UNIVERSITY OF CALIFORNIA SAN DIEGO

Gene editing of iPSC-derived natural killer (NK) cell and head and neck squamous cell carcinoma to improve NK cell anti-tumor activity

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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2021

The thesis of Jiyoung Yun

is approved, and it is acceptable in

quality and form for publication on microfilm and electronically.

University of California San Diego

2021

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ABSTRACT OF THE THESIS

Gene editing of iPSC-derived natural killer (NK) cell and head and neck squamous cell carcinoma to improve NK cell anti-tumor activity

by

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Master of Science in Biology

University of California San Diego, 2021

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Despite advancements in hematopoietic stem cell transplant (HSCT), graft rejection and graft versus host disease (GvHD) are leading cause of post-transplant mortality. To prevent side effects and to minimize graft rejection, glucocorticoids such as dexamethasone are often administered to patients after transplantation. However, the use of steroids suppresses the proliferation and cytotoxic function of natural killer (NK) cells. To overcome this limitation, I knocked out nuclear receptor subfamily 3 group C member 1 (NR3C1), gene that encodes for the glucocorticoid receptor, on induced pluripotent stem cell (iPSC)-derived NK cells. The NR3C1 knock out (KO) NK cells demonstrated higher proliferation in the presence of high concentration of dexamethasone in vitro while the proliferation of wild type iPSC-NK was inhibited. These studies demonstrate engineered-NR3C1 KO iPSC-NK cells can provide a novel therapeutic benefit in patients undergoing cell therapies that might involve glucocorticoid treatment to ameliorate toxicities.

Even with the improvement in iPSC-NK cell therapy, tumor cells become resistant to, and escape NK cell mediated immune surveillance. Previous screening data from our group identified Charged Multivesicular Body Protein 2A (CHMP2A) as a gene that mediates tumor cell resistance to NK cell cytotoxicity. We confirmed that the deletion of CHMP2A in glioblastoma and head and neck squamous cell carcinoma (HNSCC) increased allogeneic NK cell mediated killing in vivo. Here, we better define the role of CHMP2A to regulate anti-tumor immune responses by deletion of CHMP2A in 4MOSC1 and 4MOSC2 HNSCC lines and testing these for tumor development in an immunocompetent syngeneic mouse model.

Introduction

Natural killer (NK) cells are an important component of the immune system and are currently used clinically to target refractory malignancies due to their intrinsic anti-tumor killing ability without HLA restriction and prior antigen priming. As a key part of the innate immune system, NK cells comprise 5-15% of peripheral blood lymphocytes and are characterized as $CD3^{-}CD56^{+}$ lymphocytes in humans[1]. While T cells recognize the antigenic peptides presented on the surface of the major histocompatibility complexes (MHC) through their T cell receptors (TCRs), NK cells do not rely on a single receptor for their activation[2]. Instead, NK cell response is controlled by multiple signals from both inhibitory and activating receptor[2, 3]. Moreover, the cytotoxic activity of NK cells is mediated by multiple pathways. Perforin and granzyme-mediated killing are the major mechanism of NK cell cytotoxicity[4]. Once NK cells interact with target cells, they form an immunological synapse where NK cells release granules containing perforin and granzymes that lyse the target cells[5]. Another pathway is through death receptors. NK cells express ligands from the tumor necrosis factor (TNF) superfamily such as Fas ligand (FasL), TNF, and TNF-related apoptosis-inducing ligand (TRAIL) that binds to the death receptor on target cells[3].

NK cell alloreactivity is another key therapeutic element which plays an important role in hematopoietic stem cell transplant (HSCT). NK cells recognize HLA class I molecules on the target. Once NK cells sense the HLA class I molecules, NK cell mediated killing is blocked through its inhibitory killer cell immunoglobulin-like receptor (KIR)[6]. However, lack of surface HLA molecule expression makes the target susceptible to NK cell killing [7]. In mismatched transplantation, the NK cell mediated graft versus tumor (GvT) effect is mediated by donor NK cells sensing missing expression of HLA class I on the recipient tumor cells and inducing lysis of the 'missing self' recipient target[8]. However, post transplantation mortality caused by graft rejection and GvHD remain a limitation. In fact, after transplantation, glucocorticoids are used on patients to prevent complications such as

GvHD[9]. While the immunosuppressive properties of steroids can prevent post transplantation side effects, it limits the cytotoxic function of NK cells and their expansion[10]. Therefore, by making NK cells resistant to glucocorticoids, we can enhance their efficacy in HSCT along with glucocorticoid treatment.

Glucocorticoids (GCs) bind to the glucocorticoid receptor (GR) and control the expression of GC target genes[11]. Specifically, GCs are known to inhibit NK cell cytotoxicity by reducing the transcription of genes that mediate NK cell effector function[10, 12]. Previous studies showed that once GCs enter the cell, they induce the translocation of the glucocorticoid receptor into the nucleus[11]. In the nucleus, GR recruits histone deacetylases (HDACs) which remove activating histones marks from the promoter region of immune effector genes which leads to change in gene expression and production of immune effector molecule like perforin[11]. This epigenetic remodeling inhibits the proliferation and function of NK cells[11]. Here, I deleted nuclear receptor subfamily 3 group C member 1 (NR3C1), gene that encodes GR protein, on induced pluripotent stem cell (iPSC) derived NK cells to engineer glucocorticoid- resistant NK cells to prevent suppression of iPSC-NK cells under dexamethasone treatment.

I hypothesized that deletion of NR3C1 will prevent steroid mediated suppression of iPSC-derived NK cell and show improved expansion and function under steroid treatment. To test the hypothesis, I used the CRISPR/ Cas9 system to knock out the NR3C1 gene in iPSC-NK cells. We demonstrate that NR3C1$^{-/-}$ iPSC-NK cells show significantly better expansion, enhanced anti-tumor activity and persistence than wild type iPSC-NK cells in culture with dexamethasone. Furthermore, deletion of NR3C1 showed increased in NK cell-mediated killing in vitro against different tumor cell lines. As expected, dexamethasone pretreated WT iPSC-NK cells were significantly decreased in NK cell-mediated killing in vitro. Together, these studies demonstrate that deletion of NR3C1 in NK cells can prevent the loss of function seen with dexamethasone.

Head and neck squamous cells carcinoma (HNSCC) is a highly aggressive solid tumor with a high rate of mortality, morbidity, and high chance for recurrence[13]. Furthermore, HNSCC is a highly immune-infiltrated cancer types, however, the response rate to immunotherapy relying on modulating T cell anti-tumor response is generally low[13, 14]. Recently, immune checkpoint inhibitors against programmed death 1-programmed death ligand1 (PD1-PDL1) pathway showed improved response from patients with metastatic HNSCC but limited compared to other malignancies[15, 16]. Recent studies on NK cell antitumor activity against HNSCC exhibits highest CD56+ NK cell infiltration of any major tumor type and high degree of infiltration of NK cells correlated significantly with patient's prognosis and survival[15, 17]

Previous screening data from our group identified Charged Multivesicular Body Protein 2A (CHMP2A*)* as a gene that mediates tumor cell resistance to NK cell cytotoxicity. Specifically, CHMP2A mediates secretion of extracellular vehicles (EVs) expressing the NK cell activating ligands MICA/B which act as a decoy to inhibit NK cells killing of tumor cells. We confirmed that the deletion of CHMP2A in glioblastoma and head and neck squamous cell carcinoma (HNSCC) increased allogeneic NK cell mediated killing in vivo. Here, we determine the impact of deletion of CHMP2A in vivo with an intact immune model using CRISPR-Cas9 technology in 4MOSC1 and 4MOSC2 HNSCC tumor lines.

CHMP2A is a subunit of the endosomal sorting complexes required for transport III (ESCRT-III), a machinery regulating membrane remodeling events such as formation of multivesicular bodies and biogenesis of extracellular vesicles (EV)[18]. As tumor cells modify the tumor microenvironment to be become immunosuppressive, HNSCC cells secrete EVs expressing MHC Class I Polypeptide-Related Sequence A/B (MICA/B), ligand expressed on tumor cells for NK activating receptor NK group2 member D (NKG2D). Expression of MICA/B on the surface of EVs serves as a decoy and inhibits NK cell antitumor activity. In addition, these EVs also express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or Fas ligand (Fas L) which induce apoptosis in NK cells. In fact, deletion of

CHMP2A in glioblastoma and HNSCC increased tumor sensitivity to NK cell antitumor activity. This was further confirmed in vivo using the HNSCC xenograft mouse model where Cal27 HNSCC cells with CHMP2A knock out increased tumor cell sensitivity to NK cell mediated killing.

Here, I developed a syngeneic mouse model to analyze the effect of CHMP2A deletion in 4NQO-induced murine oral squamous cells (4MOSC). 4MOSC cells are derived by inducing 4-nitroquinoline-1 oxide (4NQO), a tobacco associated carcinogen, in the tongue of C57Bl/6 mice[19]. Tobacco smoking is the leading cause of the HNSCC, making 4MOSC a suitable model to study HNSCC. We deleted the *CHMP2A* gene in the 4MOSC1/2 lines and then implanted 4MOSC1/2 wild type (Wt) or 4MOSC1/2 mCHMP2A Knock out (KO) cells orthotopically into the tongue of immunocompetent C57BL/6 mice. these studies suggest that CHMP2A plays an important role in NK cell mediated anti- tumor activity.

Methods

Cell culture

K562, Molm13, and RPMI 8226 were maintained in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, 11875085) with 10% fetal bovine serum (FBS), supplemented with 2mM L-glutamine (Invitrogen), 0.1mM MEM non-essential amino acids (NEAA) (Invitrogen), and 1% Pen-Strep (Invitrogen). Cal27 were cultured in DMEM-F12 1:1 (Gibco), 10% fetal bovine serum, supplemented with 2mM L-glutamine (Invitrogen), 0.1mM MEM non-essential amino acids (NEAA) (Invitrogen) (HNSCC complete media). 4MOSC cell lines were cultured in Defined Keratinocyte SFM (ThermoFisher) supplemented with 5ng/mL EGF Recombinant Mouse Protein (Thermofisher). iPSC-NK cells were cultured in RPMI 1640 (Invitrogen), 10% health inactivated FBS, 2mM L-glutamine, 1% PenStrep and supplemented with 50IU/mL of hIL-2 every three days or media change. NK cells were co-cultured once a week with irradiated K562-mbIL-21-4-1BBL artificial antigen presenting cells developed from our lab.

CRISPR-Cas9 gene editing

NR3C1 knock out (KO) was performed on iPSC-NK cells using the ribonucleoprotein (RNP) complex. I used 2 CRISPR RNAs (crRNAs) targeting exon2 of the human glucocorticoid receptor gene: crRNA#1, GAACACTGGTCGACCTATTG; crRNA#2, GGCCAGACTGGCACCAACGG. First, I prepared the crRNA and ATTO-labeled transactivating crRNA (tracrRNA) duplex for each crRNA. Then I incubated the duplex in 95°C for five minutes on a heat block. Cas9 protein (Integrated NA Technologies [IDT]), electroporation enhancer, and guide RNA (gRNA; crRNA plus tracrRNA combination) were incubated in room temperature for 15minutes. The incubated complex was than used to electroporate 1million iPSC-NK cells using the Nucleofector TM 2b device (Lonza, AAB-1001) with Cell line Nucleofector TM Kit R (Lonza, VVCA-1001). 24 hours after transfection, cells were selected with dexamethasone (30μM) (look for brand) for 2 weeks.

CHMP2A KO was performed on 4MOSC1 and 4MOSC2 cells provided by the Silvio Gutkind

lab at Moores Cancer Center using the RNP complex. I used 3 CRISPR RNAs (crRNAs) targeting exon 1 of the mouse CHMP2A gene: crRNA#1, CCAACGACAACCCCTCGGTT; crRNA#2, GTACGCTTGAGTAGTCAAAA; crRNA #3: GTTGTGAGGCCCCTCGACAA.

PCR gel electrophoresis

DNA was extracted and purified (QIAamp DNA Blood Mini Qiagen Inc) from iPSC-NKs. I used GoTaq green Master Mix from Promega. For polymerase chain reaction (PCR) amplification, I used the following primers spanning Cas9-single-guide RNA cleavage site of exon 2 of the GR gene: exon forward primer, ACAAAAGTGATGGGAAATGACCTGG; exon 2 reverse primer, GAGACGAATGAGAGTCCTTGGAGAT. The same DNA extraction kit and method was used to amplification of mCHMP2A gene. The following primers were used spanning Cas9-single-guide RNA cleavage site of exon1 of the CHMP2A gene: exon 1 forward primer, TAGCACTTCCCAGCATTCCCGGA; exon 1 reverse primer, TGCTGGTGGAGTCTTACCACCATGG.

Western Blot

Immunoblotting was performed according to Invitrogen protocols for Mini Gel Tank 30 and iBlot2 dry system. Briefly, cell lysates were prepared incubating cells with RIPA buffer (Invitrogen) 31 containing Halt Protease Inhibitor Cocktail (Invitrogen) and quantified using BCA assay (Thermo Fisher 32 Scientific). Lysates were prepared incubating cells with 300µl of RIPA buffer (Invitrogen) containing 33 Halt Protease Inhibitor Cocktail (Invitrogen) and sonicated for 10 minutes. 25 µl of lysate was loaden on gel. Proteins were separated using NuPAGE Bis-Tris 4%-12% gels (Invitrogen) and transferred to nitrocellulose membranes using the iBlot2 dry method. Membraned were blocked for 40 minutes in 4% milk and incubated with the primary antibodies in 4% milk 1.5 hours at room temperature for anti NR3C1/ CHMP2A antibody or ON at 4°C for all the other antibodies used. After incubating the membrane with the appropriate secondary antibody conjugated to horseradish peroxidase, protein levels were detected 6 using Immobilon Western

Chemiluminescent HRP Substrate (EMD Millipore).

Flow cytometry NK cells killing assay

Target cells were pre-stained with CellTrace Violet (Thermo Fisher Scientific) at a final concentration of 5mM in PBS for 15min at 37°C. Cells were incubated in complete culture medium containing FBS for 5 minutes and harvested by centrifugation. Cells were resuspended in culture media prior to being mixed with NK cell cultures at the indicated effector to target (E:T) ratios. Co-cultures were incubated at 37°C and after 3.5 hours CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific) was added for an additional 30 minutes of culture for a total incubation time of 4-hours. SYTOX AADvanced dead cell stain solution (Thermo Fisher Scientific) was added during the final 5 minutes of staining. Cells were then analyzed by flow cytometry. NK cells killing was calculated by subtracting the background of untreated target cells to all the other samples of the same experimental group. Experiments were performed with 3 independent biological triplicates. To perform the killing assay with dexamethasone pretreatment, both NK cells and target tumor cells were cultured with 3µM of dexamethasone 48hours prior to assay.

IncuCyte Caspase 3/7- apoptosis assay

NK cells were pre-stained with CellTrace Far Red (Thermo Fisher Scientific) at a final concentration of 5mM in PBS for 15 min at 37°C. Cells were incubated in complete culture medium containing FBS for 5 minutes and harvested by centrifugation. NK cells were resuspended in media containing IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent (Essen Bioscience) diluted by a factor of 1:1000 and $1x10^4$ NK were seeded in a 96 well plate. IncuCyte ZOOM was used to acquire images every hour for 24 hours. Experiments were performed with 3 independent biological triplicates. The apoptosis was quantified by analyzing the area of Caspase 3/7 (green) within the red labelled NK cells.

Mouse NK cell isolation

Spleens from C57Bl/6 mice were collected placed into a moisturized cell strainer

(100 µm nylon mesh) into a medium containing petri dish. The spleens were mashed through the cell strainer by a plunger. The cell strainer was placed on the top of a 50 ml conical tube and the single cell suspension was transferred through the strainer into the tube. The petri dish and the strainer were rinsed with fresh medium. 5mL of LCK lysis buffer were added and mixed for 5minutes to remove red blood cells. Separation of mouse NK cells were performed using magnetic beads on the autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany). After the different cell isolation procedures, I harvested the NK cell fractions, counted the cell numbers by use of trypan blue, and performed FACS analysis to determine the percentage of NK cells.

In vivo mouse experiments and analysis

All mice were housed, treated, and handled in accordance with the guidelines set forth by the University of California, San Diego Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. 4MOSC1 and 4MOSC2 cells were transplanted (1million per mouse) into the tongue of female C57Bl/6 mice (4-6 weeks of age and weighing 16-18g). Tumor volume was monitored using a caliper every 2-3 days. Mice were sacrificed when necrosis of tongue tumor tissue was observed or at the experiment end point with rapid and humane method of euthanasia

Results

Chapter1: Deletion of NR3C1 in human iPSC-Derived NK cells promotes resistant to dexamethasone and enhances anti-tumor activity combined with steroid treatment.

Generation of NR3C1$^{-/-}$ iPSC-NK using CRISPR-Cas9 technology

Previous studies from our group demonstrated that human iPSCs can be differentiated into NK cells that have similar function and phenotype as the Peripheral Blood natural killer (PB-NK) cells[20]. To generate NR3C1$^{-/-}$ iPSC-NKs, iPSC-derived NK cells were cultured in vitro in the presence of 50 IU/mL IL-2. I used RNP-mediated CRISPR-Cas9 gene editing to delete NR3C1 on iPSC-NK cells. Two crRNA sequences were used to target exon 2 of the human NR3C1 gene. Transfection efficiency, as indicated by ATTO 550- labeled tracrRNA fluorescence, was high (98%) and deletion of the gene was further confirmed by PCR, western blot, and DNA sequencing technique. Then I treated the transfected iPSC-NK cells with 15μM of dexamethasone to select only the NR3C1$^{-/-}$ iPSC-NK cells. The knockout efficiency was high as determined by PCR (Figure 1C) and western blot analysis (Figure 1D)

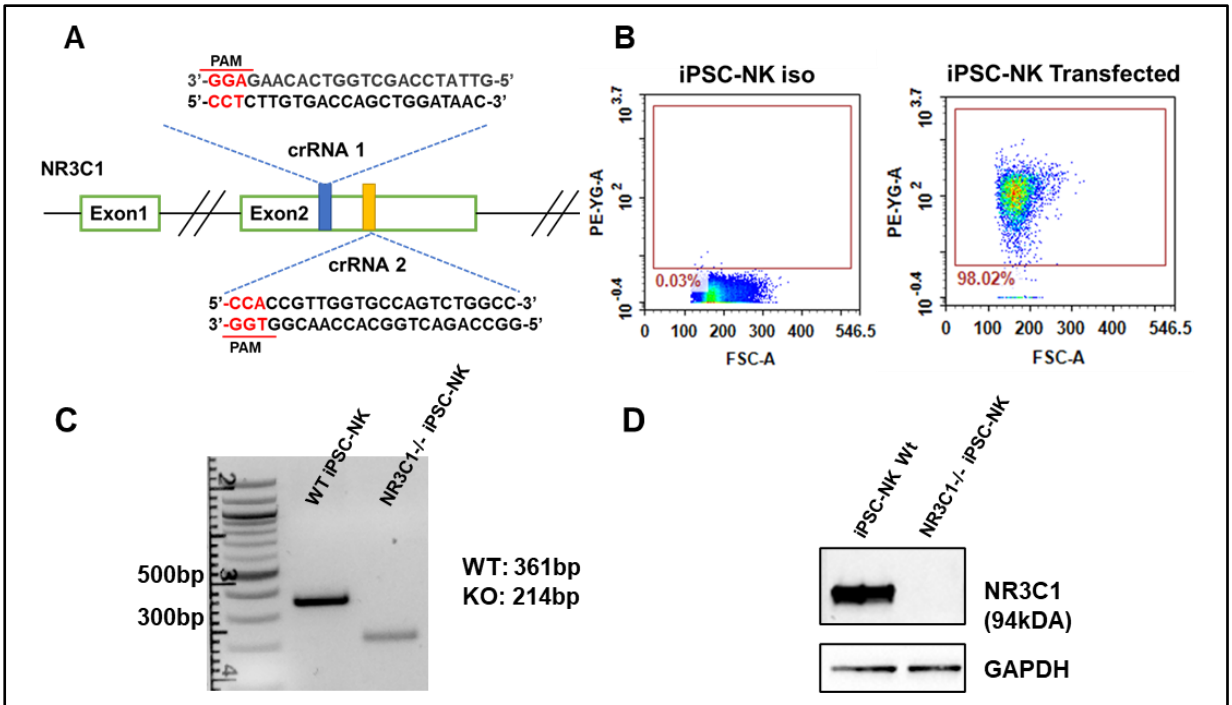


Figure 1: Generation of NR3C1$^{-/-}$ iPSC-NK cells (A)Schema of CRISPR-Cas9 mediated NR3C1 KO using two short guide crRNAs located in exon2 of the NR3C1 gene. (B)The RNP containing ATTO550-conjugated tracrRNA was transfected with the crRNA complex by electroporation. Fluorescence- activated cell sorter (FACS) plots showing the percentage of fluorescent signal. (C) NR3C1 KO efficiency of iPSC-NK cells after electroporation was determined by PCR at day3 or (D) western blot analysis at day 7. For this western blot, GAPDH was used as a loading control.

Deletion of NR3C1 protects iPSC-NKs from glucocorticoid mediated immune suppression without altering their phenotype

Then, I determined whether deletion of NR3C1 protects iPSC-NK cells from the immunosuppressive effect of glucocorticoid. I compared the expansion of cells both in short term (9days) and long term (21days) under 3μM of dexamethasone treatment. In the short term, iPSC-NK WT treated with dexamethasone showed limited expansion compared to the iPSC-NK WT untreated. However, NR3C1$^{-/-}$ iPSC-NK treated with dexamethasone maintained similar rate of expansion with the NR3C1$^{-/-}$ iPSC-NK untreated. Similarly, long term culture of iPSC-NK WT cells showed significantly lower cell growth compared to the NR3C1$^{-/-}$ iPSC-NK cells, confirming that NR3C1$^{-/-}$ iPSC-NK cells are resistant to glucocorticoids. Next, I assessed whether the deletion alters the phenotype of NR3C1$^{-/-}$ iPSC-NK cells. I analyzed expression of NK cell markers by flow cytometry. NR3C1$^{-/-}$ iPSC-NK cells consist of a homogeneous population of CD56+ NK cells that co-express typical NK cell surface antigens, including CD16, NKp44, NKp46, NKG2D, and CD45 (Figure 2C), similar with WT iPSC-NK cells.

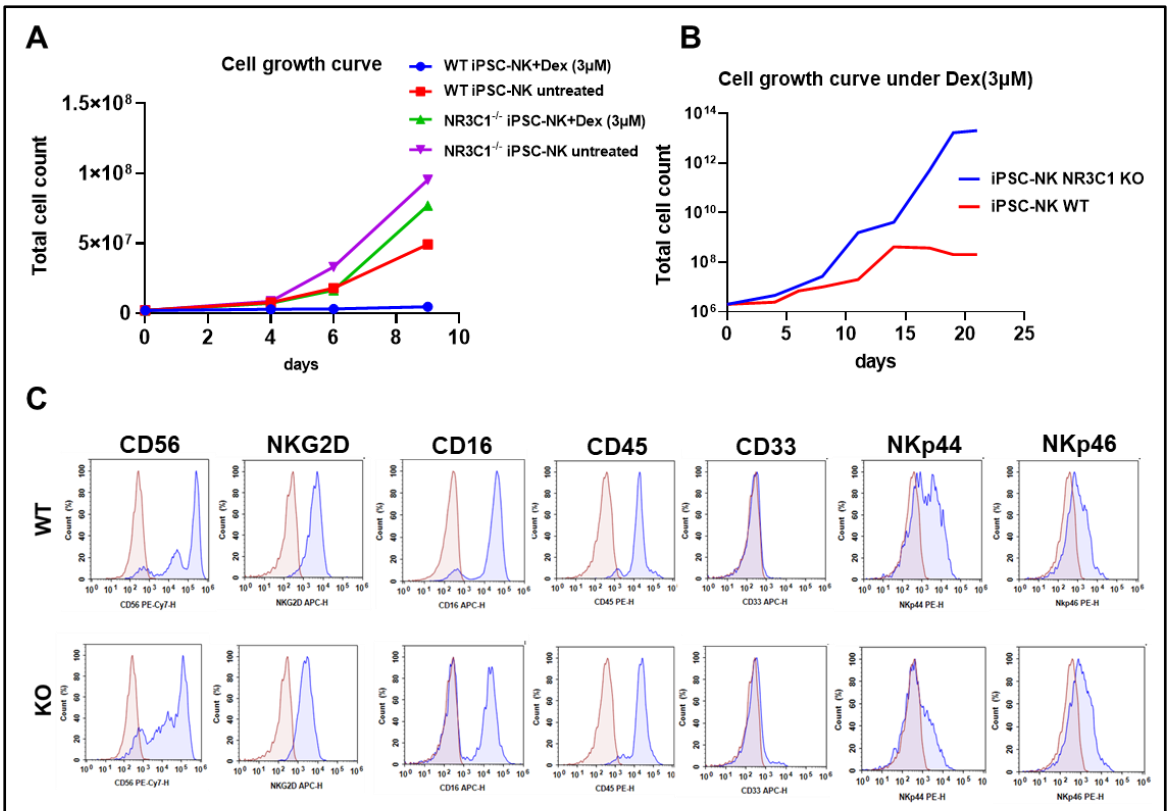
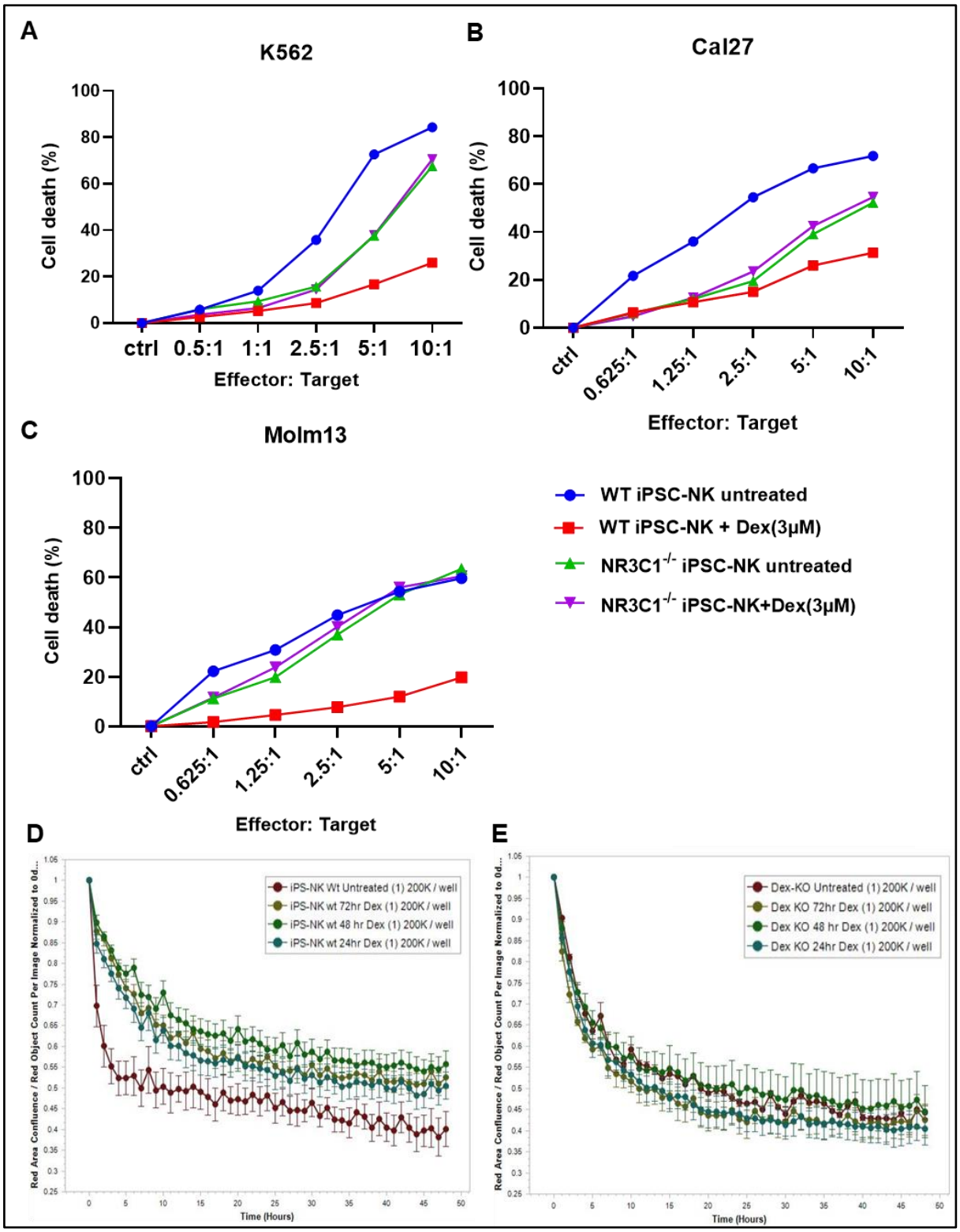


Figure 2: Deletion of NR3C1 protects iPSC-NKs from glucocorticoid mediated immune suppression(A) Expansion of in vitro WT iPSC-NK cells and NR3C1$^{-/-}$ iPSC-NK cells with and without dexamethasone (3μM). NK cells were stimulated with aAPC once a week and for the treated group, dexamethasone was added every 3days. (B)Long term effect on NK cell expansion was measured under 3μM of dexamethasone treatment. Dexamethasone was added to the culture media every 3 days. (C) WT iPSC-NK and NR3 C1$^{-/-}$ iPSC-NK cells analyzed by flow cytometry for CD56 and tother indicated typical NK cell-surface receptors.

NR3C1$^{-/-}$ iPSC-NK cells display better function compared with WT iPSC-NK with dexamethasone treatment.

Anti-tumor activity was evaluated using cytotoxicity assays against K562, MOLM-13 (two myeloid leukemia lines), and Cal27 (Head and neck squamous cell line) with and without culturing with 3μM of dexamethasone. Notably, after culturing with 3μM of dexamethasone for 48hours, NR3C1$^{-/-}$ iPSC NK cells maintained potent anti-tumor activity and exhibited better killing activity against tumor targets in both short-term (4 h) and long-term (>20 h) cytotoxicity assays than WT iPSC-NK cells (Figures 3A–3E). Furthermore, we used NK cell degranulation (indicated by cell-surface expression of CD107α) and IFN-γ expression as parameters for NK cell function. Consistent with cytotoxicity results, WT iPSC-NK cells pretreated with dexamethasone expressed minimal amounts of both CD107α and IFN- γ when stimulated with the tumor cells, indicating loss of function (Figures 3F–3G). In

contrast, NR3C1$^{-/-}$ iPSC NK cells pretreated with dexamethasone maintained similar level of cytotoxic granule release (CD107 α expression) and IFN- γ production (Figures 3F–3G) with untreated NR3C1$^{-/-}$ iPSC NK cells. Together, these results demonstrate that deletion of NR3C1 in NK cells improved cytotoxic function when treated with dexamethasone.


A
K562
Cell death (%)
100
80
60
40
20
0
ctrl
0.5:1
1:1
2.5:1
5:1
10:1
Effector: Target
B
Cal27
Cell death (%)
ctrl
0.625:1
1.25:1
2.5:1
5:1
10:1
Effector: Target
C
Molm13
Cell death (%)
ctrl
0.625:1
1.25:1
2.5:1
5:1
10:1
Effector: Target
WT iPSC-NK untreated
WT iPSC-NK + Dex(3μM)
NR3C1-/- iPSC-NK untreated
NR3C1-/- iPSC-NK+Dex(3μM)
D
Red Area Confluence / Red Object Count Per Image Normalized to 0d...
iPS-NK Wt Untreated (1) 200K / well
iPS-NK wt 72hr Dex (1) 200K / well
iPS-NK wt 48 hr Dex (1) 200K / well
iPS-NK wt 24hr Dex (1) 200K / well
Time (Hours)
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Red Area Confluence / Red Object Count Per Image Normalized to 0d...
Dex-KO Untreated (1) 200K / well
Dex KO 72hr Dex (1) 200K / well
Dex KO 48 hr Dex (1) 200K / well
Dex KO 24hr Dex (1) 200K / well
Time (Hours)

Figure3 continued

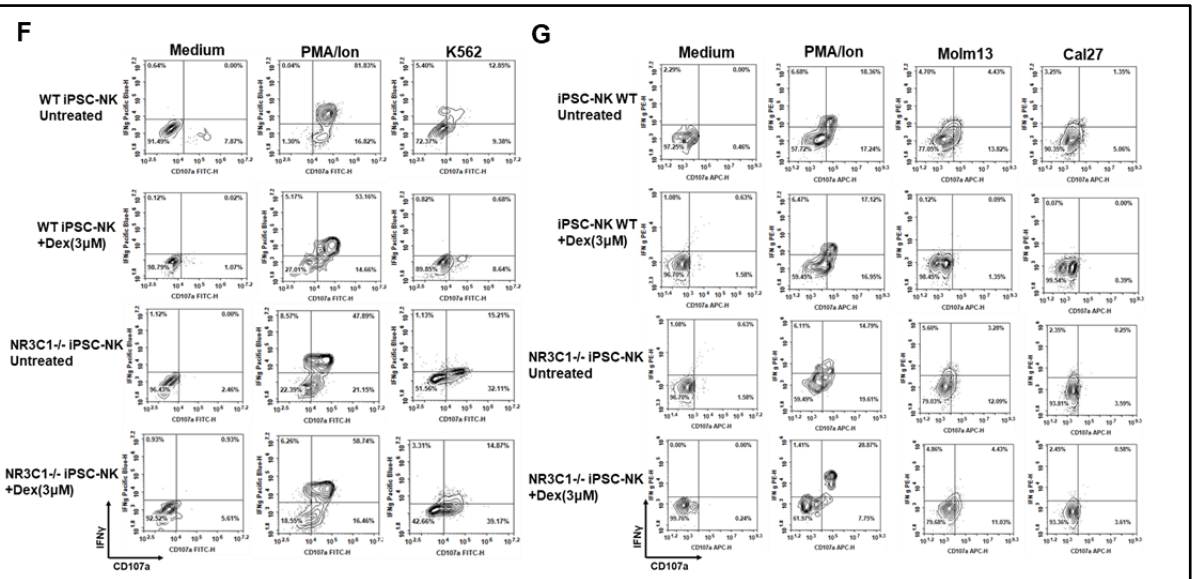

Figure 3: NR3C1-/- iPSC-NK cells display better function compared with WT iPSC-NK with dexamethasone treatment. 4-hours cytotoxicity assay to determine the effect of NR3C1 and dexamethasone on WT iPSC-NK and NR3C1 KO iPSC-NK. Cell death and apoptosis by caspase 3,7 activation and 7-AAD staining of (A)K562, (B)Cal27, and (C)Molm13 with NK cells after 4hour co culture at effector: target ratios from 0.5:1 up to 10:1. (D) WT iPSC-NK Killing against K562 cells was quantified over an extended time course using the IncuCyte real-time imaging system at effector-to target (E:T) ratio of 10:1. 3μM of dexamethasone was added 48hours prior to the assay. (E) NR3C1$^{-/-}$ iPSC-NK Killing against K562 cells (F) WT iPSC-NK cells and NR3C1$^{-/-}$ iPSC-NK cells were cultured with and without dexamethasone, and production of CD107 α and IFN- γ in response to K562 was measured. For the drug treated group, 3 μM of dexamethasone was added 48hrs prior to running the assay. WT iPSC-NK cells and NR3C1$^{-/-}$ iPSC-NK cells were left unstimulated or stimulated with a 1:1 ratio of target cells and stained for CD107α and IFN- γ 4hours later. (G) production of CD107 α and IFN-γ in response to Molm13 and Cal27 was measured.

Chapter 2: Loss of mouse CHMP2A leads to improved Natural Killer (NK) cell anti-tumor activity.

Deletion of CHMP2A on 4MOSC1 and 4MOSC2 head and neck squamous cancer cell

Previous screening identified human CHMP2A as a gene that suppresses NK cell mediated cytotoxicity. To validate the screening, I deleted mouse CHMP2A using CRISPR-Cas9 in syngeneic, tobacco-signature murine HNSCC model, 4MOSC (PMID: 31804466) developed by the Gutkind group[19]. Specifically, I used 4MOSC1 and 4MOSC2 cell lines that exhibit typical HNSCC histology[19]. I used RNP-mediated CRISPR-Cas9 gene editing, and three crRNA sequences were used to target exon 1 of the mouse CHMP2A gene. Transfection efficiency, as indicated by ATTO 550- labeled tracrRNA fluorescence, was high and deletion of the gene was further confirmed by PCR (Figure 4C) and western blot(Figure 4D).

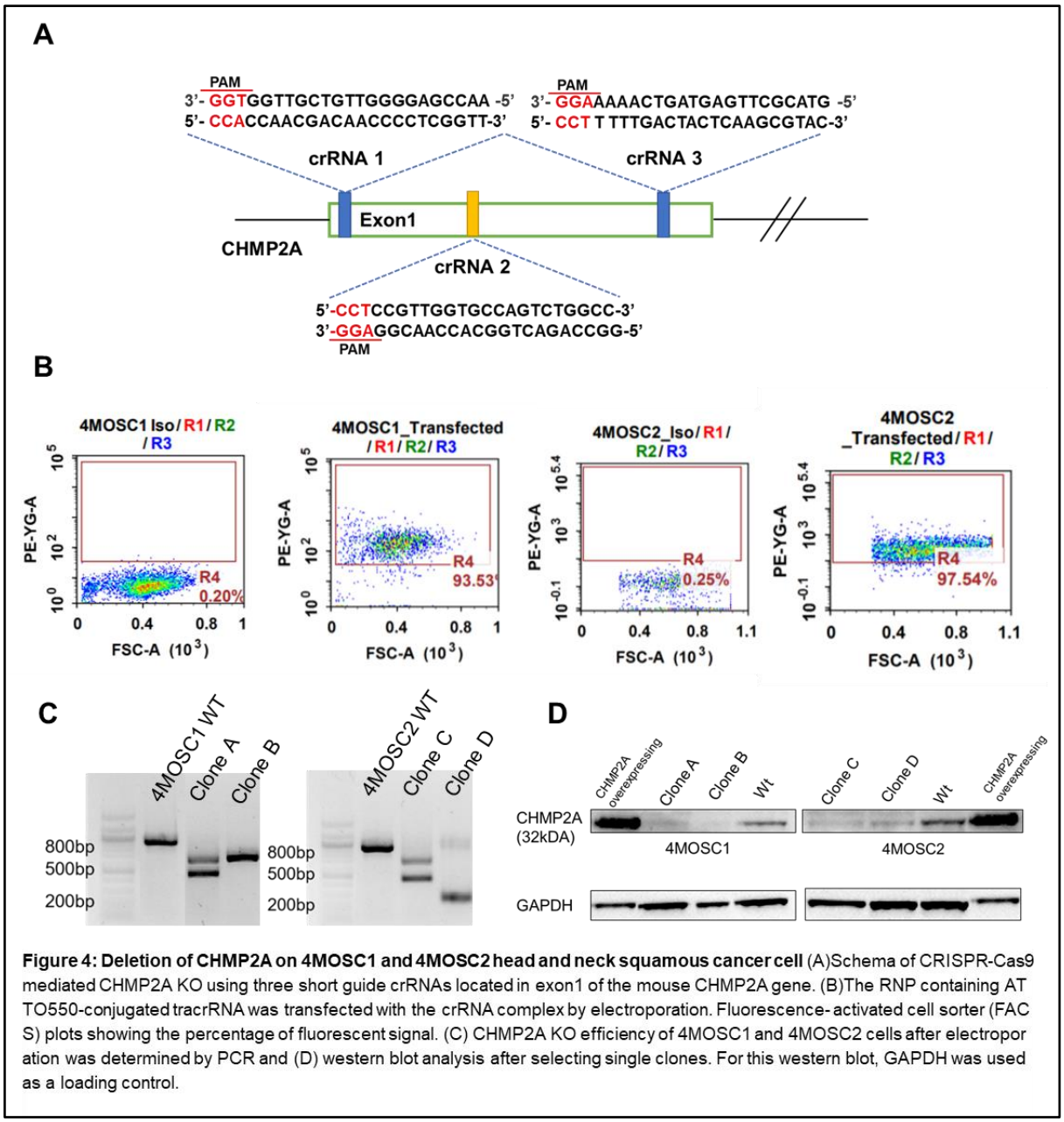

Figure 4: Deletion of CHMP2A on 4MOSC1 and 4MOSC2 head and neck squamous cancer cell (A)Schema of CRISPR-Cas9 mediated CHMP2A KO using three short guide crRNAs located in exon1 of the mouse CHMP2A gene. (B)The RNP containing AT TO550-conjugated tracrRNA was transfected with the crRNA complex by electroporation. Fluorescence- activated cell sorter (FAC S) plots showing the percentage of fluorescent signal. (C) CHMP2A KO efficiency of 4MOSC1 and 4MOSC2 cells after electropor ation was determined by PCR and (D) western blot analysis after selecting single clones. For this western blot, GAPDH was used as a loading control.

Deletion of CHMP2A showed increased NK cell cytotoxicity in vitro against 4MOSC1 cells, but no difference in 4MOSC2 tumor cells.

To test NK cell sensitivity on 4MOSC WT and KO tumor cells, I isolated mouse NK cells from the spleen and directly used for cytotoxicity assay. Cells were isolated from mouse spleen and mouse NK cells were sorted using the immunomagnetic negative selection Kit. Isolated mouse NK cells exhibited better killing activity against 4MOSC1 CHMP2A KO tumor target than 4MOSC1 CHMP2A WT cells in vitro (Figure5B). However, there was no

difference in NK cell cytotoxicity between 4MOSC2 CHMP2A KO and 4MOSC2 WT tumor cells (Figure 5C). This result is parallel to findings from Gutkind group where 4MOSC2 tumors failed to respond to anti-Programmed cell death protein 1(PD-1) treatment while 4MOSC1 tumors showed response[19].

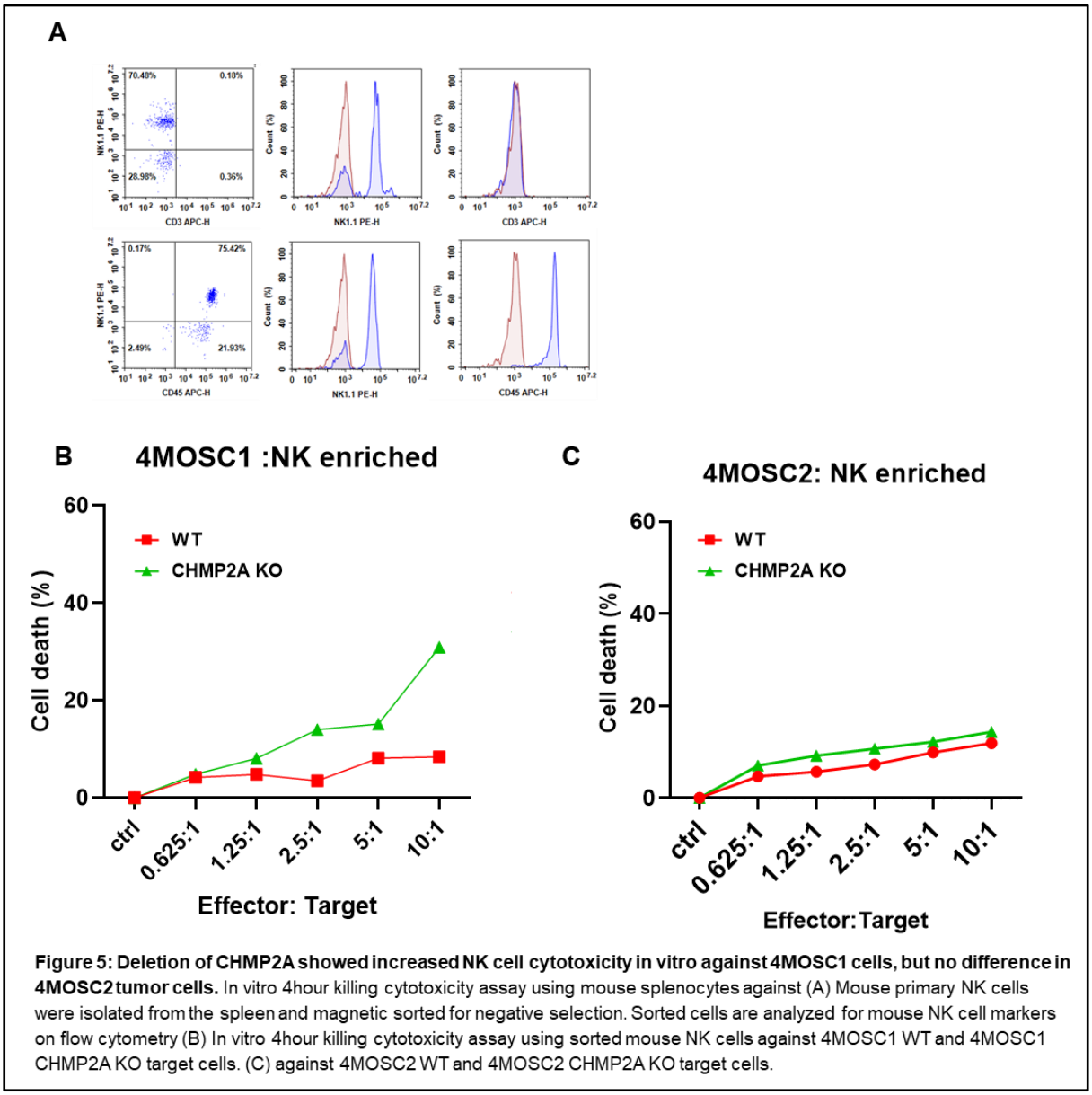

Figure 5: Deletion of CHMP2A showed increased NK cell cytotoxicity in vitro against 4MOSC1 cells, but no difference in 4MOSC2 tumor cells. In vitro 4hour killing cytotoxicity assay using mouse splenocytes against (A) Mouse primary NK cells were isolated from the spleen and magnetic sorted for negative selection. Sorted cells are analyzed for mouse NK cell markers on flow cytometry (B) In vitro 4hour killing cytotoxicity assay using sorted mouse NK cells against 4MOSC1 WT and 4MOSC1 CHMP2A KO target cells. (C) against 4MOSC2 WT and 4MOSC2 CHMP2A KO target cells.

4MOSC1-KO, but not 4MOSC2-WT, tumors spontaneously regress in vivo compared to wild type tumors

To evaluate whether CHMP2A KO has a similar impact on tumor sensitivity to NK cells in vivo, I developed a mouse syngeneic model. I implanted 4MOSC1 WT, 4MOSC1 CHMP2A KO, 4MOSC2 WT, and 4MOSC2 CHMP2A KO cells into the tongue of C57bl/6 immunocompetent mice. Consistent with the role of CHMP2A in vitro, mice bearing 4MOSC1 CHMP2A KO, but not 4MOSC2 CHMP2A KO, showed spontaneous tumor regression in vivo.

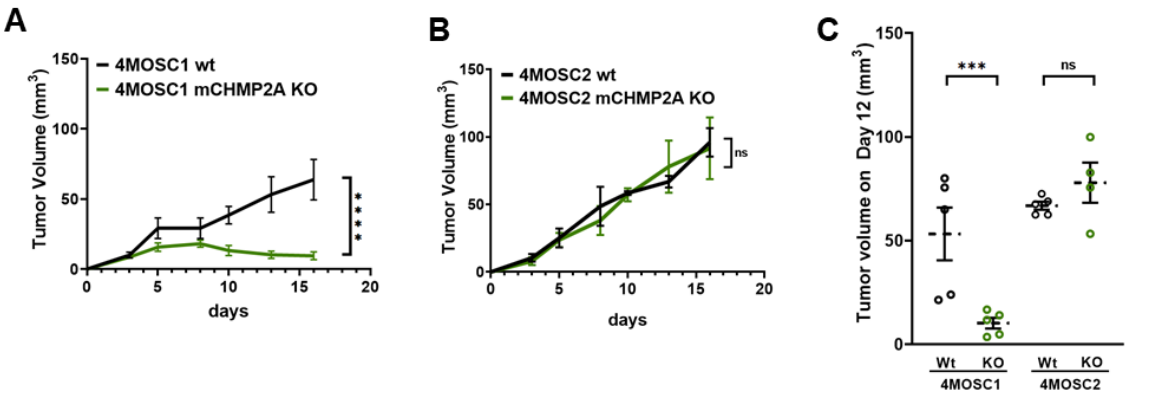

Figure 6: CHMP2A-KO shows increased sensitivity to NK cells in 4MOSC1 tumor but not in 4MOSC2 tumor. (A)C57Bl/6 mice were implanted with one million of 4MOSC 1Wt and 4MOSC 1 CHMP2A KO cells into the tongue. Growth curves of 4MOSC1 tumor-bearing mice are shown. Shown is the average volume of each tumor (n=5 mice per group; two sited Student's t test) (B) C57Bl/6 mice were implanted with one million of 4MOSC 2 Wt and 4MOSC 2 CHMP2A KO cells into the tongue. Growth curves of 4MOSC1 tumor-bearing mice are shown. Shown is the average volume of each tumor (n=5 mice per group) (C) Average volume of tumor on day 12 depicted with a dotted line. Individual dots represent volume of each tumor (n=5 mice per group)

Discussion

NK cells play a key role in immunosurveillance and controlling the development of malignancies[8]. However, due to the immune inhibitory properties of the tumor microenvironment, solid tumors are generally less sensitive than hematological malignancies to NK cell mediated killing[21].Multiple mechanisms have been proposed, including tumor cells shedding NK activating ligands from their cell surfaces halting immune cells migration to the tumor[21, 22]. Particularly in HNSCC, the tumor microenvironment is known to be immunosuppressive. Our group used “two-cell type” CRISPR-Cas9 screens to discover new protein targets important for NK cell cytotoxicity in HNSCC tumors. We ranked the top 25 hits that increased sensitivity or resistance to NK cell killing from screening and identified CHMP2A as a regulator of tumor cell sensitivity to NK cell mediated cytotoxicity. To further validate the role of CHMP2A, CHMP2A was deleted in immunocompetent C57BL/6J mouse HNSCC xenograft models and significant differences in the tumor growth were shown between wild type and CHMP2A knock out in 4MOSC1 cell line. Overall, the deletion of CHMP2A using CRISPR-Cas9 resulted in decreased tumor volume compared to the WT cells in the 4MOSC1 line. In vitro mouse NK cell cytotoxicity assays supported this finding and demonstrated that CHMP2A deletion in 4MOSC1 tumor had increased NK cell mediated killing. These studies confirm that CHMP2A contributes to resistance to NK cell killing in HNSCC and supports target meant strategies targeting this immune suppressive gene.

There was no difference in tumor growth in the CHMP2A knockout 4MOSC2 cell line which correlates with previously published data [19]. Wang and colleagues showed that with the anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) treatment, 90% of the mice injected with 4MOSC1 tumor cell responded with a complete tumor rejection while 4MOSC2 tumors failed to respond[19]. The same group further suggested that 4MOSC2 serves as a resistant model that expressed higher levels of multiple chemokines and growth factors that may contribute to the recruitment of myeloid-derived-suppressor (MDSCs), inflammatory cells, and Vascular endothelial growth factor (VEGF)[19]. The current study supports the idea that 4MOSC2

tumors are resistant to NK cell mediated killing as they demonstrate faster growth in tumor volume compared to CHMP2A knockout 4MOSC1 tumors. To verify that the proliferation of wildtype and CHMP2A knockout cells are controlled by anti-tumor activity of the intact mouse immune system and no other roles of CHMP2A, tumor growth in immunocompromised NSG mice will be investigated. Additionally, to fully understand the role of CHMP2A in inhibiting NK cell meditated cytotoxicity, it will be necessary to analyze the immune infiltration and cytokine/chemokine profile within the tumor microenvironment and these studies are ongoing.

The second project I completed was to engineer NK cells to become resistant to corticosteroids. To control side effects such as graft- versus-host disease (GVHD), corticosteroids are often administered to patients following HSCT[9]. Also, use of glucocorticoids controls inflammation and progression of Coronavirus disease 2019 (Covid-19) reducing the risk of developing respiratory failure[23]. A problem with use of glucocorticoids use to treat GVHD, severe drug refractory infections as well as Covid-19 is that corticosteroids hinder the efficacy of virus specific T cells (VSTs) and NK cells[11]. To overcome this limitation, Basar et al. deleted the glucocorticoid receptor which created resistance to the lymphocytotoxic effect of glucocorticoids[24]. Here, I showed that dexamethasone, a potent glucocorticoid, inhibits the expansion and function of iPSC derived NK cells. Deletion of the glucocorticoid receptor enhanced NK cell proliferation under dexamethasone treatment and the knockout cells retained similar cytotoxic ability as the untreated NK cells. Further studies are ongoing to make NR3C1 knock out on iPSCs and differentiate into NK cells. Engineering NK cells can be limited by cell availability after multiple passages. NK cells start losing cytotoxicity when it is maintained long term in culture[25]. However, making the deletion on iPSC level and differentiating into NK cells will allow continuous production of NR3C1$^{-/-}$ iPSC-NK. In addition, in vivo studies to assess cytotoxicity of the knockout cells will be conducted in the near future. Overall, this approach will improve NK cell activity under dexamethasone treatment in the clinic. This approach can be extended to engineer regulatory T cells to resist glucocorticoids to improve control of

GVHD. Also, glucocorticoids are widely used in therapies against brain cancer[26]. NK cells are integral in the anti-tumor response in patients with brain tumors and being able to combine dexamethasone with NK cell therapy may be beneficial[27]. By engineering glucocorticoid resistant NK cells, the cells can be used to treat brain cancer administered with glucocorticoid to allow for synergic killing of glioblastoma cells.

CRISPR Cas9 mediated gene editing allows engineering of both tumor cells and NK cells that leads to improved cytotoxicity of NK cells by different mechanisms. However, the risk of off-target gene editing remains as a challenge. One of the major causes of the off-target effect of the CRISPR gene editing is the expression of Cas9 nuclease within the cells[28]. To reduce continuous expression of Cas9, CRISPR-Cas9 ribonucleoproteins (RNPs), where Cas9 protein and sgRNA are delivered together in a complex, were used. Since the half-life RNP complex is shorter than the time required to transcribe a plasmid, the off target is known to be lower[28]. Still, further analysis will be done using GUIDE-seq to analyze off target effects[29]. By engineering tumor cells and NK cells, I aim to improve the current iteration of immunotherapies. I also aim to improve anti-tumor activity and therapeutic response of immune cells and further explore the question of why the immune system can respond to and control malignant cells in some contexts but not in others. Through this understanding we will obtain better insight into how the host immune system can be modified to eradicate cancer.

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