Targeting MHC-I related proteins for cancer diagnosis and therapy

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Chapter 6:

Nanobody-based CAR T cells for selective cytotoxicity towards MICA^{+} cancer cells in vitro

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Abstract

The stress-induced surface glycoproteins MICA and MICB are MHC-I related proteins that are upregulated on the surface of virus-infected cells or malignant cells. MICA and MICB act as ligands for NKG2D, one of the activating receptors on NK cells, $CD8^+$ T cells, and γδ T cells. When MICA binds to NKG2D, these cytotoxic immune cells become activated and can eradicate the MICA/B-positive targets through cytotoxicity and secretion of cytokines. Nanobodies, also referred to as VHHs, are the variable regions of camelid heavy chain-only immunoglobulins. We previously created nanobodies that recognize MICA and used these nanobodies as building blocks for the construction of chimeric antigen receptors (CAR) for expression in human CAR NK-92 cells. Here, we use these nanobodies to establish VHH-based CAR T cells and show that these cells recognize and selectively kill MICA positive tumor cells *in vitro*.

Introduction

The MHC class I chain-related proteins A and B (MICA and MICB) are upregulated on the surface of human cells undergoing stress, for instance due to virus infection or malignant transformation[224]. MICA/B are ligands for NKG2D, an activating receptor on NK cells, $CD8^+$ T cells, and γδ T cells, which can eradicate MICA-positive targets through cytotoxicity and secretion of cytokines[218]. Elevated levels of MICA/B on the surface of hematopoietic and epithelial solid tumors are associated with better prognosis[225–231].

Nanobodies, or VHHs, are the recombinantly expressed variable regions of camelid heavy chain-only immunoglobulins[301] which are characterized by their solubility, stability, and ease of production[309–311]. Due to their small size of 15 kD (versus 150 kD for conventional full-sized antibodies), nanobodies have a short circulatory half-life and show excellent tissue penetration. Nanobodies are attractive building blocks for the construction of chimeric antigen receptors for cell-based therapies[210,474–478,546–551].

We have developed nanobodies, VHH A1 and VHH H3, that target MICA on the surface of tumor cells. We used these nanobodies to establish anti-MICA VHH-based CAR NK-92 cells and showed that these cells recognize and selectively kill MICA positive targets *in vitro* and *in vivo*[620].

Although CAR NK cells may have an advantage in terms of safety and versatility, research on CAR NK cell-based therapy is still in the early stages of development. CAR T cell therapy has been more widely studied, with several CAR T cell therapies approved for treatment of hematopoietic cancers, such

as relapsed or refractory B-cell lymphoma or acute lymphatic leukemia based on CD19 targeting (Axicabtagene ciloleucel, brexucabtagene autoleucel, lisocabtagene maraleucel, and tisagenlecleucel), and relapsed or refractory multiple myeloma, based on B-cell maturation antigen (BCMA) targeting (idecabtagene vicleucel and ciltacabtagene autoleucel)[202]. Here, we report the generation of nanobody-based CAR T cells that recognize and selectively kill $MICA^+$ cells *in vitro*.

Materials and methods

Cell culture

MICA-expressing B16F10 murine melanoma cells and EL-4 T cell lymphoma cells, and their wild type (WT) counterparts, were a gift from K. Wucherpfennig (Dana Farber Cancer Institute). B16F10 cells and HEK293T cells were both cultured in complete DMEM (high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin). To avoid proteolytic cleavage of membrane-bound MICA, we dissociated the adherently grown B16F10 cells from the plates using an EDTA-based versene solution (Gibco). EL-4 cells were cultured in complete RPMI 1640 (RPMI 1640 supplemented with 10% FBS + 100 U/mL penicillin/streptomycin). Mouse primary T cells were cultured in BMDC medium (RPMI 1640 supplemented with 10% FBS, 1mM sodium pyruvate, 10 mM MEM-NEAA, 50 mM β-mercaptoethanol, and 100 U/mL penicillin/streptomycin). To ensure active cell proliferation, 100 IU/mL murine IL-2 (Peprotech) was added. All cells were cultured to maintain optimal densities, unless otherwise specified, and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Mice

C57BL/6J mice were purchased from the Jackson Laboratory or bred in-house. Mice were used at 8-12 weeks of age. Experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Boston Children's Hospital. Mice were housed under specific pathogen-free conditions in a controlled environment with a 12-hour light-dark cycle and ad libitum access to standard laboratory chow and water. Health status and welfare of the mice were monitored regularly throughout the study.

VHH CAR construct design and virus production

We designed a GBlock™ Gene fragment that encodes GFP, followed by a P2A proteolytic processing site to separate the GFP portion from the CAR itself. The CAR antigen recognition domain is encoded by the amino acid sequence of VHH A1 or VHH H3, separated by a hinge from the transmembrane segment of CD8 and the cytoplasmic signaling and costimulatory domains of CD28 and CD3ζ. The gene fragments for the CAR were inserted by 'sticky-end' cloning into a retroviral backbone with a mammalian MSCV promotor, modified in-house to include the desired sticky-end restriction sites (MSCV-IRES-GFP was a gift from Tannishtha Reya (Addgene plasmid #20672; http://n2t.net/addgene:20672; RRID: Addgene_20672). For retroviral production, we transfected HEK-293T cells with 7.5µg of CAR plasmid and 7.5µg of pCL-Eco (pCL-Eco was a gift from Inder Verma (Addgene plasmid #12371; http://n2t.net/addgene:12371; RRID: Addgene_12371[621]) in 1000 µl of OptiMEM. This DNA mixture was added to 500 µl of OptiMEM with 45 µl of TransIT®-LT1 (Mirus Bio LLC) and incubated for 30 minutes at room temperature. The mixture was added to ~70% confluent HEK293T cells in 10mL of DMEM + 10% FBS and retrovirus was harvested 48 hours after transfection.

Isolation of mouse T cells

Freshly isolated and transduced T cells were used for each CAR T cell experiment. T cells were isolated from the spleens of 8- to 12-week-old C57/B6 mice. Spleens were collected aseptically, homogenized, and filtered through a 40 µm cell strainer. Red blood cells were lysed with 0.8% ammonium chloride for 10 minutes on ice. T cells were isolated using the Dynabeads Untouched T cell isolation kit (Invitrogen) according to manufacturer's protocol. T cells were resuspended at $1x10^8$ cells/mL in isolation buffer (PBS with 2% FBS and 2mM EDTA). Heat-inactivated FBS (200 mL) and 200 mL antibody mix was added and incubated on ice for 20 minutes. Cells were washed with and resuspended in isolation buffer and added to the Mouse Depletion Dynabeads™. The cell-bead mixture was incubated for 15 minutes at room temperature on a rotating platform and diluted with isolation buffer prior to placement in a neodymium magnet for 2 minutes to retrieve the magnetic beads. The supernatant containing the untouched T cells was transferred to a fresh tube. T cells were activated with Dynabeads™ Mouse T-Activator CD3/CD28 (Invitrogen) at 1 ml of beads per 40,000 cells. Cells were cultured at $1.5x10^6$ cells per mL in BMDC medium with murine IL-2 (100 IU/mL) until further applications.

Retroviral transduction and selection of transduced murine T cells

For T cell transduction, a non-tissue culture treated, plastic bottom 6-well plate was coated with RetroNectin® according to the manufacturer's directions. Retroviral particles were adsorbed to the plate by centrifugation for 2 hours at 2000xg at 30°C. Viral supernatant was removed and T cells were added to the well and transduced by centrifugation for 1-1.5 hours at 2000xg at 30°C. After 24 hours the expression of GFP was measured by flow cytometry. If a transduction efficiency of at least 25% was achieved, CAR T cells were then used for further experiments.

In vitro cytotoxicity and cytokine production assays

For co-culture experiments, $3x10^5$ WT or $MICA^+$ B16F10 or WT or $MICA^+$ EL-4 cells were plated per well on a tissue culture-treated 96-well flat-bottom plate in complete RPMI supplemented with IL-2 (50 IU/ml). A1 CAR T cells or untransduced T cells were added to each well at different effector-to-target [E:T] ratios, keeping the number of target cells constant and varying the number of effector cells. After 24 hours, the IFN-γ concentration in the medium was determined using a mouse IFN-γ ELISA kit (Thermofisher scientific, #88-7314-22) according to the manufacturer's instructions. Relative cell death was determined with a lactate dehydrogenase (LDH) Cytotoxicity Assay (Abcam, Ab65393) performed according to the manufacturer's instructions.

Results

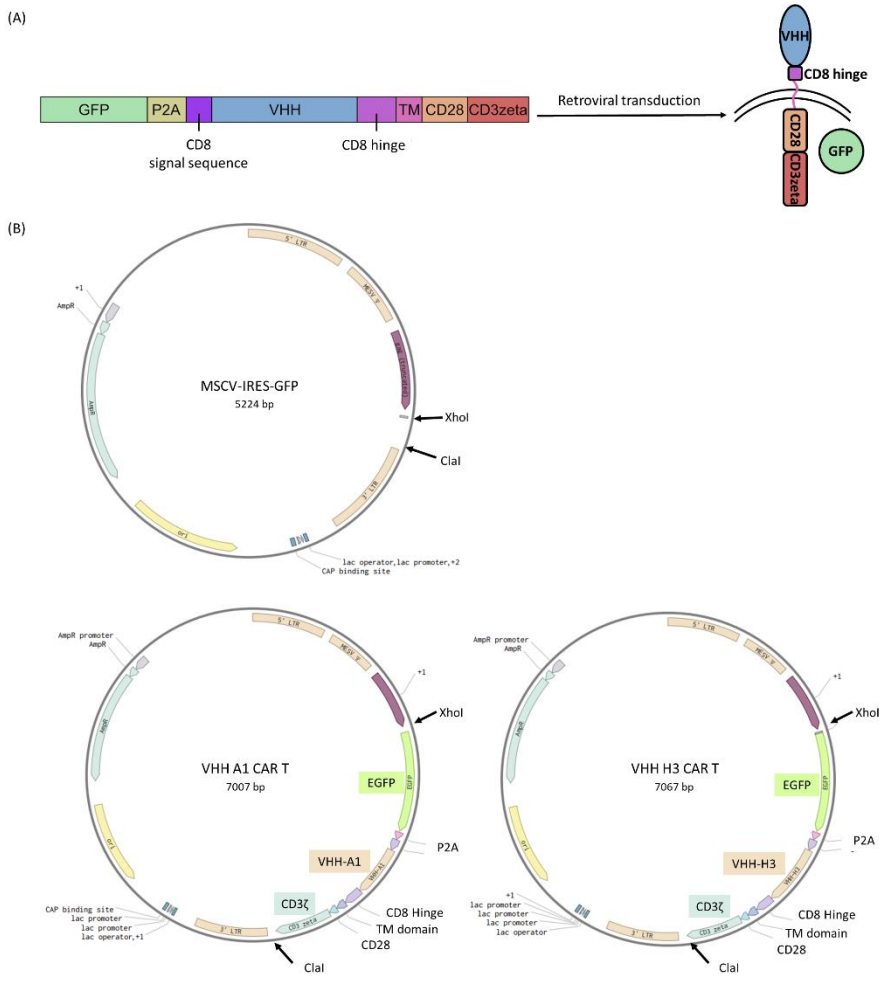
Transduction with retroviral VHH-based CAR constructs yielded MICA-specific CAR T cells

Design of CAR T cells was based on previously established VHH-based CAR T cells[476]. We designed a GBlock™ Gene fragment that encodes GFP followed by a P2A domain separating the actual CAR construct. The extracellular CAR antigen recognition domain is encoded by the amino acid sequence of VHH A1 or VHH H3, separated by a hinge from the transmembrane segment and the cytoplasmic signaling and costimulatory domains of CD28 and CD3ζ (Figure 1A). The gene fragments for the CAR were inserted by 'sticky-end' cloning into a retroviral backbone carrying a mammalian MSCV promotor (Figure 1B) and used for retroviral production in HEK-293T cells. We transduced freshly isolated T cells from mouse splenocytes by spinoculation using RetroNectin® reagent. Cells bearing these VHH-based CARs will be referred to as A1 and H3 CAR T cells. As a negative control for CAR T cells, we used untransduced T cells that underwent the same transduction protocol but in the absence of retrovirus. Because primary T cells have a finite lifespan

in culture, we produced a fresh batch of transduced T cells for each experiment. On average, we observed a transduction efficiency between 25% and 50% for A1 CAR T cells and between 20% and 30% for H3 CAR T cells. Flow cytometry data for one representative transduction are shown in Figure 1C.

A1 CAR T cells are activated by and selectively kill MICA$^+$ tumor cells

We incubated A1 and H3 CAR T cells, or untransduced T cells as negative control, with WT B16F10 or EL4 cells, or B16F10 or EL4 cells that stably express MICA at different effector to target ratios ([E:T]), keeping the amount of target cells constant and varying the number of effector cells.



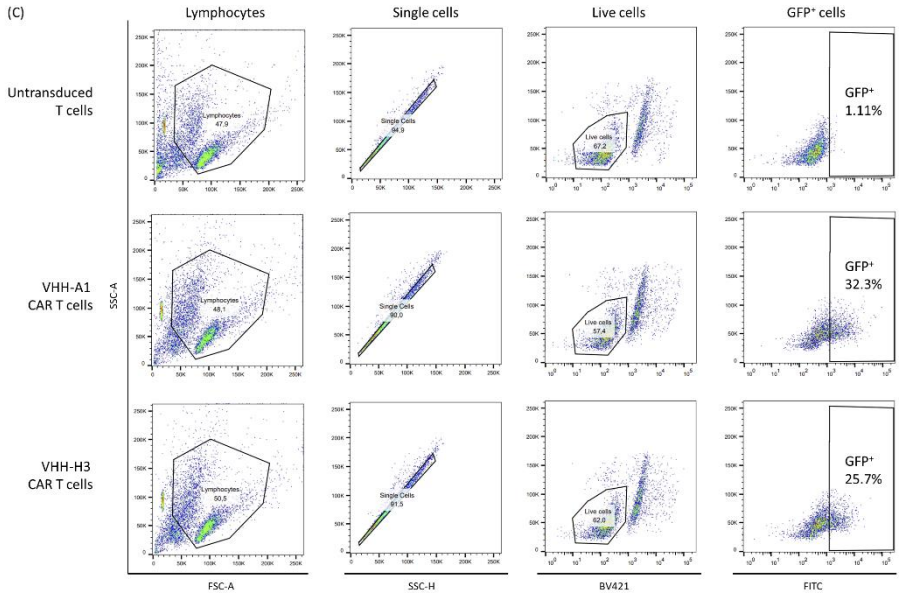


Figure 1. Design of the CAR construct and production of CAR T cells. *(A) Schematic overview of the CAR construct as transduced into the mouse primary T cells. We ordered GBlock™ gene fragments encoding for EGFP, followed by a P2A proteolytic cleavage domain separating the EGFP from the CAR construct. The CAR construct contains the amino acid sequence of VHH A1 or VHH H3 as extracellular targeting domain, and the costimulatory and activation domains of CD28 and CD3ζ. (B) We used a retroviral backbone with a mammalian MSCV promoter, modified in-house to contain XhoI and ClaI restriction sites. We cloned the GBlockTM gene fragments with 'sticky-end' cloning. These plasmids were used to create retrovirus for the transduction of isolated primary mouse T cells. (C) 24 hours after transduction, we determined the transduction efficiency by flow cytometry. Viability was determined using the LIVE/DEAD Fixable Dye (Invitrogen). We used T cells that underwent the same retroviral transduction protocol, but in the absence of retrovirus, as negative control. GFP positive cells were deemed transduced successfully. If a transduction efficiency of >25% was reached, cells were used for downstream applications.*

We measured cell death by determining the release of lactate dehydrogenase (LDH) in the culture medium. We normalized the level of cytotoxicity by subtracting the LDH released by the T cells due to reduced viability, determined by using wells containing only T cells at the appropriate cell densities. At a [E:T] ratio of [15:1], we observe significant cell death of the MICA⁺ B16F10 cells when co-cultured with A1 CAR T cells. We do not observe significant cell death when co-culturing the B16F10 WT cells with A1 CAR T cells. We do not observe significant cell death in MICA⁺ cells when co-cultured with untransduced T cells, or when co-cultured with H3 CAR T cells. For the MICA⁺ EL-4 cells, we observed significant cell death when co-cultured

with A1 CAR T cells at an [E:T] of [5:1] and [15:1]. We do not observe significant cell death in EL-4 WT cells co-cultured with A1 or H3 CAR T cells, or EL-4 MICA^{+} cells co-cultured with untransduced T cells (Figure 2A).

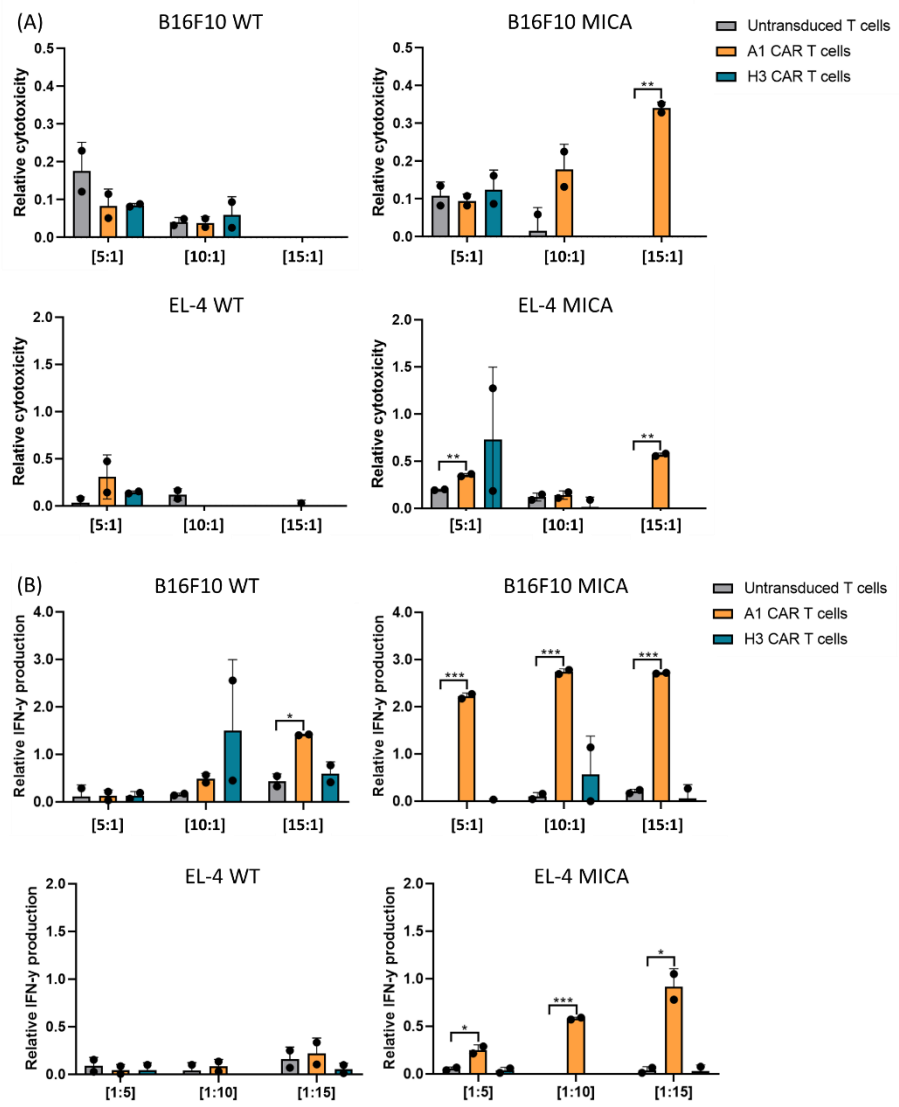


Figure 2. In vitro cytotoxicity of CAR T cells co-cultured with MICA^{+} targets.
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We incubated untransduced T cells, and A1 and H3 CAR T cells with WT B16F10 or EL-4 cells, or B16F10 or EL-4 cells that stably express MICA. We incubated for 24 hours at effector-to-target ratios [E:T] of [5:1], [10:1], or [15:1], keeping the number of target cells consistent and varying the number of effector cells. (A) Cytotoxicity was determined by measuring the LDH released in the medium. LDH concentration was normalized for the concentration of LDH released by the T cells alone due to reduced viability. At an [E:T] ratio of [15:1], we observe significant cell death of the MICA$^+$ cells when co-cultured with A1 CAR T cells ($p = 0.01$). We observe significant cell death of EL-4 MICA$^+$ cells when co-cultured with A1 CAR T cells at an [E:T] of [5:1] ($p = 0.007$) and [15:1] ($p = 0.001$). We observe no significant cell death in MICA$^+$ cells when co-cultured with untransduced T cells. No significant increase in cell death was observed when WT B16F10 or EL-4 cells were co-cultured with untransduced T cells, or A1 or H3 CAR T cells. (B) The relative levels of IFNγ were determined by sandwich ELISA with a matched murine IFNγ antibody pair. We observed significant increase in IFNγ secretion in the A1 CAR T cells when co-cultured with B16F10 and EL-4 MICA$^+$ cells at all [E:T] ratios (B16F10 MICA$^+$: [5:1] $p = 0.0005$; [10:1] $p = 0.0007$; [15:1] $p = 0.0002$, EL-4 MICA$^+$: [5:1] $p = 0.04$; [10:1] $p = 0.0003$; [15:1] $p = 0.02$). No significant release of IFNγ was observed in untransduced T cells or H3 CAR T cells co-cultured with any type of target cell, or A1 CAR T cells co-cultured with WT B16F10 or EL-4 cells. All significance was determined with multiple T-test.

To attribute the cytotoxicity to effector cell activation, we measured the secretion of IFNy in the culture medium. We normalized IFNy secretion by subtracting the spontaneous IFNy secretion by the T cells, determined by using wells containing only T cells at the appropriate cell densities. We observed a significant increase in IFNy secretion by A1 CAR T cells when co-cultured with B16F10 MICA$^+$ cells and EL-4 MICA$^+$ cells at all [E:T] ratios, but not when co-cultured with WT cells (Figure 2B). We did not see activation of H3 CAR T cells in the presence of either WT or MICA$^+$ target cells. Our previous data shows less cytotoxicity for the H3 CAR NK-92 cells as well, likely due to the lower expression of the H3-based CAR[620]. Although we have not determined and compared the CAR expression levels for the A1 and H3 CAR T cells, the data for H3-based CAR T cells is consistent with the previous findings for H3-based CAR NK cells.

Discussion

The Class I MHC-related proteins MICA and MICB, expressed on the surface of cells undergoing stress, can serve as both a diagnostic marker for certain cancers, and as a target for cancer therapy. We have produced high-affinity nanobodies A1 and H3, both of which target MICA on the surface of cells[575]. Nanobodies have been used as the targeting portion of CAR T cells, with the first nanobody-based CAR T cell (Carvykti) approved for treatment of relapsed or refractory multiple myeloma[476].

We have shown that the MICA-targeting nanobodies VHH-A1 and VHH-H3 can serve as antigen-recognition domains in CAR NK cells[620]. While advantages of using CAR NK cells over CAR T cells for therapy have been claimed[217,622], the success of CAR T cell therapy makes the production of nanobody-based CAR T cells an interesting possibility. Here, we developed VHH-based CAR T cells by retroviral transduction of T cells isolated from the spleens of mice. When co-culturing these CAR T cells with WT or $MICA^+$ cells of the murine-derived B16F10 melanoma line or EL-4 T-cell lymphoma line, we observed an increase in cytotoxicity of the $MICA^+$ cells compared to the WT cells, when co-cultured with high doses of A1 CAR T cells. Furthermore, we see a dose-dependent increase in IFN-γ release in the A1 CAR T cells co-cultured with $MICA^+$ B16F10 or EL-4 cells, compared to WT B16F10 or EL-4 cells. We see no such effect when co-culturing these cells with untransduced T cells. These results indicate that the A1 CAR T cells selectively target and kill $MICA^+$ B16F10 and $MICA^+$ EL-4 cells *in vitro*.

Although the *in vitro* data are promising, the efficacy of these VHH-based CAR T cells *in vivo* remains to be tested. A major limitation is the relatively low transduction efficiency of the T cells. Despite extensive troubleshooting steps, we have been unable to reach a transduction efficiency of, on average, more than 35%. Our method of isolation of T cells from splenocytes does not discriminate between $CD4^+$ and $CD8^+$ T cells, only the latter of which are responsible for cytotoxicity. Because the T cell fraction of mouse splenocytes consists for ~20% of $CD8^+$ T cells[623], we hypothesize that the total population of VHH-based CAR T cells capable of cytotoxicity may be no more than ~7%. This number limits the effectiveness of CAR T cell-based cytotoxicity *in vitro,* since high [E:T] are necessary to obtain an effective dose.

For *in vivo* experiments, it may be necessary to increase the number of cytotoxic CAR T cells transferred. Considering that $5x10^6$ successfully transduced CAR NK-92 cells were effective at eliminating $MICA^+$ tumors *in vivo*[620], it would be desirable to attain a similar number of successfully transduced $CD8^+$ CAR T cells. Based on transduction efficiency, we need to inject ~$72x10^6$ cells per mouse per injection. Each spleen yields approximately $40x10^6$ T cells, thus requiring several spleens as the starting material. At 3 injections per week, treatment of one mouse for one week requires the use of 6 spleens.

The troubleshooting steps we have undertaken have increased the average transduction efficiency from 12% to 35%, while simultaneously increasing cell viability. This improvement was mainly caused by switching from a

polybrene-based retroviral transduction to RetroNectin®, since polybrene is known to inhibit T cell viability[624]. We have also experimented with the addition of different stimulating cytokines, like varying concentrations of IL-2 and IL-15, the latter of which decreased the average cell viability and transduction efficiency.

An alternative option is to switch from retroviral to lentiviral transduction of T cells. Lentiviruses can infect non- or slowly dividing cells and establish long-term gene expression[625]. Lentiviral-based transduction has several other advantages, including a higher viral titer and virion stability, which could improve transduction efficiency.