DynaMag-spin magnet (Life technologies). 8ug of Fc-NKG2D was then added to the 1mg of particles, and the volume of mixture was adjusted to 500uL by adding PBS. The mixture was then gently shaken at room temperature (RT) for 1 h. The unbound Fc-NKG2D and PBS were removed from the mixture by magnetic separation. Nanoparticles were washed with 1mL PBS three times and re-suspended in PBS at a concentration of 1mg/mL.

CFSE labeling of cells

Tumor cells were harvested and washed with PBS. Tumor cells ($3 - 5 \times 10^6$) were re-suspended in 500uL of PBS containing 0.5uM CFSE (Life technologies) and incubated for 8 min at RT in the dark. RPMI 1640 complete media was added to neutralize residual CFSE. Cells were then washed with FACS buffer (PBS+1% FBS) three times.

NKG2D/NP specificity test

To determine whether msNKG2D/NP specifically bound to mouse NKG2D ligand+ tumor cells, RMA cells (NKG2D ligand-, EGFP-) and RMA/RG cells (NKG2D ligand+, EGFP+) were mixed at a 1:1 ratio ($5 \times 10^4:5 \times 10^4$). Various amount of msNKG2D/NP or msIgG/NP were added to the cell mixture, the volume was adjusted to 500uL by adding FACS buffer, and cells were incubated on ice for 30 min. Magnetic separation was performed and pre-separation, bound, and unbound fractions were collected. Cell identity was analyzed by EGFP signal using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). The percentage of ligand+ tumor cells captured was calculated according to the following equation: percentage of ligand+ tumor cells captured = $[d \times (af - eb)] / [b \times (cf - ed)]$ (a and b represent the percentage of the ligand- peak and the percentage of the ligand+ peak in pre-separation fractions, respectively. c and d represent the percentage of the ligand- peak and the percentage of the ligand+ peak in the bound fractions, respectively. e and f represent the percentage of the ligand- peak and the percentage of the ligand+ peak in unbound fractions, respectively).

To determine whether huNKG2D/NP specifically bound to human NKG2D ligand+ tumor cells, P815 cells (NKG2D ligand-) and P815/MICA cells (NKG2D ligand+, pre-labeled with 0.5uM CFSE) were mixed at a 1:1 ratio ($5 \times 10^4:5 \times 10^4$). Various amounts of huNKG2D/NP or huIgG/NP were added to the cell mixture, the volume was adjusted to 500uL by adding FACS buffer and incubated on ice for 30 min. Magnetic separation was performed on the cell mixture and pre-separation, bound, and unbound fractions were analyzed by flow cytometry. The absolute number of cells in each fraction was counted by flow cytometer under a normalized running time for each sample. The percentage of ligand+ tumor cells captured was calculated by the following equation: percentage of ligand+ tumor cells captured = absolute number of ligand+ tumor cells in bound fraction / (absolute number of ligand+ tumor cells in bound fraction + absolute number of ligand+ tumor cells in unbound fraction).

Flow cytometry

To identify mouse NKG2D ligand expression on tumor cells, cells were stained with Fc-msNKG2D, followed by secondary staining with APC conjugated anti-mouse IgG Abs (clone: Poly4053, Biolegend, San Diego, CA). To identify human NKG2D ligand expression on tumor cells, cells were stained with Fc-huNKG2D, followed by secondary staining using APC conjugated anti-human IgG Fc Abs (clone: HP6017, Biolegend). Captured splenocytes were stained with APC conjugated anti-Gr1 mAbs (clone: RB6-8C5, Biolegend) and PE conjugated anti-B220 mAbs (clone: RA3-6B2, eBioscience, San Diego, CA). Captured PBMCs were stained with FITC conjugated anti-CD14 mAbs (clone: M5E2, Biolegend) and APC conjugated anti-CD20 mAbs (clone: 2H7, Biolegend).

Results

Production of Fc-NKG2D conjugated iron oxide nanoparticles (NKG2D/NPs)

To test whether NKG2D can be used to conjugate NPs and target NPs to NKG2D ligand+ tumor cells, a mouse version immunoglobulin fragment of crystallizable (Fc) fusion NKG2D (Fc-msNKG2D) and a human version Fc fusion NKG2D (Fc-huNKG2D) were created, and these can be readily purified by protein A affinity column and conjugated to protein A coated NPs. NKG2D is a type 2 membrane protein that has its signal sequence required for secretion embedded in transmembrane portion. Therefore, in order to produce soluble Fc-NKG2Ds which can be secreted by mammalian cells, the Fc-msNKG2D was created by fusing mouse Dap10 signal peptide (sp) with mIgG2a Fc, followed by msNKG2D extracellular (EC) domain. The Fc-huNKG2D was created by fusing the mouse Dap10 signal peptide with human IgG1 Fc, followed by huNKG2D EC domain (Fig. 1a). The Fc fusion design facilitated the production and purification of Fc-NKG2Ds. Both Fc-NKG2Ds form disulfide bond dimers and could be purified with protein A affinity column (Fig. 1b). To produce Fc-msNKG2D and Fc-huNKG2D conjugated iron oxide NP (msNKG2D/NPs and huNKG2D/NPs), Fc-NKG2Ds were mixed with protein A coated iron oxide NPs. The conjugation reaction was performed at room temperature for 1 h. After removing unconjugated Fc-NKG2Ds from the reaction mixture, NKG2D/NPs were stored in PBS at 4°C.

NKG2D/NP specifically targeted NKG2D ligand+ tumor cells in a dose dependent manner

To test whether NKG2D/NP can specifically target NKG2D ligand+ tumor cells, NKG2D ligand+ and ligand- cells were pre-mixed and NKG2D/NPs or control particles were added into the cell mixture (Fig. 1c). The mixtures were incubated on ice for 30 min to facilitate targeting. Because the iron oxide NPs are magnetic, magnetic force was used to separate the bound and unbound cell fractions. To examine msNKG2D/NP targeting specificity, RMA/RG (NKG2D ligand+, EGFP+) cells and RMA (NKG2D ligand-, EGFP-) cells were mixed at a 1:1 ratio and different amount of msNKG2D/NPs or control NPs (mIgG/NPs) were added. The cell identity of pre-separation, unbound, and bound cell fractions was analyzed by flow cytometry. There was a specific enrichment of NKG2D ligand+ tumor cells (RMA/RG) in bound cell fractions in msNKG2D/NP groups but not in mIgG control particle group (Fig. 2a). The percentage of RMA/RG cells captured from the cell mixtures was determined, and there was a msNKG2D/NP dose dependent capture of RMA/RG cells, which was $83.6\% \pm 13.8\%$ and $93.3\% \pm 3.2\%$ (average $\pm$ standard deviation) for 10ug and 50ug of msNKG2D/NP, respectively. Statistical analysis was done using one-way ANOVA to compare each condition with control NP group and showed statistical significant in both groups (p value < 0.005). To assess whether huNKG2D/NP can specifically target human NKG2D ligand+ tumor cells, P815 (NKG2D ligand-) cells and P815/MICA (NKG2D ligand+, CFSE labeled) cells were mixed at a 1:1 ratio. Different amount of huNKG2D/NPs or control huIgG/NPs were added to the cells and magnetic separation was performed. The data showed an enrichment of P815/MICA cells in bound cell fractions by huNKG2D/NPs (Fig. 2b). There was also a dose dependent capture of P815/MICA cells from the cell mixture, which was $2.2\% \pm 1.8\%$ and $31.1\% \pm 11.7\%$ (average $\pm$ standard deviation) for 10ug and 50ug of huNKG2D/NP, respectively. Statistical analysis was done using one-way

ANOVA to compare each condition with control NP group and showed statistical significant in 50ug huNKG2D/NP group (p value < 0.05)

msNKG2D/NP targeted multiple types of mouse NKG2D ligand+ tumor cells in vitro even under a low tumor cell to normal cell ratio

NKG2D ligands are expressed on many kinds of tumors, therefore NKG2D/NP have the potential to target multiple tumor types. Since tumor cells are generally surrounded by normal cells *in vivo*, a more stringent test of whether msNKG2D/NP can efficiently target tumor cells but not normal cells under a low tumor cell to normal cell ratio was used. Several different types of tumor cell lines were selected to perform the specificity test. First, RMA-RG (lymphoma), ID8 (ovarian carcinoma), and RMA (lymphoma) cells were analyzed for NKG2D ligand expression (Fig. 3a). NKG2D ligand+ (RMA-RG, ID8) or ligand- (RMA) tumor cells were mixed with wild type mouse splenocytes at a 1:19 ratio (5% tumor cells), followed by addition of msNKG2D/NPs and magnetic separation. The data showed a specific enrichment of ligand+ tumor cells to a level of 35% – 50% in the bound cell fractions from the initial 5% in pre-separation cell fractions (Fig. 3b), indicating a 30% – 60% capturing of NKG2D ligand+ tumor cells. This enrichment was not seen in control (msIgG/NP) groups (data not shown) or when the RMA (ligand-) tumor cells were used. The extent of tumor cells captured from the cell mixture showed a direct correlation to NKG2D ligand expression (Fig. 3a & 3b).

huNKG2D/NP targeted multiple types of human NKG2D ligand+ tumor cell lines in vitro even under a low tumor cell to normal cell ratio

A similar approach was used to test whether huNKG2D/NPs target multiple tumor types from normal cells under conditions where there were few tumor cells and many normal cells. K562 (chronic myeloid leukemia), P815/MICA (mastocytoma), RPMI8866 (chronic myeloid leukemia), and P815 (mastocytoma) cells were analyzed for human NKG2D ligand expression (Fig. 4a). NKG2D ligand+ (K562, P815/MICA, and RPMI8866) or ligand- (P815) tumor cells were mixed with human PBMCs at a 1:19 ratio (5% tumor cells), followed by addition of huNKG2D/NP and magnetic separation. The huNKG2D/NP specifically enriched ligand+ tumor cells to 20% – 45% in the bound cell fractions from the initial 5% of tumor cells in the pre-separation cell fractions, indicating a 10% – 80% capturing of NKG2D ligand+ tumor cells (Fig. 4b). The percentage of tumor cells captured from the cell mixture showed a direct correlation to NKG2D ligand expression on the tumor cells (Fig. 4a & 4b).

Splenocytes and PBMCs captured by NKG2D/NP were not due to NKG2D binding

Cell capture data showed msNKG2D/NPs captured some splenocytes (Fig. 3b) and huNKG2D/NPs captured some PBMCs (Fig. 4b). To test whether capturing of splenocytes and PBMCs was dependent on an interaction between NKG2D and a ligand or whether it was a non-specific interaction due to the NPs themselves. The splenocytes captured by msNKG2D/NPs and msIgG/NPs were compared. A comparable amount of splenocytes were captured by the msNKG2D/NPs and the msIgG/NPs, ($2.58\% \pm 1.01\%$ and $2.85\% \pm 1.43\%$ respectively), suggesting that the capture of splenocytes was not NKG2D dependent. The

identity of splenocytes captured by each type of particles was characterized by flow cytometry, and this analysis showed a specific enrichment of B220+ B cells (Fig. 5a). The PBMCs captured by huNKG2D/NPs and huIgG/NPs were directly compared, and there was a comparable level of PBMCs captured by huNKG2D/NPs and huIgG/NPs ($11.26\% \pm 3.25\%$ and $10.56\% \pm 2.31\%$ respectively), suggesting that the capture of PBMCs was also not NKG2D dependent. PBMCs captured by these particles were specifically enriched with CD20+ B cells and CD14+ monocytes (Fig. 5b). The capture of B cells may be due to free protein A molecules on the NPs binding to surface IgG on B cells, and monocytes may have been captured due to Fc receptors on monocytes binding to the IgG-Fc portion on NKG2D/NPs and IgG/NPs.

Discussions

Using targeted-nanoparticles (TNPs) to deliver drugs or to directly kill tumor cells hold great potentials for cancer therapy [24]. However, it is not easy to identify the ideal physiochemical parameter to design TNPs that can simultaneously confer optimal targeting, immune evasion, controlled drug release, or direct tumor killing [25]. Recently, high-throughput technology and combinatorial approaches have provided effective and systemic methods to optimize TNPs leading to several early-phase TNP clinical trials [5, 26]. However, these TNPs targeted tumor-associated antigens, such as transferrin receptor, α -folate receptor, or prostate-specific membrane antigen (PSMA) [5] which are also widely expressed on normal tissues.

To create more tumor-specific TNPs, the NK cell activating receptor NKG2D was used to conjugate iron oxide nanoparticles (IONPs). NKG2D ligands are expressed on about 90% of human tumor types and are generally not expressed on the cell surface of normal cells under steady state conditions. This tumor-specific and restricted ligand expression pattern may enable NKG2D conjugated IONPs (NKG2D/NPs) to target multiple tumor types with high specificity. Another advantage of using NKG2D/NPs for cancer therapy is that NKG2D ligands are not only expressed on tumor cells, but they are also up-regulated on tumor-associated immune suppressor cells, such as regulatory T cells [27, 28] and myeloid-derived suppressor cells [29]. Furthermore, some tumor vasculature may also express NKG2D ligands [30]. This enables NKG2D/NPs to target tumor cells, tumor vasculature, and the tumor microenvironment simultaneously, and this may result in greater therapeutic efficacy.

Other than expressed on tumor cells, NKG2D ligands are known to be upregulated under certain autoimmune disease and chronic inflammation conditions [31]. This can potentially decrease the specificity of NKG2D/NPs and result in some off-tumor damage to normal tissues. The long-term toxicity of such conjugated particles remains to be determined, and the extent that these might induce innate immune responses, such as an interferon response, is unknown. Since IONPs can be used as MRI imaging contrast agent, it is possible to confirm the extent of NKG2D/NPs accumulation *in vivo*. The imaging potential of IONPs can enhance the safety profile of NKG2D/NP treatment. On the other hand, NKG2D/NPs may be designed for targeted delivery of immunomodulatory drugs to autoimmune tissues.

The Fc-fusion protein approach enabled us to generate soluble NKG2D with a high yield and a good stability. Since Fc-fusion proteins can be easily purified to high purity using a single step affinity column, and protein A or protein G conjugated IONPs are commercially available, the conjugation of NKG2D to NPs for *in vitro* analysis was easily accomplished. This approach provided an example of efficiently studying any given Fc-fusion targeting moieties at lab scale. The disadvantage of Fc-fusion approach was the non-specific binding of the Fc receptor to normal cells, which could be overcome by removing the Fc region.

In conclusion, this study provides proof-of-concept evidence that NKG2D/NPs can be used as a pan-tumor targeting strategy for nanoparticle based cancer therapy. It also provides a practical example and an efficient platform which can potentially accelerate the screening of potential target moieties and the optimal IONPs for targeted therapy.

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Figure 1. Production of Fc-NKG2D conjugated nanoparticles

(a) Mouse version Fc-NKG2D (Fc-msNKG2D) and human version Fc-NKG2D (Fc-huNKG2D) DNA construct. Fc-msNKG2D was constructed by fusing mouse Dap10 signal peptide (s.p) with msIgG2a Fc sequence, followed by msNKG2D extracellular (EC) domain. Fc-huNKG2D was constructed by fusing mouse Dap10 s.p with huIgG1 Fc sequence, followed by huNKG2D EC domain. (b) SDS-PAGE analysis of purified Fc-NKG2Ds. Fc-msNKG2D and Fc-huNKG2D can be efficiently purified by rProtein A columns. M indicate protein marker, ms and hu indicate Fc-msNKG2D and Fc-huNKG2D, respectively. (c)

Experimental design for testing conjugated NP specificity. NKG2D ligand positive and negative cells were mixed before experiment. NKG2D/NPs or control NPs were added to the cell mixture and incubated on ice for 30 min. Pre-magnetic separated, unbound, and bound cell fractions were harvested during magnetic cell separation process. The identity of harvested cells in each fraction were analyzed by flow cytometry.

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Figure 2. NKG2D/NP specifically targeted mouse NKG2D ligand positive tumor cells in a dose dependent manner

(a) RMA (NKG2D ligand-) and RMA-RG (NKG2D ligand+, EGFP+) tumor cells were mixed and different amounts of msNKG2D/NPs or control NPs were added to the cell mixtures. After magnetic separation, the bound, unbound, and no separation fractions (Fr) were analyzed by flow cytometry. The percentage of each cell type is shown. Histograms shown are representative data of two independent experiments. (b) P815 (NKG2D ligand-) and P815/MICA (NKG2D ligand+, CFSE labeled) cells were mixed and incubated with different amounts of huNKG2D/NPs or control NPs. Pre-separation, bound, and unbound

fractions were analyzed by flow cytometry. Histogram shown are representative data of three independent.

Figure 3. Multiple mouse NKG2D ligand+ tumor cell lines can be targeted by msNKG2D/NP *in vitro* even under a low tumor to normal cell ratio

(a) RMA-RG, ID8, and RMA tumor cells were stained with Fc-msNKG2D and goat anti-mouse IgG secondary antibodies. Open histograms represent specific stainings and filled histograms represent isotype controls. Mean fluorescent intensity (MFI) of specific staining is as indicated. (b) RMA-RG, ID8 (NKG2D ligand+), and RMA (NKG2D ligand-) tumor cells were labeled with 0.5 μ M CFSE and spiked into splenocytes (unlabeled) at a 1:19 tumor:splenocyte ratio. MsNKG2D/NPs (10 μ g) were added to the cell mixture. Pre-separation, bound, and unbound fractions were harvested and cells were analyzed by flow

cytometry. Histogram shown are representative data of two independent experiments and the percentage of tumor cells captured was calculated from pooled results of two independent experiments. Statistical analysis was performed using a Student's *t* test to compare percentage of tumor cells captured from ligand+ groups to ligand– group (n.s. indicates non-significant, * indicates $p < 0.05$).

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Figure 4. Multiple human NKG2D ligand+ tumor cell lines can be specifically targeted by huNKG2D/NP *in vitro* at a low ratio of tumor cells to normal cells

(a) K562, P815/MICA, RPMI8866, and P815 tumor cells were analyzed for expression of NKG2D ligands. Open histograms represent specific staining with huNKG2D-Fc and filled histograms represent isotype controls. Mean fluorescent intensity (MFI) of specific staining is as indicated. (b) K562, P815/MICA, RPMI8866, and P815 tumor cells were labeled with 0.5uM CFSE. Tumor cells were spiked into PBMCs at a 1:19 tumor cell:PBMC ratio and huNKG2D/NPs (50ug) were added to the cell mixtures. Bound and unbound fractions were harvest and analyzed by flow cytometry. Histogram shown are representative data of three

independent experiments and the percentage of tumor cells captured was calculated from pooled results of three independent experiments. Statistical analysis was performed using a Student's *t* test to compare percentage of tumor cells captured from ligand+ groups to ligand – group (n.s. indicates non-significant, ** indicates $p < 0.005$).

Figure 5. NKG2D/NP binding to splenocytes or PBMCs is not due to NKG2D

(a) Murine splenocytes (5×10^6 cells) were incubated with 50ug Fc-msNKG2D/NPs, msIgG/NPs, or unconjugated NPs. Bound fractions were harvested and stained with anti-GR1 mAbs and anti-B220 mAbs. Cells were analyzed by flow cytometry. Flow plots shown are representative results of two independent experiments. (b) Human PBMCs (5×10^6 cells) were incubated with 50ug Fc-huNKG2D/NPs, huIgG/NPs, or unconjugated NPs. Bound fractions were harvested and then stained with anti-CD14 mAbs and anti-CD20 mAbs. Cells were analyzed by flow cytometry. Flow plots shown are representative results of two independent experiments.