From CAR-T to CAR-NK cell therapy, the promise of a next generation of cancer immunotherapy

Introduction

Natural killer (NK)-cell therapies use NK cells to detect and destroy target cells that cause disease, such as cancer cells. Early clinical studies highlight the safety of NK cell therapies, but the clinical success of unmodified cells is less than clear. Due to a relatively short lifespan as well as the tumor microenvironment NK cells can struggle to tackle solid tumors. While this short lifespan can improve safety, low persistence levels can limit the therapeutic effects of NK-cell therapies. Modifying NK cells, such as with chimeric antigen receptors (CARs), offers a strategy to improve the clinical success of NK-cell therapy.[1,2]

CARS are cell surface structures that bind to antigens on target cells. When expressed on cytotoxic immune cells–such as T cells or NK cells–CARs can help direct them to kill cancer cells. CAR T-cell therapy has been clinically approved for the treatment of multiple hematological cancers. However, among its limitations, various safety issues have led the FDA to stop all 'universal' CAR T-cell therapy clinical trials.[1] CAR NK-cell therapy has benefits over CAR T-cell therapy, offering better safety and potentially providing an 'off-the-shelf' product. Consequently, renewed interest in CAR NK-cells in recent years has led to its use in various phase I and II clinical trials to tackle hematological and solid cancers (Figure 1).[3] Here, we focus on NK cells and their use as a cancer treatment when modified with CARs.

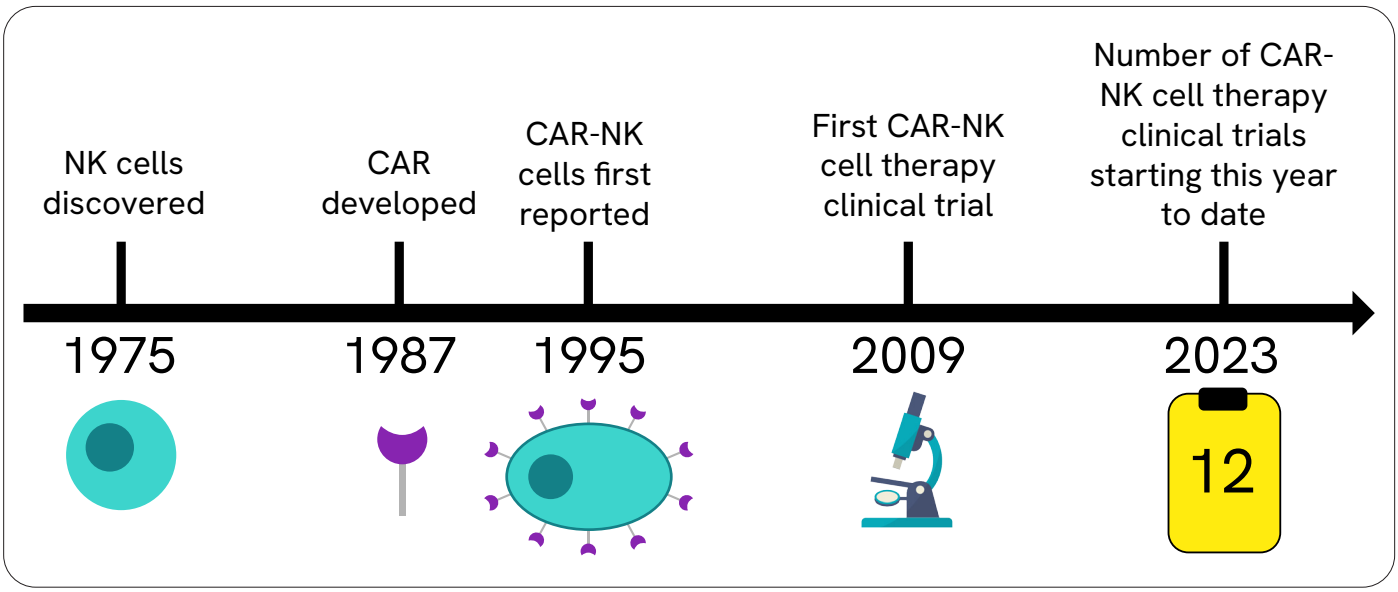

Figure 1: Timeline of CAR NK-cell therapy development.[1,3] Clinical trial data obtained from www.clinicaltrials.gov.

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NK cell background

NK cells are lymphocytes found in the blood and various organs and tissues throughout the body, such as the spleen, liver, and lungs.[4] These cells have both immune modulating and cell killing roles, forming a key part of the innate immune system—the non-specific branch of the immune system that acts as the host's 'first line of defense' against targets such as cancer cells and pathogens.[5-7]

NK cells primarily develop in the bone marrow but can also develop in organs such as the spleen and lymph nodes.[4] Developing from $CD34^+$ hematopoietic stem cells, NK cells gain surface receptors as they move through the different development stages in a process controlled by multiple transcriptional factors. The density of the CD56 receptor at the cell surface is used to categorize the two subsets of NK cells found in the peripheral blood:

- CD56Dim: These cells have a high density of CD56 and are traditionally classed as a more developed, highly cytotoxic NK cell subset. This subtype has higher levels of perforin, granzyme B, CD16 IgG Fc receptor, and KIRs, making it a highly cytotoxic NK cell subset. CD56Dim makes up the majority of NK cells found in the blood.
- CD56Bright: These cells have a low density of CD56 and are traditionally classed as a less developed, highly proliferative NK cell subset with lower levels of perforin and granzyme B than CD56Dim. CD56Bright cells produce more cytokines than CD56Dim and can generate CD56Dim cells.[3,8]

More recently, additional NK cell subtypes have been found in the blood and tissues that are functionally distinct from CD56Bright and CD56Dim.[3,4]

For their immunomodulatory role, NK cells release a variety of inflammatory cytokines that modulate the immune system and are capable of destroying a range of target cells, such as infected, stressed, or cancer cells.[9] This cytotoxic role relies on various NK cell surface receptors and lytic granules.[4]

NK cell cytotoxicity

Unlike cytotoxic T cells, NK cells do not need to be exposed to antigens to kill target cells.[10] Instead, NK cells use multiple surface receptors, which are either activating or inhibitory, to detect and kill target cells (Figure 2).[4] NK cells bind to target cells using surface adhesion proteins (selectins and integrins). Once initial contact occurs, NK cell surface receptors interact with activating or inhibitory ligands displayed on the target cell surface, generating signals that activate or inhibit the NK cell cytotoxic response.8 NK cell cytotoxicity is activated when the activating signaling outweighs the inhibitory signaling, causing NK cells to form a 'lytic immunological synapse' with target cells.[11] This synapse refers to the interface between NK and target cells, where surface receptors, proteins, and intracellular structural molecules are rearranged to allow NK cells to destroy target cells.[12,13]

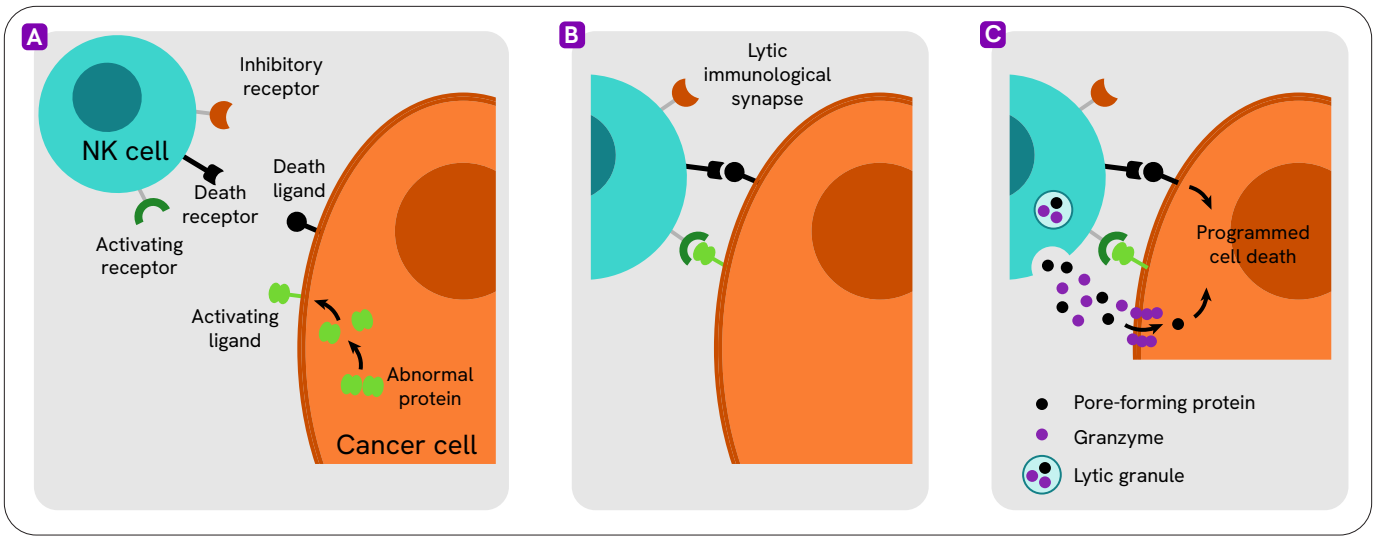

Figure 2: Overview of NK cell-mediated cytotoxicity: A–B) NK cells use surface receptors to detect cancer cells; C) activated NK cell kills cancer cells through death ligands and granule exocytosis.

NK cells can kill cells through the use of death ligands and the granule exocytosis pathway (Figure 2). NK cells express the following death ligands on their surface: TNFα, TRAIL, and FasL. Death ligands can activate programmed cell death pathways by binding to death receptors on target cells.[3,4]

The granule exocytosis pathway involves NK cells releasing secretory organelles called lytic granules. These contain cytotoxic proteins, such as pore-forming proteins (perforin and granulysin) and serine proteases (granzymes).[3,4,14]

Pore-forming proteins bind to the cell surface, where multiple monomers of the same protein aggregate and form pores in the membrane. While these pores can prove fatal for cells–causing osmotic lysis and damaging mitochondria–they also provide a method for granzymes to enter the target cell.[15] Once inside, granzymes act on serine residues in proteins to activate programmed cell death pathways.[8]

Targeting NK cell cytotoxicity

Inhibitory receptors are displayed on the NK cell surface to help detect and avoid harming healthy cells (Figure 3). Both healthy and abnormal cells–such as cancer cells–can display ligands that trigger activating receptors on NK cells. However, if sufficient levels of NK cell inhibitory receptors are stimulated, the cytotoxic response is inactive.

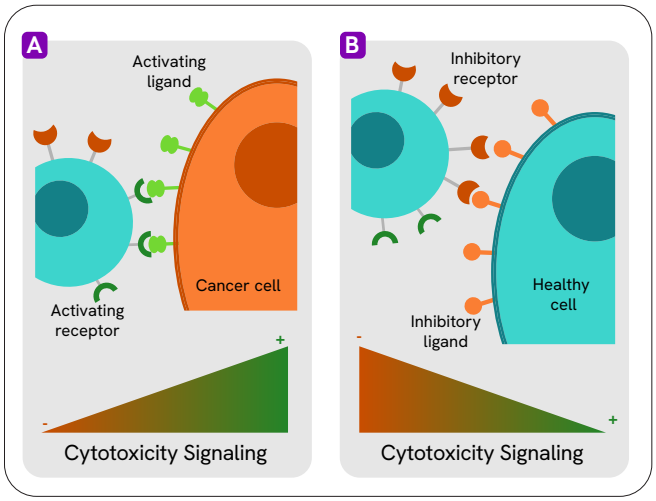

Figure 3: Overview of NK cell recognition: A) activating signaling outweighs inhibitory signaling when NK cells detect target cells, activating cytotoxicity response; B) inhibitory signaling outweighs activating signaling when NK cells recognize healthy cells, inactivating cytotoxicity response.

NK cells also target cancer cells that have attempted to evade the active immune system by lowering their major histocompatibility complex (MHC) levels. MHC-I proteins display peptides from proteins that have been broken down inside the cell on the cell surface. Because cancer cells generate abnormal proteins, MHC proteins display abnormal peptides at the cancer cell surface. The adaptive immune system uses T cells to detect cancer cells through these abnormal peptides. T cell surface receptors bind with abnormal peptide-MHC complexes, activating T cell cytotoxicity. Cancer cells can avoid immune detection by lowering their MHC levels.[16] However, as MHC-I proteins bind to inhibitory receptors on NK cells, NK cells can be activated by cancer cells that have missing MHC-I proteins.[17]

CAR NK-cell therapy

CAR cell therapy relies on cells–typically T cells or NK cells–being transformed to display a protein (CAR) on the cell surface that detects target cells. CARs detect cells by binding to antigens, such as antibodies, receptors, or other molecules on the target cell surface.[3]

Generating CAR NK-cells requires NK cells to be isolated, genetically modified, and then expanded (see Figure 4). NK cells are first isolated from a source, such as blood samples from patients, and then modified with genes that generate CARs at the cell surface. Cells are then grown or expanded to build up large enough numbers to be used as a therapeutic. These cells are then administered to the patient for cancer treatment.[18]

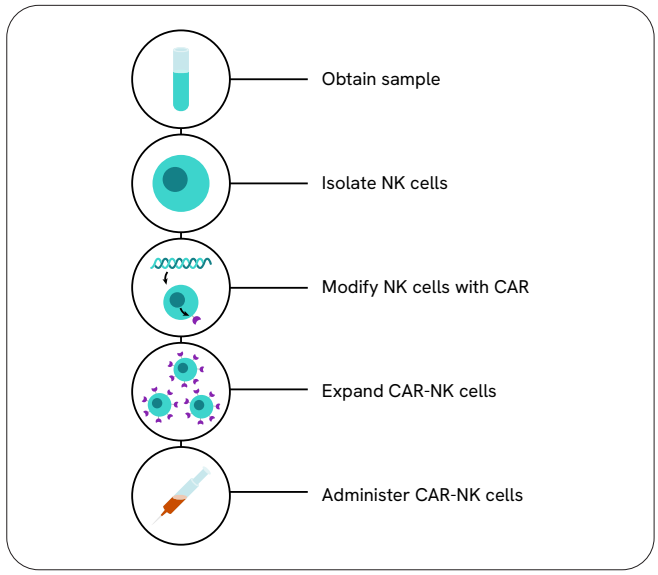

Figure 4: Overview of CAR NK-cell generation for cell therapy.

Cells for NK-cell therapy can be obtained from a range of sources:[7]

- Cell lines: Immortalized cell lines are homogenous. NK-92 has high cytotoxicity against cancer cells and is the only NK cell line approved for clinical use.[18] However, the NK-92 cell line needs to be irradiated before use as it was isolated from a patient with lymphoma, which can impair cell-killing activity.[18]
- Peripheral blood: This is easy to obtain but displays variability, with PB NK cells being a mature form of NK cells that have adapted their activating receptors to their environment. This variability in NK cells means there is not a single NK cell phenotype, which can complicate dosing.
- Umbilical cord blood: This is also easy to obtain and displays lower variability than PB NK cells. However, they are a more immature form of NK cell, leading these cells to have poor cell-killing activity against cancer cells. Cytotoxicity can be improved by expanding and transducing cells (homogenous).
- Stem cells: A homogenous cell population, but they may lack cell-killing activity against cancer cells.[6]

Genetic modification techniques

A variety of viral (transduction) and non-viral techniques have been used to deliver and modify genetic material in NK cells to generate CAR NK cells. Once the genetic material is inside the cells, it is either used by the cells or inserted into the NK cell DNA. This insertion can occur through the homologous repair of double-strand DNA breaks, the use of CRISPR-Cas9, or other techniques such as transposon systems.[3]

A common strategy for introducing genetic material into NK cells is transduction—where viral vectors are used to insert genes into cells. Retroviruses and lentiviruses are typically used to transduce NK cells.[4,19] Viral vectors are used in approved CAR T-cell therapies,[20] but the high viral quantity needed for NK cell transduction risks insertional mutagenesis. In addition, transduction methods can be expensive and complex.[21]

Non-viral or transfection strategies to deliver genes to NK cells have also been explored in CAR NK-cell therapy, such as:[3]

- Electroporation: Electric pulses are applied to cells to generate pores and increase the permeability of the cell membrane, allowing the uptake of genetic material into the cell.[19] Electroporation is an efficient transfection method for NK cells, while also being safer than viral transduction.[19,21]
- Lipofection: Liposomes are used to deliver genes inside cells. DNA-containing liposomes release the material into the cell after joining and fusing with the cell membrane. Lipofection offers a cost-effective approach and can be used to deliver a range of materials to the cell. However, viability can depend on the genetic material being delivered.[19,22]
- DNA transposon systems: Transposons are repetitive sections of DNA that can move from one region of a genome to another.[19] In DNA transposon systems, a transposon vector delivers the genetic material into the cell along with transposase. The enzyme excises and inserts the transposon into the genetic material.[23] Transposons are capable of delivering larger DNA sections to cells compared to viral vectors with similar transfection efficiencies while offering good stability. In addition, transposons may be cheaper and safer than viral vectors.[19,20]

CAR generation

CARs typically have an extracellular domain responsible for binding to antigens on target cells, a transmembrane domain, and an intracellular activation domain.[7] Traditionally, the extracellular binding domain was a single-chain variable fragment (scFv) of an antibody.[4,6] However, different molecules can be used as part of the CAR construct to target antigens, such as receptors and nanobodies.[3,18]

The intracellular signaling domain is responsible for activating CAR NK cells.[7,24] First-generation CAR constructs have a single signaling domain (typically a CD3ζ domain3), with second- and third-generation CARs having one or two additional co-stimulatory domains, respectively.[3,18,24] Fourth-generation CAR constructs are equipped with additional roles, such as suicide genes or generating cytokines.[3]

Advantages and limitations of CAR NK-cell therapy

While CAR T-cell therapy has successfully treated a range of hematological cancers, it is limited by:

- Toxicity (such as cytokine release syndrome, neurotoxicity, and graft-versus-host disease (GvHD))
- Expensive and lengthy manufacturing processes.[6,25]

GvHD is where CAR T-cells (the 'graft') attack host tissue.[26] GvHD occurs when using allogeneic T cells—cells that are taken from one person and given to another. Consequently, CAR T-cell therapies typically use autologous cells—those collected from and used in the same individual. While autologous cells may be safer, collecting and modifying cells can be expensive and lengthy procedures. Autologous CAR cell therapies also rely on patients having adequate cells at sufficient numbers, which can be an issue for patients that have undergone cancer treatment.[18,27]

NK cells are thought to be safer than T cells and research has shown NK cells do not cause adverse events like GvHD and neurotoxicity—although, cytokine release syndrome was observed in one CAR NK-cell therapy report.[3,6] The reduced risk of GvHD means allogeneic CAR NK-cell therapies could be used in anti-cancer treatments.[18] NK cells have shorter lifespans than T cells, which could contribute to the lower risk of adverse events and make such events easier to manage.[1] Consequently, the safety of allogeneic NK cells may allow CAR NK-cell therapy to be developed as an 'off-the-shelf' product for cancer patients.[6,18]

Unlike T cells, NK cells do not need to be sensitized to cancer cells to kill them because NK cells use activating and inhibitory receptors to detect and kill abnormal cells.[3] CAR NK-cell therapy may also be more robust as CAR NK-cells have both the CAR construct and their inhibitory and activating receptors to detect and kill cancer cells.[21]

While CAR NK-cell therapy can offer many advantages over CAR T-cell therapy, there are still limitations. CAR NK cells can be difficult to generate due to the challenge of isolating and expanding NK cells. Transduction of NK cells with CAR can also be an inefficient method.[7] In addition, CAR NK-cell therapy needs further optimization to better penetrate the tumor microenvironment to treat solid tumors.[25]

Clinical progress of CAR NK-cell therapies

With the benefits and potential of CAR NK-cell therapy being realized, there has been a steady increase in clinical trials using CAR-NK cells in recent years (Figure 5). These phase I–II CAR NK-cell therapy clinical studies have targeted a range of hematological cancers (B- and T-cell malignancies, multiple myeloma, myeloid leukemia) and solid tumors (such as glioblastoma and ovarian, prostate, lung, gastric, and head and neck cancers).[1] A range of CAR constructs have been developed to target the wide variety of hematological and solid cancers in these clinical studies—with some constructs aiming to target multiple hematological and/or solid cancers. For example, CAR constructs targeting the activating ligand NKG2D have been developed in clinical studies to treat different cancers, such as colorectal cancer (NCT05213195) and acute myeloid leukemia (NCT05247957).

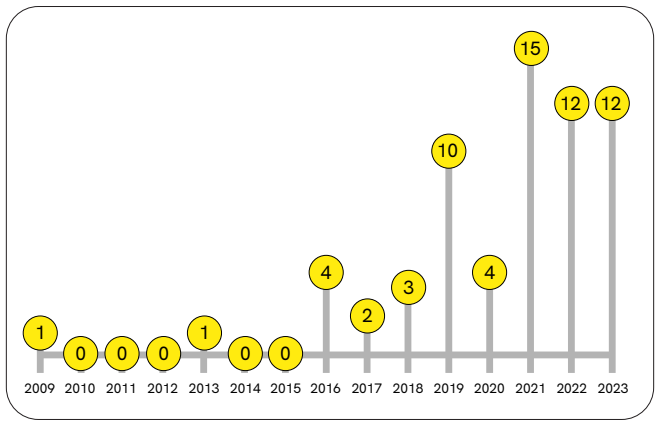

Figure 5: Graph showing the number of clinical trials started per year based on the study start date. Clinical trial data obtained from Li *et al.*1 and www.clinicaltrials.gov.

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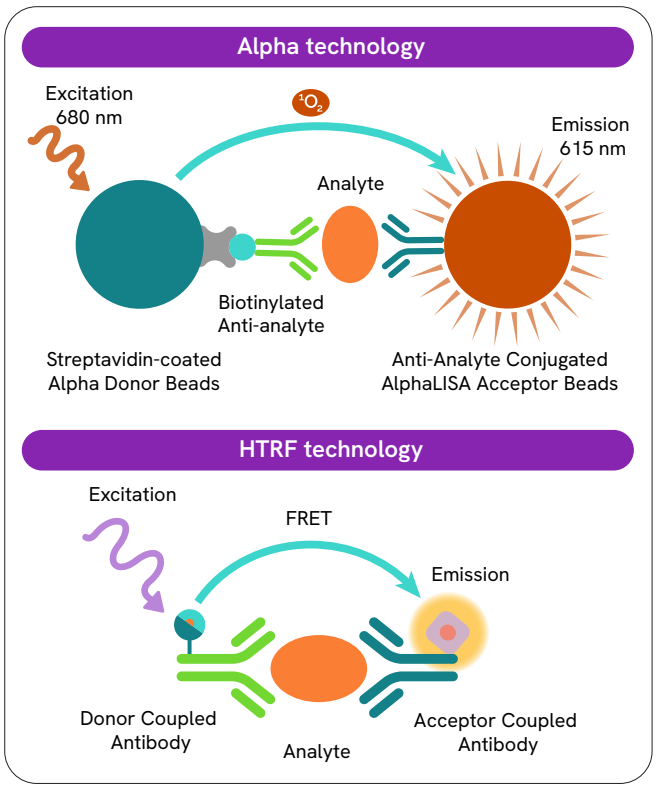

Figure 6: Alpha is a versatile, bead-based platform that enables you to assay the most complex samples in one well and with no wash steps. HTRF and LANCE are fast, sensitive, homogeneous, and ready to use assay platforms with no wash steps.

Summary

CAR NK-cell therapy has shown great promise as a targeted cancer treatment to tackle both hematological and solid cancers. Advances in CAR NK-cell therapy safety, efficacy, and clinical applications may be achieved by incorporating next-generation CAR constructs.

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