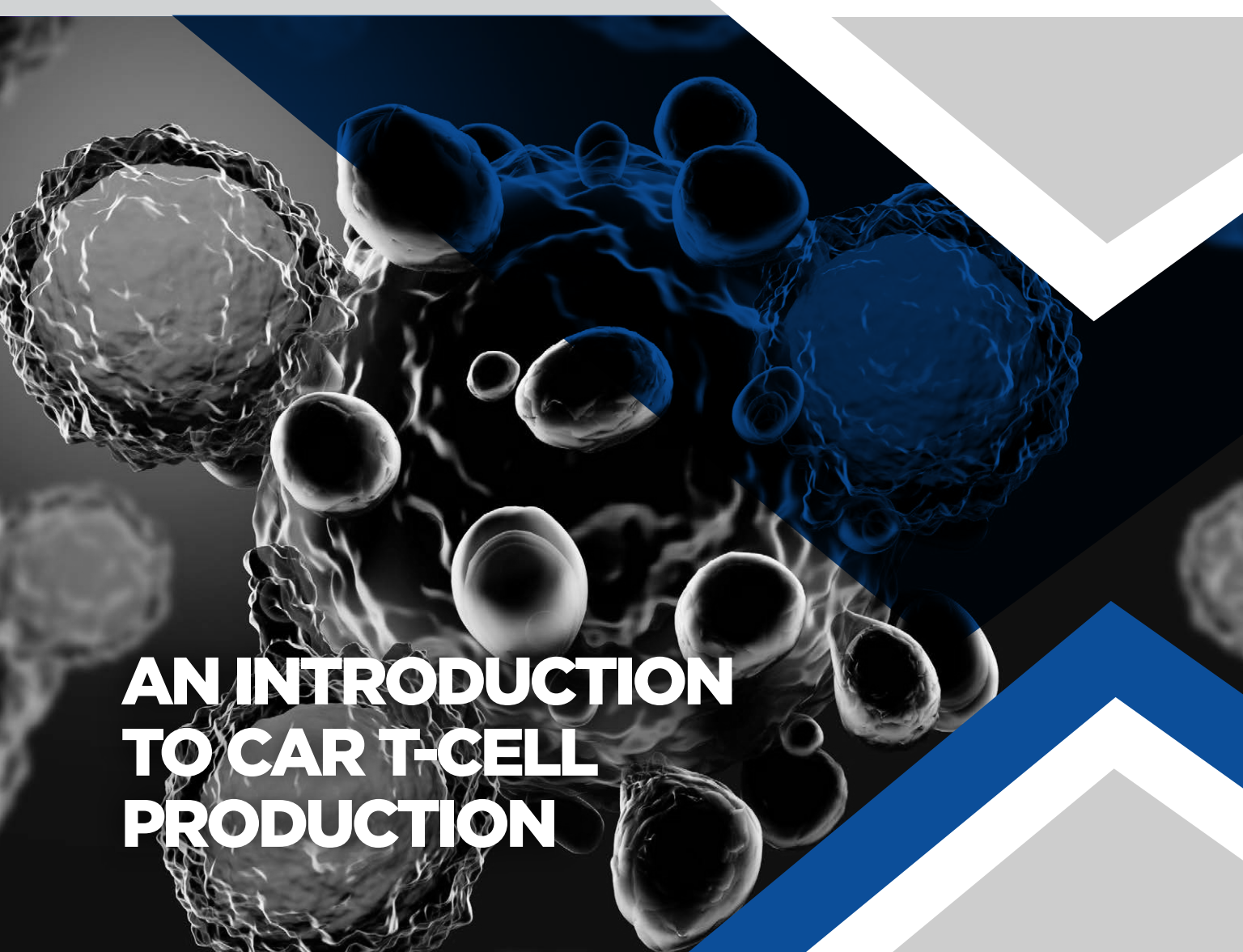


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¹) Baker, Monya. "1,500 scientists lift the lid on reproducibility." *Nature*, no. 533 (May 26, 2016): 452-54. doi:10.1038/533452a.

Locked on Target: T-Cell Receptor-Mediated Cancer Cell Killing

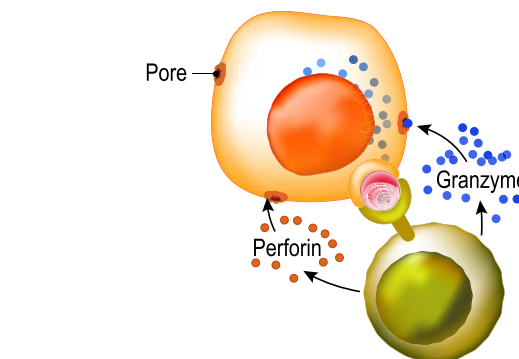
Immunotherapy enlists and empowers components of an individual's own immune system to attack cancer cells, in contrast to traditional cancer treatment plans that involve surgery, drugs, and radiation. Among several types of adoptive cell transfers (ACTs) where the patient's immune cells are collected, genetically modified, and re-infused to attack and eliminate cancer cells, CAR-T immunotherapy shows exceptional promise against both blood cancers and solid tumors.¹ In a clinical trial using CD19-targeted CAR T-cells,² durable remission was achieved in 27 of 30 patients with acute myelocytic leukemia. However, the dynamic heterogeneity of cancer cells and their complex microenvironment pose hurdles in the success of CAR-T immunotherapy.

Role Of T Cells In Mounting An Immune Response Against Cancer

T lymphocytes, a type of multifunctional white blood cell, play an essential role in mounting adaptive immune responses. T lymphocytes can gauge the state of a cell's interior by scanning for antigens on its surface. Cytotoxic lymphocytes (CTLs) or killer T cells, hunt down and eliminate compromised cells (e.g., infected, cancerous, etc.) while helper T cells regulate other T- and B-lymphocyte subsets.

CTLs, a subset of the $\alpha\beta$ T lymphocytes expressing CD8 cell-surface co-receptors, harbor on their surface copies of a multi-subunit receptor molecule, the T-cell receptor (TCR). TCRs, when triggered by Major Histocompatibility Complex 1 (MHC1), activate T lymphocytes. This, in turn, activates signaling cascades leading to cell proliferation, differentiation, cytokine production, and ultimately, the death of cancerous cells. Cancer cells must present processed antigens bound to MHC1 on their surface, together with co-stimulatory molecules (CD8 and CD28), to activate CTLs.

Upon CTL activation, the microtubule organizing center (MTOC) polarizes secretory granular traffic so that these granules fuse with the plasma membrane and release degrading enzymes



like granzymes and perforins that effectively digest and eliminate cancer cells. FasL and TRAIL expressed on reactivated CTLs are also able to kill susceptible cancer cells through interaction with death receptors or by inducing apoptotic pathways.

TCR: Components, Structure, Co-Receptors, Signaling

The TCR heterodimer is composed of six peptide chains that detect the antigen presented on cells by MHC molecules. The alpha and beta chains have amino-terminal variable and constant regions and are linked by disulfide bonds. Each TCR provides a single antigen-binding site. Since the cytoplasmic domains of TCR are relatively short, the intracellular signaling steps that follow TCR binding to the antigen are primarily carried out by CD3. CD3 molecules assemble together with the TCR and include immunoreceptor tyrosine-based activation motifs (ITAMs).

Tumor Immune Evasion

Cancer cells are recognized by TCRs through the expression of neo-antigens. In order to grow, cancer cells use multiple strategies to neutralize and evade the host's immune response.³ Cancer cells escape immune recognition by generating neo-antigens with weak immunogenicity, downregulating and modulating the expression of neo-antigens on cancer cells, and synthesizing immune suppressants that inhibit effector cell function and generate a milieu of tumor tolerance. The immunosuppressive microenvironment limits and impairs therapeutic CAR-T cells as well. Studies show tumor-mediated blocking of indoleamine 2,3-dioxygenase (IDO), an enzyme that converts tryptophan into metabolites, can impair CAR T-cell function.⁴ Therefore, one of the primary considerations in designing CAR-T cells is to find means to overcome the various ploys tumor cells use to avoid detection and thrive.

For references, please see page 7.

Start the CAR: Constructing CAR-T Cells

CARs, short for chimeric antigen receptors, are engineered molecules that consist of an extracellular ligand binding domain responsible for target specificity, a domain that inserts the receptor into the cell membrane, and one or more signaling domains that face the cell's cytosol and are responsible for activating T-cell division. Signaling domains in CARs may include CD3 ζ , CD27, CD28, ICOS, 4-1BB, and OX-40T. Cells that express CARs are essentially engineered to kill cancer cells and persist to patrol for emerging signs of tumor cells. CAR-T cells were first cultured in 1989, when first generation chimeric TCR genes were functionally expressed in T cells giving recipient T cells the ability to recognize and respond to antigens in a non-MHC-restricted manner.¹ Newer generations of CARs have multiple co-stimulatory domains that enhance T-cell proliferation and circulatory lifespan while curbing toxicity.

CAR-T Cells Counter Tumor Evasion

Tumor cells can evade T cells in the absence of robust tumor recognition mechanisms. In CAR-T cell-mediated immunity, a CAR's antibody-like single-chain variable fragment (scFv) engages antigens on tumor cell surfaces without the necessity for MHC presentation. Moreover, tumor microenvironments are characterized by T-cell exhaustion, a condition where tumor-secreted immunosuppressive factors cause reduced T-cell proliferation, increased expression of inhibitory receptors, decreased production of stimulatory cytokines, and compromised cytotoxicity. CAR-T cells designed to target multiple tumor cell antigens can dramatically improve recognition specificity for tumor-cells. In addition to directly killing tumor cells, CAR-T cells are also engineered to deliver anti-tumor agents to kill cancer clones or modify the tumor microenvironment.²

CAR: Design, Assembly, and Introduction Into T Cells

T lymphocytes are induced to express CARs by delivering and incorporating the CAR gene into the T-cell genome using electroporation or disabled viral vectors from murine retroviruses or lentiviruses. It's imperative to screen viral vectors to ensure they do not replicate in the human host. Electroporation results in transient expression but avoids the risk of developing

cancerous cells due to an insertional mutation. Advances in CAR-T cells include other modifications in the T-cell genome through gene editing and CRISPR-guided nucleases.

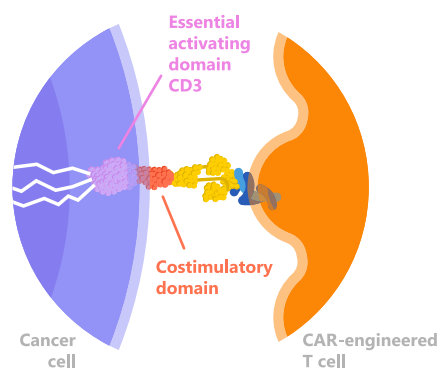
Modified T cells are then induced to divide clonally using an artificial antigen-presenting system (aAPC) consisting of anti-CD3/anti-CD28 beads or lentiviral-aAPCs. Contamination, a risk in all ex vivo cultures, can be minimized by using closed culture systems. Expansion and proliferation of CAR-T cells, if uncontrolled in the circulation, can lead to toxicity and severe adverse effects, including off-target attacks. Therefore, CAR T-cell design must include feedback regulatory systems for the optimization of therapeutic timing, strength of anti-tumor activity, and target specificity.

CAR: Testing and Screening

CAR T-cells are tested for five major classes of functional challenges to their therapeutic application: first, the capacity of these cells to infiltrate tumor sites, particularly in the case of solid tumors; second, their capacity to proliferate and persist in circulation; third, their ability to recognize cancer cells; fourth, their ability to function in an immunosuppressive environment; and fifth, their capacity to limit their expansion through feedback loops to curb toxicity and other adverse effects. Additionally, CAR-T cells are screened for contaminants that may include other T-cell subsets or tumor cells.

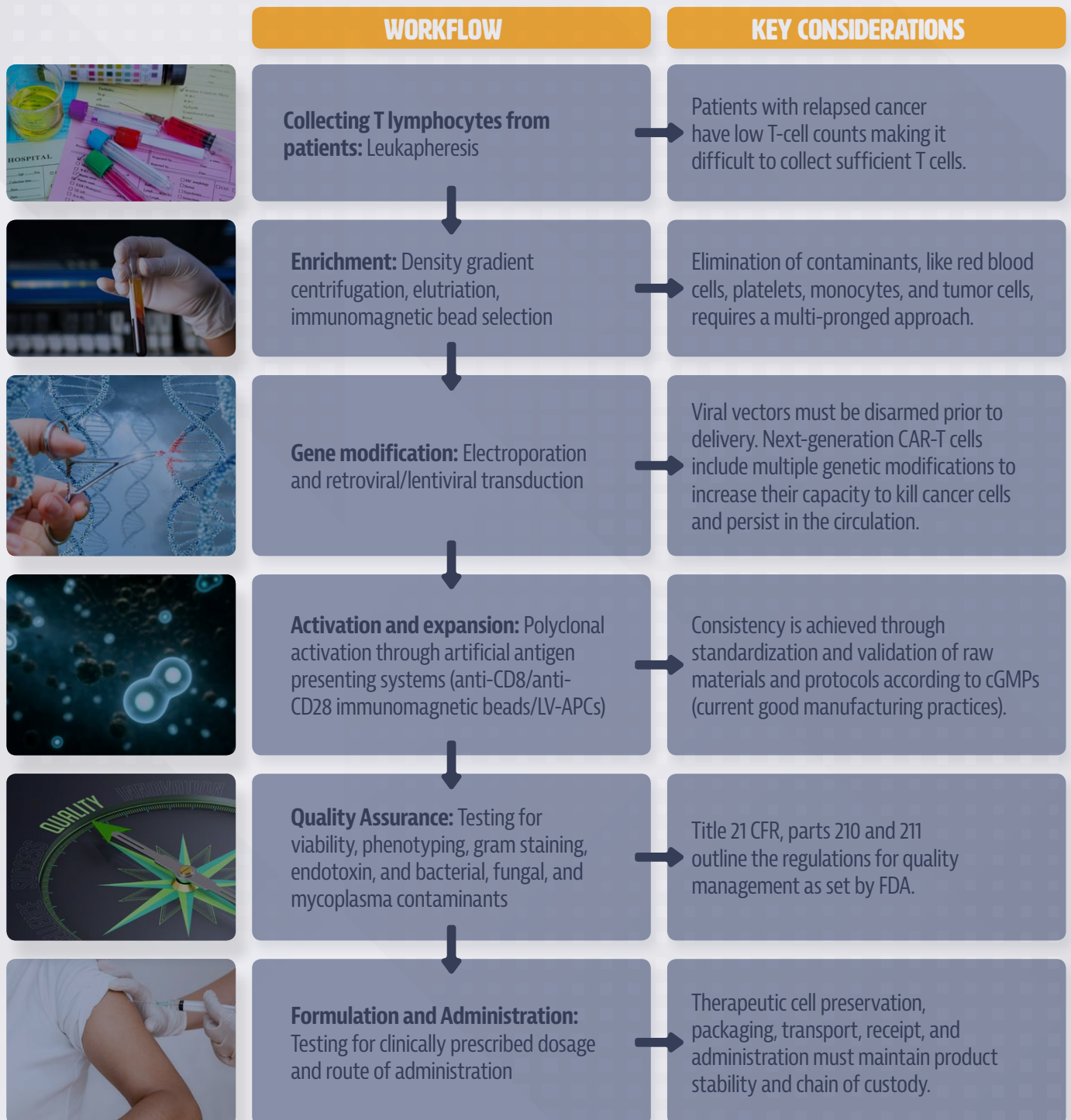
Multiple factors contribute to the variability observed in clinical responses to CAR-T cell therapy. Preparative conditioning regimes are administered to patients in order to reduce the number of circulating T cells (lymphodepletion), promoting the in vivo expansion of transferred CAR-T cells. Other factors include the dosage of infused cells, the final steady-state number of cells, the loss of tumor antigens, and the number of regulatory T cells in the tumor microenvironment, among others.³ The development of innovative strategies to regulate these diverse factors will determine the long-term clinical benefits of CAR-T cells.

For references, please see page 7.



PRODUCTION PIPELINE OF CHIMERIC ANTIGEN RECEPTOR T LYMPHOCYTES

The Chimeric Antigen Receptor (CAR) is an artificial assembly of an extracellular antigen binding domain, a transmembrane domain, and an intracellular domain(s) that enables engineered T cells to home in on tumor targets with enhanced specificity, proliferate rapidly in circulation, terminate tumor cells, and persist to patrol the body for emerging tumors.



Efficiency and Quality: Manufacturing CAR-T Cells for the Clinic



Manufacturing of CAR-T cells for clinical applications holds significant and unique challenges. The manufacturing workflow begins with collecting the patient's T lymphocytes and ends with an amplified population of therapeutic CAR-T lymphocytes ready to be infused into the patient as a living drug. The modification, activation, and expansion of T lymphocytes require a high level of expertise and sophisticated equipment that must meet the stringent standards of quality control. Moreover, production of CAR-T cells requires careful storage and handling to maintain stability of the therapeutic cells and traceability.¹

Improving CAR-T Production

Leukapheresis is the process by which mononuclear cells (MNCs), including subsets of T cells, are collected from a target patient, and it involves sustained bloodflow through closed-loop, continuous or intermittent collection systems, and centrifugation.¹ This is difficult in patients in advanced stages of malignancy who have low peripheral blood access. Central venous catheterization provides a steadier blood flow but introduces greater risk of infection and trauma. In addition, chemotherapy and radiation reduce lymphocyte count, and fewer T cells can be collected from relapsed or refractory patients who have already passed through multiple cycles of cytotoxic therapies.

The collected sample then needs to be enriched for T cells. Density gradient centrifugation efficiently removes erythrocyte and granulocyte contaminants. However, commonly used Ficoll-Paque gradients are unable to separate T cells from monocytes. Methods based on cell size and density (elutriation) remove monocyte contaminants but tumor cells and unwanted lymphocytes may be retained. Immunomagnetic beads can isolate T-cell subsets with a high degree of specificity. Optimal methods for enrichment depend on the quality of the starting sample. Scaling up requires the validation of essential culture ingredients and protocols and development of standard operating procedures to ensure consistency and dosage specificity as per cGMPs.

The phenotypic heterogeneity of solid tumors makes them difficult for CAR-T cells to detect. Fourth generation CARs bypass this limitation by including co-stimulatory domains and

CAR-inducible IL-12.² This allows NK cells and macrophages to mount a second wave of attack against cancer cells that would be undetectable by earlier generations of CAR-T cells. Another recent advance involves the modification of CARs to induce phagocytosis (CAR-P), directing macrophages to engulf target cells including cancer cells.²

Quality Concerns in Clinical Applications

Quality management is regulated by the Foundation for the Accreditation of Cellular Therapy (FACT) or the Joint Accreditation Committee (JACIE), governed by the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA) of the United States. Specifications delineated in the FDA's investigational new drug (IND) application ensure continuous control, traceability, documentation, standards, product safety, identity, purity, sterility, and potency through large-scale clinical trials. Although clinical trials are costly and time consuming, lengthening the time from bench to bedside, they play a vital role in identifying product contaminants and potency and lend insight into underlying mechanisms. Two CAR T-cell therapies have been approved by the FDA, one for pediatric acute lymphoblastic leukemia, and the other for adults with advanced non-Hodgkin's lymphomas.

Once the therapeutic cellular product is formulated, samples are tested for optimal dosage, route of delivery, and archiving. Cells can be introduced either directly into cancerous tissue or through intravenous infusions. Logistical concerns in this final stage include optimizing methods of cryopreservation, storage, packaging, transport, thawing, and infusion that preserve the stability and efficacy of the therapeutic cells.³ Impressive clinical responses have led the FDA to designate several CAR-T cell therapies as breakthroughs in cancer treatment.⁴ For CAR-T cell therapy to move from the echelons of frontier research to being adopted as a viable option in cancer treatment globally, CAR-T cellular products will need to be manufactured under standardized, industry-grade conditions.

For references, please see page 7.

Article 1 - Locked on Target: T-Cell Receptor-Mediated Cancer Cell Killing

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Article 2 - Start the CAR: Constructing CAR-T Cells

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Article 3 - Efficiency and Quality: Manufacturing CAR-T Cells for the Clinic

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