Research Paper

ROLE OF S309-CAR-NK CELLS IN NEUTRALIZING SARS-CoV-2

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PROBLEM

In SARS-CoV-2, the variant's properties influence the effectiveness of public health interventions, some even increase their transmissibility. Other properties of the variants are that they can also change the virus's antigenicity (the ability of an antigen to induce an immunological response when it encounters the human body), avoid the immunity caused by previous infection/vaccination, and evade the immune response. All these characteristics increase the virus's severity.

Omicron, characterized by 17 mutational changes, increased the number of cases dramatically by having a short generation time for transmission with a 59% higher transmissibility and 45% higher mortality rate, with a substantial antigenic change. The Omicron variant has efficient growth in the upper airway, and a bigger pool of children susceptible compared to adults, resulting in enhanced SARS-CoV2 evolution, and declined vaccine efficacy.

HYPOTHESIS

I hypothesize that S309-CAR-NK cells can bind to Omicron subvariant XBB.1.5 Spike protein; therefore, neutralizing the pseudoviral SARS-CoV2 XBB.1.5 particles *in vitro* and can be used as a potential therapeutic for immunocompromised COVID patients.

ABSTRACT

The properties of SARS-CoV-2 influence the effectiveness of public health interventions allowing them to: increase transmissibility, change antigenicity, avoid the immunity caused by previous infection/vaccination, and evade the immune response which all leads to an increase in the virus's severity. Omicron, increased the number of cases dramatically (59% higher transmissibility and 45% higher mortality rate) with a substantial antigenic change. In the virus, there are two functional regions: S1 which is the receptor binding domain (RBD), and S2. Antigens against RBD have been seen as being able to stop the virus from binding to infect human cells. Antibodies for the coronavirus recognize the spikes on the surface and bind to them to prevent the virus from binding with a healthy host cell, keep it from penetrating the cell membrane, and prevent them from releasing its genome into a cell for replication. Neutralizing antibodies such as S309 could diffuse not only this specific viral strain but also offshoots that occur because of natural mutations in the virus. Natural killer (NK) cells are a type of immune white blood cell that has granules that can destroy tumors and other virus-infected cells. Chimeric antigen receptor (CAR)-NK cells are a safer alternative to CAR-T cell therapy with advantages including less antigen loss relapse, minimal on-target, off-tumor toxicity, and possesses antibody-dependent cellular cytotoxicity (ADCC). Therefore, CAR-NK cells are good candidates for "off-the-shelf" cellular immunotherapy treatment development. The Liu Lab previously generated CAR-NK92MI (an NK cell line) that expresses S309 single-chain fragment variable (scFv) domain. Their previous data showed that S309-CAR-NK cells could bind to SARS-CoV2 wildtype pseudovirus and killing target cells expressing the spike wildtype

protein. Collectively, I hypothesize that S309-CAR-NK92MI cells can also bind to the SARS-CoV2 Omicron XBB.1.5 variant by demonstrating that it binds to the spike protein of the SARS-CoV2 Omicron XBB.1.5.

TERMINOLOGY

a. **Chimeric antigen receptor (CAR):** recombinant receptors designed for antigens that redirect the specificity and function of T lymphocytes and other immune cells in a single molecule.

b. **Single-chain variable fragment (scFv):** Artificial construct consisting of immunoglobulin heavy chain and light chain variable regions linked by peptides. In a scFv, a short peptide linker that is usually 15-20 amino acids long connects the VH and VL, while in diabodies two scFv are assembled.

c. **Allogeneic:** Relating/involving cells that are genetically different or immunologically incompatible even though they are from individuals of the same species

d. **Autologous**: Cells obtained from the same individual

    i. According to *Graft Versus Host Disease* (National Library of Medicine, Vaillant, Modi, Mohammadi, 2022), **Graft versus host disease (GvHD):** Systemic disorder that occurs when the graft's immune cells recognize the host as a foreign and attack the recipient's body cells

        .Graft = transplanted tissue

e. According to *Cytokine Release Syndrome* (Cleveland Clinic), **Cytokine storm/cytokine release syndrome (CRS)** is when the immune system responds too aggressively to infection (NK cells releasing too much cytokine)

f. According to *Immune* effector cell-associated neurotoxicity syndrome (ICANS) (UpToDate Dietrich, Frigault, 2022) and *CAR T-Cell-Associated Neurotoxicity* (Critical Care Nursing Quarterly, 2020), **Neurotoxicity/immune effector**

cell-associated neurotoxicity syndrome (ICANS): clinical and neuropsychiatric syndrome occur following types of immunotherapies (IEC and T cell) or due to disruption of blood-brain barrier or effects of an increase in cytokine in their central nervous system.

g. **Antibodies**: y Y-shaped proteins produced by the immune system after bacteria/virus invades. Helps the body remember the invader to be prepared in case they invade again.

    i. **Neutralizing antibodies:** could diffuse not this specific viral strain but also offshoots that occur because of natural mutations in the virus.

INTRODUCTION

IMPACT AND PROPERTIES OF SARS-CoV2

According to *Evaluating the Impact of SARS-CoV-2 Variants on the COVID-19 Epidemic and Social Restoration in the United States: A Mathematical Modelling Study* (Front Public Health 2022) and Properties of the Omicron Variant of SARS-CoV-2 Affect Public Health Measure Effectiveness in the COVID-19 Epidemic (PMC Yuki Furuse 2022). In SARS-CoV-2, the variant's properties influence the effectiveness of public health interventions, some even increase their transmissibility. Other properties of the variants include that they can also change the virus's antigenicity (the ability of an antigen to induce an immunological response when it encounters the human body), avoid the immunity caused by previous infection/vaccination, and evade the immune response. All these characteristics increase the virus's severity. For example, a variant of concern B.1.1.7 first identified in the UK was dominant in London and southeast England. It had the characteristic of being 50% more transmissible than previous variants.

SARS-CoV2: OMICRON

SARS-CoV2 Omicron, characterized by 17 mutational changes, increased the number of cases dramatically by having a short generation time for transmission with a 59% higher transmissibility and 45% higher mortality rate, with a substantial antigenic change. The Omicron variant has efficient growth in the upper airway, and a bigger pool of children susceptible compared to adults, resulting in enhanced SARS-CoV2 evolution, and declined vaccine efficacy. Characteristics of the omicron variant: shorter

than the Delta variant, efficient growth in the upper airway, bigger pool of children susceptible compared to adults. Studies also show that the SARS-CoV2 omicron variant has the ability to escape many currently available monoclonal antibody therapies. The effectiveness of the vaccine has also been declining. There must be a reduction in interpersonal contact between children. Child vaccination cancels the influence of the increase of infected children on vaccination effectiveness, but its ability to escape immunity continues to decrease the effect of the vaccinations. By having a short generation time, it enhances the effect of reducing in-person contact and decreasing susceptible people. However, it also diminished the effect of early isolation by TTI (test-trace-isolate).

SARS-CoV2 IMPACT ON PUBLIC HEALTH

The public health's way of limiting contact between infected individuals and others include mostly adult social activities: closing restaurants, and bars, restricting mass gathering events, and encouraging working from home. Most tighten personal, procedural, engineering, and societal control measures to decrease transmission, and control social contacts, effective testing, and tracing, robust outbreak identification, ensuring quarantine, and vaccination. Other methods of limiting interaction included reinforcing using face masks in indoor and public spaces, temporary closure of non-essential indoor areas, as well as expanding the distance rule between people. Despite these measures, there was a need for such protocols and mitigation to be enforced earlier in high-risk settings such as nursing homes, shelters, detention centers, dormitories, warehouses, and manufacturing facilities. It would also have been worth considering implementing public health measures that are child-targeted including mass

vaccination and closing schools. The potential population impact of vaccine effectiveness reduction of new variant: to reduce to 75% with similar transmissibility and mortality ~ 667,075 cumulative infections and 19, 249 deaths over the next 12 months. However, if it is reduced to 50% and 25%, there are an estimated 1.7 million and 19 million cumulative infections and 42,265 and 426,869 cumulative deaths. Random subsets of positive tests should be sequenced to monitor for mutations or other variants. There are already many surveillance programs including COG-UK, however, genomic surveillance is not available in most countries due to a lack of infrastructure, technical expertise, political will, and dedicated resources.

NK CELLS AND S309 IN LIMITING SARS-CoV2 INFECTION

Attacking the defense: SARS-CoV-2 can infect immune cells (Borsa, M., Mazet, J.M 2020) describes the structural and functional mechanism of SARS-CoV-2 cell entry (Abcam 2022), and *Natural Killer Cells in SARS-CoV-2 Infection: Pathophysiology and Therapeutic Implications* (Frontiers in immunology 2022). Spike protein is one of the proteins on the SARS-CoV-2 surface utilized to develop serology assays. Immune cells such as monocytes, types of T cells, and B cells are susceptible to SARS-CoV-2 infection. In the virus, there are two functional regions: S1 which consists of the receptor binding domain (RBD), and S2. Please refer to figure 2 in my Plan of Action. S1 is responsible for the ability of the virus to bind to human cells to facilitate infection. Antigens against RBD have been seen as being able to stop the virus from binding to infect human cells. The spike proteins bind to the ACE2 receptors at the surface of healthy cells, then S2 fuses with the cell membrane of the healthy cell. And since monocytes and lymphocytes do not express ACE2, the virus might use alternative ways

to enter the cell. During binding, S1 undergoes conformational rearrangement between up and down conformation states. The down conformation depicts how it hides the receptor binding domain (closed), and up exposes it but temporarily destabilizes the protein subunit (open). NK cells are also affected in the bloodstream by SARS-CoV2, they can interfere with their functions and overcome antiviral responses by modulating NK cell receptor expression, signaling, and cytokine secretion. According to *SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies* (Barnes, Christopher 2020) and *How antibodies neutralize the novel coronavirus: Science, Simplified* (Youtube, Scripps Research 2020), antibodies for the coronavirus recognize and bind the spike protein to prevent the virus from infecting a healthy host cell. Neutralizing antibodies such as S309, isolated from a SARS patient, could diffuse not only this specific viral strain but also offshoots that occur because of natural mutations in the virus. S309 targets the RBD epitome which is mainly VH (Immunoglobin heavy chain variable region) mediated.

INNATE IMMUNITY

The additional sources, *Innate Immunity* (Molecular Biology of the Cell. 4th edition) and *What is Innate Immunity* (center innate immunity and innate disease), clarify that Innate Immunity is the first response of the body's immune system to a harmful foreign substance. Innate Immunity contains cells that are nonspecific, and although it can distinguish invaders, from human cells, it doesn't distinguish one invader from another. The innate immunity works rapidly--usually within minutes--and causes fevers. Due to the speed, however, there are no memories associated with innate responses. The innate response will respond to the same pathogen in the exact same way no matter the

number of times it sees the pathogen. It includes chemical barriers (lysozymes in tears and lower pH in stomach), physical barriers (epithelium in skin and gut and cilia in airways), skin, mucous membranes, tears, and stomach acid. Innate immunity depends on the group of proteins and phagocytic cells that recognize the features of the pathogens, then become activated to help kill the bacteria. Once they detect the invaders, they activate cells to attach and destroy the invader and, simultaneously, inform the adaptive immune response. There are two types of innate immune responses: inflammatory response and phagocytosis by cells like neutrophils and macrophages. The process of the innate immune system starts when the bacteria move into the body, pass through airways, penetrate the epithelium of the lungs, and divide. Macrophages then ingest the bacteria and release cytokines which start an inflammatory process making blood vessels leaky, attracting eosinophils, basophils, and mast cells which also release cytokines, amplifying inflammation. Neutrophils in blood and bone marrow will join in as well, and if the invader is a virus, Natural Killer cells will help kill the infected cells.

NATURAL KILLER CELLS

As discussed by *Exhausted NK cells and cytokine storms in COVID-19: Whether NK cell therapy could be a therapeutic choice* (Ghasemzadeh and Hosseini. 2021), NK cells, also known as Natural Killer Cells, are a type of immune white blood cell that have granules that can destroy tumors and other virus-infected cells. They are cells of the innate immune system with cytolytic activity against different targets (tumor-derived or virus-infected cells). They play a key role in the host's immune defense against pathogens preventing the establishment of infection and the viral spreading through the

body. Mature NK cells constitute about 10-15 % of circulating lymphocytes and are found in the spleen, bone marrow, liver (pit cells), lung, intestinal mucosa, uterus, and small amounts of lymph nodes. They can travel in and out of tissue and bloodstream and are fundamental in the host's immune defense against pathogens, preventing the establishment of infection and the viral spreading through the body. Their functions include releasing cytotoxic granules which punch holes in the target cell's membrane by binding directly to phospholipids, creating pores, then releasing some molecules such as perforin, granzymes, and granulysin, that get inside the target cell causing them to undergo apoptosis. According to *the molecular mechanism of SARS-CoV-2 evading host antiviral innate immunity* (Viral Journal 2022). NK cells are affected in the bloodstream by SARS-CoV2, which SARS-CoV2 interrupts equations of immune responses, disrupting cytolytic antiviral effects of NK cells, and inducing a "cytokine storm" by activating infected immune cells. Adoptive transfer of proper cytolytic potentiated and lowest capacity of cytokine released NK cells and CAR-NK cells may be effective for this problem. However, SARS-CoV-2 has the ability to distort the main function or disable key players like the NK cells in the innate immune system inducing a "cytokine storm" which causes a widespread inflammatory problem.

EFFECTS OF SARS-CoV2 ON NK CELLS (CYTOKINE STORM)

The first phase of COVID pathogenesis and the involvement of NK cells in COVID pathogenesis is when the virus infects airway epithelial cells, alveolar macrophages, and recruited inflammatory monocyte macrophages and neutrophils leading them to release arrays of cytokines and chemokines. This attracts NK cells and other cells to the infection site. The second phase involves the release of cytokines from these newly

attracted cells. NK cells will infiltrate into the lungs by the releasing of innate cytokines by infected cells they might also play a role in the earlier development of inflammatory conditions in COVID-19. The cytokine storm is caused by the increase in inflammatory mediators (IL-6, IL-7, IL-10, GM-CSF, CXCL10, MCP-1, CIP-1A, etc.). IFN-g is released from NK cells, and they induce the cytokine story by macrophages and other inflammatory cells. The NK cells that are affected by the inhibitory effects of the virus lead to small amounts of cytokine release in patients with COVID-19. When the NK cell is exhausted, the causes of severe sickness are usually from a defect in the function of NK cells. It will no longer be able to destroy infected inflammatory cells that cause the cytokine storm.

ROLE OF NKG2A RECEPTOR IN SARS-COV2 PATHOGENESIS

The defensive cells of the innate immune system recognize the virus and trigger the innate response. The NK cell affects the immune response type and intensity since it is one of the cells in the first line of defense. However, in patients with severe COVID-19, there is a lower presence of NK cells. CD94/NKG2A is a heterodimeric inhibitory receptor expressed by NK cells. Two inhibitor immune receptor tyrosine inhibitor molecules allow them to bind to non-classical HLA-E molecules and inhibit NK cell toxicity and cytokine secretion. In the earlier stages of SARS-CoV-2, NKG2A also plays a role in exhausting the NK cells. The expression of the spike 1 protein of the SARS-CoV-2 in lung epithelial cells leads to a reduction of degranulation of NK cells and an increase in NKG2A/CD94 regulation. Blocking NKG2A in cancer will restore NK functions, therefore, controlling tumor growth. Overruling the innate immune response of the host can be caused by the failure of the immune response toward viral pathogens.

This is because SARS-CoV-2 up-regulates the level of NKG2A in NK cells. Not only that, but a lack of DC signals also caused by antigen-presenting cells infected with MERS-CoV can lead to the promoting NK cells attending in apoptosis. SARS-CoV-2 can encode a wide range of viral structural and non-structural proteins having roles in viral replication and packing. This affects the IFN signaling pathway, which impairs the IFN-mediated antiviral responses.

T- CELLS AND CAR-T CELL THERAPY

The immune system creates an army of T cells to protect the body from cancer cells, infections, and other viruses and bacteria. T cells inspect any suspicious cells in the body, and if they are recognized as dangerous, they attack it to limit the damage to healthy cells. They achieve this by having protein receptors that can latch onto antigens (fragments of proteins found on the surface of all cells). When it latches onto an abnormal antigen, releasing toxic chemicals to damage the antigen while other immune cells come. Abnormal cells could disguise themselves as healthy cell or sprout so many antigens on the surface that the T cells can’t achieve an effective attack because it is stymied or find ways to turn off the immune response against them. CAR T cell therapy is a way to see through the disguises of the cells. In a process like blood donation where a patient’s T cells are collected to reprogram T cells to produce special receptors on their surface known as chimeric antigen receptors (CAR) to recognize specific antigens on the cancer cells and destroy them. After the CAR-T cells are grown in a lab, they are infused back into patients.

REPLACING CAR-T CELL THERAPY WITH CAR-NK CELLS

As explained by *Novel insights in CAR-NK cells beyond CAR-T cell technology; promising advantages* (International Immunopharmacology 2022), CAR-NK cells are a safer alternative to CAR-T cell therapy. Their advantages include less antigen loss relapse, minimal on-target, off-tumor toxicity, and antibody-dependent cellular cytotoxicity (ADCC). By having various activating and inhibitory receptors, it allows the CAR-NK cells to have stronger and less off-target reactions. CAR-T cells also have IL-1,2,6,8,10,15, TNF- MCP-1. Not only that, CAR-NK cells have a low risk of grafts versus host disease (GvHD), cytokine release syndrome (CRS), and immune effector cell-associated neurotoxicity syndrome (ICANS), where these side effects are often seen in CAR-T therapy due to allogeneic donors. Allogeneic or haploidentical NK cells can be used and they will have no serious side effects after administration. They are also able to be isolated and established from various sources. With CAR-dependent and CAR-independent ways to identify cancer cells, there will be less antigen loss relapse. This makes CAR-NK cells good candidates for “off-the-shelf” cellular immunotherapy treatment development because no evidence of GvHD is found in almost all clinical studies of NK cells. They release IFN-γ and GM-CSF that causes fewer toxic complications and reduces the occurrence of cytokine storms, making it a safe way of cell-based immunotherapy.

S309 ANTIBODIES IN NEUTRALIZING SARS-CoV2

The antibody for the coronavirus recognizes the spikes on the surface and binds to the surface preventing the virus from binding with a host cell. If it does bind, antibodies can help keep them from penetrating the cell membrane even if they do antibodies can prevent them from releasing their genome into the cell for replication.

Please refer to my Figure 1 for reference. After binding they also signal to other parts of the immune system to destroy the invader. Doctors transfer the blood of covid patients with antibodies that have already encountered covid the preset convalescent plasma or isolate specific antibodies with concentrated antiviral properties then multiply it in the lab (monoclonal antibodies). Other scientists want to know more information on how antibodies interact with the spike protein on covid viruses and how the vaccine for our body generates antibodies that are effective against the virus. According to *SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies* (Barnes 2020). The S309 neutralizing antibody targets mainly receptor binding domain epitome which is mainly VH mediated. Smaller C135 relative to S309 focus on interactions with R346 (RBD) and N440 (RBD) and is engaged by residues from heavy-chain and light-chain CDRs. These are not conserved between SARS-CoV2 and SARS-CoVRBDs. When combined with Nabs that block ACE2 while also limiting viral escape binding outside of the ACE2 binding site could potentially provide additional neutralizing effects.

Given this information about SARS-CoV-2, their variants' impact, especially Omicron, on public health, and the physical structure of the current variant of the SARS-CoV-2 virus, we have learned about the critical position the public is in. With this information along with learning about Natural Killer cells' role in the immune system and S309-CAR-NK's part in immunotherapy for this virus, we hypothesize that S309-CAR-NK cells can bind to Omicron subvariant XBB.1.5 Spike protein; therefore, neutralizing the pseudoviral SARS-CoV2 XBB.1.5 particles *in vitro* and can be used as

a potential therapeutic for immunocompromised COVID patients. I will design three experiments to test my hypothesis. A brief rationale for each experiment is stated in the

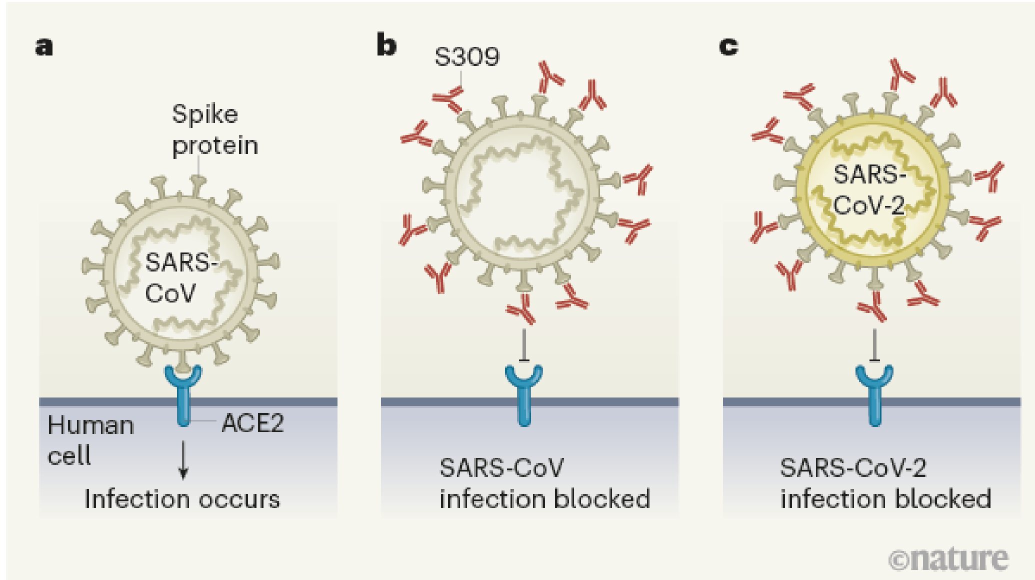

Figure 1: Figure of mAbs (monoclonal antibodies), also known as “blocking antibodies that are designed to prevent the progression of diseases, actively neutralizing the SARS-CoV-2 virus (Rossheim, Brooke, and Nancy Perilstein, 2021).

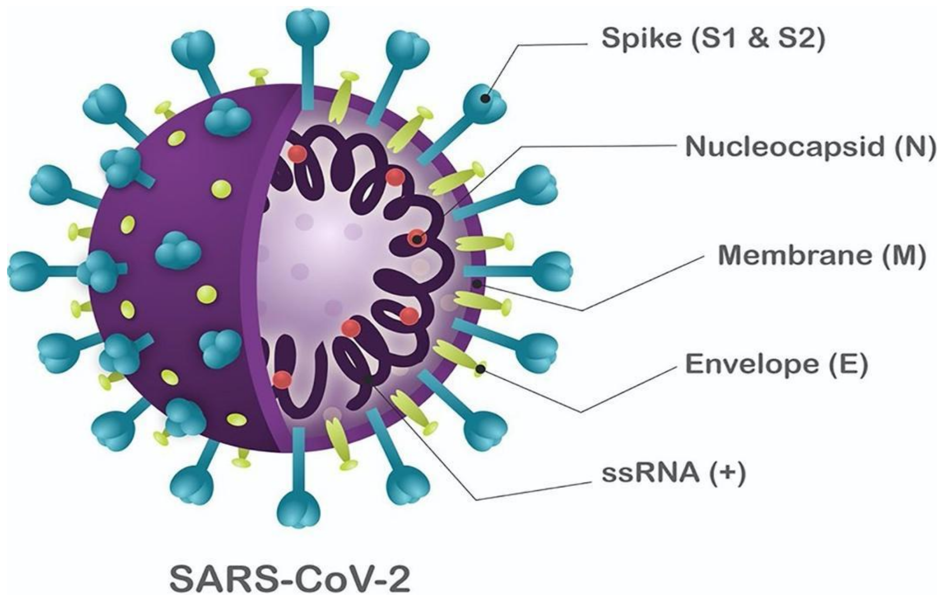

Figure 2: Schematic structure of a SARS-CoV -2 virus. The structure illustrates the spike (S1 & S2), nucleocapsid, membrane, and envelope proteins. They are all

contained within the lipid bilayer of the host membrane, also known as the viral envelope (De Andrade Santos, Igor, et al., 2020)

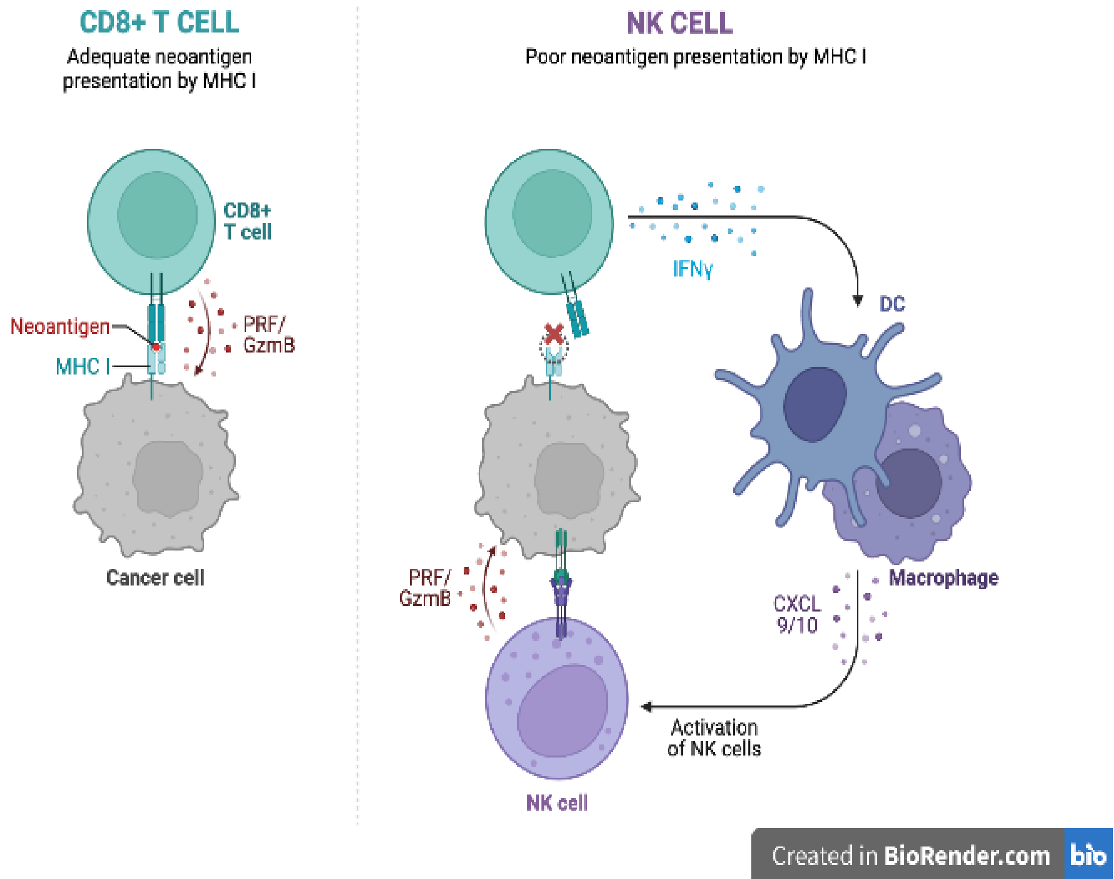

Figure 3: Neoantigen presentation by MHC in two conditions. One with an adequate presentation by CD8 and T Cells and one with a poor presentation with NK cells. The CD8 and T cells need to use MHC to recognize cancer cells and deliver perforin/granzyme B into the cancer cell. On the right, the MHC is no longer needed for the NK cell to recognize the cancer cell. (Created by Biorender).

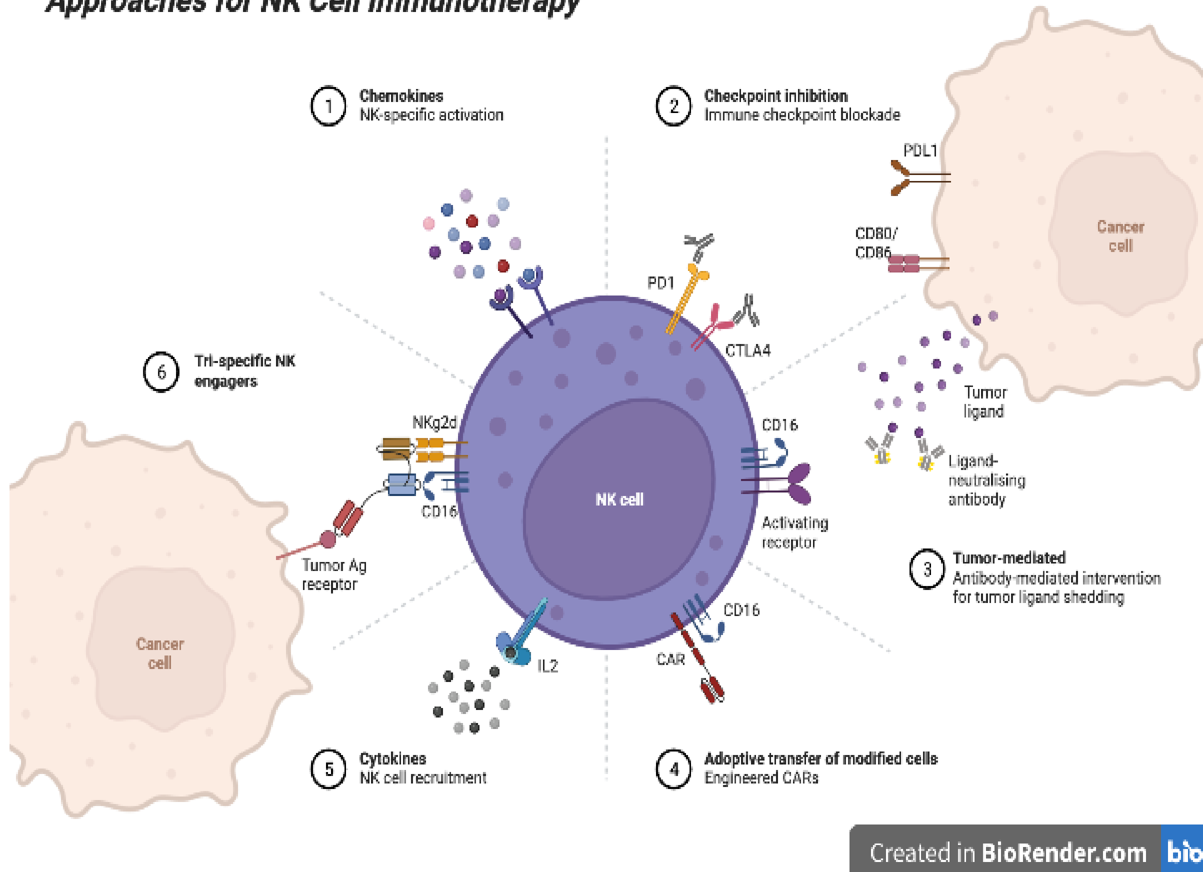

Figure 4: Six different approaches for NK cell immunotherapy. These approaches include 1. Chemokines 2. Checkpoint inhibition 3. Tumor-mediated 4. Adoptive transfer of modified cells 5. Cytokines 6. Tro-specific NK engagers. The figure illustrates the areas of the NK cells when the approach is used. (Created by Biorender).

PLAN OF ACTION:

To confirm that the S309-CAR-NK92MI cells are successfully genetically modified, I will use both western blot and flow cytometry described in experiments 1 and 2. The first experiment is to determine the intracellular expression of S309-CAR-NK cells by using a western blot. The cells are genetically modified as third generation scFv-based CARs to carry both intracellular and extracellular domains (Figure 5). The intracellular domain consists of CD3ζ that will be immunoblotted against using

SDS-PAGE followed by a western blot. The second experiment is to determine the surface expression of S309-CAR-NK cells using flow cytometry. Even when the intracellular domain is presented inside the S309-CAR-NK92MI cells, it does not necessarily mean that the surface S309 scFv domain is expressed. The second experiment will be performed by flow cytometry to determine the surface expression. Once I confirm that the NK92MI cells are successfully modified, I next investigate and test the functionality of S309-CAR-NK92MI cells in binding to the Spike Omicron XBB.1.5 protein in the last experiment. Based on published data by the Liu Lab, the binding of S309-CAR-NK92MI cells to the spike protein is a strong indication that the cells can neutralize the SARS-CoV2 virus. Therefore, it can be reasonably hypothesized that once S309-CAR-NK92MI cells are shown to bind to the protein, it suggests the S309-CAR-NK92MI cells will also neutralize XBB.1.5.

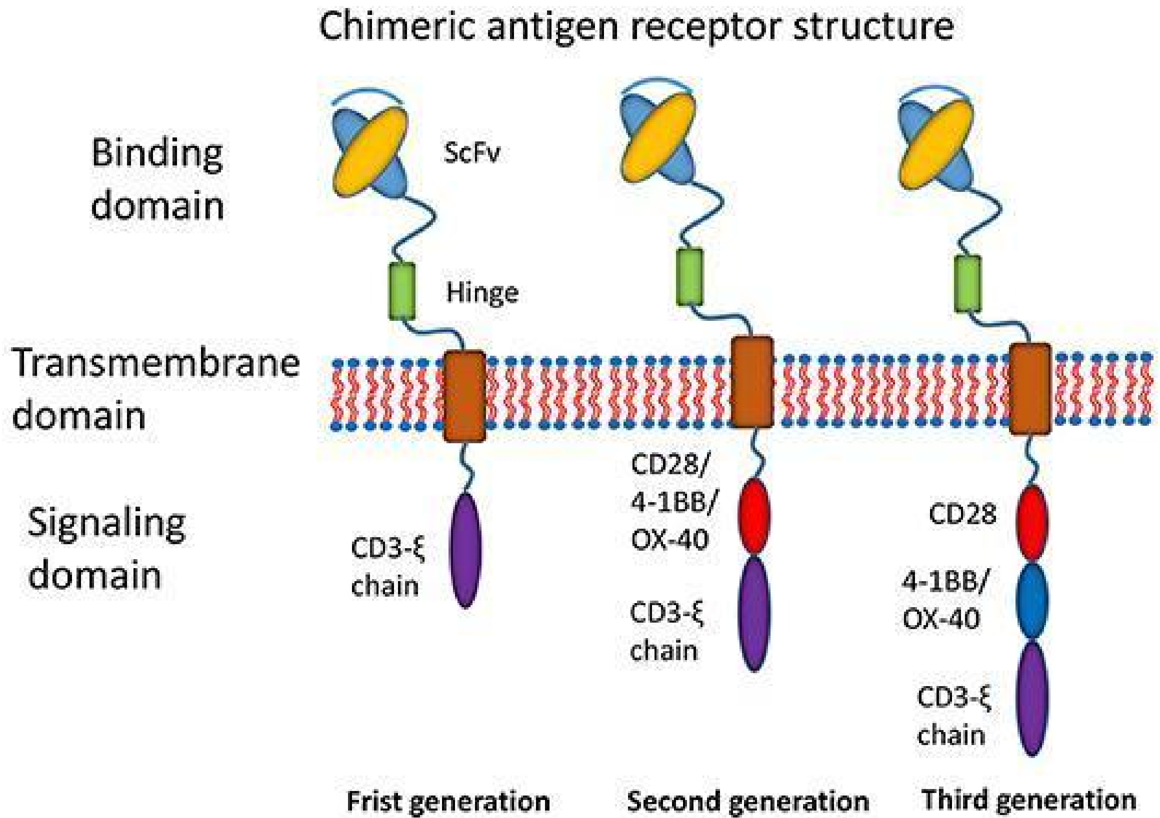

Figure 5: Schematic figure of different CAR generations. S309-CAR-NK92MI belongs to the third generation which contains the scFv binding domain, the transmembrane domain, and the signaling domain. The intracellular or signaling domain for the third-generation CAR consists of CD28, 4-1BB/OX-40, and CD3ζ (Liu, Jie, et al., 2019)

MATERIALS

Materials for Flow Cytometry

NK92MI cells (purchased from ATCC, catalog number CRL-2408) and genetically modified S309-CAR-NK92MI cells used for flow cytometry are maintained by Liu Lab. PE anti-His Tag (Clone: J095G46, BioLegend, Catalog number 362603), Alexa Fluor 647 anti-human CD56 (Clone: HCD56, BiolLegend, Calalog number 318314), FITC anti-human CD3 (Clone: HIT3A, BioLegend, Catalog number 300306), Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen, catalog number A11034), SARS-CoV-2 Spike Trimer Protein, His Tag (Biosystem Acro, catalog number SPN-C522T).

Materials for SDS-PAGE and Western Blot

Purified anti-CD247 (TCRȥ) (Clone: 6B10.2, Biolegend, catalog number 644102), Pierce BCA Protein Kit (Thermo Scientific, Catalog number 23225), 10x Tris/Glycine Buffer (Bio-Rad, catalog number 161-0771), 10x Tris/Glycine/SDS Buffer (Bio-Rad, catalog number 161-0772), Anti-mouse IgG HRP- linked Antibody (Cell Signaling Technology, catalog number, 7076S), SuperSignal WestFemto Maximum Sensitivity Substrate (Thermo Scientific, catalog number 349095), and b-Actin Rabbit mAb (HRP Conjugate Cell Signaling, catalog number 5125), Non-fat dry milk (Cell Signaling, Catalog number 9999), Phosphate buffer saline powder (Sigma, Catalog number P13813), Tween20 (Sigma, Catalog number P1379), 10% Mini-PROTEAN TGX Precast Gels (Bio-Rad, Catalog number 4561034), Precision Plus Protein Dual Color Standards (Bio-Rad, Catalog number 1610374).

PROCEDURE

There are three experiments to be done to conclude the role of S309-CAR-NK cells in neutralizing SAR-CoV-2. This section describes the steps taken to achieve proving my hypothesis. To test that S309-CAR-NK cells can bind to Omicron subvariant XBB.1.5 Spike protein; therefore, neutralizing the pseudoviral SARS-CoV2 XBB.1.5 particles *in vitro* and can be used as a potential therapeutic for immunocompromised COVID patients, I will perform the following experiments to test my hypothesis:

1. Determine the intracellular expression of S309-CAR-NK cells using western blot
2. Determine the surface expression of S309-CAR-NK cells using flow cytometry
3. Investigate whether S309-CAR-NK cells bind to Omicron subvariant XBB.1.5 Spike protein

DETERMINE INTRACELLULAR EXPRESSION OF S309-CAR-NK CELLS USING WESTERN BLOT:

Generation of a protein standard curve:

I used a BCA kit to generate a protein standard curve. First, perform a 1:2 serial dilution to dilute the BSA protein provided in the BCA kit using PBS from 2 mg/ml to 0.015625 mg/ml. Then, pipette 20 mL of the diluted protein, including PBS as blank, into a well of a 96-well plate (with duplicates). Carefully pipette 200 mL of the working solution of BCA into each well. The plate is covered in aluminum foil to protect it from the light and it is incubated in a 37◦C incubator for 30 minutes. The absorption values are read at 562 nm wavelength to generate a standard curve, calculated as follows: The average of each concentration is calculated. Then, subtract all values from the PBS blank value. Using Excel to graph the standard curve using concentration (mg/ml) as

the x-axis and absorption value as the y-axis. A linear progression trendline is then applied to generate an equation. This equation can then be applied to determine the protein concentration for the SDS-PAGE.

SDS-PAGE:

First, lyse 1 x 10^6 cells with 100 µL RIPA buffer in the presence of protease

inhibitors on ice for 30 minutes. Then centrifuge the cells at max speed, at 4°C, for 20 minutes. The supernatant is then transferred to a new tube. I used some of the supernatants from each sample to quantify protein concentrations using a BCA kit as described in the previous section. Add 6x SDS containing beta-mercaptoethanol into the remaining supernatant and put it into a heat block to boil for 10 minutes. Assemble the 10% protein gel into an SDS-PAGE apparatus. Using the micropipette, carefully load 20 µg of protein per lane. Proceed by filling the apparatus with a running buffer. Attach the box's lid with the power supply and turn on the desired voltage. Watch to see if bubbles appear to be rising, indicating that the buffer tank has started and is working. Run the gel until the blue line reaches the bottom. Proteins are now negatively charged because they are coated by the SDS. They will move from the negative (top) to the positive terminal (bottom). The proteins are stacked on top of the resolving gel, and they resolve according to the function of their molecular weight. Stop the voltage once the loading dye line reaches the bottom of the gel.

Gel Transferring and Western Blotting:

While waiting for the gel to finish running, soak the sponges needed for western blotting in the transfer buffer. Cut pieces of the PVDF membrane and place them in a box to be treated with methanol to and transfer to the buffer. Remove the gel once it's done, and place the sponge on the clear side of the chamber, then, on top, add a piece of wet filter paper. Proceed by layering the gel, activated membrane, a piece of wet filter paper, and the sponge. After everything is layered, close the chamber with the "sandwich" inside. Place the chamber into a box and cover it with a transfer buffer. Add transfer buffer to the outer box, close the lid, and turn on the voltage at 100V for 1 hour in a cold room. Once complete, disassemble the module and place the membrane in a box. Pour the 5% milk-blocking buffer over the membrane and place it on the rocker at room temperature for 1 hour. Then add anti-CD3z primary antibodies at 1:1000 dilution and proceed to incubate it at room temperature for 1 hour. With PBST buffer, wash blot 5 times, 5 minutes each time. After adding 1:2000 dilution of the anti-rabbit secondary antibody, incubate for 1 hour at room temperature. Then again, using PBST buffer, wash the blot 5 times, 5 minutes. Add ECL, then expose the membrane using a Chemidoc. Next, briefly rinse the blot with PBST once and add conjugated anti-β-actin (housekeeping protein) at 1:10,000 dilution. Incubate at room temperature for 1 hour. Wash the blot 5 times, 5 minutes each with PBST buffer for the final time. Add ECL and then expose the membrane using a Chemidoc.

THE SURFACE EXPRESSION OF S309-CAR-NK CELLS: FLOW CYTOMETRY:

To determine the surface expression of S309-CAR-NK cells, parental non-modified NK cells will be used as the negative control. To prepare for the experiment, all centrifuging in the protocol should be completed at 1250-1500 RPM or

300-500 g for five minutes. Label FACS tubes to be arranged in a tube rack. Once complete, harvest cells and on the last wash step and resuspend the cells at 1 million cells/100 µL of flow cytometry staining buffer (1 x 10^6/100 µL). Then, aliquot 100 µL into each FACS tube. Add conjugated antibody concentrated at 5-10 mL per million cells (5-10 µL/1 x 10^6) and vortex.

1. CD56-PE-Cy7 for NK cells
2. CD3-PE for T cells
3. hIgG-APC for CAR marker

Proceed to incubate cells for 30 minutes in the dark on ice. Wash cells in 2 milliliters of flow cytometry staining buffer to remove unbound antibodies. Then centrifuge the cells and vortex to resuspend the cells. Add 2 mL of flow cytometry staining buffer and repeat centrifugation. Resuspend the cells, in 200-400 µL of flow cytometry staining buffer for flow cytometry analysis.

INVESTIGATE WHETHER S309-CAR-NK CELLS BIND TO OMICRON

To investigate whether S309-CAR-NK cells bind to Omicron subvariant XBB.1.5 Spike protein, parental non-modified NK cells will be used as negative control and all centrifuging in the protocol should be completed at 1250-1500 RPM or 300-500 g for five minutes. Again, label FACS tubes to be arranged in a tube rack. Harvest cells and on the last wash step resuspend the cells at 1 million cells/100 µL of flow cytometry

staining buffer (1 x 10^6/100 µL). Then aliquot 100 µL into each FACS tube and add 1 µg of Spike omicron protein. Vortex and proceed to incubate on ice for 30 minutes. Wash cells with FACS buffer. After, add conjugated antibody concentrated at 5-10 µL per million cells (5-10 µL/1 x 10^6) and vortex.

1. CD56-PE-Cy7 for NK cells
2. CD3-PE for T cells
3. hIgG-APC for CAR markers
4. anti-His to detect spike protein binding to CAR-NK cells

Next, incubate cells for 30 minutes in the dark at room temperature and wash cells in 2 milliliters of flow cytometry staining buffer to remove unbound antibody. Centrifuge the cells and vortex to resuspend them. Add 2 mL of flow cytometry staining buffer and repeat centrifugation. Then resuspend cells in 200-400 µL of flow cytometry staining buffer for flow cytometry analysis.

RESULTS

DETERMINE INTRACELLULAR EXPRESSION OF S309-CAR-NK CELLS USING WESTERN BLOT:

Generation of a protein standard curve:

The method described above is used to calculate the standard curve values (Table 1). Once these values are generated, a standard curve linear progression along with an equation is created so that this equation can be applied for future protein quantifications. Figure 6 shows the protein standard curve, with an equation of y= 1.2823x + 0.0256 indicating that the standard curve is a linear progression with a slope of 1.2823 and an R^2 = 0.993 indicating the reliability of the standard curve.

Concentration (mg/ml)	2	1	0.5	0.25	0.125	0.062 5	0.0312 5	Blank
Abs 562 nm (raw values)	2.573	1.376	0.801	0.49	0.305	0.194	0.141	0.102
	2.778	1.513	0.684	0.455	0.295	0.195	0.144	0.099

Concentration (mg/ml)	2	1	0.5	0.25	0.125	0.062 5	0.0312 5	Blank
Abs 562 nm (Avg)	2.675 5	1.444 5	0.742 5	0.472 5	0.3	0.194 5	0.1425	0.100 5

Concentration (mg/ml)	2	1	0.5	0.25	0.125	0.062 5	0.0312 5	Blank
Abs 562 nm (Subtracted blank)	2.575	1.344	0.642	0.372	0.199 5	0.094	0.042	0

Table 1: Table of values calculated for the standard curve. These concentrations and absorption values are plotted to generate an equation that will be applied to future protein quantifications.

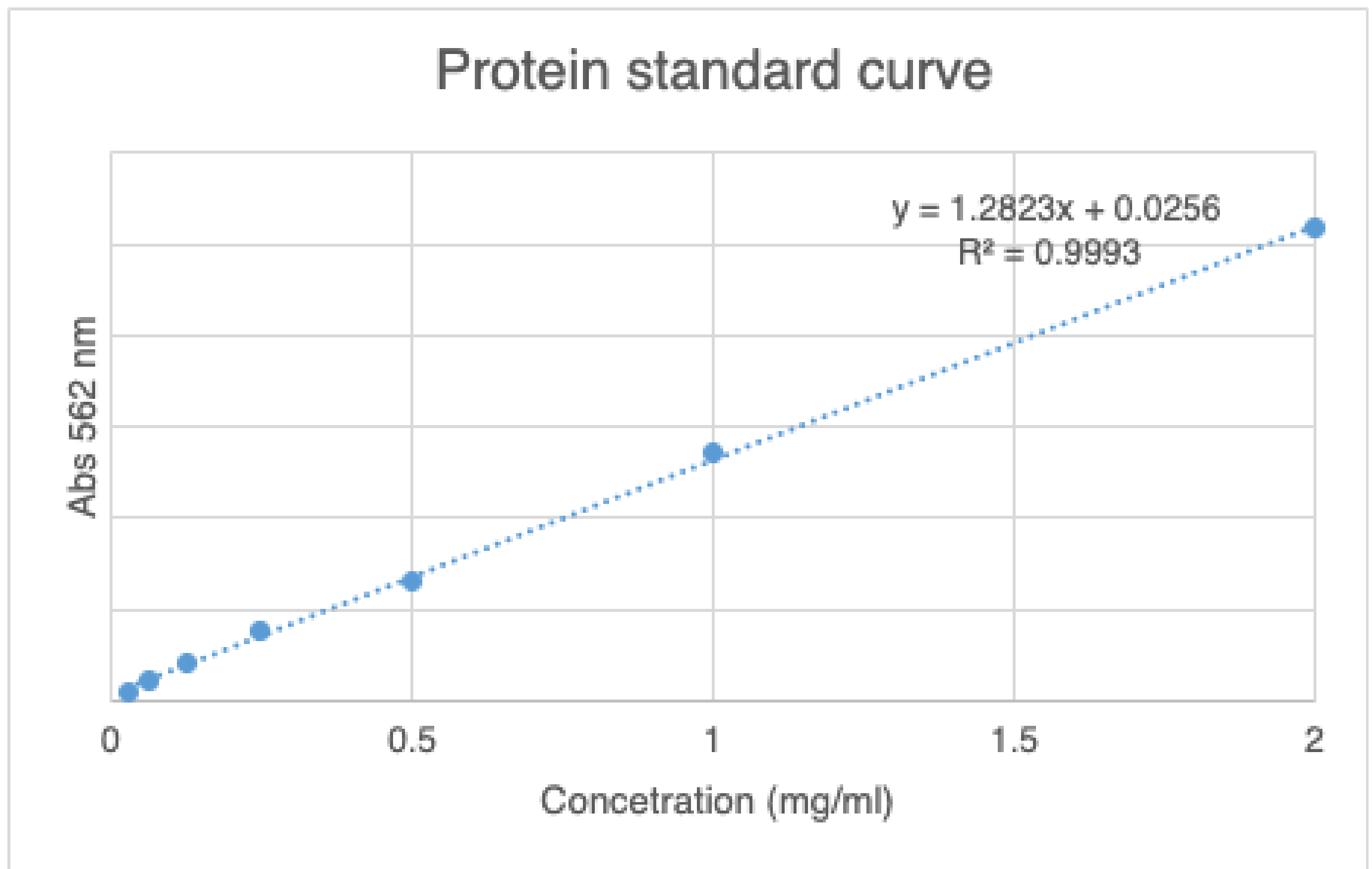

Figure 6: Representative of a protein standard curve to the values found in table one. A linear progression trendline was applied to generate an equation that determine the concentration of protein used later for SDS-PAGE. The x-axis in concentration is measured in mg/ml and the y-axis is the average absorbance 562 value measured in nm.

SDS-PAGE:

Prior to loading the processed protein samples as described in the Method, I first used the BCA kit to quantify the protein concentrations of the two samples. To ensure that the protein concentrations do not exceed the limit of the standard curve, I first diluted the samples to 1:2 dilution with RIPA buffer. By using RIPA buffer as blank and applying the previously generated standard curve equation (Figure 6), I was able to determine the protein concentrations. As the total loading volume is 20 µg (or 0.02 mg), I next divide 0.02 to the protein concentration to determine the volume needed to load on the SDS-PAGE gel, which equates to 0.00663 mL and 0.00636 mL for NK92MI cells and S309-CAR-NK92MI cells, respectively (Table 2). Appropriate volumes of 6x SDS were added to the samples and boiled for 10 minutes.

Samples	Abs 562nm		Average	Subtracted blank	Dilution factor	Equation applied (mg/ml)	Concentration (mg/ml)	Amount loaded (µg)	Volume needed (in ml)
NK92MI	2.04200	2.11300	2.07750	1.93300	2	1.50745	3.01490	0.02000	0.00663
S309-CAR-NK92MI	2.13000	2.19100	2.16050	2.01600	2	1.57217	3.14435	0.02000	0.00636
Blank	0.14200	0.14700	0.14450	0.00000					

Table 2: A walk-through of protein concentration determination using the standard curve generated in Figure 6.

Gel Transferring and Western Blotting:

Once the 20 µg of each sample is loaded as indicated, I then transferred the gel followed by a western blot against both CD3ζ (intracellular domain of the S309-CAR-NK92MI) cells and β-actin (housekeeping protein, or a protein that is abundantly expressed in all cells). As expected, I observe a band in S309-CAR-NK92MI cells migrated at around 19kDa, or the molecular weight of CD3ζ, but not non-modified NK92MI, suggesting a successful modification of S309-CAR-NK92MI cells. β-actin protein is presented in both samples to show that the NK92MI sample was loaded, but CD3ζ was not expressed since NK92MI cells do not endogenously express CD3ζ (Figure 7). The intensities of the bands were calculated using Fiji (or ImageJ) as described in the **ANALYSIS** (Table 3). To generate fold change relative to β-actin, divide the CD3ζ intensity of NK92MI or S309-CAR-NK92MI to corresponding β-actin intensities. The experiment was repeated three times to ensure technical reproducibility. A graph is generated in Figure 8 showing that S309-CAR-NK92MI cells quantitatively express higher CD3ζ than that of NK92MI cells. The β-actin is not plotted as the values were used to normalize the CD3 ζ intensities. t-test was used for the statistics.

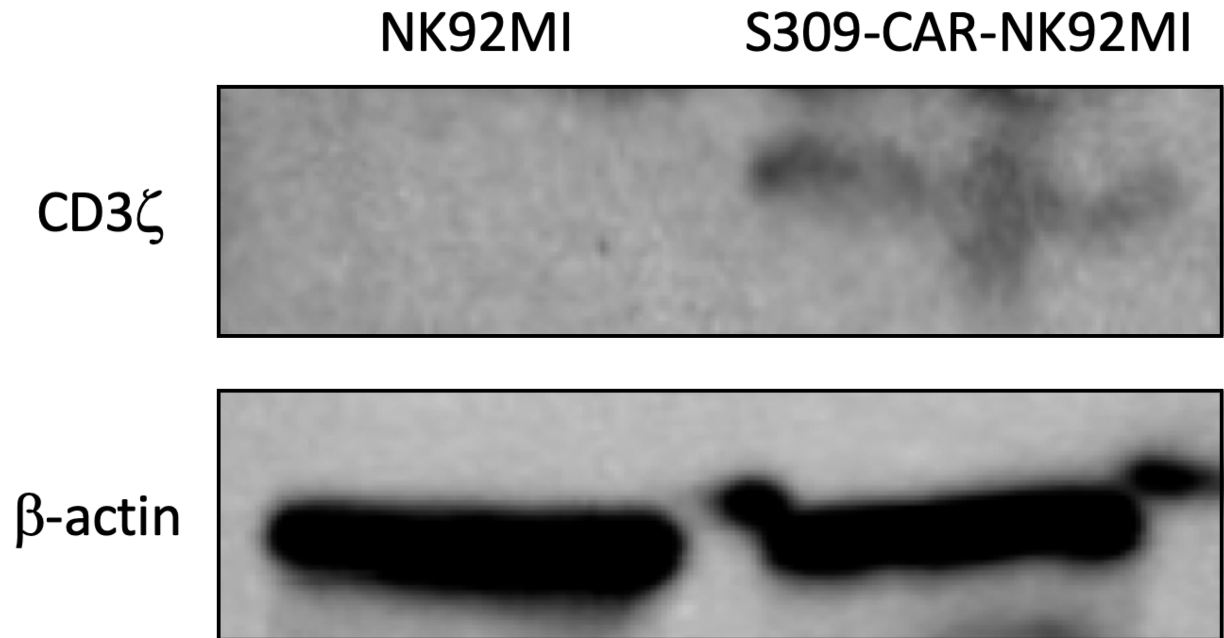

Figure 7: Snapshot of the intensity of the bands from S309-CAR-NK92MI and NK92MI expression after gel transferring and western blot when proteins β-actin and CD3ζ were presented.

	Samples	Raw intensities	Fold change relative to b-actin
CD3ζ_repeat 1	NK92MI	5.129	0.0366
	S309-CAR-NK92 MI	18.934	0.1571
β-actin_repeat 1	NK92MI	140.019	1
	S309-CAR-NK92 MI	120.489	1
CD3ζ_repeat 2	NK92MI	6.957	0.0472
	S309-CAR-NK92 MI	55.001	0.3714
β -actin_repeat 2	NK92MI	147.185	1
	S309-CAR-NK92 MI	148.073	1
CD3ζ_repeat 3	NK92MI	9.62	0.0608
	S309-CAR-NK92 MI	38.821	0.3007
β -actin_repeat 3	NK92MI	158.217	1
	S309-CAR-NK92 MI	129.071	1

Table 3: Data of calculated intensity of the bands using Fiji (or ImageJ). Three trials ran for both β-actin and CD3ζ with both samples S309-CAR-NK92MI for each. Values calculated for raw intensities and intensities relative to b-actin.

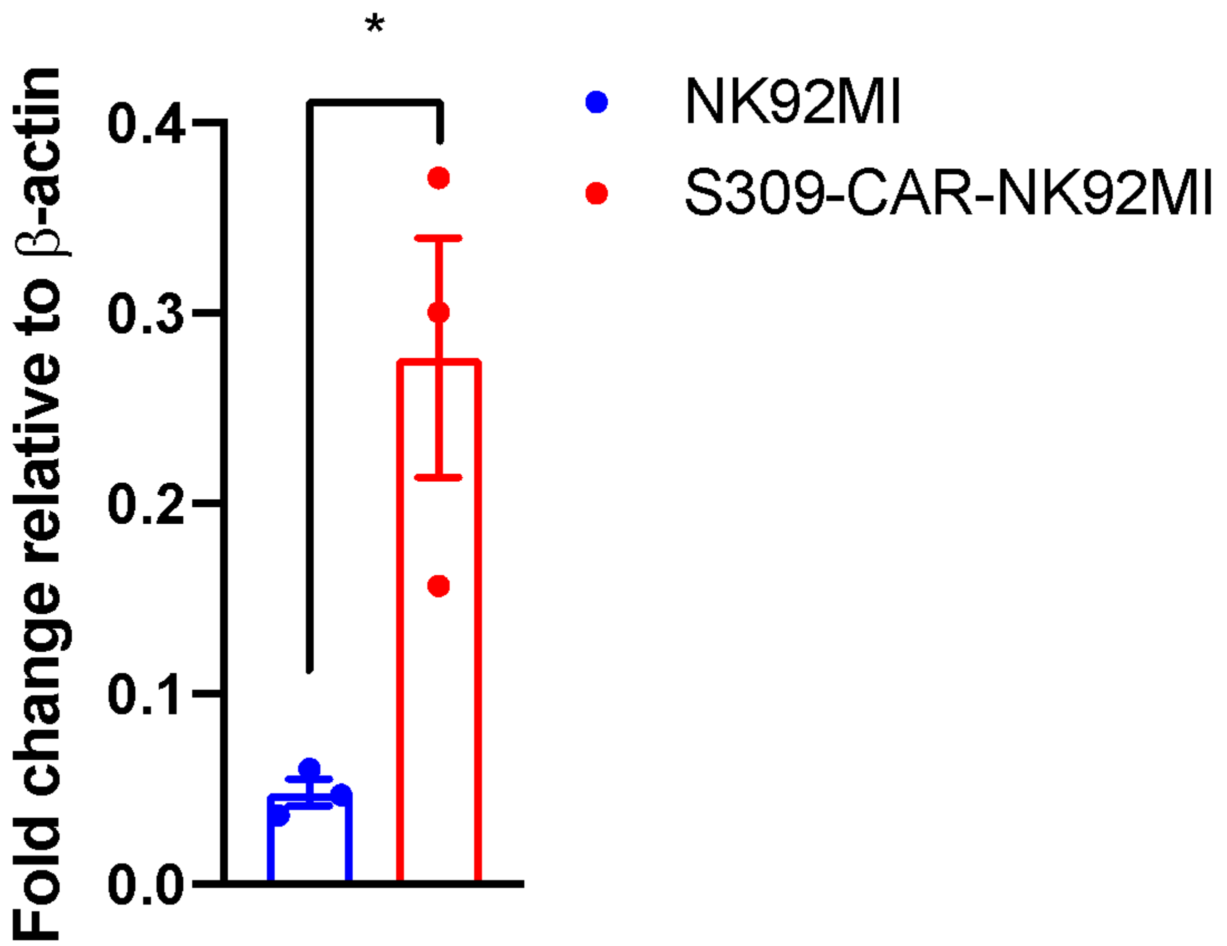

Figure 8: Error bars formed from repeated experiment to ensure reproducibility depicting the fold change of NK92MI and S309-CAR-NK92MI relative to β-actin (y-axis).

THE SURFACE EXPRESSION OF S309-CAR-NK CELLS: FLOW CYTOMETRY:

After flow cytometry, data were analyzed by FlowJo software. Cells were first selected for the live population in the lymphocyte panel represented by SSC-A (y-axis) and FSC-A (x-axis). Cells were then selected for single cells followed by surface S309-CAR, where the y-axis is for CD56 (NK cell marker) and the x-axis represents the CAR expression. As expected, all cells shift upward in the y-axis indicating that they all express CD56 because they are NK cells. For the S309-CAR-NK92MI cells, around 63% of cells shift rightward indicating that 63% of this population express the surface S309-CAR compared to 0% for the NK92MI control (Figure 9). This flow data indicates successful modification of S309-CAR-NK92MI cells.

This experiment was repeated three times to ensure reproducibility. Since the experiment was performed multiple times, we can now generate two different graphs with error bars to show the reproducibility of the experiment and the stability of the S309-CAR expression (Figure 10). The left panel shows the percentage by which these cells express S309-CAR and the right panel represents how highly expressed the S309-CAR is by calculating the mean fluorescent intensity (MFI). t tests were used to analyze the statistics, *, $p \leq 0.05$ and ***, p   0.0005.

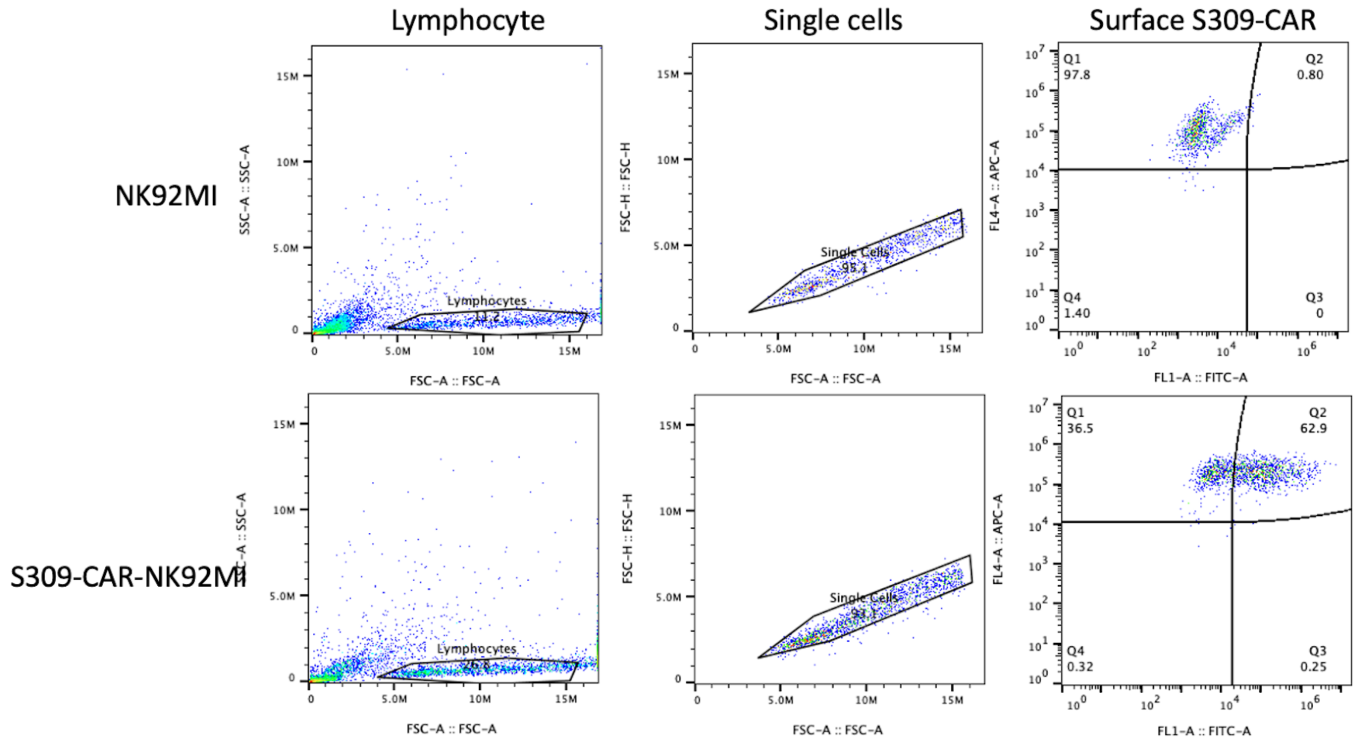

Figure 9: Representative dot plots showing the expressions in NK92MI and S309-CAR-NK92MI. Confirmed by flow cytometry. Data are shown with three panels each, the live population in lymphocytes (SSC-A as the y-axis and FSC-A as the x-axis), selected single cells, then the surface of S309-CAR (y-axis is for CD56 and CAR expression as the x-axis).

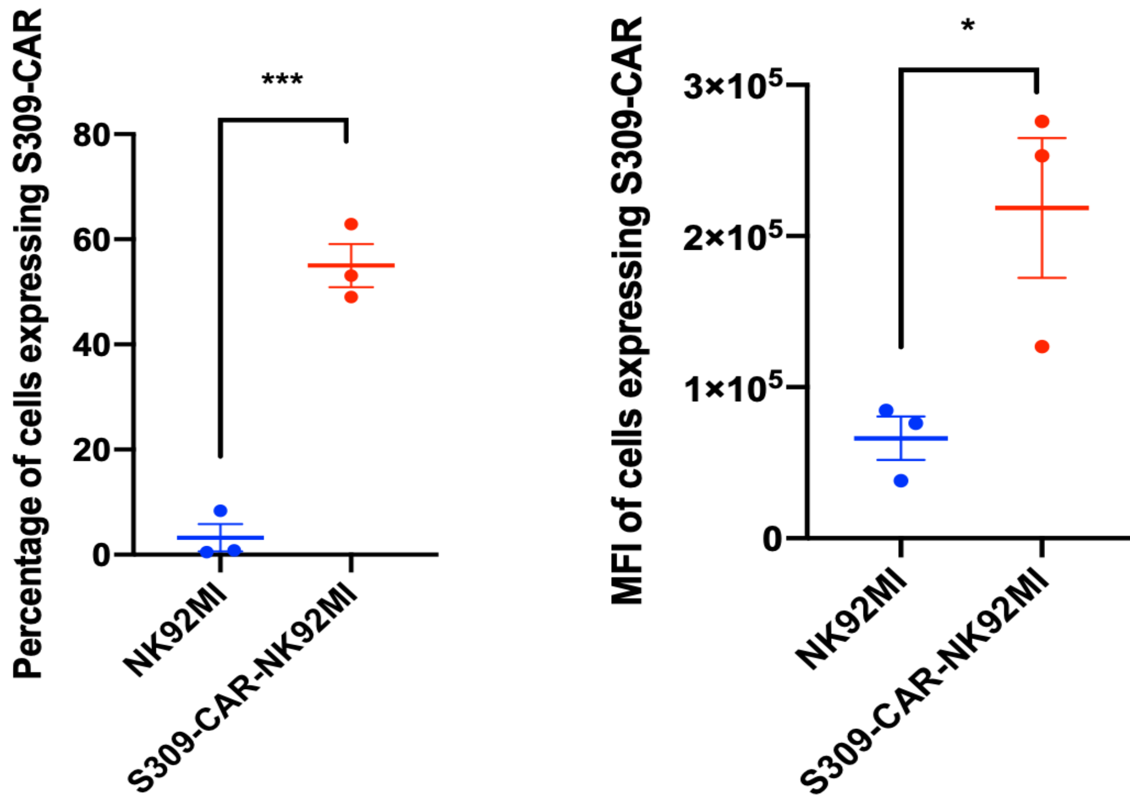

Figure 10: Quantitative data of the percentage of cells expressing S309-CAR (left panel) and using MFI (mean fluorescent intensity) of cells to indicate the expression of S309-CAR (right panel). The error bars show the reproducibility of the experiment and the stability of the S309-CAR expression. NK92MI and S309-CAR-NK92MI are represented in both panels on the x-axis.

INVESTIGATE WHETHER S309-CAR-NK CELLS BIND TO OMICRON

FlowJo software was used to analyze the data after flow cytometry. Cells were first selected for the live population in the lymphocyte panel represented by SSC-A (y-axis) and FSC-A (x-axis). Following the selection of single cells, the S omicron protein was added, where the y-axis represents FL4-A and the x-axis represents FL2-A. The y-axis of all cells shifts upward as expected, showing that all of them bind to S omicron. For the S309-CAR-NK92MI cells, around 69% of cells shift rightward indicating that 69% of this population bind to the S omicron protein compared to 0% for the NK92MI control (Figure 12). This flow data demonstrates the successful binding of S309-CAR-NK92MI cells to the Spike omicron protein. It is important to note that the S309-CAR-NK92MI cells were not homogenously expressing the S309-scFv domain (Figure 11 only shows around 60% expression), and meanwhile we observe 69% of S309-CAR-NK92MI cells to the Spike omicron protein. These data indicate that S309-CAR-NK92MI cells actually have 100% binding efficiency to the Spike omicron protein.

Reproducibility was ensured by repeating this experiment three times. Due to the fact that the experiment was repeated multiple times, we can now generate two different error bars to quantify the reproducibility of the experiment and the stability of the binding of the cells to the S omicron protein (Figure 12). The left panel shows the percentage by which these cells bind to the S omicron protein and the right panel represents how much the cells bind to the S omicron protein by calculating the mean fluorescent intensity (MFI). t tests were used to analyze the statistics, **, $p \leq 0.05$ and ***, p 0.0005.

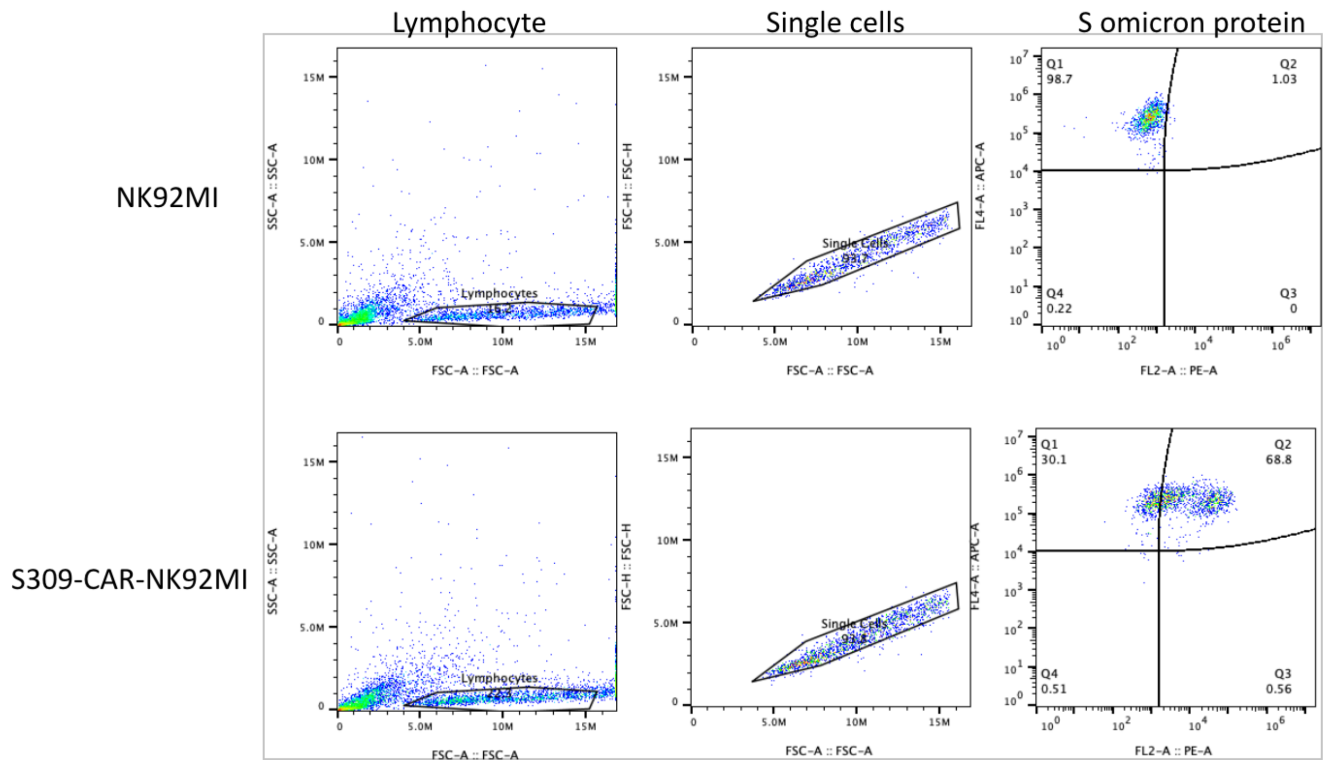

Figure 11: Representative dot plots showing the expressions in NK92MI and S309-CAR-NK92MI. Confirmed by flow cytometry. Data are shown with three panels each, the live population in lymphocytes (SSC-A as the y-axis and FSC-A as the x-axis), selected single cells, then the S omicron protein (FL4-A = APC-A for the y-axis and FL2-A::PE-A for the x-axis).

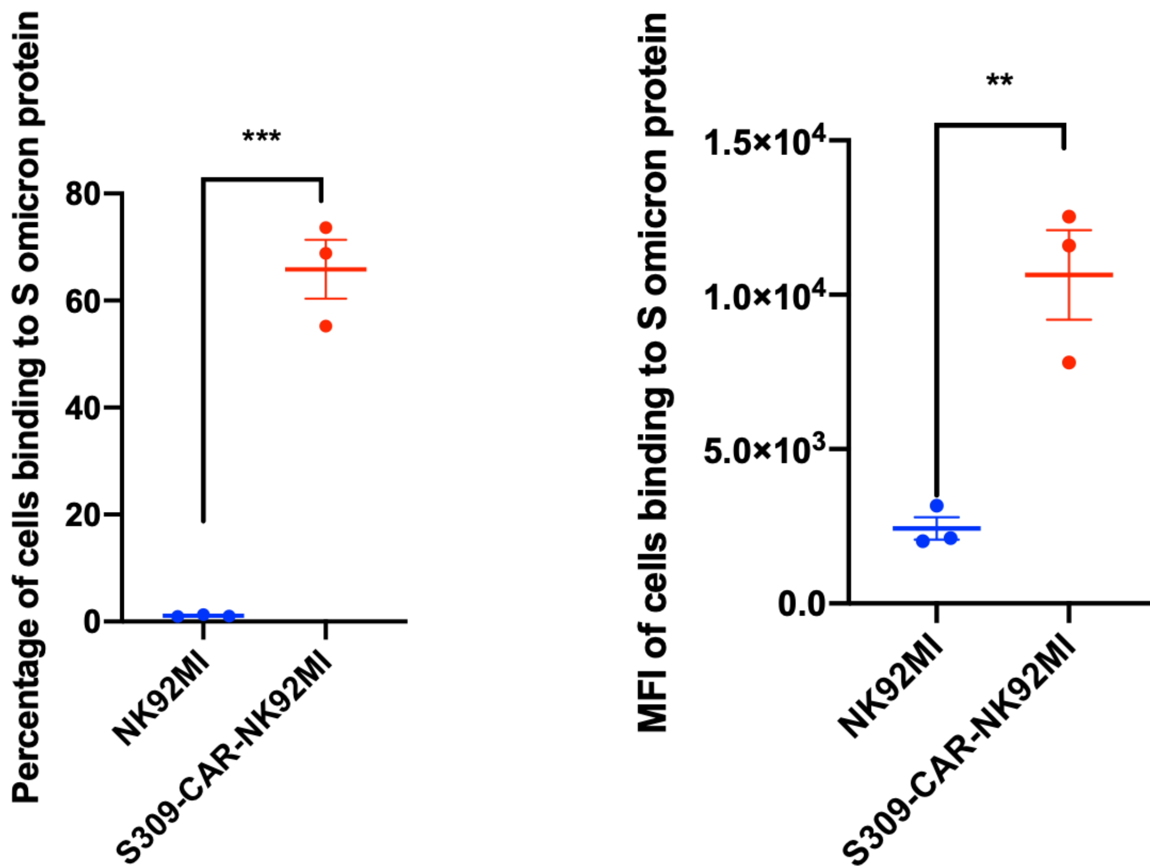

Figure 12: Quantitative data of the percentage of cells binding to S omicron protein (left panel) and using MFI (mean fluorescent intensity) indicate the cells binding to S omicron protein (right panel). NK92MI and S309-CAR-NK92MI are represented in both panels on the x-axis.

ANALYSIS:

The values by the BCA assays were obtained from Biotek Synergy HT Microplate Reader. The values were plotted in Excel as shown in Tables 1 and 2 for the generation of the standard curve and protein concentrations determination.

The Western blots images were captured by ChemiDoc Imaging System by Bio-Rad and converted into TIFF (Figure 7). The intensities of the bands were quantified using Fiji or ImageJ by first subtracting any background noise followed by creating rectangles (with the same area) around the bands. The raw intensity values are shown in Table 3 with appropriate normalization to β-actin, which the is graphed in Figure 8.

Flow cytometry data were obtained by Dr. Liu's lab member using an Accuri C6 Plus Flow Cytometer by BD Biosciences. The flow data analyses were assisted by Dr. Liu's lab member. The mean fluorescent intensities (MFI) and percentage were graphed in Prism version 8 to calculate for the statistics using unpaired t-tests.

Analysis Software includes Excel, FlowJo version 8 by BD Biosciences, Fiji, and Prism version 8.

CONCLUSION:

Public health interventions are influenced by the properties of SARS-CoV-2. The number of cases increased dramatically with Omicron, which had a substantial antigenic change, resulting in declined effectiveness of both monoclonal antibody therapies and vaccines. In the virus, there are S1 which is the receptor binding domain (RBD) and S2. Antibodies for the coronavirus recognize the spikes on the surface and bind to them to prevent the virus from binding with a healthy host cell. Neutralizing antibodies such as S309 could diffuse not only this specific viral strain but also offshoots that occur because of natural mutations in the virus. SARS-CoV2 interrupts equations of immune responses, disrupting cytolytic antiviral effects of NK cells and inducing a "cytokine storm" by activating infected immune cells. The advantages of CAR NK cells include less antigen loss relapse, minimal on-target, off-tumor toxicity, and antibody-dependent cellular cytotoxicity (ADCC). They have stronger and less off-target reactions and have a low risk of grafts versus host disease (GvHD), cytokine release syndrome (CRS), and immune effector cell-associated neurotoxicity syndrome (ICANS), which are often seen in CAR-T therapy due to allogeneic donors.

My hypothesis is that S309-CAR-NK cells can bind to Omicron subvariant XBB.1.5 Spike protein; therefore, neutralizing the pseudoviral SARS-CoV2 XBB.1.5 particles *in vitro* and can be used as a potential therapeutic for immunocompromised COVID. This hypothesis was a success.

A western blot, as well as flow cytometry, were used to confirm the success of genetic modification of S309-CAR-NK92MI cells. They were used to determine S309-CAR-NK cell intracellular expression and the surface expression of S309-CAR-NK

cells respectively. Once the NK92MI cells have been successfully modified, S309-CAR-NK92MI cells were tested on their ability to bind to the Spike Omicron XBB.1.5 protein in the last experiment. As expected, NK92MI cells do not express the CD3ζ domain, but S309-CAR-NK92MI cells do. I also validated my western blot results by using flow cytometry. My data show that the S309-CAR-NK92MI cells, around 63% of cells shift rightward indicating a successful modification of S309-CAR-NK92MI cells. Since the experiment was performed multiple times, two different graphs with error bars were made and show the reproducibility of the experiment and the stability of the S309-CAR expression (Figure 8). t-tests were used to analyze the statistics.

Next, I determined the ability that S309-CAR-NK92MI cells binding to the Spike omicron protein by incubating the cells with the protein followed by flow cytometry. I demonstrated that approximately 69% of S309-CAR-NK92MI cells shift rightward, indicating successful binding to the spike omicron protein. It is important to note that only around 60% of cells are S309-CAR-NK92MI cells, and concomitantly, around 60% of S309-CAR-NK92MI cells bind to the spike omicron protein. These data indicate that S309-CAR-NK92MI cells have a 100% binding efficiency to the spike omicron protein. Two different error bars were generated to quantify the reproducibility of the experiment and the stability of the binding of the cells to the S omicron protein (Figure 10). t-tests were used to analyze the statistics as well.

As previously demonstrated by the Liu Lab, a binding of S309-CAR-NK92MI to the protein suggest binding to the SARS-CoV2 virus, resulting in neutralization. It is therefore reasonable to postulate that the S309-CAR-NK92MI cells have the ability to neutralize the SARS-CoV2 omicron variant. The SARS-CoV2 omicron variant is known

to escape the majority of existing SARS-CoV2 neutralizing antibodies and monoclonal antibody therapies. However, S309-CAR-NK92MI cells are shown to still conserve the ability to bind to spike omicron XBB.1.5 protein, suggesting the superiority of the S309 neutralizing antibody in recognizing and neutralizing the future SARS-CoV2 variants.

RISK AND SAFETY

Biohazard chemicals include the use of sodium dodecyl sulfate (SDS), methanol, and beta-mercaptoethanol. To mitigate the risks of exposure, these chemicals will be handled inside a fume hood. As NK92MI is a cancer cell line and is derived from a cancer patient, additional precautions will be needed such as staining cells for flow cytometry in the hood. Personal protective equipment (PPE) including goggles, gloves, a lab coat, and closed-toe shoes will be always worn during experiments. The use of some equipment, such as a flow cytometer and heat block (used to denature proteins for the SDS-PAGE) may be dangerous and will be done by a supervised adult. All activities performed in the lab will be closely monitored by a supervisor adult.

BIBLIOGRAPHY:

1. A., Angel, et al. "Graft versus Host Disease - Statpearls - NCBI Bookshelf." *Graft Versus Host Disease*,10 Oct. 2022, https://www.ncbi.nlm.nih.gov/books/NBK538235/.
2. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Innate Immunity. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26846/
3. Animal Biotechnology. "Single-Chain Variable Fragment." *Single-Chain Variable Fragment - an Overview | ScienceDirect Topics*, 2014, https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/single-chain-variable-fragment.
4. Barnes, Christopher O., et al. "SARS-COV-2 Neutralizing Antibody Structures Inform Therapeutic Strategies." *Nature News*, Nature Publishing Group, 12 Oct. 2020, https://www.nature.com/articles/s41586-020-2852-1.
5. Borsa, Mariana, and Julie M. Mazet. "Attacking the Defence: SARS-COV-2 Can Infect Immune Cells." *Nature News*, Nature Publishing Group, 21 Aug. 2020, https://www.nature.com/articles/s41577-020-00439-1.
6. Ghasemzadeh, Mehran, et al. "Exhausted NK Cells and Cytokine Storms in COVID-19: Whether NK Cell Therapy Could Be a Therapeutic Choice." *Human Immunology*, U.S. National Library of Medicine, Jan. 2022, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8423992/.

7. Gu, Wenjing, et al. "The Molecular Mechanism of SARS-COV-2 Evading Host Antiviral Innate Immunity - Virology Journal." *BioMed Central*, BioMed Central, 19 Mar. 2022, https://virologyj.biomedcentral.com/articles/10.1186/s12985-022-01783-5.
8. Ebrahimiyan , Hamidreza, et al. "Novel Insights in Car-NK Cells beyond Car-T Cell Technology; Promising Advantages." *International Immunopharmacology*, Elsevier, 9 Feb. 2022, https://www.sciencedirect.com/science/article/pii/S1567576922000716?via%3Dihub.
9. Ma, Minh Tuyet, et al. "Car-NK Cells Effectively Target SARS-COV-2-Spike-Expressing Cell Lines in Vitro." *Frontiers*, Frontiers, 30 June 2021, https://www.frontiersin.org/articles/10.3389/fimmu.2021.652223/full.
10. Pinto, Dora, et al. "Cross-Neutralization of SARS-COV-2 by a Human Monoclonal SARS-COV Antibody." *Nature News*, Nature Publishing Group, 18 May 2020, https://www.nature.com/articles/s41586-020-2349-y.
11. Furuse, Yuki. "Properties of the Omicron Variant of SARS-COV-2 Affect Public Health Measure Effectiveness in the COVID-19 Epidemic." *International Journal of Environmental Research and Public Health*, U.S. National Library of Medicine, 19 Apr. 2022, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9099739/#:~:text=Although%20the%20short%20geneation%20time,the%20effectiveness%20of%20early%20isolation.

12. Li, Rui, et al. “Evaluating the Impact of SARS-COV-2 Variants on the COVID-19 Epidemic and Social Restoration in the United States: A Mathematical Modelling Study.” *Frontiers in Public Health*, U.S. National Library of Medicine, 10 Jan. 2022, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8786080/.
13. “What Is Innate Immunity?” *WHAT IS INNATE IMMUNITY? | Center for Innate Immunity and Immune Disease*, https://ciiid.washington.edu/content/what-innate-immunity.
14. “Structural and Functional Mechanism of SARS-COV-2 Cell Entry.” *Abcam*, 19 May 2022, https://www.abcam.com/content/structural-and-functional-mechanism-of-sars-cov-2-cellentry#:~:text=SARS%2DCoV%2D2%20S%20protein,to%20the%20host%20cell%20membrane.
15. Di Vito, Clara, et al. “Natural Killer Cells in SARS-COV-2 Infection: Pathophysiology and Therapeutic Implications.” *Frontiers in Immunology*, U.S. National Library of Medicine, 30 June 2022, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9279859/.
16. “Cytokine Release Syndrome: Symptoms, What It Is & Treatment.” *Cleveland Clinic*, https://my.clevelandclinic.org/health/diseases/22700-cytokine-release-syndrome.
17. Dietrich, Jorg, and Matthew j Frigault. “Immune Effector Cell-Associated Neurotoxicity Syndrome (ICANS).” *UpToDate*, 7 Dec. 2022, https://www.uptodate.com/contents/immune-effector-cell-associated-neurotoxicit

y-syndrome-icans#:~:text=Immune%20effector%20cell%2Dassociated%20neurotoxicity%20syndrome%20(ICANS%20is%20a,and%20T%20cell%20engaging%20therapies.

18. Rivera, Drs. “Car T-Cell-Associated Neurotoxicity: Current Management and ...: Critical Care Nursing Quarterly.” *LWW*, Apr. 2020, https://journals.lww.com/ccnq/Abstract/2020/04000/CAR_T_Cell_Associated_Neurotoxicity__Current.7.aspx.
19. de Aguiar, Rodrigo Barbosa, et al. “Generation and Functional Characterization of a Single-Chain Variable Fragment (ScFv) of the Anti-FGF2 3F12E7 Monoclonal Antibody.” *Nature News*, Nature Publishing Group, 14 Jan. 2021, https://www.nature.com/articles/s41598-020-80746-8.
20. Zhang, Chen, et al. “Home - Books - NCBI.” *National Center for Biotechnology Information*, U.S. National Library of Medicine, 3 Oct. 2022, https://www.ncbi.nlm.nih.gov/books.