

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Development and optimization of HER2-specific chimeric antigen receptor (CAR)-engineered natural killer cells for targeted therapy of solid tumors

By Bence Gergely

Supervisor: Dr. Árpád Szöőr



UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2026.

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By Bence Gergely, Molecular Biology MSc

Tutor: Dr. Árpád Szöör, PhD

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Defense Committee:** Prof. Dr. László Virág, DSc

Reviewers: Dr. Árpád Lányi, PhD

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Dr. József Tóvári, PhD

The PhD Defense takes place at the Lecture Hall of Building A of the Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:00 on 7th September 2026.

Introduction

Malignancies remain among the leading causes of death worldwide. Despite substantial advances in surgery, chemotherapy, and targeted molecular therapies over the past decades, durable long-term remission remains elusive for many tumor types. This challenge is particularly pronounced in cancers that evade conventional treatment strategies through molecular heterogeneity, adaptive resistance mechanisms, and the establishment of an immunosuppressive tumor microenvironment.

Immunotherapy has emerged as one of the most rapidly advancing fields in cancer research, harnessing and augmenting the intrinsic antitumor capacity of the host immune system. Within this context, increasing attention has been directed toward the use of natural killer (NK) cells in adoptive immunotherapy. NK cells are key effector cells of the innate immune system, characterized by their inherent cytotoxic activity and their ability to eliminate transformed cells independently of antigen presentation.

Genetic engineering of NK cells with chimeric antigen receptors (CARs) enables integration of innate and adaptive immune features, thereby providing a promising, potentially safer therapeutic platform. Importantly, CAR-NK cells offer distinct advantages, including allogeneic applicability and a more favorable toxicity profile, which may translate into clinical benefits compared to CAR-T cell-based therapies.

The objective of this dissertation is to develop and functionally optimize HER2-specific CAR-NK cells, with a particular focus on the role of costimulatory signaling domains in regulating cellular proliferation, persistence, and antitumor activity. This work specifically investigates how the incorporation of NK cell-specific signaling pathways influences the generation of effective immune responses against resistant HER2-positive solid tumors.

The results of this study are expected to contribute to the rational design of CAR-based cellular therapies and to provide new insights into the treatment of HER2-positive malignancies, particularly in clinical settings where current therapeutic options demonstrate limited efficacy.

Introduction to immunotherapies

The immune system plays a critical role in tumor development and progression, contributing to both the recognition and elimination of malignant cells. Tumor immunity is founded on the dynamic interplay between innate (nonspecific) and adaptive (antigen-specific) immune responses, which together form a complex and highly coordinated defense network against malignant transformation.

The innate immune system serves as the first line of defense and includes epithelial barriers, phagocytic cells, and natural killer (NK) cells. These components provide rapid, broad-spectrum responses and can recognize and eliminate transformed cells without prior sensitization. In contrast, the adaptive immune system mediates highly specific and long-lasting antitumor responses characterized by immunological memory. Its principal effectors are T and B lymphocytes, which recognize tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) through antigen-specific receptors. Cytotoxic CD8⁺ T cells directly eliminate tumor cells through perforin- and granzyme-mediated apoptosis, while CD4⁺ helper T cells coordinate immune responses by producing cytokines that support both cellular and humoral immunity. In addition, B-cell-derived antibodies contribute to tumor control through opsonization and the induction of antibody-dependent cellular cytotoxicity (ADCC).

Despite the effectiveness of immune surveillance in early tumor control, cancer cells can develop multiple mechanisms to evade immune detection and destruction, thereby enabling disease progression. These immune evasion strategies include impaired antigen presentation, secretion of immunosuppressive cytokines and expression of inhibitory molecules. Furthermore, tumors actively remodel the tumor microenvironment by recruiting immunosuppressive cell populations, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which inhibit the function of effector immune cells. Collectively, these processes are captured by the concept of cancer immunoediting, which encompasses three distinct phases: elimination, equilibrium, and escape.

Advances in the understanding of these mechanisms have laid the foundation for modern cancer immunotherapy. Unlike conventional treatments that directly target tumor cells, immunotherapies aim to restore or enhance the antitumor capacity of the immune system. Current immunotherapeutic strategies - including monoclonal antibodies, immune checkpoint inhibitors,

immunomodulatory agents, oncolytic viruses, cancer vaccines, and adoptive cell therapies - are designed to overcome immune evasion, reinvigorate immune responses, and reprogram the tumor microenvironment toward an immunostimulatory state.

CAR T cell therapy

Chimeric antigen receptor (CAR) T cell therapy represents a major advancement in cancer immunotherapy, based on the genetic engineering of a patient's own T lymphocytes to express synthetic receptors that redirect their specificity toward tumor-associated antigens. These receptors combine the antigen-binding properties of monoclonal antibodies with the signaling and effector functions of T cells, thereby enabling highly targeted and potent antitumor activity independent of major histocompatibility complex (MHC)-mediated antigen presentation.

The therapeutic process involves several key steps. First, peripheral blood T cells are collected from the patient and activated *ex vivo*. These cells are then genetically modified - most commonly using retroviral or lentiviral vectors - to introduce the gene encoding the CAR construct. Following expansion to clinically relevant numbers, the engineered CAR T cells are reinfused into the patient. Before infusion, patients typically undergo lymphodepleting conditioning, which enhances therapeutic efficacy by reducing endogenous lymphocyte populations and creating a favorable environment for the expansion and persistence of the transferred cells.

The structural design of CAR molecules is critical to their function and comprises four components. The extracellular antigen-recognition domain is usually a single-chain variable fragment (scFv) derived from a monoclonal antibody, which confers target specificity. This domain is connected via a flexible hinge region to a transmembrane domain that anchors the receptor within the cell membrane. The intracellular portion contains signaling domains required for T cell activation, including the CD3 ζ chain as the primary activation motif, along with one or more costimulatory domains, such as CD28 or 4-1BB, which enhance proliferation, persistence, and effector function.

Clinical aspects and perspectives of CAR T cell therapy

Clinical outcomes in hematological malignancies have clearly demonstrated the transformative potential of CAR T cell therapy. However, responses are not uniform across all disease types. For example, in relapsed or refractory acute myeloid leukemia (AML), therapeutic efficacy has thus

far been modest. This limitation is attributed in part to the lack of well-defined, tumor-specific target antigens and, in part, to the functional exhaustion of infused T cells. These observations underscore that the clinical success of CAR T cell therapy is highly dependent on target antigen selection, the immunobiological characteristics of the tumor environment, and the capacity of engineered T cells to persist long term.

In contrast, the efficacy of CAR T cell therapy in solid tumors has remained limited to date, reflecting the influence of multiple interconnected barriers. A major challenge is the heterogeneous and often low-level expression of tumor-associated antigens, which facilitates immune escape. In addition, the tumor microenvironment exerts strong immunosuppressive effects through cellular components such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), as well as soluble factors including transforming growth factor- β (TGF- β) and interleukin-10 (IL-10). These factors collectively impair the proliferation, persistence, and effector function of infused CAR T cells.

Furthermore, T cell intrinsic limitations - including progressive functional exhaustion and increased susceptibility to apoptosis - restrict the durability of antitumor responses. These challenges are particularly pronounced in solid tumors and contribute substantially to therapeutic failure, highlighting the need for next-generation strategies to improve the efficacy of cellular immunotherapies.

CAR NK-Cell Immunotherapy

Chimeric antigen receptor (CAR) NK-cell therapy represents a rapidly emerging and innovative approach in immuno-oncology, combining the antigen-specific targeting capability of CAR-engineered T cells with the intrinsic cytotoxicity and favorable safety profile of natural killer (NK) cells. This hybrid strategy integrates key features of both adaptive and innate immunity within a single cellular platform.

In CAR NK cells, the introduction of a chimeric antigen receptor enables antigen-specific recognition and elimination of tumor cells. At the same time, NK cells retain their native ability to detect and respond to transformed cells through major histocompatibility complex (MHC)-independent mechanisms mediated by activating receptors such as NKG2D, DNAM-1, NKp30, NKp44, and NKp46. This dual recognition capacity enhances the robustness of the antitumor

response and reduces the likelihood of immune escape through antigen loss or HLA downregulation.

An additional advantage of CAR NK cells is their favorable safety profile. Unlike CAR T cell therapies, NK cells do not require MHC-restricted antigen recognition and therefore do not induce graft-versus-host disease (GvHD) in allogeneic settings. This property enables the development of universal, “off-the-shelf” CAR NK-cell products that can be manufactured in advance, cryopreserved, and made readily available for clinical use. Such an approach has the potential to significantly reduce production time and cost while improving patient access to cellular immunotherapies.

Furthermore, NK cells exhibit a cytokine secretion profile that is generally associated with improved clinical tolerability. Early-phase clinical studies have reported a lower incidence and reduced severity of cytokine release syndrome (CRS) compared to CAR T cell therapies. Notably, a phase I/II clinical trial conducted at MD Anderson Cancer Center (NCT03056339) demonstrated that cord blood-derived CD19-specific CAR NK cells induced significant antitumor responses in patients with refractory hematological malignancies without causing CRS or neurotoxicity.

Collectively, these characteristics highlight the potential of CAR NK-cell therapy as a safer and more versatile alternative to CAR T cell-based approaches, particularly in the context of solid tumors, where toxicity and immunosuppressive microenvironments remain major challenges.

Clinical Successes of CAR-NK Cell Therapies in Hematological Malignancies

The clinical development of CAR NK-cell therapies has advanced considerably over the past decade, with the most significant progress observed in hematological malignancies. Early translational efforts in the mid-2010s established proof of concept by demonstrating the efficacy of CD19-specific CAR NK cells in preclinical models of B-cell leukemias and lymphomas. A major milestone in the field was achieved by investigators at MD Anderson Cancer Center, who reported the first clinical evidence of safety and efficacy in 2020. In this phase I/II clinical trial (NCT03056339), umbilical cord blood–derived CD19-specific CAR NK cells were administered to patients with relapsed or refractory B-cell malignancies, including diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), and indolent non-Hodgkin lymphomas (NHL).

The clinical outcomes were highly encouraging and represented a significant advancement in the field of cellular immunotherapy. Among the 11 treated patients, 8 achieved an objective response, including 7 complete remissions. Notably, no cases of cytokine release syndrome (CRS), neurotoxicity, or graft-versus-host disease (GvHD) were observed, underscoring the favorable safety profile of CAR NK-cell therapy. Furthermore, the infused CAR NK cells remained detectable in the peripheral blood for over 12 months, challenging earlier assumptions regarding their limited *in vivo* persistence and suggesting the potential for sustained therapeutic activity.

Importantly, this approach employed an “off-the-shelf” strategy using allogeneic, cryopreserved NK cells rather than patient-derived autologous cells. The engineered cells were further modified to express interleukin-15 (IL-15), which enhanced their survival and proliferative capacity *in vivo*. Collectively, these findings provided strong clinical validation for CAR NK-cell therapy and established a foundation for its continued development and optimization.

Challenges of CAR NK-Cell Therapy in Solid Tumors

Despite the clear advantages of CAR NK-cell therapy over CAR T cell approaches in terms of safety and manufacturability, its clinical efficacy has thus far been demonstrated predominantly in hematological malignancies. In contrast, outcomes in solid tumors have been considerably more limited. This discrepancy reflects both the intrinsic biological properties of NK cells and the complex, immunosuppressive nature of the tumor microenvironment. Major challenges include limited *in vivo* persistence, insufficient tumor infiltration, and reduced resistance to inhibitory signals and metabolic stress within the tumor niche.

Among these barriers, limited persistence represents a central constraint on therapeutic efficacy. Unlike T cells, which can undergo long-term clonal expansion and form memory populations following appropriate costimulation, NK cells are inherently short-lived effector cells that lack classical immunological memory. Peripheral blood-derived NK cells typically persist for only a few days to, at most, one to two weeks *in vivo*, thereby restricting the duration of their antitumor activity. Although cytokine support - such as interleukin-2 (IL-2) or interleukin-15 (IL-15) - can transiently enhance NK-cell survival and function, this strategy alone is insufficient to achieve durable tumor control and may, in some cases, lead to systemic toxicity.

One promising strategy to address this limitation is to optimize intracellular signaling domains within the CAR construct. In CAR T cells, costimulatory domains such as CD28 and 4-1BB activate key pathways, including PI3K/AKT and NF- κ B, thereby promoting proliferation, persistence, and resistance to apoptosis. However, these signaling modules are not fully aligned with the native activation mechanisms of NK cells. Unlike T cells, NK cells do not rely on T cell receptor (TCR) signaling; instead, they are regulated by a network of activating and inhibitory receptors that signal through adaptor proteins such as DAP10, DAP12, and 2B4. As a result, CAR constructs based on conventional T cell designs often induce only transient activation in NK cells, without supporting sustained proliferation or the development of durable, memory-like responses.

Objectives

Over the past decade, anticancer immunotherapies have undergone rapid and transformative development, with approaches based on the targeted reprogramming of immune effector cells—particularly T and natural killer (NK) cells—emerging at the forefront. Chimeric antigen receptor (CAR) T cell therapies have achieved remarkable success in the treatment of hematological malignancies; however, their efficacy in solid tumors remains limited. This is largely due to the immunosuppressive tumor microenvironment, heterogeneous antigen expression, and barriers to effective tissue infiltration.

In light of these challenges, increasing attention has been directed toward alternative effector cell populations with distinct biological advantages. NK cells, characterized by their intrinsic cytotoxic activity, major histocompatibility complex (MHC)-independent target recognition, and reduced risk of graft-versus-host disease, represent a promising platform for next-generation cellular immunotherapies. The therapeutic potential of CAR-NK cells may be further enhanced through the rational design of CAR constructs incorporating optimized intracellular signaling domains that promote cellular persistence, proliferation, and antitumor function.

Accordingly, the primary objectives of this study were:

1. To design and generate CAR constructs optimized for NK cells, to enhance activation, proliferation, and tumor infiltration within the solid tumor microenvironment.
2. To evaluate and compare the functional properties of CAR-NK cells containing different costimulatory signaling domains using *in vitro* two-dimensional (2D) and three-dimensional (3D) co-culture systems.
3. To assess the *in vivo* antitumor efficacy of the most promising CAR-NK cell constructs in preclinical models of solid tumors that are resistant to conventional monoclonal antibody therapies.

Materials and Methods

Cell lines

The CD16.176V.NK-92 cell line (hereafter referred to as NK-92) was used as the effector cell population and was kindly provided by Dr. Kerry S. Campbell (Brink Biologics, Inc., San Diego, CA, USA). This cell line is derived from the parental NK-92 line, which lacks endogenous CD16 expression, and was genetically modified via retroviral transduction to express the high-affinity CD16 variant (FcγRIIIA-176V).

The *in vitro* antitumor activity of HER2-specific CAR-engineered NK-92 cells was evaluated using multiple HER2-positive tumor cell lines. These included: (i) MDA-HER2, a cell line stably expressing HER2 generated via retroviral transduction from the parental triple-negative MDA-MB-468 cell line (lacking ER, PR, and HER2 expression); (ii) the NCI-N87 human gastric carcinoma cell line; and (iii) the JIMT-1 breast cancer cell line.

The JIMT-1 cell line, originally established from a pleural metastasis of a trastuzumab-resistant breast cancer patient, represents a clinically relevant resistance model. Its xenografts form a dense extracellular matrix enriched in hyaluronan/MUC4 polymer complexes, which can sterically hinder antibody binding. The parental MDA-MB-468 cell line (hereafter referred to as MDA) was used as a HER2-negative control.

Production of retroviruses and transduction of CAR-NK-92 cells

Retroviral particles were generated using HEK293T packaging cells transiently transfected with the pSFG plasmid encoding HER2-specific CAR constructs. Co-transfection included the pMax.RD114 plasmid (encoding the RD114 envelope protein) and gag-pol MoMLV Peg-Pem plasmids (providing viral replication machinery), using jetPRIME transfection reagent.

After 72 hours, viral supernatants were collected, filtered through a 0.22 μm sterile filter, and stored at –80 °C until use.

For transduction, NK-92 cells were cultured at 5×10^5 cells/mL in complete NK medium supplemented with 400 IU/mL IL-2. Cells were transduced in RetroNectin-coated vessels (20 μg/mL) in the presence of viral supernatant and IL-2. Non-transduced NK-92 cells were maintained under identical conditions as controls.

Following transduction, cells were expanded in complete NK medium containing 400 IU/mL IL-2. CAR-expressing populations were enriched by fluorescence-activated cell sorting (FACS) to achieve $\geq 90\%$ CAR expression. Only these enriched populations were used in subsequent experiments, and CAR expression levels were routinely verified.

HER2-CAR constructs

First-generation CAR constructs consisted of a HER2-specific FRP5-derived single-chain variable fragment (scFv), an IgG1 short hinge region, a CD28-derived transmembrane domain, and a CD3 ζ intracellular signaling domain (HER2.z).

Second-generation constructs included an additional costimulatory domain derived from CD28, 4-1BB, or 2B4 (HER2.CD28.z, HER2.4-1BB.z, HER2.2B4.z).

Third-generation constructs incorporated dual costimulatory domains, including CD28 and 4-1BB (HER2.CD28.41BB.z) or 2B4 and 4-1BB.

Selected CAR constructs (HER2.z, HER2.CD28.z, HER2.41BB.z, HER2.CD28.41BB.z) were kindly provided by Dr. Stephen Gottschalk (St. Jude Children's Research Hospital, Memphis, TN, USA).

Flow cytometry

CAR expression was confirmed by direct immunofluorescent labeling using Alexa Fluor 647–conjugated recombinant HER2 extracellular domain (5 $\mu\text{g/mL}$) for 10 minutes on ice.

Samples were analyzed on a NovoCyte RYB flow cytometer, and data were processed using NovoExpress v1.2.1 software (Agilent Technologies). At least 10,000 events were acquired per sample.

Cytokine secretion assay

To assess functional activation, 1×10^5 CAR-NK-92 or non-transduced (NT) NK-92 cells ($\pm 10 \mu\text{g/mL}$ trastuzumab) were incubated either on plates coated with recombinant HER2-Fc (1 $\mu\text{g/mL}$) or co-cultured with HER2-positive target cells (MDA-HER2, JIMT-1, N87) at a 1:1 effector-to-target (E:T) ratio for 24 hours.

Interferon-gamma (IFN- γ) levels in supernatants were quantified by ELISA using a Tecan Spark® microplate reader. MDA cells served as HER2-negative controls, and NT NK-92 cells served as effector controls.

***In vitro* cytotoxicity assay**

Cytotoxic activity was assessed using luciferase-expressing tumor cell lines (MDA-HER2-ffLuc, JIMT-1.ffLuc, N87.ffLuc). Target cells (3×10^4) were seeded in 96-well plates and incubated for 4 hours before the addition of effector cells.

CAR-NK-92 or NT NK-92 cells were added at varying E:T ratios (1:1 to 0.003:1). After 24 hours, D-luciferin substrate was added, and luminescence was measured using a Tecan Spark® plate reader.

Experiments were performed in duplicate across three independent replicates.

Proliferation assay

Proliferative capacity was evaluated by serial cell counting. CAR-NK-92 cells (1×10^4) were cultured in duplicate in IL-2-supplemented medium. Cell numbers were determined every 3.5 days by flow cytometry.

After each measurement, viable cells were reseeded at the initial density, and cumulative proliferation was calculated across successive growth cycles.

Three-dimensional (3D) tumor model

Tumor spheroids were generated by culturing 5×10^3 JIMT-1.ffLuc or N87.ffLuc cells in U-bottom plates for 72 hours.

Spheroids were co-cultured with 2×10^5 CAR-NK-92 or NT NK-92 cells for 24 hours. Cell death was assessed by propidium iodide staining and quantified using confocal microscopy (Zeiss LSM 880).

***In vivo* xenograft model**

Antitumor efficacy was evaluated using immunodeficient NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1 Wjl/SzJ}). Mice were subcutaneously inoculated with 3×10^6 JIMT-1.ffLuc or N87.ffLuc cells in PBS/Matrigel.

Tumor growth was monitored by bioluminescence imaging (IVIS Spectrum CT) following intraperitoneal injection of D-luciferin (150 mg/kg).

Mice were treated with CAR-NK-92 cells (5×10^6 , i.v., biweekly), NT NK-92 cells (control), or NT NK-92 + trastuzumab (ADCC control).

All groups received IL-2 supplementation (50,000 IU, i.p., twice weekly).

All procedures complied with FELASA guidelines and were approved by the University of Debrecen Animal Ethics Committee (#5-1/2017/DEMÁB).

Immunohistochemistry

Tumor infiltration by CAR-NK cells was assessed using immunofluorescence. Frozen tumor sections (7 μ m) were stained with Alexa Fluor 647–conjugated anti-human IgG antibody to detect CD16 expression.

Nuclei were counterstained with DAPI, and samples were imaged using confocal microscopy.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5. Data are presented as mean $\pm$ SD or SEM.

Comparisons between two groups were performed using a two-tailed Student's t-test, while multiple groups were analyzed using one-way ANOVA followed by Bonferroni post hoc testing.

Differences were considered statistically significant at $p < 0.05$.

Results

HER2-specific CAR-NK-92 cells containing T cell–derived costimulatory domains mediate transient, targeted antitumor activity in xenograft models

Generation of NK-92 cell lines expressing HER2-specific CARs

The extracellular antigen-recognition domain of the CAR constructs used in this study consisted of an FRP5-derived single-chain variable fragment (scFv), connected to a CD28 transmembrane domain through a short IgG1 hinge region. The intracellular signaling domains included CD3 ζ alone or in combination with the T cell–associated costimulatory domains CD28 and/or 4-1BB. Although CD28 primarily contributes to early activation signaling, 4-1BB promotes pathways associated with cell proliferation, survival, and sustained effector function.

Using retroviral transduction, we generated NK-92 cell lines expressing a first-generation HER2.z CAR lacking a costimulatory domain, second-generation HER2.CD28.z and HER2.41BB.z CARs, and a third-generation HER2.CD28.41BB.z CAR containing both CD28 and 4-1BB intracellular signaling domains.

HER2-specific CAR-NK-92 cells recognize and eliminate trastuzumab-resistant HER2-positive tumor cells in 2D cultures

The *in vitro* activation of HER2-specific CAR-NK-92 cells was assessed by measuring IFN- γ secretion and by performing bioluminescence-based cytotoxicity assays. HER2-CAR-NK-92 cells exhibited robust activation-induced cytokine release in response to immobilized recombinant HER2 protein and following co-culture with HER2-positive tumor cells, including MDA-HER2, JIMT-1, and N87 cells. Among the tested constructs, HER2.CD28.41BB.z CAR-NK-92 cells induced the highest IFN- γ secretion across all co-culture conditions.

The cytotoxic activity of HER2-specific CAR-NK-92 cells was evaluated in the presence and absence of IL-2. In the presence of 400 IU/mL IL-2, non-transduced NK-92 cells displayed substantial cytolytic activity, comparable to both HER2-CAR-NK-92 cells and trastuzumab-treated non-transduced NK-92 cells. In contrast, in the absence of IL-2, the cytotoxic activity of non-transduced NK-92 cells was minimal, even at high effector-to-target ratios. Under these conditions, no significant differences were detected among the individual HER2-CAR constructs

or between HER2-CAR-NK-92 cells and trastuzumab-treated NK-92 cells used as an ADCC control.

HER2-specific CAR-NK-92 cells harboring 4-1BB efficiently penetrate CD44-negative N87 spheroids, whereas CD44-Positive JIMT-1 spheroids remain resistant

Previous work from our group demonstrated that the CD44-rich extracellular matrix of trastuzumab-resistant JIMT-1 tumors provides substantial physical and functional protection against antibody-mediated immune attack. This compact extracellular matrix restricts antibody access to the inner regions of tumor spheroids, thereby limiting trastuzumab-mediated NK-cell cytotoxicity. In contrast, conventional HER2-specific CAR T cells were previously shown to penetrate this barrier, recognize HER2-expressing cells within the spheroid core, and induce pronounced cytotoxicity.

Based on these observations, we evaluated the antitumor activity of HER2-specific CAR-NK-92 cells in 3D tumor spheroid models using trastuzumab-resistant CD44-positive JIMT-1 cells and trastuzumab-sensitive CD44-negative N87 cells.

CD44-positive JIMT-1 spheroids remained largely resistant to HER2-CAR-NK-92 cell treatment. Only minimal propidium iodide uptake was observed, predominantly at the spheroid periphery. Similarly, trastuzumab-directed non-transduced NK-92 cells induced only limited peripheral cytotoxicity.

In contrast, HER2-specific CAR-NK-92 cells efficiently recognized and penetrated CD44-negative N87 spheroids. CAR-NK-92 cells expressing HER2.41BB.z or HER2.CD28.41BB.z induced particularly strong cytotoxic effects, leading to marked disruption of spheroid architecture within 24 hours. By comparison, N87 spheroids treated with trastuzumab-directed non-transduced NK-92 cells or untreated control NK-92 cells showed negligible antitumor effects.

HER2-CAR-NK-92 cells fail to eliminate CD44-positive, trastuzumab-resistant JIMT-1 xenografts *in vivo*

To determine whether the limited penetration observed in 3D cultures translated into reduced therapeutic efficacy *in vivo*, we established subcutaneous xenografts using HER2-positive, CD44-positive, trastuzumab-resistant JIMT-1.flLuc cells in immunodeficient NSG mice.

HER2-specific CAR-NK-92 cells or non-transduced NK-92 cells were administered intravenously at a dose of 5×10^6 cells, beginning on day 14 after tumor inoculation and repeated twice weekly. In the ADCC control group, mice received non-transduced NK-92 cells together with trastuzumab, administered intraperitoneally at 100 μ g twice weekly. Tumor progression was monitored longitudinally by bioluminescence imaging, and survival was analyzed using the Kaplan–Meier method.

Neither HER2-specific CAR-NK-92 cell treatment nor the combination of trastuzumab and non-transduced NK-92 cells induced detectable tumor regression in the JIMT-1.flLuc xenograft model. In addition, none of the treatment groups showed a significant improvement in overall survival compared with controls.

HER2-CAR-NK-92 cells exert significant antitumor activity against CD44-negative, trastuzumab-sensitive N87 xenografts

We next evaluated HER2-CAR-NK-92 cells in a trastuzumab-sensitive, CD44-negative xenograft model. HER2-positive/CD44-negative N87.flLuc tumor cells were injected subcutaneously into NSG mice using the same experimental design as in the JIMT-1 model.

Both second- and third-generation HER2-specific CAR-NK-92 cells efficiently recognized and inhibited the growth of N87 xenografts. However, no significant difference was observed in antitumor efficacy between HER2-CAR-NK-92 cells and trastuzumab-directed NK-92 cells, suggesting that antibody-mediated ADCC and CAR-mediated targeting were similarly effective in this trastuzumab-sensitive model.

Both treatment strategies significantly prolonged survival compared with untreated controls, although neither approach resulted in complete tumor eradication. These findings support the therapeutic potential of HER2-specific CAR-NK-92 cells and indicate that further optimization is required to achieve durable tumor control.

The combination of 2B4 and 4-1BB costimulatory domains enhances the antitumor activity of HER2-specific CAR-NK-92 cells in CD44-positive JIMT-1 xenografts

Generation of HER2-specific CAR-NK-92 cell lines containing NK cell-specific signaling domains

To improve the cytotoxic efficacy of genetically modified NK cells, we generated additional HER2-specific CAR constructs containing NK cell-relevant intracellular signaling domains. Using the same retroviral transduction strategy, NK-92 cells were engineered to express HER2.2B4.z, HER2.41BB.z, or HER2.2B4.41BB.z CARs, the latter combining 2B4 and 4-1BB signaling domains.

HER2-specific CAR-NK-92 cells containing NK cell-specific signaling domains efficiently recognize and kill HER2-positive tumor cells in 2D cultures

The functional activity of HER2-CAR-NK-92 cells containing NK cell-associated signaling domains was assessed using complementary *in vitro* assays. Effector cell activation was measured by IFN- γ secretion, while cytotoxicity was evaluated using a short-term bioluminescence-based killing assay across multiple effector-to-target ratios.

Among the tested constructs, HER2.2B4.41BB.z CAR-NK-92 cells consistently produced the highest levels of IFN- γ , suggesting that the combination of 2B4 and 4-1BB provides additive or synergistic enhancement of NK-cell activation.

All HER2-CAR-expressing NK-92 populations efficiently eliminated HER2-positive MDA-HER2, JIMT-1, and N87 tumor cells at a 1:1 effector-to-target ratio. Cytotoxicity decreased progressively at lower effector-to-target ratios, indicating a dose-dependent killing response. Notably, CAR constructs containing 4-1BB exhibited enhanced cytotoxic activity at lower effector-to-target ratios compared with constructs lacking 4-1BB.

HER2.2B4.41BB.z CAR-NK-92 cells exhibit sustained proliferative capacity during repeated antigen stimulation.

The proliferative capacity of the different HER2-specific CAR-NK-92 cells was assessed using a repeated antigen stimulation assay. This approach enabled evaluation of expansion potential following sequential exposure to HER2-positive target cells.

HER2.2B4.41BB.z CAR-NK-92 cells demonstrated significantly greater and more sustained proliferation than second-generation CAR-NK-92 cells following stimulation with HER2-positive MDA-HER2 cells. However, this proliferative advantage was not accompanied by a proportional increase in cytotoxic activity.

Moreover, repeated exposure to HER2-positive target cells progressively reduced the antitumor responsiveness of the effector cells, particularly after the sixth stimulation cycle. This decline in cytolytic activity suggests the development of functional exhaustion during prolonged antigenic stimulation.

HER2.2B4.41BB.z CAR-NK-92 cells efficiently penetrate both CD44-positive and CD44-negative tumor spheroids

Because HER2.2B4.41BB.z CAR-NK-92 cells displayed superior proliferative capacity during repeated stimulation, we next investigated whether this advantage translated into improved tumor penetration and cytotoxicity in 3D tumor models.

In CD44-positive, trastuzumab-resistant JIMT-1 spheroids, HER2.2B4.41BB.z CAR-NK-92 cells efficiently penetrated the compact spheroid structure and reached the inner tumor layers. Extensive and homogeneous propidium iodide uptake throughout the spheroid indicated widespread tumor cell death. These findings suggest that the dual 2B4/4-1BB signaling configuration enables CAR-NK-92 cells to overcome the physical and functional barriers imposed by the CD44-rich extracellular matrix.

In contrast, non-transduced NK-92 cells exhibited no detectable cytolytic activity against JIMT-1 spheroids, confirming that tumor cell killing was CAR-dependent. CAR-NK-92 cells expressing only HER2.2B4.z or HER2.41BB.z induced moderate cytotoxicity, primarily restricted to the spheroid periphery. These results indicate that either signaling domain alone was insufficient to support deep tumor penetration in this resistant 3D model.

In CD44-negative, trastuzumab-sensitive N87 spheroids, all three HER2-specific CAR-NK-92 variants—HER2.2B4.z, HER2.41BB.z, and HER2.2B4.41BB.z—induced strong cytotoxicity. Propidium iodide staining was observed throughout the spheroids, indicating that, in the absence of a dense CD44-rich extracellular matrix, CAR-NK-92 cells could access the tumor core regardless of the costimulatory configuration.

HER2.2B4.41BB.z CAR-NK-92 cells exhibit enhanced *in vivo* antitumor efficacy and deep intratumoral penetration

Finally, we evaluated the *in vivo* antitumor efficacy of HER2-specific CAR-NK-92 cells in HER2-positive/CD44-positive JIMT-1 and HER2-positive/CD44-negative N87 xenograft models.

CAR-NK-92 cells expressing the HER2.2B4.41BB.z construct demonstrated pronounced antitumor activity in both the ADCC-resistant JIMT-1 and ADCC-sensitive N87 models. In both settings, treatment with HER2.2B4.41BB.z CAR-NK-92 cells resulted in significant tumor regression and significantly prolonged survival compared with CAR-NK-92 cells expressing only HER2.2B4.z or HER2.41BB.z.

Immunohistochemical analysis of excised xenografts further confirmed the superior tumor infiltration capacity of HER2.2B4.41BB.z CAR-NK-92 cells. In trastuzumab-resistant JIMT-1 tumors, only HER2.2B4.41BB.z CAR-NK-92 cells were detected throughout the tumor parenchyma, whereas CAR-NK-92 cells containing single costimulatory domains showed little or no intratumoral presence.

In N87 xenografts, all HER2-specific CAR-NK-92 populations showed diffuse tumor infiltration; however, HER2.2B4.41BB.z CAR-NK-92 cells exhibited the most extensive intratumoral distribution.

Together, these findings demonstrate that the dual 2B4/4-1BB costimulatory configuration enhances CAR-NK-92 cell proliferation, tumor penetration, and *in vivo* antitumor efficacy. This signaling architecture may therefore represent a promising strategy for improving CAR-NK cell therapies against solid tumors with dense and structurally restrictive tumor microenvironments.

Summary

The treatment of HER2-positive malignancies remains a significant clinical challenge, largely due to the emergence of therapeutic resistance and the immunosuppressive nature of the tumor microenvironment. Adoptive cell therapies, particularly those based on chimeric antigen receptor (CAR) engineering, offer new opportunities to enhance targeted antitumor immunity. While CAR T cell therapies have demonstrated remarkable success in hematological malignancies, their efficacy in solid tumors remains limited. In this context, CAR-engineered natural killer (NK) cells represent a promising alternative, especially considering their favorable safety profile and potential for allogeneic application.

In this study, we generated and functionally characterized NK-92 cell lines expressing HER2-specific CAR constructs. CAR-NK cells incorporating T cell-derived costimulatory domains exhibited robust cytotoxic activity *in vitro* against HER2-positive tumor cells, including trastuzumab-resistant models. However, their antitumor efficacy *in vivo* was limited and transient, particularly in CD44-positive, therapy-resistant xenografts, highlighting the challenges posed by tumor architecture and microenvironmental barriers.

To address these limitations, we developed a third-generation CAR construct incorporating NK cell-relevant signaling domains. CAR-NK-92 cells expressing the 2B4.41BB.z construct demonstrated enhanced proliferative capacity, sustained activation, and improved functional persistence upon repeated antigen stimulation. Importantly, in three-dimensional tumor spheroid models, these cells exhibited superior tumor penetration in both CD44-negative and CD44-positive settings.

Consistent with these findings, *in vivo* studies revealed that CAR-NK cells harboring combined 2B4 and 4-1BB costimulatory domains displayed significantly improved antitumor activity and deeper intratumoral infiltration, even in previously resistant xenograft models. These results underscore the importance of CAR design and demonstrate that the rational selection of intracellular signaling domains is a critical determinant of CAR-NK cell efficacy.

Collectively, our findings indicate that the simultaneous engagement of 2B4 and 4-1BB signaling pathways synergistically enhances NK cell proliferation, cytotoxic function, and tumor infiltration. This study provides strong preclinical evidence that NK cell-specific optimization of CAR

signaling architecture is a promising strategy to improve the efficacy of cellular immunotherapies in solid tumors. Furthermore, these results establish a foundation for future translational and clinical investigations to overcome resistance in HER2-positive malignancies.

Novel Findings

In this study, we developed an engineering strategy to expand the therapeutic potential of cancer immunotherapy using genetically modified natural killer (NK) cells.

In the first part of the dissertation, we demonstrated that NK-92 cells expressing conventional, T cell–derived chimeric antigen receptors (CARs) exhibit robust activation and increased IFN- γ secretion under *in vitro* conditions. However, our findings also revealed key limitations associated with the treatment of solid tumors. Although HER2-specific CAR-NK-92 cells delayed tumor progression *in vivo*, they failed to achieve complete tumor eradication, particularly in CD44-positive tumor models. These results highlight the critical role of the tumor microenvironment and extracellular matrix in mediating resistance and confirm that conventional CAR designs are insufficient to overcome these physical and biological barriers.

In the second phase of the study, we focused on optimizing CAR constructs tailored to NK cell biology. We demonstrated that the combined incorporation of 2B4 and 4-1BB costimulatory domains into the intracellular signaling region significantly enhances the functional properties of CAR-NK cells. In three-dimensional tumor spheroid models, this third-generation construct (HER2.2B4.41BB.z) exhibited superior intratumoral penetration, including in models characterized by heterogeneous CD44 expression.

Importantly, in preclinical NSG mouse models, activation of the combined 2B4 and 4-1BB signaling pathways resulted in a more robust and durable antitumor response than earlier CAR designs, leading to significantly prolonged survival in treated animals.

Collectively, these findings define a novel direction for the clinical development of CAR-NK therapies. They demonstrate that rational, NK cell–specific optimization of intracellular signaling domains can overcome key resistance mechanisms associated with solid tumors, thereby enhancing therapeutic efficacy and supporting future translational applications.

Appendix



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Registry number: DEENK/240/2026.PL
Subject: PhD Publication List

Candidate: Bence Gergely
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Gergely, B.**, Vereb, M. A., Rebenku, I., Szöőr, Á., Vereb, G.: Breaking the barrier: Synergistic 2B4.4-1BB signaling potentiates HER2-CAR NK cells to penetrate and destroy ADCC-resistant tumors.
Cancer Lett. 651, 1-9, 2026.
DOI: <http://dx.doi.org/10.1016/j.canlet.2026.218534>
IF: 10.1 (2024)
2. **Gergely, B.**, Vereb, M. A., Rebenku, I., Vereb, G., Szöőr, Á.: Targeting HER2-Positive Solid Tumors with CAR NK Cells: CD44 Expression Is a Critical Modulator of HER2-Specific CAR NK Cell Efficacy.
Cancers. 17 (5), 1-17, 2025.
DOI: <http://dx.doi.org/10.3390/cancers17050731>
IF: 4.4 (2024)

List of other publications

3. Barden, M., Elsenbroich, P. R., Haas, V., Ertelt, M., Pervan, P., Velas, L., **Gergely, B.**, Szöőr, Á., Harrer, D. C., Bezler, V., Holzinger, A., Friis, R. U. W., Vereb, G., Schütz, G., Schoeder, C. T., Hombach, A. A., Abken, H.: Integrating binding affinity and tonic signaling enables a rational CAR design for augmented T cell function.
J. Immunother. Cancer. 12 (12), 1-14, 2024.
DOI: <http://dx.doi.org/10.1136/jitc-2024-010208>
IF: 10.6





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4. Kola, J. B., Turarova, B., Csige, D., Sipos, Á., Varga, L., **Gergely, B.**, Refai, F. A., Uray, I. P.,
Docsa, T., Uray, K.: Stretch-Induced Down-Regulation of HCN2 Suppresses Contractile
Activity.

Molecules. 28 (11), 1-14, 2023.

DOI: <http://dx.doi.org/10.3390/molecules28114359>

IF: 4.2

Total IF of journals (all publications): 29,3

Total IF of journals (publications related to the dissertation): 14,5

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

28 April, 2026

