# Novel CAR design dually targets HER2$^+$ breast cancer and MDSCs to improve efficacy in solid tumors

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## Abstract

CAR T cell therapy is a promising immunotherapy that has been approved by the FDA for use in blood cancers, but has yet to be efficacious in solid tumors due to limitations including antigen escape, on-target off-tumor effects, tumor infiltration challenges, toxicities, and the immunosuppressive tumor microenvironment. Current strategies to overcome these obstacles are ongoing and include dual target CARs, combination therapy with checkpoint blockade, local CAR administration, and targeting immunosuppressive microenvironment cells. However, additional studies are still needed to continue to improve the efficacy of CAR T. In this proposal, we first review the function of T cells in the immune system, and discuss how CAR T cell therapy enhances immune response to cancer. We also review current strategies being tested to overcome limitations in CAR T cell therapy on solid tumors. We then propose a novel CAR design that targets HER2 on breast cancer and secretes a NBD peptide that is receptive to endocytosis into MDSCs through CD73. The NBD peptide blocks the Nf-Kb signaling pathway which reduces the immunosuppressive qualities of MDSCs in the tumor microenvironment. This design requires preclinical studies to validate its efficacy and safety before being used in clinical trials. If successful, this proposal could present a novel therapeutic option for patients with HER2 positive breast cancer that would then need to be followed up with clinical studies.

## Cancer and its societal impacts

Cancer is a disease that occurs from an accumulation of mutations, causing cells to build up into a tumor or travel to the blood. Cancer cells grow much faster than normal cells, as they are no longer responding to controlled growth signals. As the cancer cells rapidly grow, they can

form a tumor, which is a condensed, uncontrollable growth of cells.Tumors can cut off nutrient and oxygen supplies to normal cells, damaging healthy tissue (*What Is Cancer? - NCI*, n.d.). These cancerous tumors affect millions each year, with 18 million new cancer cases recorded worldwide in 2020 alone. It is estimated that by 2040, there will be 28 million new cases of cancer each year (*Worldwide Cancer Statistics*, 2015). Despite the commonality of cancer, current treatments cost billions each year. In 2015, the U.S. alone spent an estimated $190.2 billion on cancer care, and this cost is estimated to exceed $245 billion by 2030 (*Financial Burden of Cancer Care | Cancer Trends Progress Report*, n.d.) (*The Future of Health System-Based Cancer Care | AHA*, n.d.). Overall, cancer has a major impact on public health and the world economy, and scientists are continuously searching for new breakthroughs in treatment and care.

**Current cancer treatments**

Currently, there are several options for cancer treatments, including surgery, radiation, chemotherapy, targeted therapy, and immunotherapy (*Types of Cancer Treatment - NCI*, n.d.). Surgery involves a surgeon physically removing a tumor, and works best on solid tumors. Radiation is a method in which beams of energy are used to destroy the genetic material of cancer cells, which prevents them from reproducing by killing them (*Radiation Therapy - Mayo Clinic*, n.d.). Chemotherapy involves the use of drugs to shrink and kill cancerous tumors, and is typically administered intravenously. Targeted therapy is a treatment that targets proteins associated with tumor growth, and interrupts cancer growth signals (*Targeted Therapy for Cancer - NCI*, n.d.). Immunotherapy boosts the immune system to enable immune cells to find and kill cancer cells (*Immunotherapy for Cancer - NCI*, 2015). Although these treatments can

lead to complete remission in some cancer patients, not all patients respond to existing methods of treatment, highlighting the continual need for treatment innovation.

**The cancer immunity cycle**

Ideally, the immune system will respond to and attack cancer cells in the body through a cycle called the cancer immunity cycle. This cycle can help kill some cancer cells, but it is often insufficient to systemically eradicate cancer, which is why additional treatments may be required (*The Immune System and Cancer*, 2014). The cancer immunity cycle is composed of 7 steps that repeat in a recurring manner (*Cancer-Immunity Cycle*, n.d.). The first step is death of cancer cells (apoptosis), which results in the release of neoantigens, or antigens formed by cancer cells due to genetic mutations. During this step, the neoantigens are released into the surrounding tumor microenvironments. The second step of the cycle occurs when the neoantigens are taken up by dendritic cells (DCs), which are professional antigen presenting cells (APCs). The DCs process the neoantigens and present them on the major histocompatibility complex (MHC) in the form of peptides. (N. Xie et al., 2023). The second step continues as the DCs migrate to nearby lymphatic organs, such as the lymph nodes, and present the neoantigens to T cells. If a cytotoxic T cell (ie: a $CD8^+$ T cell) has the corresponding T cell receptor (TCR) to recognize the antigen being presented by the DC, the T cell and DC will form an immunological synapse. This is a crucial step in the cancer immunity cycle, as it determines whether or not the T cell gets activated. In order to be activated, a T cell must bind to the correct DC that presents a peptide recognized by the TCR. In addition, the costimulatory receptors on the T cell (such as CD28) must bind with corresponding costimulatory molecules on the DC (such as CD80 or CD86) If the T cells are activated, they will clonally expand and migrate to the organ containing the tumor, which is the 4th step of the cancer immunity cycle.

This migration is aided by cytokines, which help guide the activated T cells to the tumor, especially if the tissue containing the tumor is inflamed. The 5th step of the cycle involves the activated cytotoxic T cells permeating the tumor itself. The T cells can then recognize the cancer cell antigens presented on MHC and bind to the cancer cells, which is the 6th step of the cycle. The final step of the cycle involves the cytotoxic T cells killing the cancer cells, which occurs while the cytotoxic T cell and cancer cell are bound. This step leads to the death of cancer cells, which will release neoantigens and start the cycle over again (*Cancer-Immunity Cycle*, n.d.).

**T cell activation**

T cell activation is one of the most important steps in the cancer immunity cycle. The goal of T cell activation is for naive T cells to become activated and elicit a response to foreign cells. There are two main types of T cells: effector and memory T cells (*T Cell Activation | Mechanism*, n.d.). Effector T cells can be helper, cytotoxic, or regulatory T cells, whereas memory T cells are responsible for eliciting a rapid immune response to previously encountered pathogens (Marketing, 2020). The most common types of T cells are $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. In order to become activated, a T cell must receive 3 necessary signals. The first signal is the binding of a T cell receptor (TCR) with a major histocompatibility complex (MHC) of an antigen-presenting cell (APC) (*T Cell Activation | Mechanism*, n.d.). APCs present antigens in the form of peptides, and receive these peptides via phagocytosis or pinocytosis. APCs then migrate to lymph nodes to present the antigen to naive T cells by binding MHC with the TCR of the T cell (*20.3E*, 2018). The binding of MHC and TCR is stabilized by either a CD4 or CD8 coreceptor protein. Once MHC and TCR have bound, a second signal is necessary for the naive T cell to be activated. This second signal is called costimulation and occurs when the costimulatory molecules of a naive T cell and APC interact. A common T cell costimulatory

molecule is CD28, and common APC costimulatory molecules include CD80 and CD86 (*T Cell Activation | Mechanism*, n.d.). If costimulation does not occur, the T cell will die or enter anergy, which is a state of inactivation (*Anergy, Exhaustion, and Clonal Deletion*, n.d.). The third signal of T cell activation is cytokine production, which can help stimulate and mobilize cytotoxic T cells (*T Cell Activation | Mechanism*, n.d.). Once a cytotoxic T cell is activated, it will migrate to the site of the tumor and kill the cancer cells through secreted proteins.

***Signal 1***

The first signal of T cell activation occurs when MHC and TCR bind. Both of these binding interactions mediate the recruitment of lymphocyte tyrosine kinase (LCK) to bind with the cytoplasmic tail of the CD4 protein. After binding, LCK then phosphorylates the immunotyrosine activating motifs (ITAM) portions of the six CD3 molecules, enabling recruitment of ZAP-70, or zeta-associated proteinLCK phosphorylates and activates ZAP-70, which then phosphorylates and activates adaptor protein LAT. The LAT protein  recruits and activates scaffold protein SLP-76, which recruits GRAP-2 andGADs. This GRAP-2/GADs protein also binds to LAT. Collectively, the phosphorylation of LAT leads to downstream activation of the PLC gamma,PI3K, and MEK/ERK pathways, which all relate to T cell proliferation and development. In addition to this, the LAT protein also activates a VAV protein, which directly activates the RAC pathway to mediate T cell differentiation. Overall, all of the previously described pathways converge and ultimately lead to the transcription of NFAT, NFKB, and AP-1, which are transcription factors that drive T cell differentiation and production of IL-2 (*T Cell Activation | Mechanism*, n.d.).

***Costimulation (Signal 2)***

The second signal of T cell activation occurs when the B7 (CD80/CD86) costimulatory molecule on an APC binds to a CD28 costimulatory receptor on a T cell. Overall, this signal allows the T cell to respond to an antigen. When the CD28 molecule is activated, it recruits a PI3K molecule,

which leads to the activation of the NF-kB pathway. CD28 activation also activates the VAV mediated pathway (*T Cell Activation | Mechanism*, n.d.).

**Cancer immune system evasion**

Cancer cells can deploy various methods to evade the immune system and break the ideal cancer immunity cycle. Cancer cell immunoediting is the process in which cancer cells evolve so they are no longer recognized by the immune system (*Immunoediting - Latest Research and News | Nature*, n.d.). There are three phases of cancer immunoediting: elimination, equilibrium, and escape (*Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion | Science*, n.d.). Elimination is the first step, and involves the cancer cells being killed or eliminated by immune cells such as T cells. If the cancer cells are not eliminated properly, they can enter a state of equilibrium,in which the immune system eliminates cancer cells at the same rate that cancer cells proliferate, and the tumor overall does not progress. During equilibrium, the interactions between cancer cells and immune cells may allow cancer cells to evolve and become unrecognized by the immune system. This evolution can lead to the state of escape, in which cancer cells break the balance of equilibrium and begin to grow into a tumor. In this state, the immune system is unable to sufficiently eliminate the cancer cells, and the disease progresses (*Cancer Immunotherapy | Tumor Microenvironment*, n.d.). One cancer cell evasion method is to display normal cell checkpoints on its surface, which

tricks T cells into thinking it's a self cell. Cancer cells can also reduce the number of MHC molecules on their surface, making it harder for T cell TCRs to bind (*CAR-T Cells*, n.d.).

**Benefits of using immunotherapy against cancer**

Immunotherapy is a cancer treatment that enhances immune cell function through bioengineering. Since cancer cells excel in adapting to evade immune cells, immunotherapy fights back by reinforcing the immune cells. Some main types of immunotherapy include monoclonal antibodies, immune checkpoint inhibitors, non-specific immunotherapy, and CAR T cell therapy. Monoclonal antibodies are synthetic antibodies and have a variety of functions, such as blocking abnormal cancer protein function and inhibiting immune checkpoints. This allows for immune system responses to become boosted or re-activated against cancer. Non-specific immunotherapies help aid the immune system in destroying cancer. Some of these immunotherapies include cytokines and Bacillus Calmette-Guerin (BCG). Cytokines are small signaling proteins that help activate immune cells, and BCG can activate the immune system to destroy cancer cells in the bladder. CAR T cell therapy involves inserting a chimeric antigen receptor (CAR) gene into a patient's T cells, allowing them to better recognize and respond to cancer cells (*What Is Immunotherapy?*, 2013).

**CAR T cell therapy**

CAR T cells are designed to overcome cancer immune evasion. CAR T cells are modified in a lab to display a chimeric antigen receptor (CAR), which can detect cancer cells even if they do not display MHC. CAR T cell therapy is individualized treatment unique to each patient. To create CAR T cells for a specific patient, the patient’s blood must be filtered through an apheresis machine that collects T cells/white blood cells and returns red blood cells to the patient. This is a process called leukapheresis (*CAR T-Cell Therapy*, n.d.-b). These T cells are

then transduced with lentivirus containing DNA that instructs the T cell to produce membrane-bound CARs. Once mixed, the lentiviruses bind to LDLR receptors on the T cell, triggering endocytosis of the lentivirus. Once inside the T cell, the lentivirus releases DNA, which is transcribed and translated by the T cell into CARs. CARs are engineered to recognize specific MHC independent domains such as CD19 on cancer cells (*CAR-T Cells*, n.d.). A region called scFv (single chain fragment variable) on a CAR is what recognizes and binds to cancer cell antigens such as CD19, allowing CARs to detect cancer cells even if they do not display MHC. CARs also have a hinge region, which can regulate the CAR signaling threshold. CARs then have a transmembrane domain, which helps anchor the entire CAR to the T cell membrane (K et al., 2020). CARs also include intracellular signaling domains, which relay signals that lead to the activation of the T cell.These intracellular signaling domains typically include a CD3ζ molecule and at least one costimulatory molecule. Typical costimulatory molecules include CD28 and 4-1BB (Wu et al., 2020). These molecules help relay a signal to activate pathways that drive T cell activation (*T Cell Activation | Mechanism*, n.d.). After the CAR T cells are created, they are expanded *in vitro* and infused back into the patient's blood, where they can bind to cancer cells and become activated. (*CAR T-Cell Therapy and Its Side Effects*, n.d.) (*CAR-T Cells*, n.d.). When a CAR T cell is activated, it degranulates, releasing granzyme and perforin to directly kill the cancer cell (B. D. Choi et al., 2013). A single CAR T cell can kill several cancer cells by attaching, killing, and moving on to consecutive cancer cells.

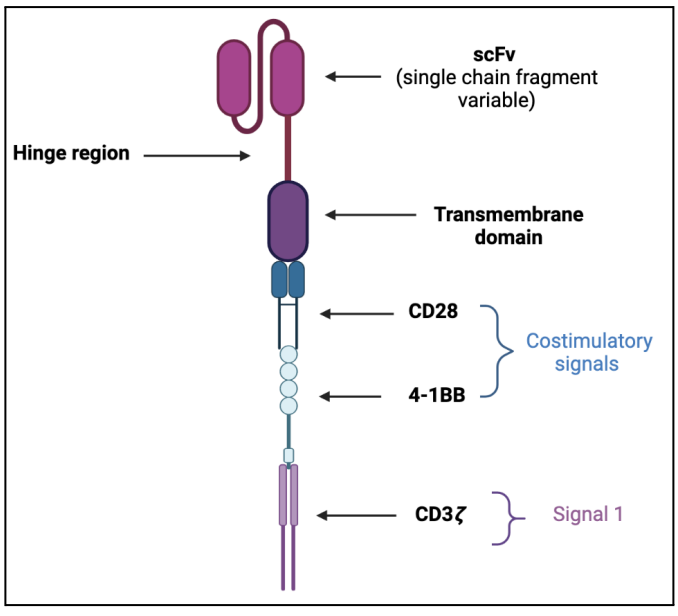



**Fig 1**. Structure of a CAR.

A typical CAR protein consists of four main regions: the intracellular signaling domain (costimulatory signals and signal 1), the transmembrane domain, the hinge region, and the antigen binding domain (scFV). The antigen binding domain is responsible for recognizing and binding to a cancer cell antigen. The hinge region gives the CAR flexibility, which enhances binding affinity. The transmembrane domain anchors the CAR to the CAR T cell. The intracellular signaling domain passes signals along a pathway that eventually leads to the activation of the CAR T cell.

**Viral transfection and transduction**

CAR T cells are typically created through the process of viral transfection and transduction, a method in which viruses are engineered to contain specific genomes that are then introduced to a target cell. The basic stages of viral transfection and transduction include transfecting packaging cells with viral plasmids, collecting and purifying virus, and transducing host cells with virus (O'Keefe, 2022). Lentiviruses are a type of virus in the retroviridae family and are commonly used for transduction due to their stability and broad tropism (*The Basics of the Recombinant Lentivirus System*, n.d.).The first step of the transfection/transduction process involves specific plasmids being transfected into packaging cells, which are often HEK 293T cells (human embryonic kidney cells) (O'Keefe, 2022). Depending on the lentivirus packaging system generation, 3 or 4 plasmids are constructed to contain the essential genes necessary for a virus to be synthesized. In the 2nd generation lentivirus packaging system, a transfer, packaging, and envelope plasmid is used. The transfer plasmid encodes for the transgene, or the artificially created gene, and also contains elements that promote RNA polymerase II to transcribe the viral mRNA (*The Basics of the Recombinant Lentivirus System*, n.d.).The packaging plasmid encodes for genes that help integrate the viral genome into to HEK

packaging cell (*Gag-Pol Polyprotein | DrugBank Online*, n.d.). The envelope plasmid contains genes that encode for envelope proteins. These plasmids can be transfected into the packaging cells through different methods and calcium phosphate-mediated or lipid-based transfection can improve transfection efficiency of HEK 293T cells. After the packaging cells have been transfected, they will begin to produce virus by transcribing and translating the plasmids. As the HEK cells produce the viruses, the viruses get pumped into the supernatant, which is then collected (O'Keefe, 2022). Transduction occurs when the viruses are introduced to the target host cells and infect the cells, inserting the transgene into the host (*The Basics of the Recombinant Lentivirus System*, n.d.). During transduction, the virus binds with receptors on the host cell and either injects its genome into the host cell or undergoes endocytosis. The virus DNA then is integrated into the genome of the host cell, and the host cell begins to express the CAR gene (says, 2020). This entire process is crucial to CAR T cell therapy, which relies on creating viruses that can infect T cells with the chimeric antigen receptor (CAR) gene. Once the T cells receive the CAR gene, they become CAR T cells that can better recognize and attack cancer cells. It is also important to note that in CAR T cell therapy, transfection is not personalized, as the CAR gene remains constant. However, transduction is always individualized to the patient, as their specific T cells must be acquired and infected with the CAR gene.The lentiviruses used to deliver genes in viral transduction have many advantages over other virus types. One benefit of using lentiviruses is that they ensure stable integration of the gene into the host cell genome. This allows the CAR gene to be expressed in the T cells for a long time, ensuring the T cells are equipped to fight cancer over time. Another benefit of using lentiviruses is that they have broad tissue tropism, which means they can infect many types of cells and tissues (*The Basics of the Recombinant Lentivirus System*, n.d.). In addition to this,

unlike adenoviral vectors, lentiviruses do not generate any immunogenic proteins, which means they will not elicit an unnecessary immune response when introduced to the host cells. Lentiviruses can also deliver transgene fragments as large as 9 kilo bases (kb), making them a versatile option for delivering CARs of different sizes (*Viral Based Gene Delivery System for CAR T Cell Engineering - Creative Biolabs (Updated Version)*, n.d.).

**Patient experience**

Currently, the Food and Drug Administration (FDA) has approved six CAR T cell therapies: Abecma (idecabtagene vicleucel), Breyanzi (lisocabtagene maraleucel), Kymriah (tisagenlecleucel), Tecartus (brexucabtagene autoleucel), Yescarta (axicabtagene ciloleucel), and Carvykti (ciltacabtagene autoleucel) (*CAR T-Cell Therapy for Cancer | OHSU*, n.d.). CAR T cell therapy has resulted in complete response (CR) averages of 40-54%, 67%, and 69-74% in patients with RR aggressive B cell lymphomas, mantle cell lymphoma, and indolent B cell lymphomas, respectively (Cappell & Kochenderfer, 2023). After a patient has been treated with CAR T cell Therapy through viral transduction, they can be hospitalized for up to two weeks, and can expect a recovery period of around 2 to 3 months (*CAR T-Cell Therapy*, n.d.-a). There are also several side effects that can occur after receiving CAR T cell therapy, including cytokine release syndrome (CRS), nervous system problems, or abnormal blood mineral counts (*CAR T-Cell Therapy and Its Side Effects*, n.d.). CAR T cell therapy typically causes toxicities such as CRS due to the large amount of cytokines produced by CAR T cells. CAR T cell therapy can also potentially cause neurological side effects, which are not completely understood by scientists (*Remodeled CAR T-Cell Therapy Causes Fewer Side Effects - NCI*, 2020). Scientists are currently developing new CARs that aim to reduce these side effects.

**Common disadvantages of CARs and current strategies to overcome them**

### *Antigen Escape*

One of the main challenges in CAR T cell therapy is antigen escape,when tumor cells develop resistance to CARs by reducing the amount of target antigens displayed on their surfaces. One potential strategy to combat antigen escape is to target multiple antigens, preventing tumor cells from evading CAR T cells by suppressing a single antigen. Multiple antigens can be targeted using dual CAR constructs or tandem CARs, which involve the co-transfection of packaging cells to produce viruses that infect T cells with two sets of genes encoding for two different CARs that target different antigens on cancer cells. The expression of two different CARs on a CAR T cell can also be achieved through cocktail or sequential infusion and bicistronic plasmids (B. Xie et al., 2022). Clinical trials have explored using CAR T cells to target both CD19 and CD22 or CD19 and BCMA. The CD19/CD22 targeted trial had mixed results, with some acute lymphoblastic leukemia (ALL) and Diffuse large b cell lymphoma (DLBCL) patients experiencing severe toxicities, but two patients achieving complete response (“Phase I Experience with a Bi-Specific CAR Targeting CD19 and CD22 in Adults with B-Cell Malignancies,” 2018). The CD19/BCMA targeted trial was more successful, as all treated patients achieved either complete response, very good partial response, or partial response. Additionally, no severe adverse effects arose during this trial (“A Bcma and CD19 Bispecific CAR-T for Relapsed and Refractory Multiple Myeloma,” 2019). Preclinical trials have also tested tandem CARs targeting EGFRvIII and IL-13Ra2 in heterogeneous glioblastoma (GBM). In this study, the tandem CARs had increased cytotoxic effects against GBM and resulted in complete and durable responses in orthotopic murine models (Schmidts et al., 2023). These tandem CARs have yet to be examined in clinical trials

### *On-target off-tumor*

One of the main challenges of using CAR T cell therapy to treat solid tumors is that solid tumor antigens can also be expressed on normal tissues. This makes it difficult for CAR T cells to differentiate between tumor cells and normal cells, causing the CAR T cells to attack healthy cells. One strategy to prevent this is to engineer CARs that express an MHC-independent TCR as well as a costimulatory CAR that functions independently of the TCR. In one pre-clinical trial, T cells were engineered to express a Vg9Vd2 TCR as well as an anti-GD2 CAR to target a GD2 model cancer antigen. By adding the MHC independent TCR to the CAR T cell, on-target off tumor effects were decreased, as the TCR ensured the T cell was targeting cancer cells only. This trial resulted in GD2-expressing neuroblastoma cells being effectively killed by Vg9Vd2 TCR and anti-GD2 CAR expressing T cells. T cells that only expressed anti-GD2 CAR were not able to bind to and kill the neuroblastoma cells (Fisher et al., 2017)).

***CAR T cell trafficking and tumor infiltration***

Another challenge of using CAR T cell therapy on solid tumors is the limited ability of CAR T cells to infiltrate the tumor. The microenvironment of solid tumors are typically immunosuppressive, meaning they can suppress the functions of CAR T cells and other immune cells. In addition, tumors have physical barriers such as the tumor stroma, which makes it harder for CAR T cells to penetrate the tumor. One method to overcome these limitations is the use of local and regional delivery routes instead of systematic delivery. Locally administering CAR T cells eliminates the need for CAR T cells to traffic to the tumor site and also limits on-target off-tumor toxicities. If CAR T cells are specifically directed to the tumor through local administration, there is less of a chance they will be exposed to normal cells, limiting the chances of the CAR T cells mistakenly attacking the normal cells. Preclinical trials experimented with locally and regionally delivering CAR T cells to target HER2+ breast cancer (PMC7685198)

and subcutaneous human medulloblastoma (MED8A)(*Delivery of CAR-T Cells in a Transient Injectable Stimulatory Hydrogel Niche Improves Treatment of Solid Tumors | Science Advances*, n.d.), respectively. In the study targeting HER2+ breast cancer, the local delivery of CAR T cells resulted in complete tumor regression and strong anti-tumor responses. The regional delivery of CAR T cells was also tested, and resulted in equal anti-tumor responses to local delivery, but slightly delayed responses to the treatment overall (Priceman et al., 2018). In the MED8A preclinical trial, locally administered hydrogel-encapsulated CAR T cells led to a complete response in 70% of the treated animals, whereas non-local administration led to a complete response in only 40% of animals (*Delivery of CAR-T Cells in a Transient Injectable Stimulatory Hydrogel Niche Improves Treatment of Solid Tumors | Science Advances*, n.d.).

Another strategy to improve CAR T cell trafficking is to engineer CAR T cells that express chemokine receptors on their surfaces. Chemokines are small signaling proteins that can help stimulate cell migration (Hughes & Nibbs, 2018). In the context of cancer, chemokines can support tumor survival or recruit immunosuppressive cells to the tumor microenvironment (Borsig et al., 2014). With chemokine receptors, CAR T cells have heightened responsiveness to tumors, resulting in more effective and direct migration to the tumor site. A preclinical study experimented with engineering CAR T cells to co-express CXCR1 or CXCR2, which are the receptors of IL-8, a chemokine that recruits immunosuppressive myeloid cells. The results from this study demonstrate that co-expression of the chemokine receptors on CAR T cells caused increased migration in response to IL-8 without affecting the cytotoxicity of the CAR T cells (PMC6728370). Furthermore, A20-28z CXCR2 CAR T cells showed heightened anti-tumor activity against antigen specific tumor xenografts (Whilding et al., 2019). Infiltrating the physical barrier of the tumor cell stroma is another challenge to the efficacy of CAR T cell therapy. One

preclinical study experimented with engineering T cells to express heparanase, which is an enzyme that breaks down the main component of a tumor cell stroma (HSPG). This study displayed that the CAR T cells that underwent gene transfer to display heparanase had enhanced survival rates in neuroblastoma xenograft models (Caruana et al., 2015).

***Immunosuppressive microenvironment***

In a tumor microenvironment, several cells can be present that possess immunosuppressive qualities, including myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and tumor-associated macrophages (TAMs). Tumor facilitating chemokines, growth factors, and cytokines are also produced by these immunosuppressive cells. The combined immunosuppressive factors present in the tumor microenvironment as well as chronic TCR stimulation cause T cells to become exhausted and lose the ability to perform cytotoxic functions. One strategy to prevent T cell exhaustion and heighten immune response is to use a combination immunotherapy of CAR T cells and checkpoint blockade (Y. Yin et al., 2018). Checkpoint blockade aims to block checkpoint proteins that can suppress the full function of T cells and other immune cells (*Definition of Immune Checkpoint Inhibitor - NCI Dictionary of Cancer Terms - NCI*, 2011). Two clinical trials attempted to use combination PD-1 blockade and CD19 CAR T cell therapy in children with pretreated B-ALL, and mesothelin targeted CAR T cells and anti-PD-lagent in mesothelioma patients, respectively (“Checkpoint Inhibitors Augment CD19-Directed Chimeric Antigen Receptor (CAR) T Cell Therapy in Relapsed B-Cell Acute Lymphoblastic Leukemia,” 2018) (Adusumilli et al., 2019). The PD-1 blockade and CD19 trial discovered that the combination therapy had positive results and improved T cell persistence and function, and the mesothelioma study culminated in a 72% response rate and complete metabolic responses from two patients. Studies are underway to engineer CARs to target

immunosuppressive cells in the tumor. One study engineered CAR T cells to target both tumor cells and MDSCs. In this study, the TRAIL receptor 2 (TR2) was identified on MDSCs, and the CAR T cells were engineered to express a costimulatory TR2. 41BB receptor to target TR2. These CAR T cells also targeted the tumor. This resulted in improved reproduction and persistence of the CAR T cells (Z. Liu et al., 2022).

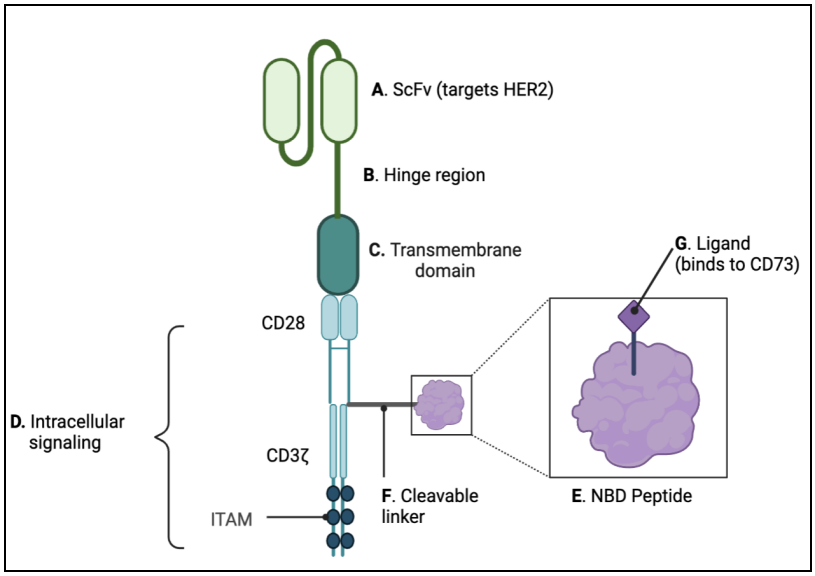
***CAR T cell associated toxicities***

One of the main side effects of CAR T cell therapy is toxicities such as cytokine release syndrome, neurotoxicities, and immune related adverse events (IRAEs). Cytokine release syndrome (CRS) occurs when CAR T cells rapidly release large amounts of cytokines into the blood, causing the immune system to go into overdrive. Cytokines are signal proteins that help activate T cells, and are also produced by T cells. They are essential for relaying immune system messages. However, when there are too many cytokines in the body, the body can start displaying unnecessary immune responses, causing toxic side effects. CRS symptoms typically begin with a fever, but can progress into life-threatening capillary leak with hypoxia and hypotension. Neurotoxicities are also common side effects of CAR T cell therapy. Scientists currently do not completely understand why neurotoxic side effects arise from CAR T cell therapy, but it has been observed that neurotoxicity only occurs in patients who have CRS, and that patients with pre-existing neurological conditions or higher tumor burdens are more susceptible to it (*Neurotoxic Side Effects of CAR T-Cell Therapy*, 2020). One possible strategy to reduce the toxic side effects of CAR T cell therapy is to reduce the affinity of its receptors such that only tumors with high antigen density could be detected by the CAR T cells, reducing unnecessary immune responses to healthy cells. The decrease of CAR T cell immune response would decrease toxicities elicited from heightened immune responses such as excess cytokine

secretion (X. Liu et al., 2015). Immunotherapies such as CAR T cell therapy can also cause IRAEs, which are autoimmune disorders that can affect any organ in the body (Conroy & Naidoo, 2022). Some IRAEs include cardiotoxicity, pulmonary toxicity, and neurotoxicity (J. Choi & Lee, 2020). CRS can be classified under the IRAE category. IRAEs are not completely understood by scientists, but some current treatments involve glucocorticoids.

**Design**

Though there are several strategies targeting current limitations of CAR T cell therapy, the most effective combinations and alterations of them have yet to be fully explored. In regards to targeting the immunosuppressive tumor microenvironment, many current treatments have not experimented with armored CAR T cell therapy, which is what our novel design is. The tumor microenvironment is the area contained inside of a tumor that has been altered by cancer cells to promote cancer growth (Nm & Mc, 2020). The tumor microenvironment includes malignant cells, immune cells, stroma cells, and extracellular molecules that can inhibit immune functions (Baghban et al., 2020). One type of cell commonly present in the tumor microenvironment are myeloid-derived suppressor cells (MDSCs). These cells suppress immune responses and support tumor progression (Lv et al., 2019). MDSC accumulation and progression in the tumor microenvironment is supported by the NF-kB pathway, which is a family of transcription factors (Z. Yin et al., 2019). Our novel CAR T cell therapy design specifically targets the Nf-kB pathway in MDSCs, along with targeting cancer cells. **Fig. 2** Novel



CAR design that targets HER2+ breast cancer and MDSCs in the tumor microenvironment. A) Single chain variable fragment (ScFv) of the CAR, which is responsible for recognizing and binding to a specific cancer cell antigen. In our design, the ScFv portion is altered to have affinity for the HER2 receptor  on breast cancer cells. This target is for proof of concept and can be modified to accommodate other cancer receptors as well. B) Hinge region and C) transmembrane domain, which remain constant with a typical CAR. The hinge region gives the CAR flexibility, which increases its binding efficacy to the target antigen. The transmembrane domain stabilizes the CAR by anchoring it to the T cell membrane (Funfrock, 2021). D) Intracellular signals necessary for the CAR T cell to become activated. A CD3ζ molecule and one or more costimulatory molecules make up the main intracellular signals in the CAR T cell. Typical costimulatory molecules include CD28 and 4-1BB (Wu et al., 2020). The role of CD3ζ in intracellular signaling is to recruit ZAP70, which initiates a phosphorylation cascade of additional signals that eventually activate the T cell (Bridgeman et al., 2014). In CAR T cells, costimulatory domains increase CAR T cell activation rates when exposed to cells expressing the target antigen. This occurs without the target cell expressing costimulatory receptor ligands (*Selecting Costimulatory Domains for Chimeric Antigen Receptors: Functional and Clinical Considerations - PMC*, n.d.). E) The peptide secreted by the CAR T cell that targets the Nf-kB pathway in MDSCs. The peptide we chose is NEMO Binding Domain peptide (NBD) peptide, which has effectively inhibited the Nf-kB pathway in preclinical studies and clinical trials by binding to the NEMO regulator (A et al., 2011). NBD peptide sequence is DRQIKIWFQNRRMKWKKTALDWSWLQTE (Anaspec, n.d.). In our design, we added a signal peptide (SP) to the N-terminus of the NBD peptide, which signals it to be secreted from the CAR T cell (“A Comprehensive Review of Signal Peptides,” 2018). In addition to the SP, a cleavable

linker was added to the design that links the CAR and the NBD peptide. The linker will be cleaved, allowing the peptide to be secreted. Once the peptide is secreted, it binds to the CD73 receptor on MDSCs. We engineered the NBD peptide to express a negatively charged ligand that can bind to the positive active site of CD73. The bound CD73 receptor then undergoes endocytosis, bringing the NBD peptide into the MDSC. We chose the CD73 protein as a target due to previous studies demonstrating its ability to be endocytosed in the context of the tumor microenvironment (Huang et al., 2022). In addition, the CD73 receptor is also present on TRegs, which can have immunosuppressive qualities in the tumor microenvironment (Ohue & Nishikawa, 2019). This means that possible off-target effects of our CAR design could inhibit additional immunosuppressive cells, contributing to our aim of targeting the immunosuppressive tumor microenvironment. Once the NBD peptide is endocytosed via CD73 into the MDSC, it can bind to NEMO and inhibit the Nf-kB pathway. Overall, the inhibition of the Nf-kB pathway should reduce the immunosuppressive qualities of MDSCs in the tumor microenvironment, allowing CAR T cells to induce apoptosis in HER2$^+$ breast cancer cells without being inhibited by the tumor microenvironment.

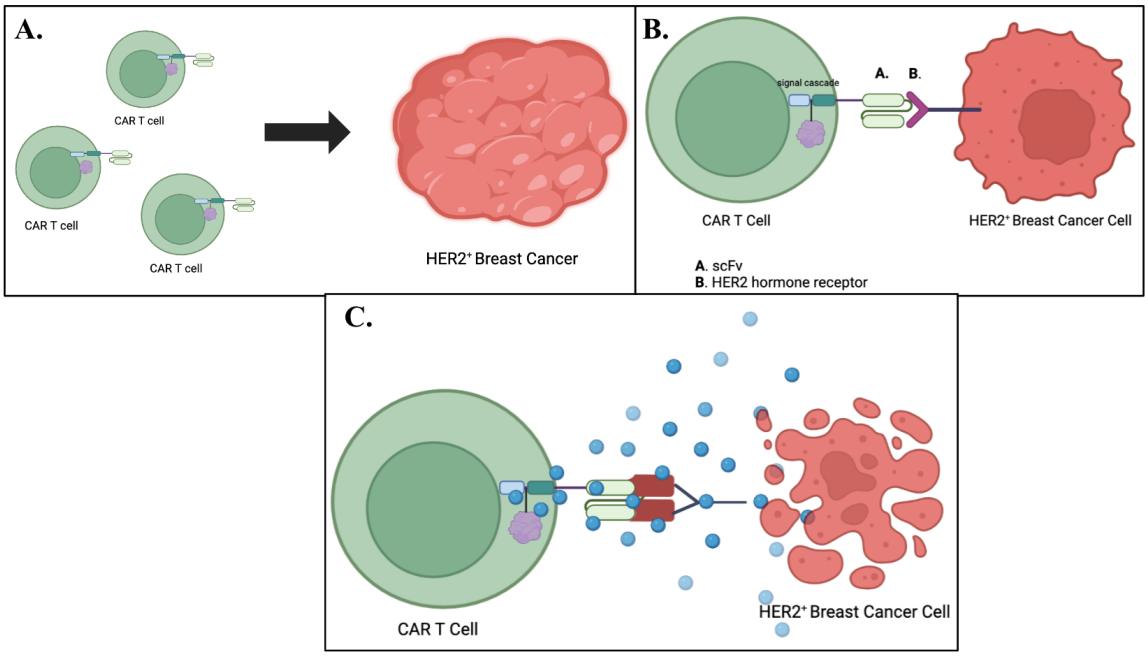


**Fig 3.** CAR T cell design inducing apoptosis in HER2⁺ breast cancer. **A)** CAR T cell with HER2⁺ receptive ScFv region approaching cancerous solid tumor. **B)** scFv on the CAR T cell binding with HER2 hormone receptor on a HER2⁺ breast cancer cell, causing intracellular signaling cascade to occur. **C)** CAR T cell releasing perforin and granzymes; cytotoxic proteins that induce apoptosis in the cancer cell.

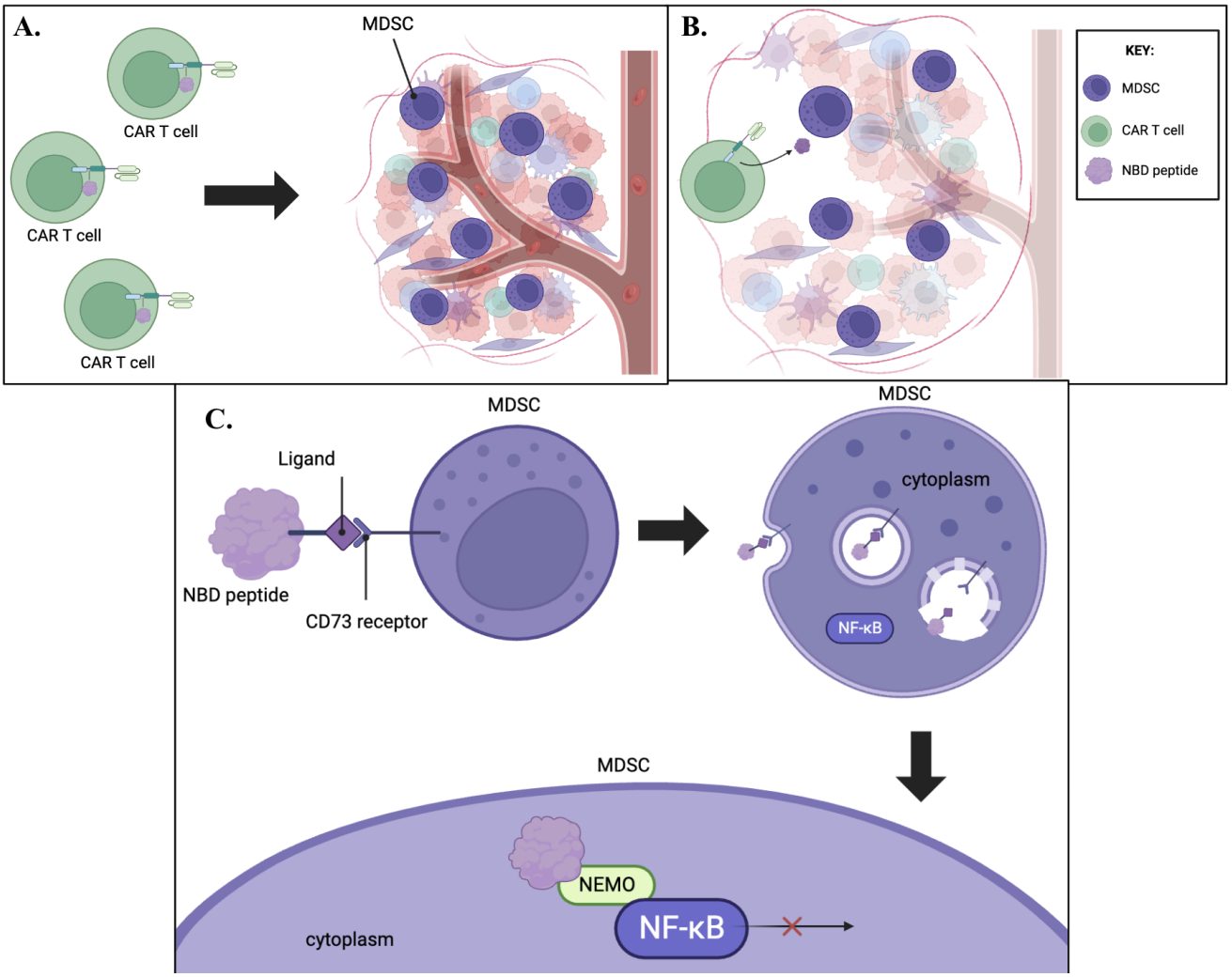



**Fig 4.** CAR design targeting Nf-kB in MDSCs. **A)** CAR T Cell approaching HER2⁺ breast cancer tumor microenvironment. **B)** Inside of the tumor microenvironment, the link between the NBD peptide and CAR intracellular signaling is cleaved by an enzyme and the NBD peptide is secreted from the CAR T cell. **C)** NBD peptide entering MDSC cell through CD73 mediated

endocytosis. NBD peptide then binds to NEMO and inhibits the Nf-kB pathway from activation and translocation into the nucleus.

**Discussion**

While CAR T cell therapy has shown some promise, it is still limited in efficacy, specifically in solid tumors, due to the immunosuppressive tumor microenvironment and MDSCs. While other groups have tried to combine existing CAR T cell strategies with chemical means of inhibition, this strategy is nonspecific. We propose a specific way to engineer CAR T while simultaneously inhibiting MDSCs. Some key necessary tests to demonstrate efficacy of the design include determining possible off-target effects of targeting CD73, testing the feasibility of CD73-induced endocytosis, and testing possible off-target effects of the NBD peptide. The HER2 targeting portion of our CAR has shown clinical efficacy in several trials. In one study, CAR T cell therapy targeting HER2 in colorectal cancer (CRC) effectively prevented CRC progression in xenograft models (Xu et al., 2021). In another study, one dose of HER2 targeting CAR T cells eliminated tumors and supported long-term survival of mice with breast tumor cells (Budi et al., 2022). In addition, the NBD peptide has successfully inhibited the Nf-kB pathway in preclinical studies and clinical trials by binding to the NEMO regulator (A et al., 2011). If our design is successful, it can provide a novel therapeutic option to treat $HER2^+$ breast cancer patients. Some potential limitations in our design include off-target effects, which can be beneficial or limiting. The targeting of Tregs through CD73, for example, could be beneficial as Tregs are immunosuppressive. If the NBD peptide inhibits the Nf-kB pathway in cells such as immune cells, this could reduce immune responses in the tumor microenvironment. Another potential limitation includes unforeseen toxicities.

**Conclusion**

CAR T cell therapy is an immunotherapy that has been approved by the FDA for use in blood cancers, but has several limitations in solid tumors such as on-target off tumor effects, toxicities, antigen escape, tumor infiltration challenges, and the immunosuppressive tumor microenvironment. Current methods addressing these limitations include dual target CARs, combination therapy with checkpoint blockade, local CAR administration, and targeting immunosuppressive microenvironment cells, but additional studies are still needed to continue developing these technologies. In this proposal, we reviewed the function of T cells in the immune system and the effects of CAR T cell therapy on cancer. We also review strategies addressing current limitations of CAR T cell therapy in solid tumors. We then propose a novel CAR design that targets $HER2^+$ breast cancer and secretes NBD peptide to infiltrate MDSCs and inhibit the Nf-kB pathway, limiting the immunosuppressive qualities of MDSCs in the tumor microenvironment. This design requires preclinical studies to validate its efficacy and safety before being used in clinical trials. If successful, our design provides a novel option for treatment of $HER2^+$ breast cancer, and can be expanded to target other cancer receptors in the future. This design contributes to the ongoing research of the use of CAR T cell therapy in solid tumors, with the overall goal of CAR T cell approval for use in solid tumors.

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