# Chimeric antigen receptor-natural killer

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## ABSTRACT

Chimeric antigen receptor (CAR) technology has transformed adoptive cell therapy, particularly through the success of CAR-T cells in hematologic malignancies. However, challenges such as toxicity, complex manufacturing, and high costs have encouraged the exploration of alternative immune cell platforms. Natural killer (NK) cells represent a promising approach due to their intrinsic cytotoxic activity, major histocompatibility complex-independent tumor recognition, and lower risk of severe immune-related toxicities. CAR-NK cells combine CAR-mediated antigen targeting with the natural antitumor functions of NK cells, supporting the development of safer and potentially "off-the-shelf" cellular therapies. This article the biological rationale of CAR-NK cells, current gene engineering strategies, and the evolving clinical landscape, highlighting their potential in hematologic malignancies, solid tumors, and autoimmune diseases.

**Keywords:** Natural killer; CAR-NK; Cell therapy.

## INTRODUCTION

Chimeric antigen receptor (CAR) technology is based on the genetic engineering of immune cells to express synthetic receptors capable of recognizing tumor-associated antigens and promoting the elimination of malignant cells. Initially developed for T lymphocytes (CAR-T cells), this approach has achieved remarkable clinical success, particularly in B-cell malignancies. However, important limitations remain, including severe toxicities such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), as well as the complexity, time, and high cost associated with autologous cell manufacturing. These challenges have driven the search for alternative immune effector cells that may provide potent antitumor activity with improved safety profiles. This article presents the clinical applications of adoptive CAR natural killer (NK) therapy, integrating the biological rationale, preclinical and clinical evidence, and translational guidance required for its safe and effective implementation.

### CAR-NK cells: biological basis and therapeutic potential

NK cells have emerged as a promising platform for CAR engineering. Unlike T cells, whose activity relies primarily on CAR-mediated antigen recognition, NK cells possess intrinsic cytotoxic mechanisms that function independently of the major histocompatibility complex, allowing them to detect and eliminate malignant cells through a balance of activating and inhibitory receptors.[1] As a result, CAR-NK cells combine CAR-directed targeting with innate immune recognition, providing an additional mechanism of tumor control. Clinically, NK cells also offer important advantages, including a minimal risk of graft-versus-host disease (GVHD) in allogeneic settings and a lower incidence of severe CRS and neurotoxicity compared with CAR-T therapies.[2]

These biological and functional distinctions highlight key differences between CAR-T and CAR-NK platforms, influencing their mechanisms of tumor recognition, safety profiles, and potential clinical applications (Table 1).

**Table 1.** Main differences between CAR-T and CAR-NK.

| Feature | CAR-T cells | CAR-NK cells |
|---|---|---|
| Mechanism of tumor recognition | CAR-mediated antigen recognition | CAR-dependent and CAR-independent recognition |
| Intrinsic cytotoxicity | Limited without CAR engagement | Strong natural cytotoxic activity against stressed or transformed cells |
| Risk of GVHD | Present in allogeneic settings due to T-cell receptor recognition | Minimal risk, enabling allogeneic "off-the-shelf" approaches |
| Toxicity profile | Higher incidence of CRS and ICANS | Generally lower risk of CRS and neurotoxicity |
| Persistence *in vivo* | Long persistence, which can increase both efficacy and toxicity | Typically shorter persistence, potentially improving safety |
| Manufacturing | Usually autologous and patient-specific | Compatible with allogeneic and off-the-shelf production |

Source: Elaborated by the authors.

These characteristics have positioned CAR-NK cells as a promising next-generation platform for cellular immunotherapy, particularly in settings where safety, rapid availability, and allogeneic use are critical. Multiple cellular sources have been explored to enable scalable CAR-NK production, each with distinct advantages and limitations.[3] Peripheral blood-derived NK cells are readily accessible but often limited by donor variability and expansion capacity. Umbilical cord blood provides a more naïve and proliferative NK population, although cell numbers may be initially restricted. Immortalized NK cell lines, such as NK-92, offer ease of expansion and manufacturing consistency but require irradiation prior to infusion, limiting *in vivo* persistence. Induced pluripotent stem cell-derived NK cells represent a highly scalable and standardized platform, yet their production remains complex and resource-intensive. Despite progress across these approaches, efficient and stable genetic engineering of NK cells remains a major challenge, underscoring the need to optimize gene delivery strategies to fully unlock the therapeutic potential of CAR-NK cells.

## Viral and non-viral gene delivery methods in CAR-NK engineering

The development of CAR-NK cells requires efficient gene delivery strategies to ensure stable CAR expression and sustained antitumor activity. However, NK cells are intrinsically resistant to genetic modification, making gene transfer a major challenge. Viral vectors, including retroviral, lentiviral, and adeno-associated virus (AAV) systems, remain the most widely used methods.[4] Retroviral vectors efficiently transduce dividing cells but carry a risk of insertional mutagenesis, whereas lentiviral vectors can modify both dividing and non-dividing cells, with improved efficiency using modified envelopes such as BaEV. AAV systems are mainly used in CAR-NK gene-editing approaches, particularly with CRISPR/Cas9.

In addition to viral methods, non-viral gene delivery approaches such as transposon systems, electroporation, lipofection, and lipid nanoparticles (LNPs) have gained attention as potentially safer and more cost-effective alternatives.[5] Transposon platforms enable stable integration of CAR constructs, while electroporation is commonly used for transient CAR expression via mRNA delivery. Lipid-based systems, including LNPs, offer another strategy for nucleic acid delivery with reduced cytotoxicity. Despite these advances, achieving efficient and stable genetic engineering of NK cells remains a major challenge in CAR-NK therapy development. The main strategies used for CAR-NK gene delivery are summarized in Table 2.

**Table 2**. Main strategies for gene delivery in CAR-NK engineering.

| Gene delivery strategy | Main advantages | Main limitations | Relevance for CAR-NK |
|---|---|---|---|
| Viral methods (retroviral, lentiviral, AAV) | High efficiency; enables stable CAR expression; widely used in clinical studies | Higher cost; complex manufacturing; potential risk of genomic insertion | Currently, the most common approach for generating CAR-NK cells with long-term CAR expression |
| Non-viral methods (transposons, electroporation, LNPs) | Lower cost; simpler production; reduced risk of viral-related safety concerns | Often lower efficiency or transient expression, more difficult to scale up | Emerging alternatives for CAR-NK engineering and scalable manufacturing |

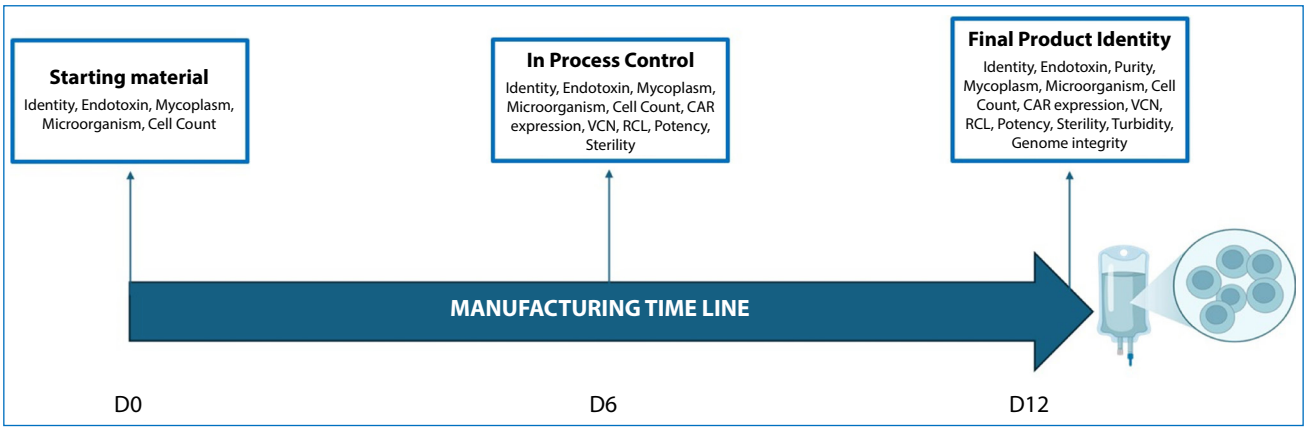
Source: Elaborated by the authors.

## General manufacture and quality control of ATMPs

In general, the manufacturing process of advanced therapy medicinal products (ATMPs), particularly CARNK cells, is strongly influenced by the regulatory framework in which the Cell Processing Center operates. This process initially involves the classification of the manufacturing environment in accordance with Good Manufacturing Practices (GMP), to appropriately define the use and the level of control required for the manufacturing systems employed.

In this context, CARNK cell production may be carried out using different technological platforms, including fully closed systems, such as the CliniMACS Prodigy, open systems, such as GRex, and semiclosed systems, exemplified by the combination of CliniMACS Plus and GRex. The selection of the manufacturing platform for advanced therapy products depends on multiple factors, including the required financial investment, process scalability, as well as the level of technical knowledge and operational expertise necessary for proper cell manipulation within the established systems.

The manufacturing processes of advanced therapy products may incorporate different quality control time points throughout production in order to monitor critical process and product parameters. As an example, the CARTHIAE1 study – Clinical trial using CART cells for the treatment of patients with refractory or relapsed CD19positive Blymphoid malignancies (NCT05705570) – conducted by Hospital Israelita Albert Einstein in São Paulo, Brazil, has a total manufacturing cycle of 12 days. In this study, manufacturing process controls are performed on days 0 (D0), 6 (D6), and 12 (D12), the latter corresponding to the final product harvesting step (Fig. 1). This workflow serves as an example of CAR-T cell manufacturing, which includes a genetic modification step, and is used here as a reference since CAR-NK therapies have not yet reached clinical trials in Brazil.




Source: Elaborated by the authors.

**Figure 1.** Example of a manufacturing process timeline for advanced therapy products, indicating strategic quality control checkpoints throughout development.

Once the manufacturing process has been duly validated and approved by the competent regulatory authorities, the final product must undergo a series of quality control assays aimed at ensuring its identity, purity, potency, and safety for patient infusion. Given that this technology predominantly relies on viral vectors for genetic modification, quality control testing includes specific assays, as described in Table 3, addressing critical aspects related to biosafety and product compliance.

The collective set of these assays forms the foundation of quality control for an ATMP, ensuring that the final product meets the critical quality attributes (CQAs) required by regulatory agencies (Agência Nacional de Vigilância Sanitária [ANVISA], European Medicines Agency [EMA], Food and Drug Administration [FDA]). Proper definition, validation, and interpretation of these criteria are indispensable for the safe release of the batch, patient protection, and the robustness of clinical development in advanced therapies.

**Table 3.** Critical aspects of ATMPs release.

| Assay | Definition | Aim |
|---|---|---|
| Identity | Assesses whether the final product corresponds to the intended cell population. This is typically performed by flow cytometry, confirming the presence of specific markers (e.g., $CD56^+/CD3^-$ for NK cells) and CAR expression. | Ensures that the product administered to the patient is indeed the correct product, as specified in the regulatory dossier. Identity is a CQA and an essential requirement for batch release. |
| Purity | Determines the proportion of target cells relative to undesired cells (e.g., residual T lymphocytes, B cells, monocytes) and process-related contaminants. | Reduces the risk of adverse events, such as unwanted immune reactions or off-target effects, and is therefore fundamental to clinical safety. |
| Viability | Determines the percentage of viable cells in the final product, typically assessed by flow cytometry (e.g., 7AAD, propidium iodide), or equivalent methods. | Cell viability directly impacts therapeutic efficacy and safety, as dead or apoptotic cells may promote inflammation and reduce clinical benefit. |
| VCN | Quantifies the average number of integrated transgene copies per cell, typically assessed by quantitative or digital PCR. | Balances efficacy and safety: a low VCN may compromise product potency, whereas a high VCN increases the risk of insertional mutagenesis and genomic toxicity. |
| RCL/retrovirus | Detects the presence of replicationcompetent viral particles inadvertently generated during manufacturing using viral vectors. | This is a critical regulatory biosafety assay. The presence of replicationcompetent lentivirus/retrovirus (RCL/RCR) represents a high risk of infection and serious adverse events in patients. |
| Turbidity | Visual assessment of the product for the presence of particles, precipitates, or abnormal coloration. | Serves as an early indicator of microbiological contamination, protein precipitation, or process failures. Although simple, it is a critical criterion for product release. |
| Genome integrity | Assesses the presence of undesired genetic alterations, such as rearrangements, mutations, or chromosomal instability arising from cell expansion or genetic modification. | This assessment is essential to mitigate the risk of cellular transformation, oncogenicity, and genotoxic effects, particularly in products with the potential for *in vivo* persistence. |
| Endotoxin | Measures levels of bacterial lipopolysaccharides, typically assessed using the Limulus amebocyte lysate test. | Endotoxins can induce severe inflammatory reactions, fever, septic shock, and organ failure. This is a mandatory parameter for injectable products. |
| Mycoplasm | Detects the presence of mycoplasma using culture-based methods, PCR, or other validated assays. | Mycoplasma contamination adversely affects cell viability, metabolism, and function, and poses a significant infectious risk, making it a critical contamination concern in cell culture–based products. |
| Sterility | Assesses the absence of viable aerobic and anaerobic microorganisms, in accordance with pharmacopeial methods. | This is a fundamental requirement to ensure the microbiological safety of the product, particularly for therapies administered via the intravenous route. |
| Microorganisms | Quantifies the microbial load prior to the final manufacturing step or in intermediate products, particularly when full sterility cannot be immediately achieved. | Enables monitoring of process control and prevention of critical sterility failures, and is particularly important for products intended for rapid release. |

PCR: polymerase chain reaction; RCL: replication-competent lentivirus; VCN: vector copy number. Source: Elaborated by the authors.

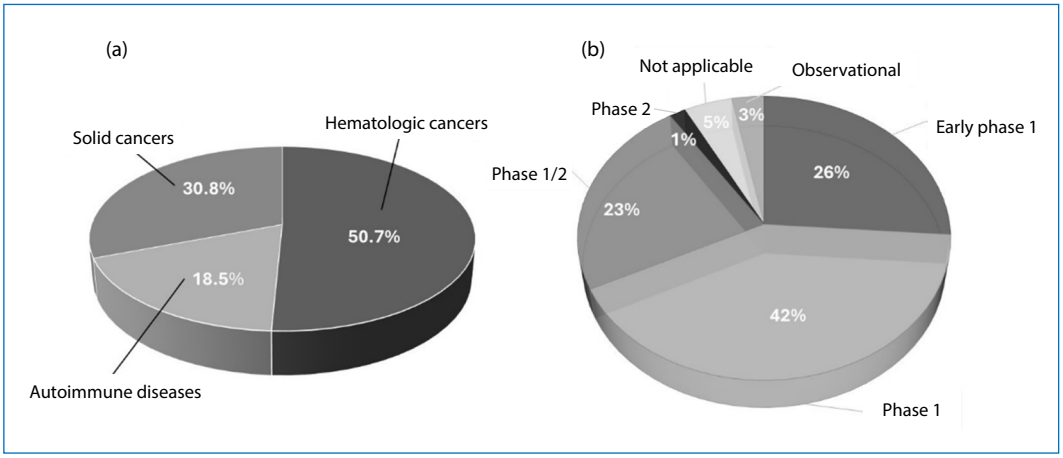
The implementation of a robust quality management system is essential in the field of advanced therapies, as it ensures consistency, traceability, and reliability throughout all stages of the manufacturing process. Regulatory agencies, through specific regulations, establish guidelines for the manufacture, control, and commercialization of these products, including the mandatory adoption of GMP. Such practices are fundamental to ensuring that products are manufactured under controlled conditions and meet stringent quality standards.

Furthermore, regulatory agencies require manufacturing centers to maintain comprehensive quality management systems, which include the conduct of rigorous clinical studies aimed at evaluating product safety and efficacy. These systems are critical for the credibility of manufacturing facilities and for fostering the confidence among healthcare professionals and patients in the therapies provided.

## Overview of current clinical evidence

Currently, 65 recruiting clinical studies involving CARNK cells are registered on ClinicalTrials.gov.[6] The search for trials in ClinicalTrials.gov was conducted using the filters "CARNK," including only studies with a "recruiting" status and eligibility criteria open to both sexes and age.

Among these, 33 (50.7%) correspond to approaches for hematologic malignancies, 20 (30.8%) focus on solid tumors, and 12 (18.5%) are directed toward autoimmune diseases (Fig. 2A). The phase distribution shows a predominance of Phase I trials (27; 42%), followed by Early Phase I (17; 26%), Phase I/II (15; 23%), and only one Phase II study (Fig. 2B), reflecting a clinically expanding field that nonetheless remains largely in early development stages.




Source: Elaborated by the authors.

**Figure 2.** Overview of diseases and clinical status. a) Overall disease groups that are addressed throughout clinical trials (recruiting status). b) Overall phase distribution related to the 65 CAR-NK clinical trials (recruiting status).

In hematology, CAR-NK therapies are being investigated for diseases such as non-Hodgkin lymphomas, AML, multiple myeloma, ALL, CLL, severe aplastic anemia, and myeloproliferative neoplasms, targeting antigens including CD123, CLL-1, CD33, CD19, CD7, BCMA, GPRC5D, CD70, and CD5. Many approaches also incorporate cytokine-armed CAR-NK cells, particularly expressing IL-15 or IL-12, to enhance cell persistence and antitumor activity. Early clinical studies have demonstrated encouraging safety and efficacy profiles. In a Phase I trial, Liu et al.[7] reported seven complete remissions and six minimal residual disease-negative responses among 11 patients with $CD19^+$ lymphoid malignancies treated with anti-CD19 CAR-NK cells, without CRS, neurotoxicity, or GVHD. Similarly, CD33-targeted CAR-NK cells derived from the NK-92 line showed good tolerability in patients with AML in a Phase I study (NCT02944162), further supporting the favorable safety profile of CAR-NK therapies. In addition, CAR-NK therapy has been explored as a "bridge-to-transplant" strategy, particularly in relapsed or refractory ALL, enabling rapid disease debulking and temporary immune control until allogeneic hematopoietic cell transplantation (allo-HCT).

Solid tumors present significant challenges for adoptive cell therapies due to their immunosuppressive and poorly penetrable tumor microenvironment (TME).[8,9] Several CAR-NK clinical trials are currently investigating pancreatic cancer, targeting molecules such as PD-L1, ROBO1, TROP2, MUC1, CLDN18.2, and stress-induced NKG2D ligands frequently overexpressed in solid tumors.[10,11] CAR-NK strategies are also being explored for glioblastoma, focusing on targets including MUC1, PD-L1, and HER2, a marker associated with poorer clinical outcomes in this disease. Additional solid tumor targets under investigation include 5T4, CD70, mesothelin, CLDN6, DLL3, AXL, and GPC3. Gene-editing strategies such as CRISPR-Cas9-mediated knockout of inhibitory receptors (e.g., TGFβRII and PD-1), together with the expression of TME-modulating molecules such as heparanase and chemokines, have been explored to enhance CAR-NK activity within the immunosuppressive TME, although these approaches are still undergoing clinical validation.

In autoimmune diseases, CAR-NK therapies focus on targeting B cells and plasma cells to induce a selective "immune reset" while avoiding broad immunosuppression. Common targets include CD19, which enables depletion of pathogenic B cells, and BCMA, which targets long-lived plasma cells producing autoantibodies.

Combined CD19/BCMA strategies have gained interest due to their ability to eliminate both compartments. Another emerging approach involves chimeric autoantibody receptors (CAARs), in which the CAR extracellular domain contains the relevant autoantigen to selectively recognize autoreactive B cells. Supporting this concept, Meng et al.[12] developed a CAAR targeting the La/SSB autoantigen, which enabled selective elimination of autoreactive B-cell clones when expressed in NK92MI cells, highlighting the potential of CAR-NK cells as a precise platform for autoimmune disease treatment.

### Current limitations and future perspectives

Despite the promising potential of CAR-NK therapy, several challenges remain before its broad clinical implementation. Key scientific limitations include the limited persistence and expansion of NK cells *in vivo*, difficulties in efficient genetic engineering, and the impact of tumor immune evasion and the immunosuppressive microenvironment. In addition, manufacturing standardization, quality control, and regulatory requirements for advanced cellular therapies remain important barriers. Nevertheless, CAR-NK cells represent a promising advancement in cellular immunotherapy, particularly in onco-hematology, combining CAR specificity with the favorable safety profile of NK cells. Continued advances in cell engineering, manufacturing, and clinical translation will be essential to integrate CAR-NK therapies into routine medical practice and expand treatment options for patients.

### Key points

- CAR-NK cells combine the specificity of CAR technology with the intrinsic cytotoxicity of NK cells.
- CAR-NK therapies are associated with a lower risk of CRS and neurotoxicity compared with CAR-T therapies.
- Advances in gene delivery and cell engineering are expected to improve CAR-NK persistence and clinical efficacy.
- CAR-NK therapies are currently being explored in hematologic malignancies, solid tumors, and autoimmune diseases, targeting antigens such as CD19, BCMA, CD33, and CD70.
- Most CAR-NK clinical trials remain in early-phase development, but initial studies have demonstrated encouraging safety and antitumor activity.

## CONFLICTS OF INTEREST

Nothing to declare.

## DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE TOOLS

Artificial intelligence-assisted tools were used exclusively for language refinement. All content was critically reviewed by the authors.

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## DATA AVAILABILITY STATEMENT

Not applicable.

## AUTHOR CONTRIBUTIONS

**Conceptualization:** Zanetti LC, Paiva RMA, Kerbauy LN. **Investigation:** Zanetti LC, Paiva RMA, Kerbauy LN. **Methodology:** Zanetti LC, Paiva RMA, Kerbauy LN. **Formal Analysis:** Zanetti LC, Paiva RMA, Kerbauy LN. **Data Curation:** Zanetti LC, Paiva RMA, Kerbauy LN. **Project Administration:** Zanetti LC, Paiva RMA, Kerbauy LN. **Funding Acquisition:** Zanetti LC, Paiva RMA, Kerbauy LN. **Writing:** Zanetti LC, Paiva RMA, Kerbauy LN. **Supervision:** Zanetti LC, Paiva RMA, Kerbauy LN. **Final Approval:** Zanetti LC.

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