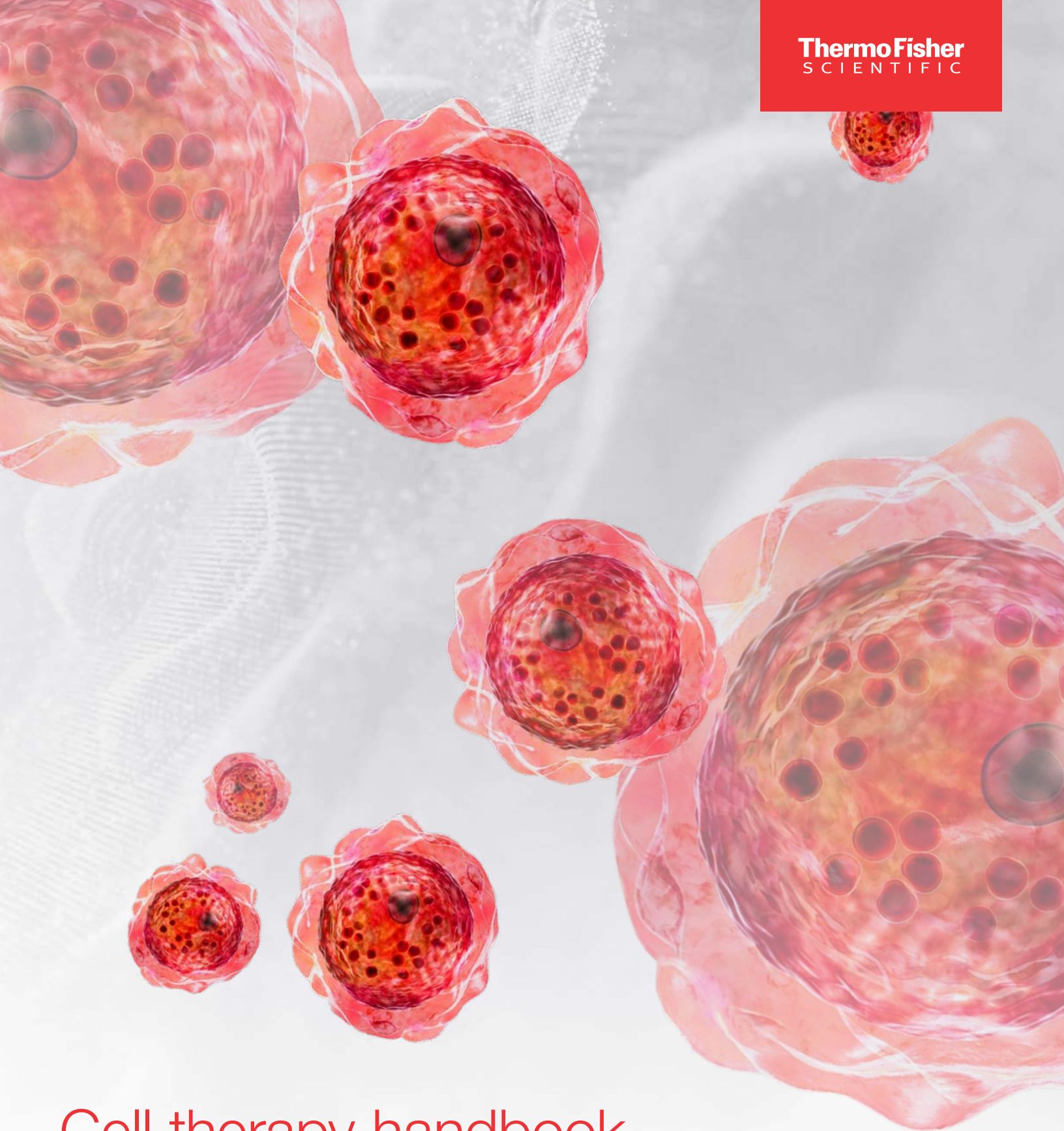
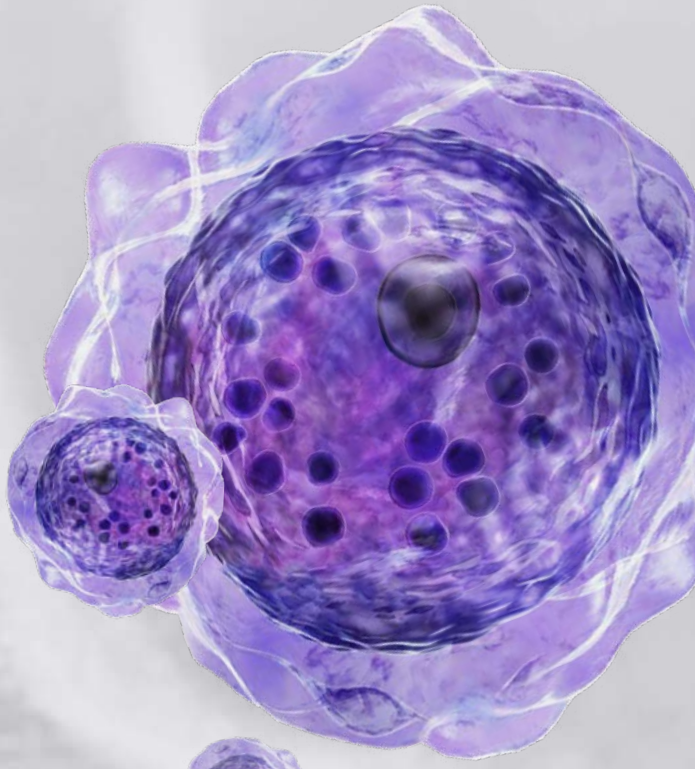


Cell therapy handbook

Considerations for cell therapy development and manufacturing

Whether you're new to cell therapy manufacturing or looking to expand your existing processes and knowledge base, this educational handbook will provide you with the major considerations for successful cell therapy manufacturing. The Cell Therapy Handbook reviews the latest methodologies, common practices, resources, applications and more, to support every step of your cell therapy manufacturing workflow.



Contents

Section 1:
Raw material considerations for cell therapy manufacturing

Introduction

Successful clinical translation of a cell therapy product hinges on early process- and material-selection decisions that impact manufacturing. Not only do materials present in the final approved drug (i.e., excipients) need to meet certain specifications, but raw materials (or ancillary materials) used in the manufacturing process must meet stringent quality standards. If the raw materials chosen early in product development do not satisfy the necessary regulatory criteria at clinical trials and commercialization stages, they will need to be replaced with materials that do. Those substitutions can result in significant increases in costs and time.

The best practice to support successful clinical trials and commercialization of a cell therapy requires a raw material strategy with the end goal in mind. This longer-term view focuses on the use of higher grades of raw materials earlier in cell therapy product development to meet the necessary regulatory qualifications for clinical trials, and ultimately commercial manufacturing of the final approved therapeutic. This strategy can increase the probability of success and head off costly surprises that could cause an untimely demise for a promising cell therapy candidate.

In this section, we will provide a high-level overview of the considerations used to select raw materials that mitigate risk and align with current regulatory guidelines. For deeper discussion on this topic, please see heading Additional resources at the end of this section.

What are raw materials

Raw materials, also referred to as ancillary materials in US regulations*, are components that come in contact with the cell therapy product during manufacturing, but are not intended to remain in the final therapeutic. Cell culture media and growth factors would be examples of raw materials employed in manufacturing a cell therapy. While not present in the final product, raw materials are still important because of their potential impact on the safety, purity, and potency of the final cell therapy product.

Generally speaking, raw materials are not regulated products. However, regulatory guidelines recommend that developers use therapeutic-grade raw materials whenever possible because of their potential influence on the characteristics and safety of the final cell therapy product. Unfortunately, therapeutic-grade versions will not exist for every type of raw material used. In these cases, the best option would be to choose raw materials manufactured under the appropriate current good manufacturing practices (cGMP). While the same materials developed for research use only (RUO) and *in vitro* diagnostics (IVD) uses might be available, they will lack some of the necessary traceability and testing that will be required, particularly as a therapeutic moves further into clinical trials and hopefully commercialization. Raw materials designed for RUO or IVD use should be avoided in a long-term cell therapeutic manufacturing strategy.

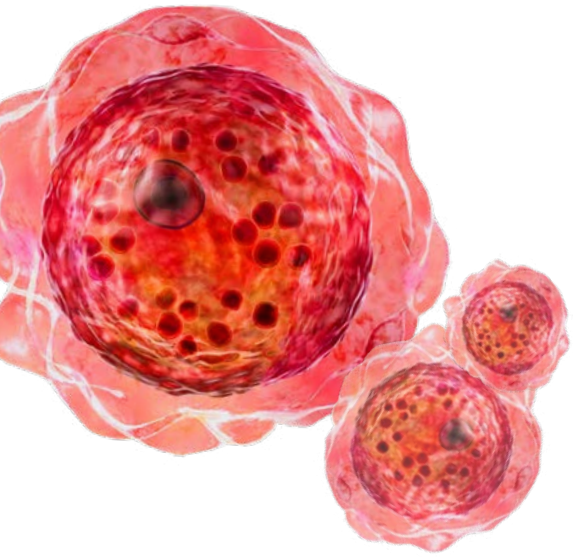
No specific cGMP guidance exists for raw material manufacturing, unlike that found for medicines and medical devices. Regulatory guidance (e.g., USP <1043> and ISO276) only recommends choosing raw materials made under an appropriate quality management system, a rather vague term. Suppliers may say their products are manufactured under cGMP conditions, with claims ranging from declarations of cGMP based on following particular cGMP guidelines; independent quality management system certification (e.g., ISO9001); or even regulatory agency inspection if the site is manufacturing regulated products. However, there is no such thing as a defined "GMP-grade" material.

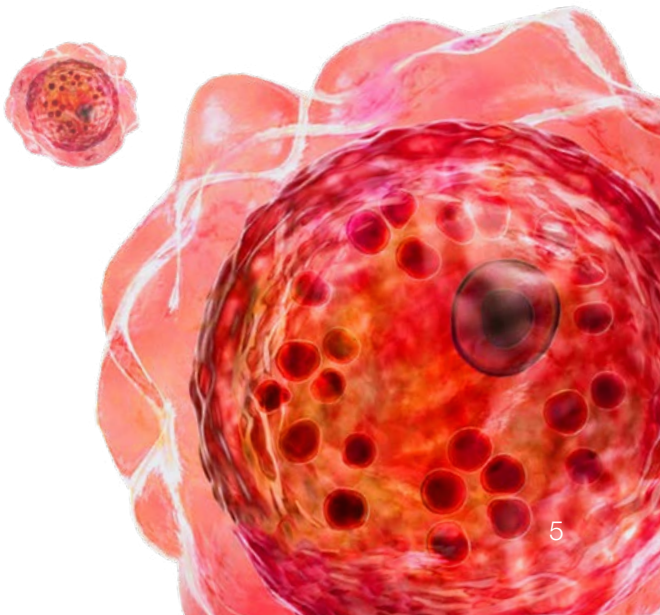
The first place to begin to understand what is meant by cGMP manufacturing of a raw material is the published regulatory guidance. Table 1 presents some of the main guidelines across different regions. Japan, Europe, and the United States have the most detailed raw material guidance, with Japan having some of the strictest.

*There can be some confusion around the terms "raw materials" and "ancillary materials" across regions -- Europe uses the term "raw materials", whereas the US uses the term "ancillary materials". Ancillary materials are also synonymous with "processing materials", as defined in 21 CFR Part 1271, and "components" in Pharma cGMP Part 211.

Table 1. Raw material regulatory guidance from major jurisdictions.

Region	Raw material regulatory guidance
International	• WHO GMP for Biological Products • Various ISO standards (ISO 9001, 13485, and TC276) • Various ICH guidelines (ICH Q5A, ICH Q5D, ICH Q, 3 ICH Q2)
Australia	• Australian regulatory guidelines for biologicals (ARGB)—critical raw materials used in manufacturing
Japan	• PMDA MHLW Public Notice No. 210—Standard for Biological Ingredients • Raw material certification process available
Europe	• ATMP Regulation (EC) No. 1394/2007 • Ph. Eur 5.2.12 Raw Materials of Biological Origin for the Production of Cell-Based and Gene Therapy Medicinal Products • EudraLex Volume 4 GMP guidelines (May 2018)
United States	• USP <1043>—Ancillary Materials for Cell, Gene and Tissue-Engineered Products • USP <92>—Growth Factors and Cytokines Used in Cell Therapy Manufacturing (limited to rh-IL4) • FDA chemistry, manufacturing, and controls (CMC) guidances • 21 CFR Part 1271 Section 1271.210—GTPs • 21 CFR Part 211 subpart E—GMPs • Master File process available

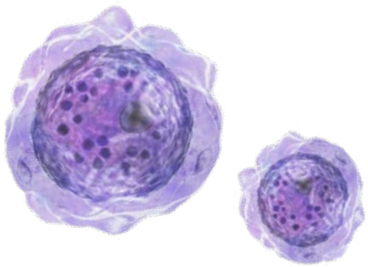




In general, cGMP refers to the "minimum requirements for the methods, facilities, and controls used in the manufacturing, processing, and packing of a drug product" to ensure the product is safe and is of the correct potency and composition [1]. These general cGMP requirements entail specifications for personnel; quality control plans and functions; facilities and equipment; control of components, containers and closures; manufacturing and records; laboratory controls; packaging, labeling, and distribution; and record keeping. Table 2 provides examples of some of these requirements.

Table 2. Some examples of essential components of cGMP manufacturing [1].

System	Example
Control of materials	Quarantine and release process at warehouse with incoming goods
GMP analytics	Quality control laboratory with quality assurance oversight
Standard operating procedures	Batch record documentation with process for deviations and corrective and preventative action (CAPA)
Qualified operators	Required training for gowning, safety, and operations
Controlled access with environmental control	Keyed access to ISO 7 environment with ISO 5 space for aseptic processing
Environmental monitoring and cleaning cycles	Drop plates, swab testing, particle monitoring, and regular disinfection and cleaning



One of the most globally recognized raw material guidance documents is USP <1043>. USP <1043> presents a risk-based model based on 4 risk categories that are used to assess each raw material (Table 3). These risk categories are defined by specific activities required of the manufacturer [2]. The required activities of each risk level are phased, with a subset required of all products (e.g., Certificates of Analysis and lot-to-lot testing). As the risks associated with the raw material increase, different activities are also required (e.g., safety testing for residual materials containing animal products). Risk also increases as the product moves into later phases of clinical testing. Product developers should aim to source tier 1 and 2 raw materials; tier 3 is less favorable; and tier 4 should be avoided for clinical work. Zero risk is unattainable, so developers will strive to maintain the lowest risk possible when selecting raw materials while still maintaining performance.

Table 3. USP <1043> raw material risk categories.

Tier	Risk level	Description	Example
1	Lowest	Highly qualified and suited for CGT* manufacturing	Rh-insulin for injection used as a cell culture medium additive
2	Low	Well-characterized, intended for use as raw material, manufactured under a quality management system in compliance with GMP	Gibco CTS media and reagent products
3	Moderate	Not intended to be used as a raw material	RUO or IVD materials such as some cell culture media
4	High	Not produced under a recognized quality management system, not intended for use as a raw material; can be animal-derived, toxic, and variable in biologic activity	Animal cells or animal sera, cholera toxin used in cell culture, or selection agents for transgene expression

*CGT = cell and gene therapy

Critical quality attributes of raw materials in cell therapy manufacturing

When choosing raw materials for use in manufacturing of cell therapeutics, developers typically focus on four key product characteristics:

- Material identity
- Purity, and presence of impurities
- Lot-to-lot consistency
- Storage and stability

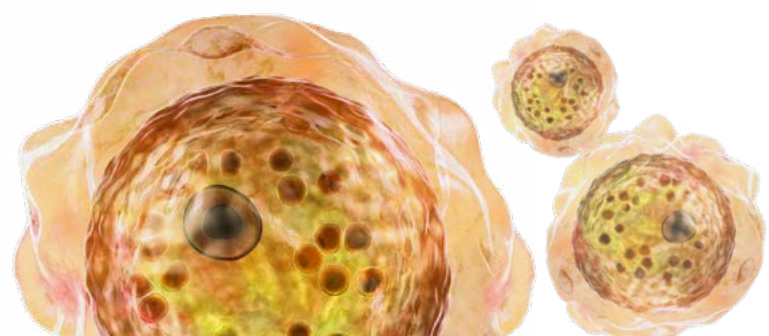
Table 4 summarizes some of the important details to consider for each of these characteristics. With the lack of global standards for critical quality attributes, cell therapy manufacturers will choose raw materials that will meet the standards of the region with the most stringent requirements.

With regard to a material's identity, cell therapy manufacturers should pay close attention to biosafety characteristics to determine any risks a material might bring to the facility, to the operator, and in the final cell therapy product. The preference is to avoid animal origin components when possible. When this is not possible, a risk-based approach to these raw materials will become important, using the following considerations:

- Possible alternatives (e.g., recombinant proteins)
- Viral inactivation process
- Upstream vs. downstream use (risk increases the further downstream a raw product is used)
- Grade of material (e.g., cGMP compliance vs. RUO)
- Demonstrated product traceability and documentation (from supplier)
- Country of origin (important for CJD, BSE and TSE risk)

Table 4. Considerations for four key raw material characteristics.

Characteristic	Look for
Identity and freedom from microbial or viral contamination	• Any information on the molecular composition or formulation • If material is proprietary, documentation on the activity of the active components • COO, health statement, and pathogen testing for animal-derived materials • Required viral testing, and donor eligibility and screening, documentation for human-derived materials
Purity and impurity	• Documentation on purity • For multiple-component products, purity of active ingredients • Identification of impurities should be documented • Assays to detect residuals
Consistency	• Supplier effort to determine lot-to-lot consistency on Certificates of Analysis • GMP-manufactured materials easier to demonstrate consistency
Storage and stability	• Supplier's recommended storage conditions (e.g., temperature, light, humidity) demonstrating that raw materials maintain consistent performance • Product shelf life backed by stability testing that reflects product use as a raw material

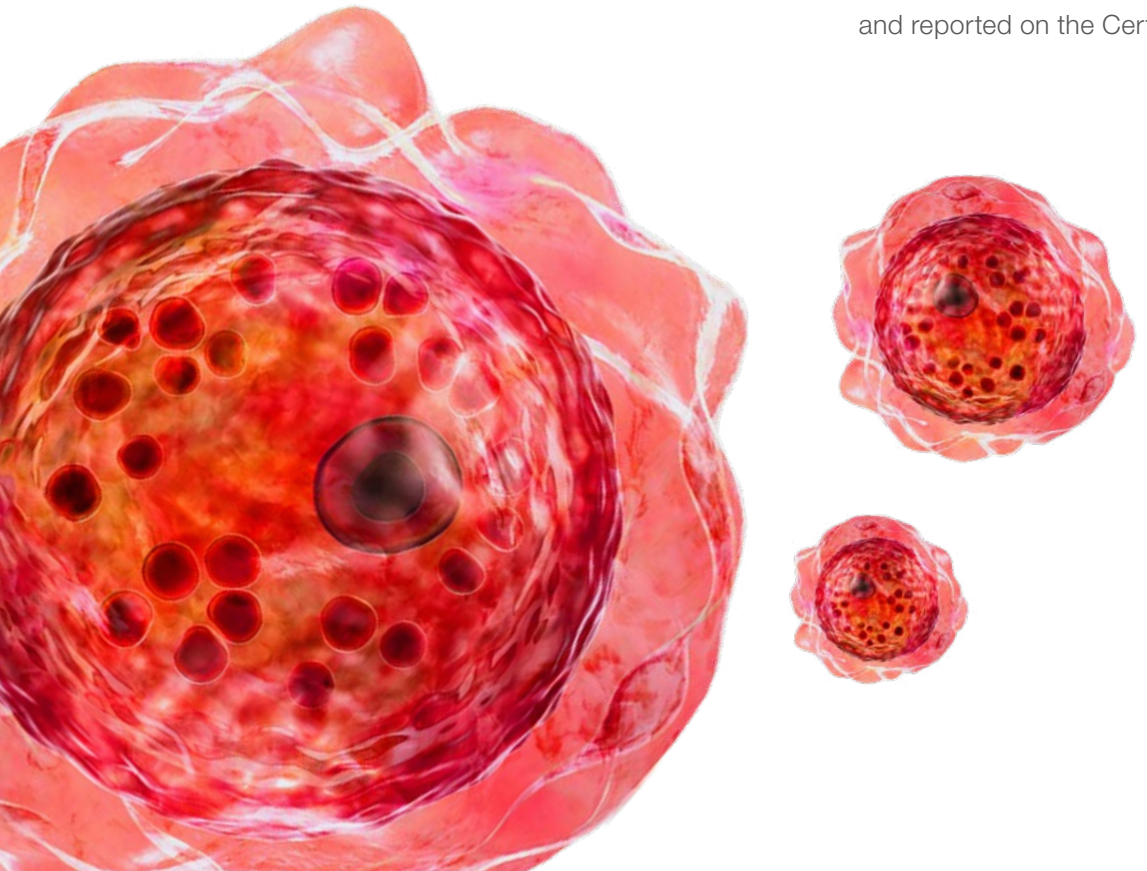


It is within this biosafety area where some of the terminology used can become confusing. When selecting affected raw materials, a cell therapy manufacturer should gain a clear understanding of a supplier's definitions for terms such as "animal origin free", "serum free", and "xenofree" to fully understand the potential risks associated with the raw material.

Cell therapy manufacturers need to also consider the performance testing of raw materials in their final material decisions. The supplier should provide performance data that are reflective of a product's intended use as a raw material. For example, a performance test for media using a CHO cell line is of little use if the intended use of the media is to grow T cells. The data should also enable developers to determine the performance consistency of a raw material, with quantitative data being better than pass/fail results. To assess the data accurately and determine their relevancy to the intended use, the supplier should provide the assay methodology used, preferably using reference test methods (e.g., United States Pharmacopeia or USP).

Whenever possible, developers should choose USP or EP grades of raw materials with monographs. Monographs ensure the raw material meets specific quality standards for identity, strength, quality, and purity determined by specific tests, procedures, and acceptance criteria. If monographs are not available, some (if not all) of the important attribute information discussed should be found in a supplier's material documentation (see below, key raw material documentation). However, it is possible that the developers will need to perform additional testing to make final robust material decisions and mitigate risks associated with any raw material. Even though no global quality standards exist, it is best for manufacturers to choose raw materials that are fully characterized in order to ascertain the risks associated with them, including:

- Numerical specifications for test methods on Certificates of Analysis to demonstrate lot-to-lot consistency
- Performance tests focused on intended use in the manufacturing process, with stability tests linked to performance
- Traceability of biologically derived raw materials to primary, secondary, and tertiary levels, provided on Certificates of Origins
- USP test methods or validated in-house methods used and reported on the Certificate of Analysis



Key raw material documentation

Much of the information on raw material quality attributes discussed above can be found in various supplier documentation (Table 5, Figure 1). Besides determining the appropriateness of a raw material for cell therapy manufacturing, some of the information found in these various documents will be necessary for a variety of regulatory filings. In cases where raw materials contain proprietary components or formulations, developers should look for suppliers who can provide that information through Regulatory Support Files, which are provided under signed confidentiality agreements. Some regions (e.g., the United States) support Master Files provided by suppliers for the sharing of proprietary materials with the appropriate regulatory agencies. Master Files do not require signed confidentiality agreements and can be a faster way to get the necessary information for regulatory filings.

- GMP manufacturing (21 CFR part 820 and certified to ISO 13485)
- Detailed Certificate of Analysis (COA) and Certificate of Origin (COO)
- Drug Master File (DMF) or Regulatory Support File (RSF)
- Aseptically sterile product (validated SAL 10-3)
- Endotoxin and mycoplasma tested
- Performance tested (T cell functional assay)
- Adventitious viral testing of human-derived proteins and access to viral inactivation data
- Proven use in cell therapy manufacturing

Figure 1. Example of important product characteristics that cell therapy manufacturers should look for in various product documentations. The Gibco™ CTS™ Immune Cell Serum Replacement is an example of a reagent specifically designed for use in cell therapy manufacturing that meets documentation requirements. It complies with the raw material guidances in the United States, Europe, and Japan. The reagent is intended to replace the use of human serum when performing ex vivo culture of human lymphocytes.

Table 5. Raw material supplier documentation types.

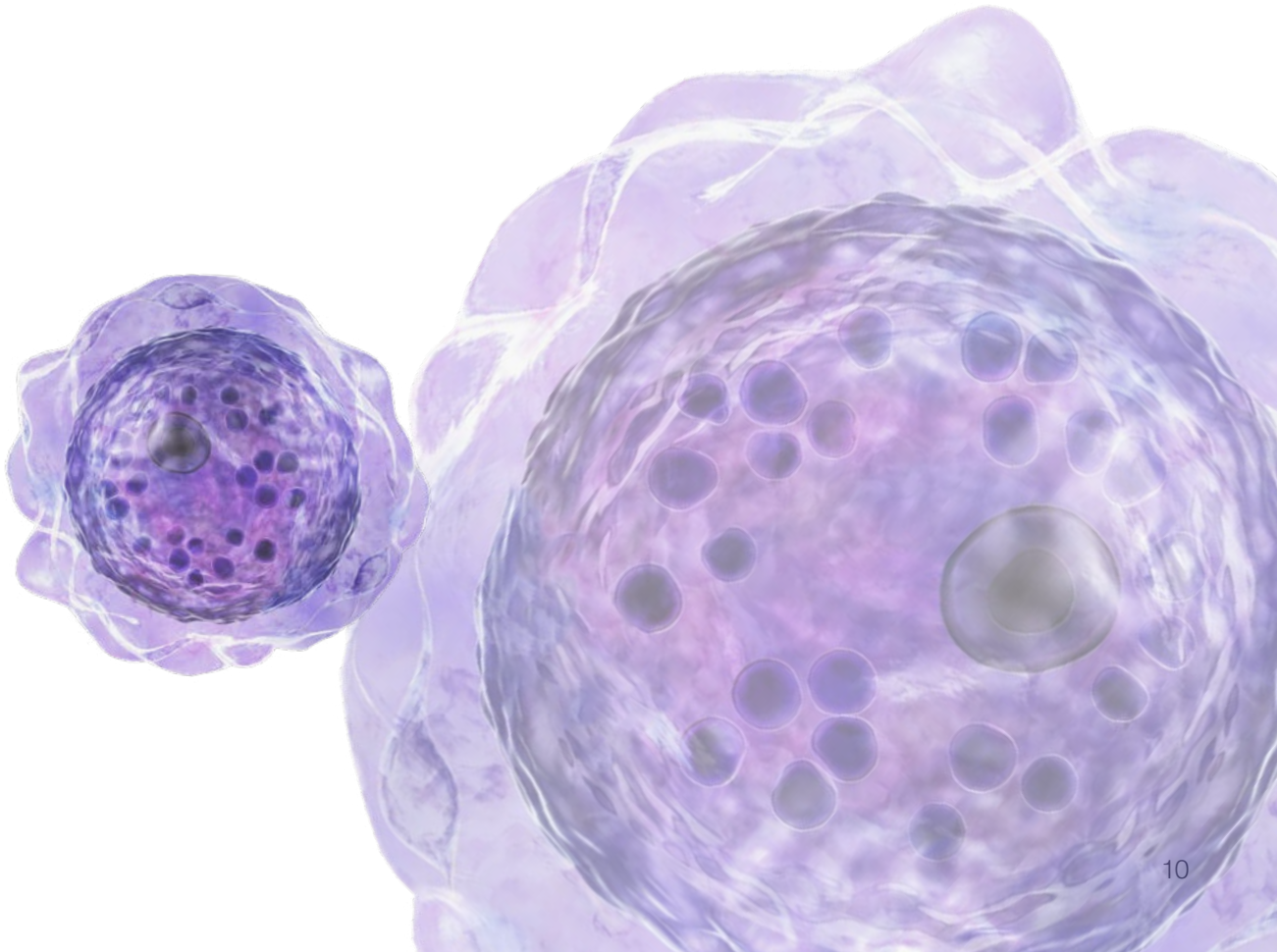
Documentation type	Description
Certificate of Analysis (COA)	COAs contain information on product lot; product shelf life and expiration; identity; quantity; purity and impurities; safety; and biological activity.
Certification of Origin (COO)	A COO demonstrates supply chain control (traceability), which is particularly important for human- and animal-derived products.
Safety Data Sheet (SDS)	SDSs, provided as applicable, contain information on the properties of each material and their physical, health, and environmental hazards and subsequent protective measures associated with them. They also contain necessary safety precautions for handling, storing, and transporting the material.
Certificate of Compliance (COC)	COCs may be provided to support compliance claims about quality systems or standards.
Regulatory Support File (RSF)	Under a confidentiality agreement, this summary provides product performance, stability, quality control, and analytical testing methods specifically designed to meet cell therapy raw material regulatory requirements. Used when Master Files are unavailable.
Drug Master File (DMF)	A detailed submission to a regulatory body that provides confidential information about facilities, processes, and raw materials used in manufacturing, testing, processing, packaging, and storage. DMFs are only available in the United States, Canada, and Japan.

Supplier and developer responsibilities

Ultimately, it is the drug manufacturer's responsibility to assess the risks associated with and suitability of the chosen raw materials, with much of this assessment occurring during the vendor qualification process (read more below about vendor qualification). Table 6 provides some of the important items to address during this process to mitigate risk associated with the cell therapy.

Table 6. Some key considerations for raw material risk assessment.

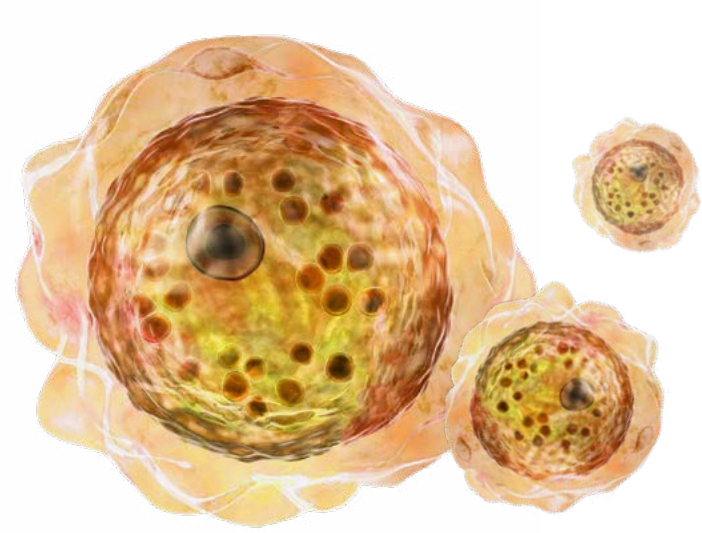
Raw material:	Considerations
Source	• Is this material human-, animal-, or recombinant-derived? • Is the source a viral concern? • Can the material be replaced with lower risk substitutes?
Manufacturing	• What are the cGMP, aseptic, and cross-contamination concerns in non-dedicated facilities? • Is there possible exposure of the material to other human and animal products during manufacturing? • Has the supplier's manufacturing site been audited by our team?
Testing	• What tests are available that demonstrate the material's identity, purity, safety, and performance? • Has any viral inactivation been performed? Is it validated?
Traceability	• Can the supplier demonstrate material traceability on all risk components and their supply chain?



The raw material supplier(s) also have obligations during the selection process and beyond. It is important that the cell therapy manufacturer and the raw material provider(s) understand their responsibilities throughout the clinical trial and commercialization process, and work together to meet these responsibilities in a timely manner (Table 7).

Table 7. Responsibilities for cell therapy manufacturers and their suppliers.

Activity	Manufacturer	Supplier
Qualify the performance of raw material for intended use	✓	
Provide COA, COO, SDS for raw material		✓
Ensure that the raw material is safe with respect to human and animal diseases		✓
Conduct a risk assessment of the raw material for use in cell therapy manufacturing	✓	
Confirm COA tests critical to final cell therapy product	✓	
Characterize the raw material and set specifications		✓
Assess lot-to-lot variation of the raw material on the final cell therapy product	✓	
Determine biocompatibility, cytotoxicity, and additional safety testing (if not available from supplier)	✓	
Assess residual raw materials in the final cell therapy product	✓	
Assess stability of the raw material		✓
Prepare regulatory support documentation (Master File or RSF)		✓
Execute quality and supply agreements		✓



Summary

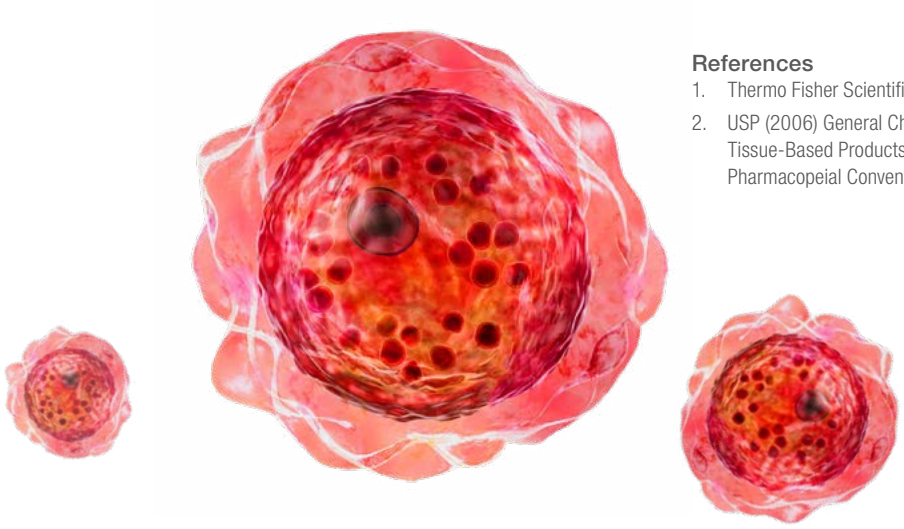
Numerous regulatory challenges exist in the selection of quality raw materials for use in cell therapy manufacturing. No global standard is available covering the critical attributes of raw materials used in a cell therapy, making it difficult to cover all regions a drug might be used. There is also no specific cGMP guidance on the manufacturing of raw materials used in cell therapies and confusion over terminology used exists, making identification of an appropriate supplier a more burdensome process. These challenges put more pressure on developers to define a strategy that balances the costs and performance of a raw material, while mitigating risks. This strategy should be formulated with a long-range view so as to avoid the need to substitute raw materials at later stages of clinical development and trials. This might include developing a product to meet the most stringent regulatory requirement of the regions for which it is intended.

A cell therapy's ingredients are critical to developing a reproducible and robust manufacturing process. Proper sourcing of materials, early in development of a cell therapy, from reliable suppliers who make products specifically for cell therapeutics can shorten the development timeline, dramatically reduce costs, and improve the likelihood of approval from regulatory authorities.

Additional resources

This article provides an overview of the numerous challenges and considerations that a cell therapy manufacturer must address while selecting appropriate raw materials. For more in-depth discussions on these topics, we recommend **Manufacturing Pluripotent Cell Therapeutics** [1], an on-demand webinar about **GMP ancillary materials for cell and gene therapy manufacturing**, and numerous publications from a variety of regulatory agencies, including:

- Ph Eur 5.2.12 Raw Materials of Biological Origin for the Production of Cell-Based and Gene Therapy Medicinal Products
- USP <1046> Cell- and Tissue-Based Products
- USP <1047> Gene Therapy Products
- USP <1043> Ancillary Materials
- USP <1024> Bovine Serum
- USP <90> Fetal Bovine Serum
- USP <89> Enzymes Used as Ancillary Materials
- USP <92> Growth Factors and Cytokines
- Japan's Standard for Biological Ingredients+
- ISO Working draft Ancillary Materials Present During the Production of Cellular Therapeutic Products



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Section 2:
Overview of the vendor qualification process

Introduction

Vendor (or supplier) qualification (VQ) is the process of determining a vendor's capability to fulfill the specified requirements of necessary products or services. For manufacturing of cell therapies, necessary goods and services can cover a broad range, including raw material selection, aseptic filling, manufacturing, formulation and cryopreservation services, analytical assays, kitting services, and cold chain distribution. The VQ process informs all involved parties that the products and services meet the acceptable criteria for identity, quality, and purity, and provides assurance that the product and service consistently meet the specified GMP requirements.

The cell therapy industry is in its nascent stage, currently with minimal standardized regulatory policies or guidelines for VQ. However, most manufacturing entities (sponsors and contract development and manufacturing organizations) adhere to standards established by the FDA, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH E6 R2 and ICH Q10), and the International Organization for Standardization (ISO). Unfortunately, these agencies provide minimal details on VQ programs specifically for cell therapy manufacturers, requiring these manufacturers to establish a robust VQ program as a first step in monitoring, identifying, and mitigating vendor-associated risks.

A typical VQ process can be divided into 4 steps:

1. Define vendor requirements and develop a vendor questionnaire
2. Compile a list of potential vendors and evaluate capabilities to identify the top candidates
3. Conduct a comprehensive audit and choose appropriate vendors
4. Develop and implement vendor requalification plan

Define vendor requirements

The first step in a vendor evaluation begins with defining vendor requirements and designing a comprehensive questionnaire. Vendor requirements should address several varied attributes (see Table 1 for some topics). The questionnaire should also address other significant vendor policies such as change control management (e.g., changes in internal suppliers and production locations; change notification policy timing and exceptions). In the end, the vendor's responses to the questions should help assign risk levels to several key areas including the vendor's process performance and quality management system, corrective action and preventive action (CAPA) system, and change management system (CMS). (See below, compile a list of potential vendors and assess their capabilities).

Table 1. Common topics covered in a vendor selection questionnaire.

• Vendor's skills to deliver the materials and services
• Vendor's open, timely, and transparent communication, with a well-defined plan to manage emergencies
• Vendor's control of its policies and procedures to ensure consistent performance
• Vendor's commitment to maintain quality and performance
• Vendor's guarantee in the form of documents that prove the ability to deliver consistent products and services for timely delivery
• Vendor's sustainability policy
• Cost associated with requested goods and services
• Capacity for timely delivery of required products
• Policies and strategies in place to anticipate and mitigate changes related to the internal supply chain, warehouse, raw materials, and manpower
• Financial standing (cash reserves) and resources to cover any future increased commercial manufacturing demand
• Alignment of supplier and customer corporate cultures and core values

Compile a list of potential vendors and assess their capabilities

The next steps include developing a list of relevant vendors of the raw materials and determining each vendor's capabilities and other attributes based on their responses to the questionnaire. While the questionnaires are being completed by potential vendors, the internal team conducts a further internal assessment focusing on a literature review, the vendor's technical capabilities, the vendor's regulatory history (e.g., FDA 483 documents, recalls, warnings, etc.), the vendor's annual reports, and any previous or current client references.

Upon receipt of the completed vendor questionnaire, the quality assurance (QA) team of the manufacturing entity reviews the questionnaire for completeness and acceptability within a specified time period from receipt. Any identified concerns arising from the internal research or the questionnaire triggers a written response to the vendor's QA team requesting specific clarifications needed to make a final selection. Once all questions are answered satisfactorily, the initial evaluation process is complete, resulting in a narrowed vendor list. The evaluation also highlights specific items that require close scrutiny in the audit phase.

An important step in this exercise includes evaluation of a vendor's own supply chain strategy. This relates to understanding the quality and origin of vendor's raw materials, business continuity, and contingency plans for uninterrupted supply. One of the worst scenarios facing a cell therapy manufacturer is the need to replace or substitute a raw material during clinical trials. Likewise, once a product is commercialized, the manufacturer needs to closely monitor the raw material supplies in order to avoid delays in production. The supply chains for manufacturing specific cell therapies can be quite complex and require overseeing of a large number of suppliers. A risk-based approach to this oversight can simplify that task [1].

A risk-based strategy would evaluate the individual raw materials based on their criticality to the manufacturing process, leading to a framework that allows the manufacturers to assign risk levels to the supplier's capabilities. This approach also allows manufacturers to better allocate time and resources to monitor the materials after commercialization. Table 2 provides an example of some general factors associated with risk, although individual manufacturers would probably have additional issues to add to each level.

Table 2. Risk levels (adapted from reference 1).

Risk level	Associated factors
High	• Custom product with no alternatives or alternatives that would be hard to qualify • Product used in critical steps (e.g., direct and/or patient contact) • Specified source in license where alternative would require additional testing (e.g., stability testing)
Medium	• Product alternatives available • Product used upstream in process; general usage; used in well-established steps • Alternative product available with agency preapproval or only moderate additional testing
Low	• Multiple qualified product alternatives available; safety stock possible • Product used in well-established steps that are common practice in the industry • Alternative product available with minor regulatory concerns requiring only notification or minimal additional testing

Conduct audits and choose final vendor(s)

This narrowed group of vendors moves to the next evaluation step. This process known as an audit, is ideally conducted by a cross-functional team that includes members from QA, process development, manufacturing, and analytical development as well as other technical experts. It is best practice for the manufacturing entity to have a standard operating procedure (SOP) to assess vendor capabilities and attributes under a variety of audit levels.

The type of audit conducted is based on many criteria including past relationships with the vendor, the longevity of the approval status, whether the vendor supplies critical or non-critical products or services, and risk assessment strategies of the manufacturing entity. Several types of auditing processes exist that are categorized by levels of stringency:

1. No audit or check list–minimal impact materials
2. Retrospective audit–qualification based on past performance
3. Paper audit–qualification by an audit check list
4. On-site audit

The types of audits and the frequency of audits required must be defined in the specific VQSOP. It is also common practice to define ongoing audit frequencies in a requalification plan (see below, develop a vendor requalification plan).

Once the audit is complete, the audit team generates an assessment covering the suitability of the supplier's facility and its quality management systems, the supplier's staff and departmental organization (both staff levels and skill sets), a review of the supplier's documentation procedures (e.g., relevant SOPs), and a review of the supplier's supply chain. In some instances, it might be appropriate to ask the supplier to manufacture a test lot of the raw material prior to final selection.

Once the assessment is complete, the QA team along with designated personnel makes the final vendor selection(s). When a vendor is "Approved", QA updates the Approved Vendor and Supplier List and issues a letter of approval to the vendor. If the vendor is deemed "Not Approved", the QA team will collaborate with relevant departments to determine what additional information and steps are required to qualify the vendor. Non-approved vendors can be reconsidered if they provide additional information or put a Corrective Action and Preventative Action (CAPA) in place. If such vendor responses are satisfactory, the vendor may be "Approved". If the responses are not satisfactory or the vendor is not willing to make appropriate changes, they will remain "Not Approved".

In certain exceptions (e.g., additional information is not available or there is no immediate alternative), a risk assessment plan is put in place to determine if the vendor can be used until further required actions are taken to avoid a shutdown of the manufacturing activities. A Quality Agreement is then put in place for all approved vendors.

Develop a vendor requalification plan

After the VQ process and final vendor selection, a plan and SOP is developed for vendor requalification using defined and preestablished intervals. While the initial VQ process involves a detailed evaluation of a vendor's attributes and capabilities, a clinical and commercial manufacturing program's success relies on VQ as an ongoing process, with regular supplier meetings and audits to maintain the highest quality of products and services. The requalification plan defines the types and frequency of audits and is shared with the vendor. The requalification plan also identifies instances that would trigger additional audits, such as a change in manufacturing location, addition of new plants or warehouses, moving operations to another country, and a change in raw materials due to a global shortage of existing raw materials.

Costs associated with VQ process

The financial impact of vendor qualification is high, potentially adding to the cost of new commercial cell therapies. An estimated $130–150 million (USD) is spent annually for on-site and remote new vendor qualification assessments [2]. Table 3 shares some typical costs for various entities. These costs do not include the cost of periodically requalifying existing vendors or the indirect costs of distributing and evaluating requests for information.

Table 3. Typical costs (in USD) associated with VQ audits (VQA) [2].

	Average cost per VQA	Average yearly VQA cost
Overall	$13,259	$270,033
Sponsors	$12,432	$197,940
CROs	$18,704	$666,883
Small companies	$12,607	$150,570
Medium companies	$17,072	$475,445
Large companies	$21,839	$1,886,308

Summary

The high-level regulatory requirements established by the FDA, ICH, and ISO are useful, but lack standardization and specificity for implementing a VQ program for cell therapy manufacturing. This results in highly variable and labor-intensive VQ programs and processes, which can lead to delays and increased cost burdens to cell therapy products. Until the cell therapy industry establishes standards to streamline the VQ process, a current best practice requires a collaborative relationship based on open and timely conversations, clearly defined expectations during the qualification process, and a plan to manage risk and achieve success for both parties.

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Section 3:
Overview of cell isolation, engineering, and expansion

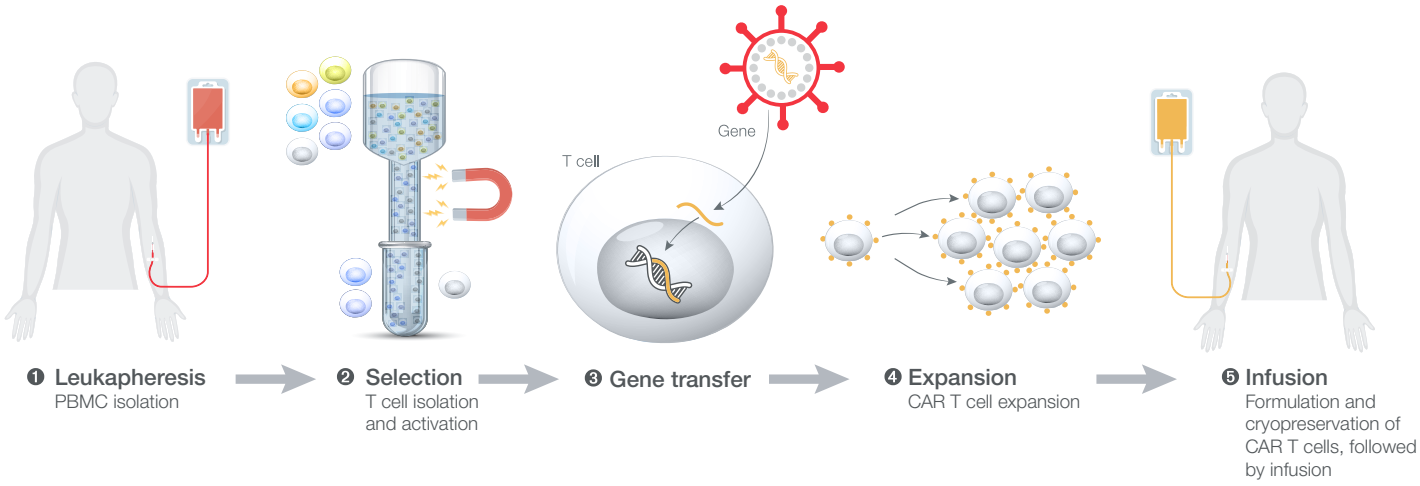
Introduction

The use of chimeric antigen receptor (CAR) technology has contributed towards significant advances in the treatment of certain types of cancer. This technology harnesses the immune defenses (e.g., T cells) to specifically target a patient's cancerous cells with modified immune cells carrying a CAR "payload". As with many new technologies, rapid progress is being made that overcomes the barriers and hurdles associated with earlier generations of the CAR T technology.

These next sections will discuss some of the more recent improvements to the development and manufacturing of CAR T cell therapies, including approaches to T cell isolation, engineering steps to produce CAR T cells, and strategies for the expansion of engineered cells for subsequent patient treatment (Figure 1). This section will also introduce some newer approaches that use natural killer (NK) cells as immunological weapons for cancer treatment. A short introduction to the biology behind CAR T therapies is provided here as a platform to discuss the manufacturing process.What is CAR?

A chimeric antigen receptor (CAR) is an artificial receptor that is engineered to be expressed on immune cells such as T cells and NK cells. For CAR T cell therapies, the T cells are engineered to express a CAR protein that recognizes unique tumor antigens. The CAR protein is composed of an extracellular domain derived from a monoclonal antibody and an intracellular domain derived from T cells. The design and construction of these components is what make CAR T therapy one of the most advanced forms of adoptive cell therapies. The CAR protein is composed of three parts (Figure 2):

1. **The extracellular domain**—a single-chain fragment variant (scFv), which is derived from a monoclonal antibody molecule specific to a unique tumor antigen (e.g., CD19 on leukemia cells)
2. **A transmembrane domain**—to serve as an anchor
3. **An intracytoplasmic domain**—the "functional" component derived from T cells



Autologous CAR T cell therapy (patient's blood)	CAR gene transferred into T cells	Same patient
Allogeneic CAR T cell therapy (healthy donor's blood)	a. Genome editing of T cells b. CAR gene transferred into edited T cells	Multiple patients

Figure 1. Similarities and differences associated with autologous vs allogeneic approaches to CAR T cell therapy workflow.

Extracellular domain (scFv)

The scFV or extracellular domain is the tumor antigen-binding domain, which specifies the CAR T target. It is located on the T cell membrane and is a single-chain antibody fragment derived from a monoclonal antibody. The scFv is made up of the variable region of a light and heavy (VL and VH) chain and is fused to the transmembrane domain with a short linker. Like any antibody, these single-chain antibodies can bind to protein, carbohydrate, and glycolipids [1].

Transmembrane domain

The transmembrane domain functions solely to stabilize the scFv portion of CAR on the T cell surface. The transmembrane domain is usually derived from CD8α but can also be based on CD4 or CD28 [2].

Intracytoplasmic domain

The intracytoplasmic domain, which is derived from the CD3 ς chain, is the functional (or signaling) end of the CAR. After the binding of the CAR scFv to the tumor antigen, the CAR intracytoplasmic (CD3 ς chain) forms a cluster, which will initiate activation signaling, ultimately leading to cytotoxicity of the tumor cells.

The design of each of these parts of the CAR is critical for the success of the anti-tumor response. As expected, there have been several improvements to make CAR T cells kill more efficiently, persist longer in vivo, and be less toxic. For example, a second-generation CAR added an immunomodulator at the intracytoplasmic domain (e.g., CD28 or CD137 (4-1BB)) and improved the killing machinery. When both immunomodulators CD28 and CD137 were added (third-generation CAR), persistency improved [3,4].

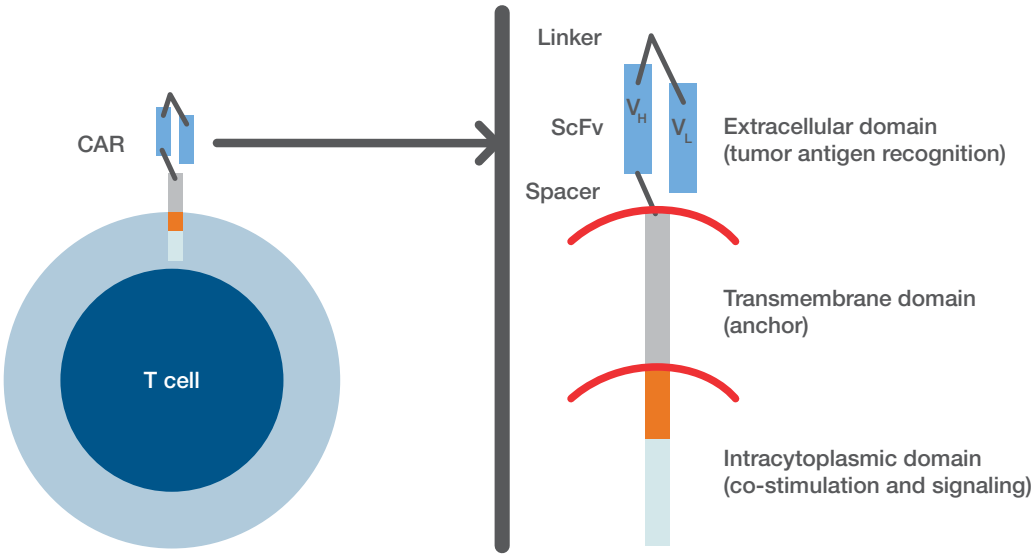


Figure 2. Anatomy of a chimeric antigen receptor.

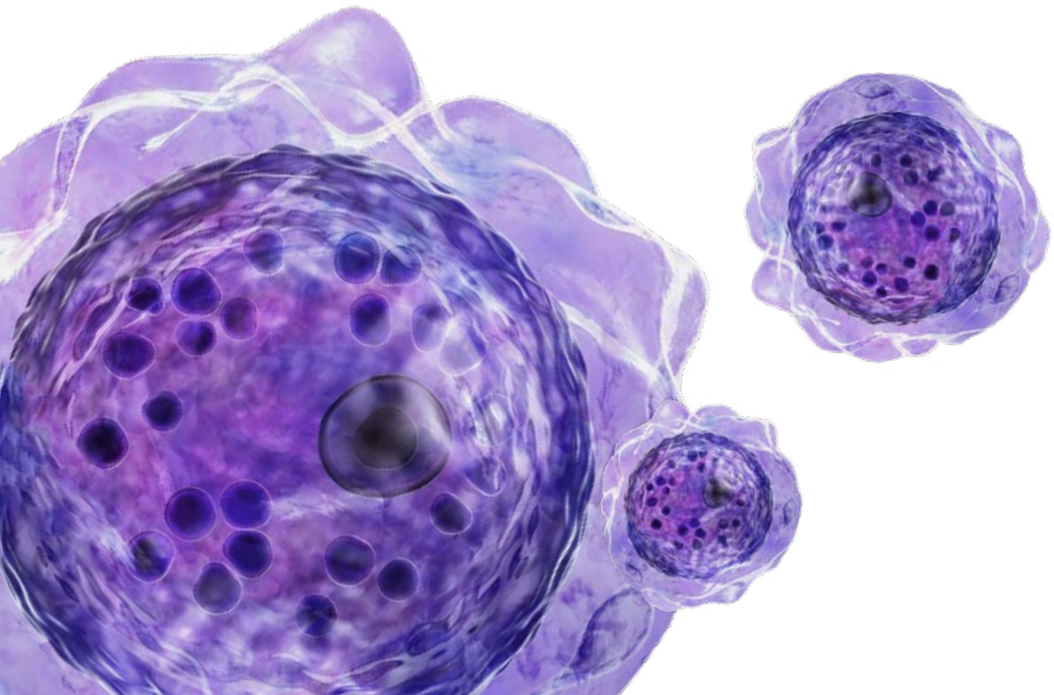
Autologous versus allogeneic CAR T therapies

Early CAR T cell therapy work relied on harnessing the power of T cells isolated from the cancer patient. The patient's T cells were then modified to target cancer cells and infused back into the patient. This process, known as autologous CAR T therapy, typically took 3–4 weeks and had an approximate failure rate of 7–10% [5]. While great success was achieved with this approach, autologous CAR T therapy manufacturing is a lengthy process that extends treatment timelines and is not scalable.

The limitations of autologous CAR T therapy can be overcome by engineering third-party T cells derived from healthy donors. These so called "off-the-shelf" or allogeneic CAR T cells can be made in advance and released for immediate use when needed by the patient. Unlike autologous therapies that directly treat one patient, allogeneic therapies can treat multiple patients. Table 1 summarizes the many benefits to using an allogeneic approach compared to an autologous approach. In addition, the allogeneic approach provides a standard drug product produced from donor sources displaying an optimal immunological profile enriched with stem cell memory T cells (TSCM). This could make allogeneic CAR T cell products a first line therapy for B cell malignancy.

Table 1. Differences between autologous and allogeneic CAR T therapies.

Autologous CAR T product	Allogeneic CAR T product
One product for one patient	One product for multiple patients
Patient donor—high variability in quality and quantity (low TSCM number)	Consistent quantity and quality from selected healthy donors with high TSCM numbers
Cannot select desired T cell phenotypes and functions	Can optimize T cell phenotypes and functions (e.g., edit in homing and growth genes)
Urgent timelines to be met for product generation to meet the need of the individual patient	Prepared and ready for patients when needed
Limited in scalability	Ease of scalability
Single cancer target	Multiple cancer targets (multiple genes edited)
Increased treatment costs (quality testing and regulatory costs specific to single patient)	Decreased treatment costs (quality testing and regulatory costs spread over many patients)



Issues with allogeneic CAR T therapy: overcoming patient rejection

While allogeneic CAR T therapy helps to address some of the issues encountered with autologous approaches, it still faces serious hurdles. Most importantly, allogeneic CAR T therapies can cause serious life-threatening reactions arising from patient rejection—where the patient's own immune system recognizes the donor cells as foreign [6,7]. Figure 3 illustrates the basic biology behind patient rejection of allogeneic T cells. This rejection is driven by the interaction of human leukocyte antigen (HLA) class I and T cell receptors (TCR) that are expressed on both the donor's and patient's T cells and can lead to three rejection scenarios:

- Graft-versus-host disease (GvHD)
- Host-versus-graft-disease (HvGD)
- A patient's NK cells attacking allogeneic CAR+ T cells with masked HLA (a modification strategy used to avoid the first two scenarios)

TCR is a membrane-bound protein consisting of α and β chains and is expressed as part of the CD3 complex molecule on the surface of all T cells (Figure 3). The surface-displayed HLA class I molecules appear ubiquitously on cells throughout the body and consist of α chains that are stabilized by β2-microglobulin (β2M, Figure 3).

An HLA class I molecule is made up of a group of 6 genes that are designated as A, B, C, E, F, and G. These genes are further divided based on their polymorphism. The genes A, B, and C are highly polymorphic with over 6,000 alleles represented in each one of them. The non-polymorphic genes are E, F, and G with allelic variants of less than 300 [8,9].

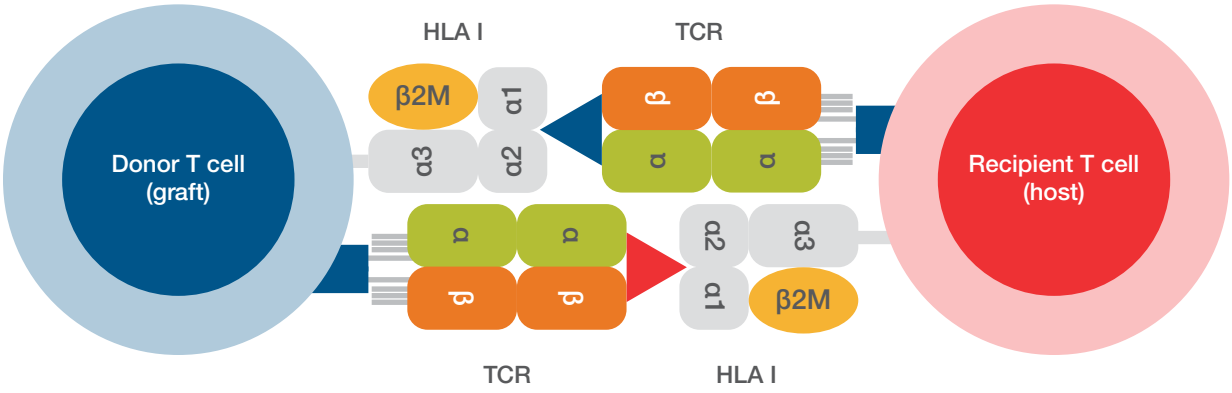


Figure 3. Biology of HLA I and TCR interaction and patient rejection. The interaction between the TCR and HLA I on both donor and patient T cells drives two rejection pathways. In graft vs. host disease, the donor TCR recognizes the allogeneic peptide/HLA I of the host T cell, resulting in rejection (i.e., killing) of the host cell. In host vs. graft disease, the opposite occurs—the host TCR recognizes the donor HLA I as foreign and targets it for killing.

The mechanism of rejection is initiated by the recognition and interaction of the TCR αβ chains of T cells with the HLA class I molecules (Figure 4). In graft-versus-host disease, the allogeneic rejection arises when the TCR on donor T cells regards the HLA class I complex on the recipient's cells (tissues) as foreign and attacks it [8]. Similarly, in host-versus-graft disease, the TCR of the recipient's T cells recognizes the HLA class I complex on donor T cells as foreign and attacks it.

To remove these rejection barriers, scientists can exploit the fundamental biology of the TCR and the HLA class I complex. More specifically, the disruption of β2M through gene editing can be used to prevent mature donor HLA class I molecules from reaching the cell surface, essentially shielding the donor T cells from recipient T cell recognition and elimination. Similarly, disruption of the donor cell TCR α or β chains through gene editing can prevent the recognition and attack of donor T cells by the recipient T cells.

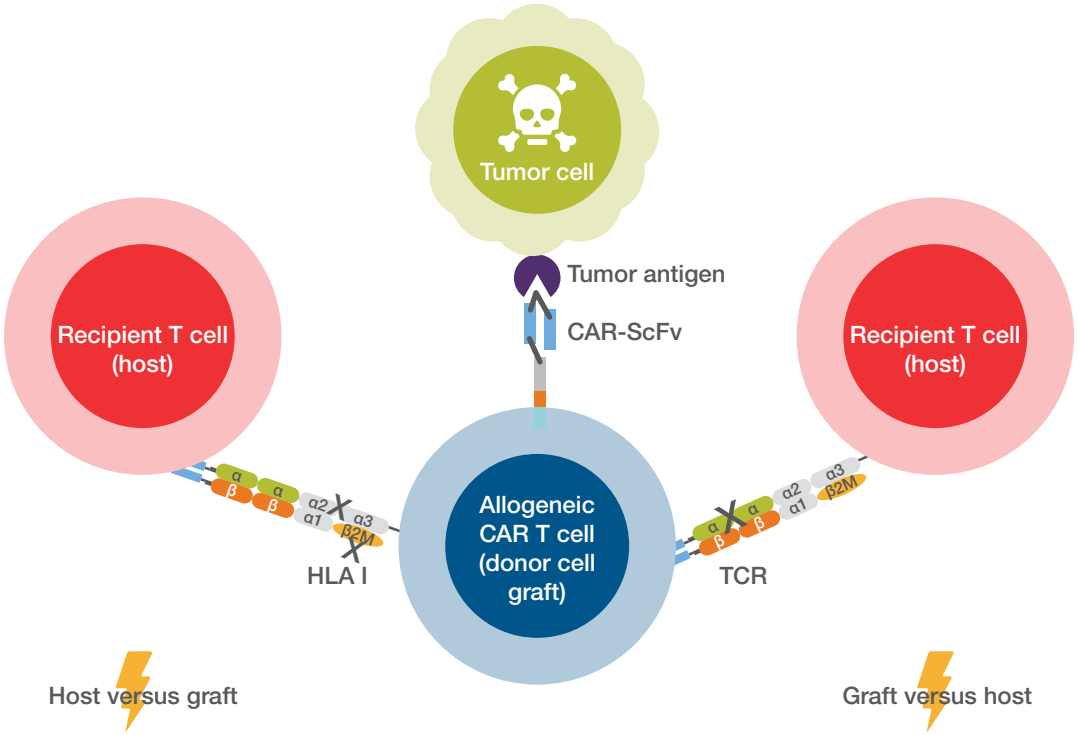
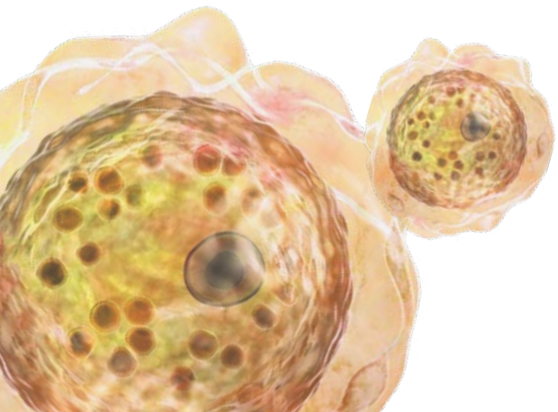


Figure 4. Off-the-shelf strategy to prevent allogeneic rejection responses. Right side: Elimination of the TCRα chain on the grafted allogeneic CAR T cells prevents graft vs. host disease (GvHD). Left side: Elimination of HLA I through disruption of β2M on the grafted allogeneic CAR T cells prevent host vs. graft disease (HvGD).



These approaches, however, can lead to a third rejection barrier caused by the loss of the HLA class I on the donor cells, rendering the donor cells susceptible to targeting by the recipient's own NK cells (Figure 5), also known as the "missing self signal" [10,11]. To overcome recipient NK cell-mediated elimination of HLA– or TCR– donor cells, researchers can genetically modify the donor cell to express an inhibitory molecule such as non-polymorphic HLA-E (Figure 5) [12,13]. This modification can be performed by inserting or "knocking-in" the sequence to HLA-E fused with β2M. This step leads to the stable expression of a type of HLA class I molecule on the donor cells and prevents recipient NK-mediated killing of those cells (Figure 5).

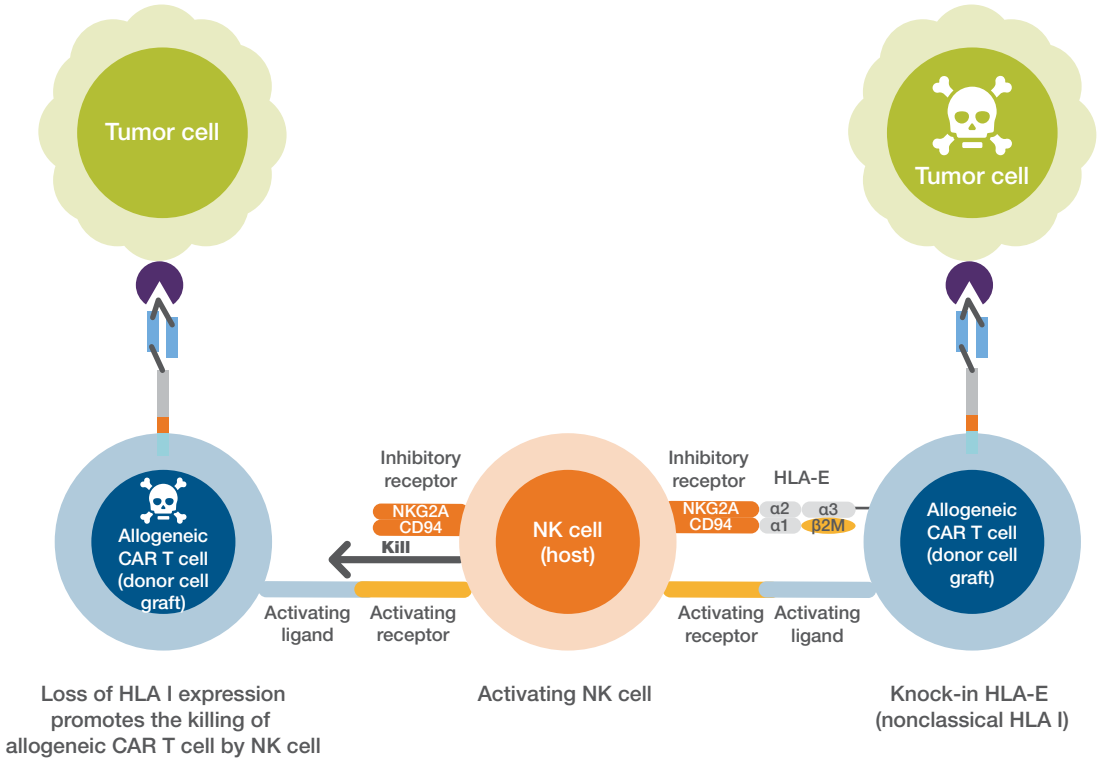
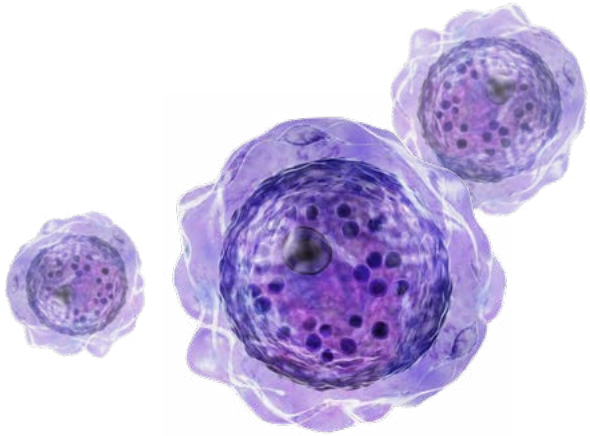


Figure 5. Addition of HLA-E on donor T cells will prevent host NK killing. An allogeneic CAR T cell that lacks the HLA I protein would be a target for the NK cells (also known as the "missing self signal"). This is a normal immune reaction to cells that do not express HLA I. Engineering cells with a knock-in HLA-E gene will prevent the killing of the allogeneic CAR T cells by the host NK cells.



Summary

The choice of allogeneic T cell sources allows for the use of material that features a higher quality starting blood from healthy third-party donors and improved immunological cell makeup, which can improve scalability of the final product. It also provides an approach to treat multiple patients unlike an autologous approach. The use of allogeneic T cell sources can lead to patient rejection outcomes (e.g., GvHD and HvGD), and technical advances are being utilized to mitigate some of these issues through the use of gene editing of HLA class I or TCR genes to overcome the "foreignness" seen in the host system. Clinical successes provide evidence that allogeneic CAR T cell therapies employing some of these masking techniques enable use of this approach for a wider number of patients and are fueling the growth in this space with multiple allogeneic CAR-focused companies already testing allogeneic CAR T therapy in the clinic (Table 2).

Table 2. Allogeneic CAR T companies with clinical trials.

Companies	Products	CAR targets	Allogeneic cell sources
Allogene and Pfizer	UCART19	CD19	T cells
Kuur Therapeutics	KUR-502	CD19	NK, T cells
Cellectis and Pfizer	UCART19, UCART123	CD19, CD123	T cells
Celyad	CYAD-211	BCMA	T cells
CRISPR Therapeutics	CTX110	CD19	T cells
Fate Therapeutics	FT819	CD19	iPSC-derived T cells
Poseida Therapeutics	P-BCMA-ALL01, P-MUC1-ALLO1	BCMA, MUC1	T cells
Precision Biosciences	PBCAR269A	CD19	T cells
Tessa Therapeutics	CD30.CAR-EBVST	CD30	EBV T cells

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Section 4:
Cell isolation

Introduction

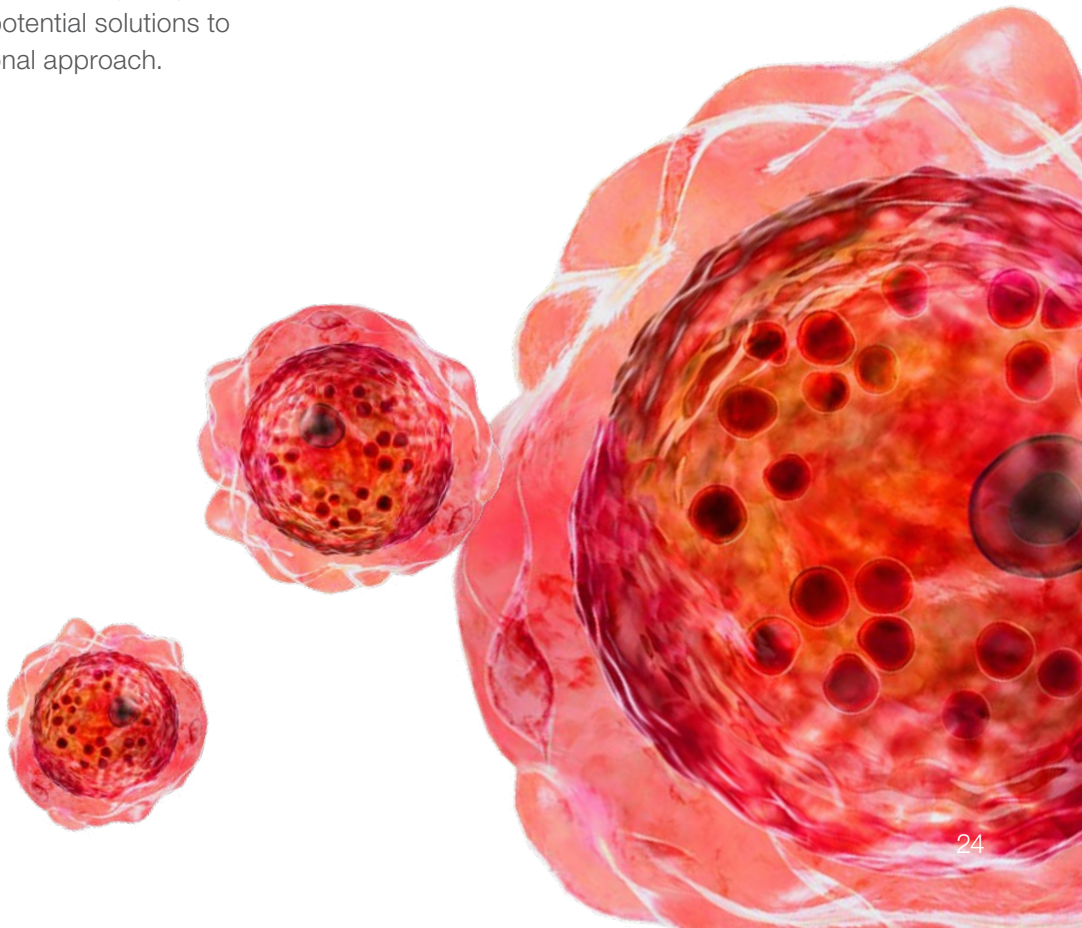
At the foundation of any cell therapy development and manufacturing workflow, the quality of the starting cellular material directly impacts the final product's viability and the efficacy of patient treatment. Most cell isolation methods, specifically peripheral blood mononuclear cell (PBMC) isolations, are currently performed using open systems, which can contribute to errors and contamination, resulting in the failure to produce a viable cell therapy. Additionally, donor product variability can lead to differences in cell composition, cell viability, and sensitivity during the expansion stages of the manufacturing process. For a multitude of reasons, not all patients, including healthy donors, are able to effectively mobilize functional T cells at the desired numbers suitable for the process. Therefore, T cell isolation workflows must be flexible to allow for various modifications, while still yielding a standardized CAR T cell product, regardless of the input material harvest from the patient or donor. The ideal isolation workflow would also be automated, closed, and consistent.

This section will discuss the isolation of PBMCs and selection and activation of the T cell populations (CD3+) to be engineered. This section will also discuss alternative cell approaches using induced pluripotent stem cell (iPSC)-derived cells and NK cells, which are potential solutions to overcome issues with the more traditional approach.

Healthy donor characteristics

The development of an allogeneic cell therapy begins with the isolation of T cells from donated blood in a process known as leukapheresis. To go to a clinical trial, a sufficient amount of donated blood is needed with the correct cell composition and phenotypes. For an allogeneic therapy, the ideal donor blood is preferably from a young individual. The donated blood composition should have a low percentage of monocytes and neutrophils (specifically granulocytes), and cells should demonstrate an efficient doubling time. The donor blood should have an immunological profile consisting of a normal CD3+ cell number, a balanced CD4/CD8 ratio, a sufficient number of stem memory cells, and expression of CD62L+CCR7+ T cells.

As expected, cell therapy developers and manufacturers want the highest purity of T cell population possible and sometimes look for specific subsets of T cells. Poor quality starting material may result in an inability to use it for cell therapy processing and ultimately, failure of the donor product reaching the clinic. However, efficient cell isolation methods that are reliable with high yields and purity can alleviate the pressure on the quality of donor blood.



PBMC isolation

Once donated blood is obtained and characterized, the next step is to isolate PBMCs. Typically, the isolation methods are characterized as either open or closed, depending on the amount of direct user interaction needed. Closed methods are preferred because of a decreased risk for contamination and user error, and for some clinical applications, might be required.

The most well-known practice to isolate PBMCs relies on density gradient centrifugation using Ficoll medium [1]. Although this method successfully isolates PBMCs from red blood cells, the isolated PBMCs retain contaminants such as granulocytes, monocytes, and even some residual red blood cells. Most density gradient centrifugation isolations are performed using an open system, which makes the procedure prone to errors, contamination, and user-to-user variability. While automated closed systems for density gradient centrifugations exist, these systems tend to lose cells, lowering yields due to the lack of system flexibility and can be significantly more expensive.

A more recent closed system approach relies on counterflow centrifugation, which separates cells based on their size and density. Systems using this technology (e.g., Gibco™ CTS™ Rotea™ system) suspend cells in a fluidized bed by exerting a constant flow force against centrifugal forces (Figure 1). The suspended cells are gently concentrated without forming a pellet and then washed with very high recoveries. Using elutriation, dead cells can be removed to optimize viability of the population. Adjustments to centrifugal speed and flow rate allow for cells to be fractionated based on size and density, with minimal shear.

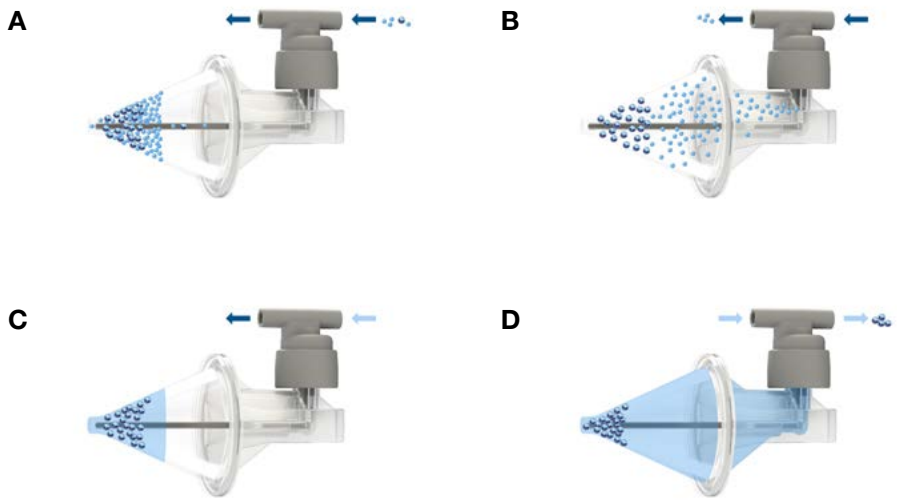


Figure 1. Principle behind counterflow centrifugation (CFC). The closed system CTS Rotea instrument relies on counterflow technology to separate cells based on size and density. **(A)** Cell loading: With "balanced" *g*-force and counterflow parameters, input material comprising media and cells is introduced to the CFC chamber via the central tube. **(B)** Elutriation: Larger or denser cells are captured in the fluidized bed, while smaller or less dense cells and debris pass through and are "eluted" through the top of the CFC chamber. **(C)** Media exchange and washing: Wash buffer is pumped through the fluidized bed, replacing the original media in the input product. Note: The fluidized bed enables very fast and efficient washing. **(D)** Cell concentration: Washed and concentrated cells are now recovered from the CFC chamber by simply reversing the pump and extracting the concentrate via the internal tube. More details and a video on counterflow centrifugation technology can be found here.

Density gradient centrifugation and counterflow centrifugation produce comparable results (Figure 2). However, distinct advantages of the counterflow centrifugation methodology are that it can be performed more quickly, and most importantly, in the more desirable closed system setting. Table 1 summarizes the PBMC isolation methods.

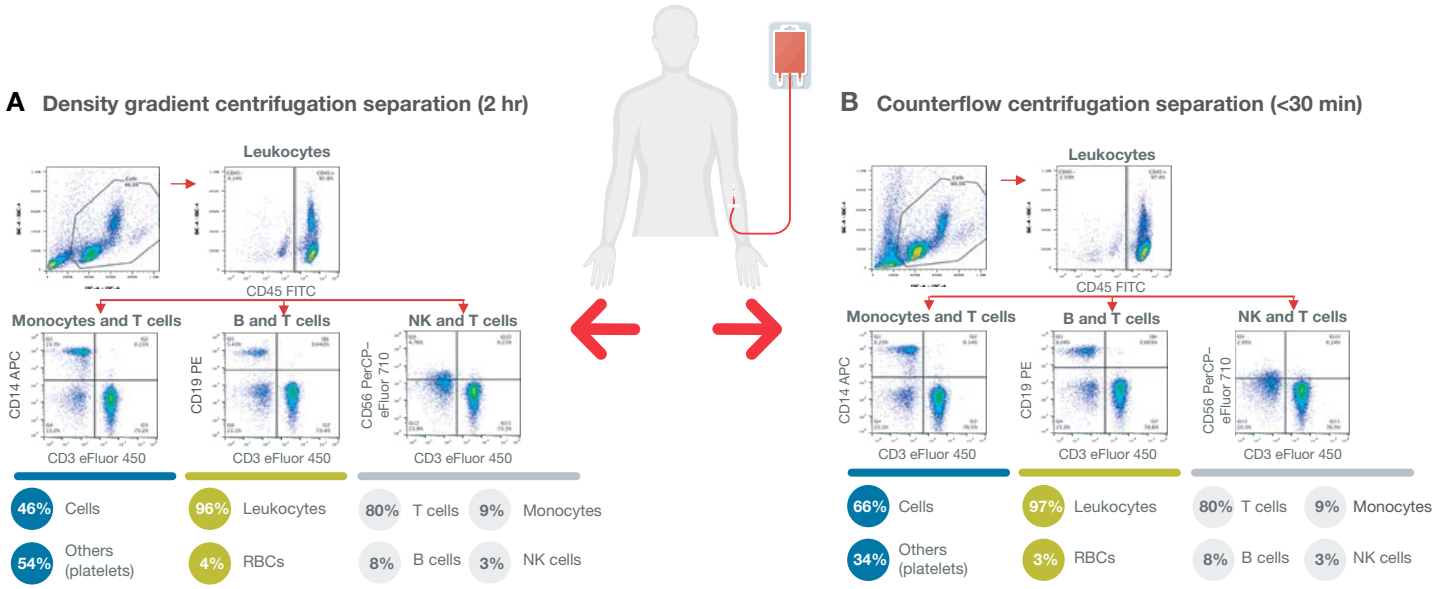


Figure 2. Closed counterflow centrifugation system versus open density gradient system for isolation of PBMCs from red blood cells. Fluid from a single-donor leukopak was divided in half, and PBMCs were separated using **(A)** density gradient centrifugation using Ficoll polymer or **(B)** counterflow centrifugation using the CTS Rotea system. The CTS Rotea system can isolate PBMCs from a leukopak in less than 30 minutes, with nearly equivalent performance to the density gradient system and the added benefit of closed processing.

Table 1. Comparison of PBMC isolation methods.

	Density gradient centrifugation	Counterflow centrifugation
Advantages	Well-known Widely used Inexpensive (open system only)	Shortened processing times Protocol flexibility Less user error and variability Less contamination Automation-capable
Disadvantages	Long and tedious process More expensive (closed system)	More expensive (closed system)

T cell isolation and activation

Following PBMC isolation, the next step in the process is the isolation of T cells to remove any lingering contamination of other cell types and to improve product specificity. This is then followed by activation. The inherent variability with allogeneic donor blood leads to an inability to differentiate between cell types. To overcome this problem, magnetic bead-based approaches for selection of T cell populations have been developed. These approaches use magnetic beads conjugated to antibodies that recognize T cell surface markers and bind to them. When placed in close proximity to a magnet, the bead-T cell complex binds and is held while unwanted cell types can then be washed away. Once isolated, the T cells can be released from the beads (see Figure 3).

Several magnetic bead products are commercially available. Some of these platforms can be used for very specific cell populations (e.g., CD4/CD8+ or CD62L+), which are activated later through other means. Another system, Gibco™ CTS™ Dynabeads™ CD3/CD28, provides both the primary and co-stimulatory signals required for activation and expansion of T cells, eliminating the need for a separate activation step and reducing the potential to introduce contamination.

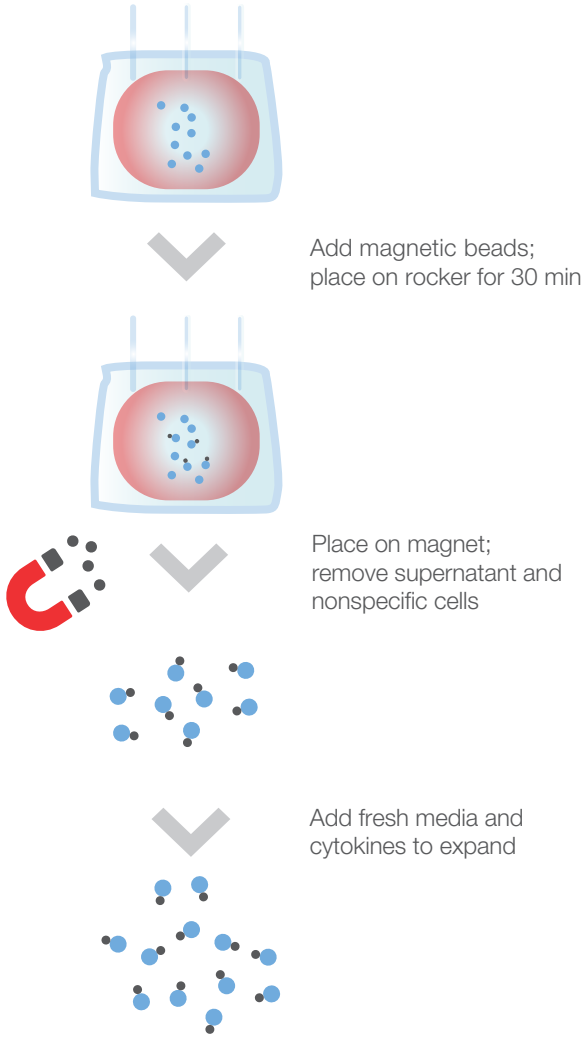
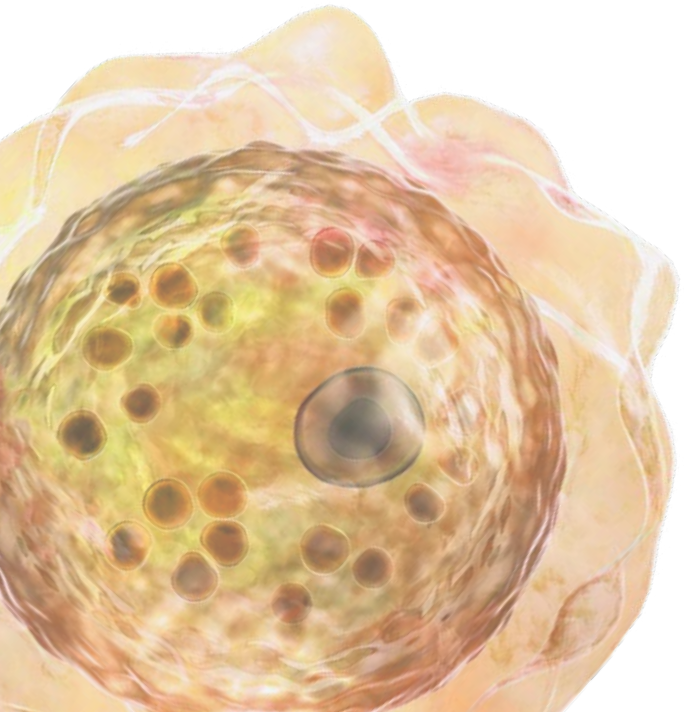


Figure 3. Use of CTS Dynabeads CD3/CD28 to isolate and activate T cells for subsequent engineering. PBMCs are harvested and are activated using the CTS Dynabeads CD3/CD28 (bead:cell ratio of 3:1) in cell culture bags. The cells and beads in culture media are incubated for 30 minutes on a rocker. After the 30 minutes, the culture bag is placed on the Gibco™ CTS™ DynaMag™ Magnet and CD3/CD28+ cells attached to beads are captured. The supernatant and non-specified cells are discarded and fresh media with cytokines is added for simultaneous activation and expansion of the T cells. Using this approach, CD3+CD28+ T cells are isolated with over 90% recovery, uniformly stimulated (>95% CD25+), and a highly pure population with over 95% CD3+, with no need for antigen presenting cells (APCs). For more details on this process, see **One-step isolation and activation of naive and early memory T cells with CTS Dynabeads CD3/CD28.**



iPSC-derived CAR NK cells

One major issue with allogeneic workflows is insufficient cell numbers or starting material to create a product for patient infusion. To overcome this limitation, a revolutionary approach has been developed to derive NK cells or T cells from iPSCs. The use of NK cells reduces rejection barriers (e.g., graft-vs.-host disease) and NK cells can be generated from several different sources such as umbilical cord blood, bone-marrow, human embryonic stem cells, and iPSCs.

A significant advantage of NK cells is that unlike T cells, they exhibit improved survival after killing multiple target cells. NK cells also produce a different profile of cytokines than T cells. The cytokines produced by T cells cause cytokine release syndrome (CRS), a life-threatening condition that has been observed in some patients using an adoptive cell therapy, and this can potentially be avoided with the use of NK cells.

The most common cause of allogeneic therapy rejection is due to differences in the HLA class I gene of iPSC-derived products, resulting in a mismatch between the donor and recipient. T cells regularly interact with HLA complexes, thus any changes to the HLA complex on the surface would be an indication that the substance is foreign. Conversely, NK cells can exhibit cytotoxicity toward different tumor targets in an HLA-independent manner, which could mitigate the mismatch between donor and recipient. The isolation of primary NK cells is difficult and complex and can lead to low yields, making iPSC-derived CAR NK cells a more attractive choice for allogeneic workflows. To this end, the main goal is to identify an iPSC line(s) that can avoid allogeneic rejection and reduce the time to move cell therapies into the clinic.

Summary

Preparation of allogeneic CAR T cells requires identification of healthy third-party donors and isolation of a sufficient number of T cells for the subsequent engineering steps. Ideally, isolation would be performed in a closed system environment that can provide flexibility for modifications to easily account for differences in source material. Recent advancements have investigated use of other sources or cell types (i.e., iSPC-derived NK cells) to further expand and improve the use of the CAR technology.

Additional resources

- **Grievink HW, et al. (2016) Comparison of Three Isolation Techniques for Human Peripheral Blood Mononuclear Cells: Cell Recovery and Viability, Population Composition, and Cell Functionality. Biopreserv Biobank 14(5):410–415.**
- **One-step isolation and activation of naive and early memory T cells with CTS Dynabeads CD3/CD28.**
- **Habib S, Tariq SM, Tariq M. (2019) Chimeric Antigen Receptor-Natural Killer Cells: The Future of Cancer Immunotherapy. Ochsner J 19(3):186–187.**
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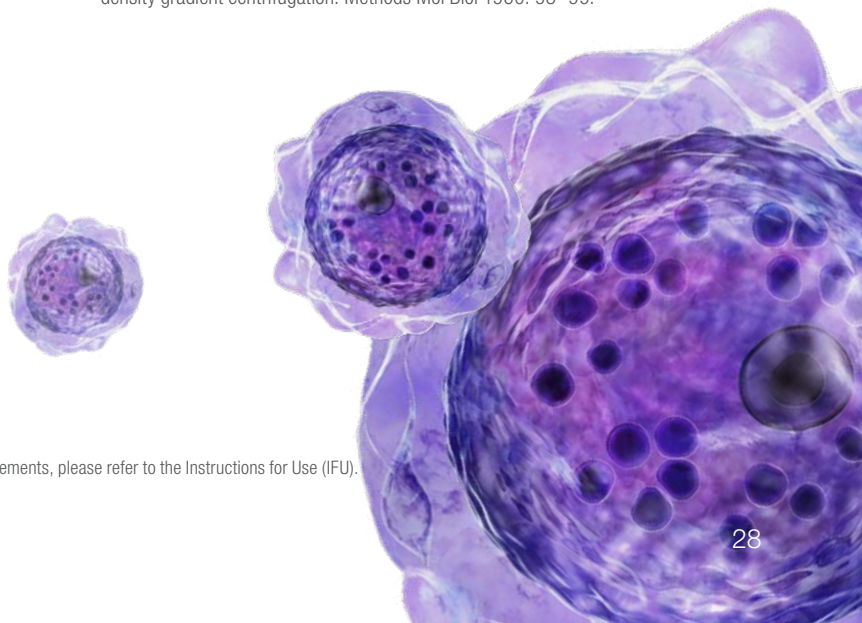


Section 5:
Cell engineering

Introduction

The next step in the development of an allogeneic-based CAR T cell therapy is engineering or making changes to the genetic makeup of the isolated T cells. These changes ultimately produce T cells that circumvent the life-threatening issues of rejection (for additional background information, **see Section 3**, overview of cell isolation, engineering, and expansion). The changes also introduce the chimeric antigen receptor (CAR) that targets antigens on the surface of tumor cells. Numerous cell engineering approaches exist and this section will describe three gene editing tools currently used: zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (CRISPR-Cas9). Several methods for delivering these tools to T cells, as well a general overview of the engineering workflow, will also be discussed.

Knock-ins versus knockouts

The design of the off-the-shelf or universal CAR T cells from third-party, allogeneic healthy donors uses gene editing tools to mutate specific genes by targeting changes in the DNA of the donor cells. These tools facilitate gene editing in allogeneic T cells in two ways (Figure 1):

1. **Knock-in**—adds a gene of interest to achieve the desired function in cells (e.g., adding HLA-E to prevent host NK killing of the allogeneic CAR T cells)

2. **Knockout**—disrupts unwanted gene functions of the donor T cells, typically through deletions of genetic sequences (e.g., eliminating the TCR αβ chains of the TCR).

To knock in or knock out genes, scientists usually rely on a DNA-specific endonuclease that is directed to a specific cut site using a "guide" protein or nucleic acid sequence. After the double-stranded DNA is cut, cellular repair mechanisms fix the cut region of the gene. These double-stranded DNA repairs can be completed via two mechanisms: nonhomologous end joining (NHEJ) or homology-directed repair (HDR). The imprecise NHEJ is error prone and can lead to small insertions or deletions (indels) at the target site. If the NHEJ repair is made precisely in the coding region of the targeted gene, an indel or knockout of that gene will be produced (Figure 1). With HDR, a donor template sequence (e.g., HLA-E T cell engineering) flanked by the sequences homologous to those surrounding the cut site is added to the reaction for insertion via recombination. This desired sequence insertion results in a precise gene addition known as knock-in mutation (Figure 1).

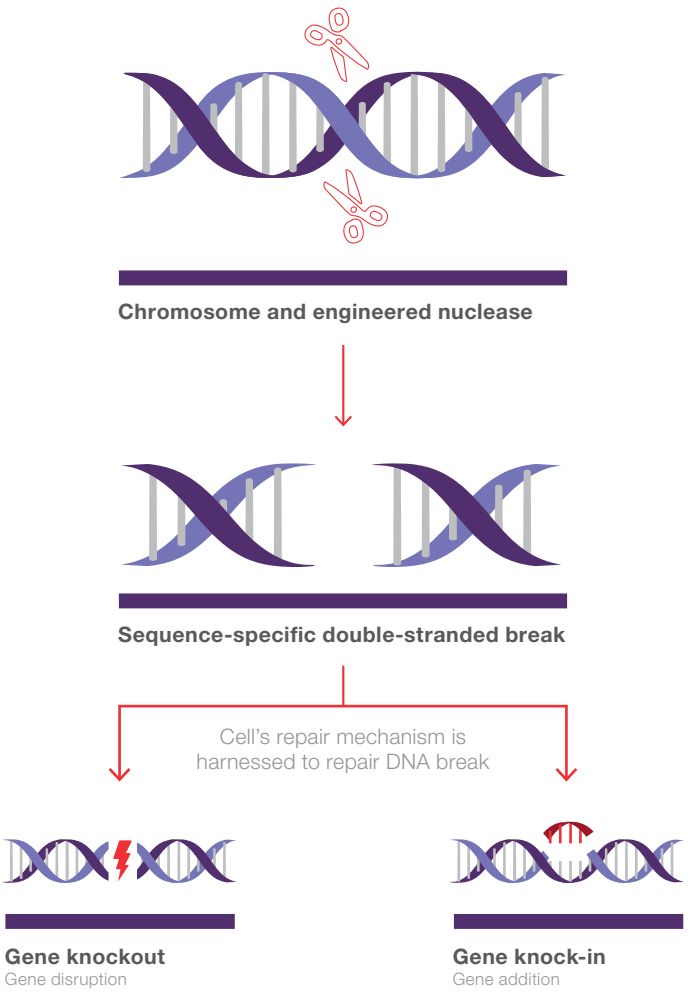


Figure 1. Type of mutation is guided by the specific cellular repair mechanisms.

Gene editing technologies used to create allogeneic T cells

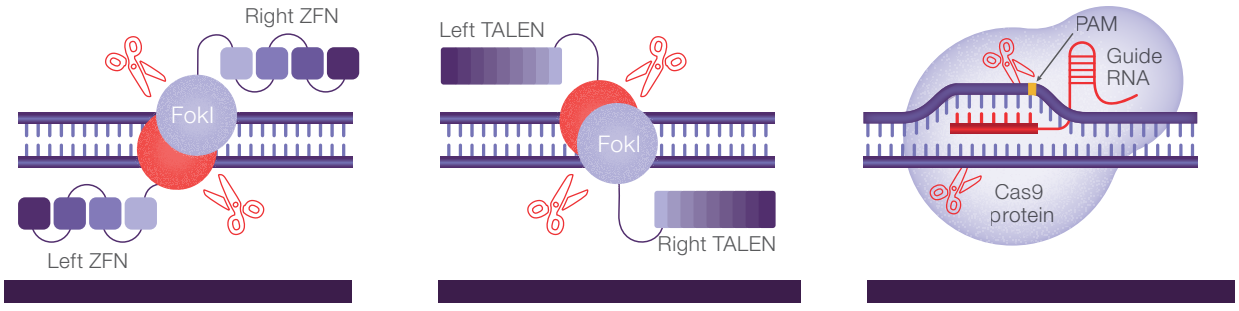
Numerous approaches to targeted genome modification can generate these permanent mutations, but three gene editing tools have been well-studied for creating allogeneic CAR T cells with **either a knockout TCR complex or knock-in HLA-E gene**: ZFN, TALEN, and cluster regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (CRISPR-Cas9) (Figure 2) [1–4].

ZFN

ZFN is a transcription factor that can be specially designed as an artificial endonuclease that cuts a specific sequence of double-stranded DNA. ZFN consists of two components: the first part is a zinc-bearing DNA-binding protein consisting of several zinc finger modules, which act as a guide to bind to a desired DNA sequence through DNA-protein interactions. The second part is the FokI nuclease, which is connected to the DNA-binding protein via a flexible peptide linker, and cuts the DNA creating a double-stranded break (Figure 2A) [5]. Each zinc finger module interacts with three consecutive nucleotides, so two zinc finger modules will interact with 6 consecutive nucleotides and so on. A fully functional zinc finger DNA-binding domain consists of a chain of 3–6 individual zinc finger modules that hybridize to a highly specific target binding site of 9–18 base pairs, which determines the specificity of the cut region of the DNA sequence. In practice, ZFNs are used as a pair with a right-hand and left-hand ZFN, and each FokI must be dimerized for it to function as a nuclease that can cut the desired double-stranded DNA region. A zinc finger DNA-binding protein can be designed to pair with a desired part of the genome, placing these DNA-binding proteins and the nuclease in the exact region to create the desired mutation or change. Designing ZFN is very time-consuming because the DNA-binding protein (a tertiary structure) needs to "fit" exactly to 3 consecutive nucleotides (Table 1). This process takes twice as long because there is a left and right ZFN requirement. This strict requirement also makes ZFN a highly specific engineering tool that has very few off-target effects.

TALEN

TALEN is another specially designed DNA nuclease similar to ZFN. TALEN also has a DNA-binding protein (TALE) that is derived from Xanthamonas and a Fok1 nuclease as the cleavage domain. Also like ZFN, TALEN works as a pair of modules (i.e., a right-hand TALEN and left-hand TALEN) and the two FokI nucleases must be dimerized to form a functional nuclease that can cleave the targeted double-stranded DNA (Figure 2B). The DNA-binding guide component is 12–20 individual TALE repeats that are arranged in a chain, where binding to a single DNA base pair is based on the repeat variable di-residues (RVDs) at position 12 and 13 of each TALE unit [6]. TALEN offers high target specificity because two TALEN complexes are required for DNA cleavage, but the final TALENs usually take four weeks to synthesize (Table 1).



ZFNs
Zine-finger nucleases (ZFNs)

TAL effector nucleases
Transcription activator-like (TAL) effector nucleases (TALENs)

CRISPR-Cas9 nucleases
Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR- associated protein 9 (Cas9) system

Figure 2. Gene-editing tools for allogeneic T cells.

CRISPR-Cas9

More recently, CRISPR-Cas9 gene-editing technology derived from *Streptococcus pyogenes* has become available and is vastly different from both ZFN and TALEN gene-editing technologies [7] (see also the **CRISPR genome editing resource guide, 3rd edition**). Similar to ZFN and TALEN, there is a desired number of approximately 20 base pairs for the target sequence. CRISPR-Cas9 technology consists of a nuclease component (Cas9) and an RNA component that acts as a guide (gRNA). Unlike ZFN and TALEN, which use protein-DNA interactions and require dimerization of the FokI nucleases for specific cleavage, the CRISPR-Cas9 system relies on RNA-DNA hybridization for target specificity. Another difference centers on acceptable target sites. The CRISPR-Cas9 target site for gene editing must have a protospacer adjacent motif (NGG; also known as a PAM site) in order for the gRNA to "locate" the specific DNA sequence, somewhat decreasing the flexibility of this system (Figure 2C). Despite this design drawback, CRISPR-Cas9 is highly efficient and takes less time to develop in comparison to TALEN and ZFN. Figure 3 presents data describing the high efficiency of creating knockouts using CRISPR-Cas9.

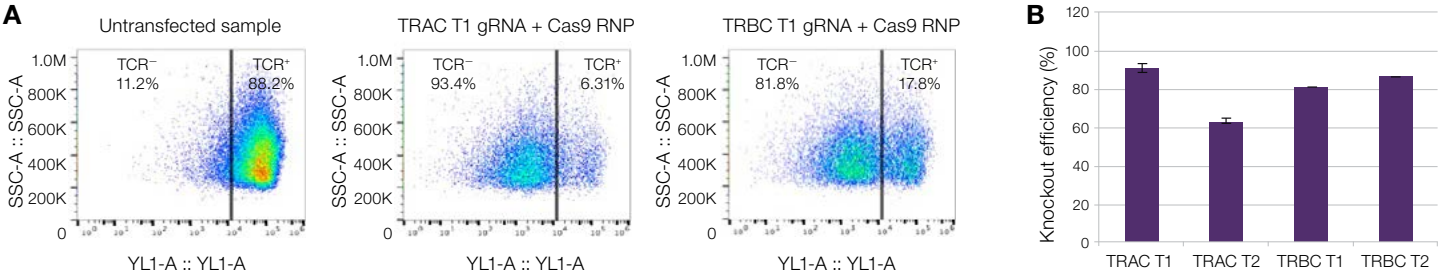
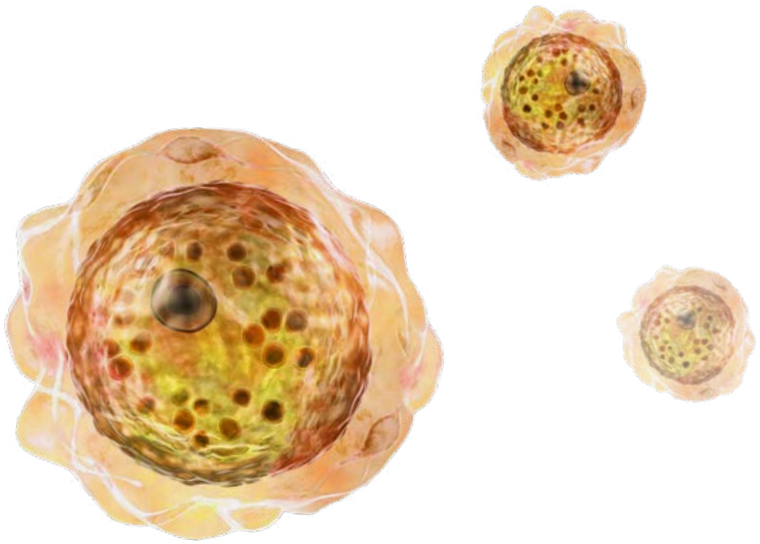


Figure 3. High efficiency functional knockout in T cells. T cells were isolated from PBMCs (from a healthy donor) using Invitrogen™ Dynabeads™ magnetic beads, and then transfected with Invitrogen™ TrueCut™ Cas9 Protein v2 and Invitrogen™ TrueGuide™ modified mynthetic sgRNAs targeting T cell receptor alpha (TRAC) or beta (TRBC) regions using the Invitrogen™ Neon™ Transfection System. **(A)** Analysis by flow cytometry following binding with antibody specific to the TCR shows >90% functional knockout of the receptor. For both TRAC and TRBC, gRNAs specific for two different genomic DNA targets (T1 and T2) were tested; results are shown only for the T1 target in each case. **(B)** Summary of next-generation sequencing (NGS)-based analysis of cleavage efficiency at two different genomic targets (T1 and T2) for both TRAC and TRBC loci. For more details on this experiment, see below, achieve functional knockout in up to 90% of human primary T cells.



Summary of gene editing nuclease systems

The two genome editing technologies, ZFN and TALEN (Figures 2A and 2B), are very specific gene-editing tools because two DNA binding proteins linked to nucleases (left- and right-handed complexes) must be designed for each editing experiment. The genetic sequences for these tools are transferred to a cell to be expressed into functional nucleases. The genome editing technology, the CRISPR-Cas9 system (Figure 2C), simply requires the *in vitro* combination of the designed gRNA and the Cas9 protein to create a single CRISPR-Cas9 ribonucleoprotein (RNP) complex that can induce the double-stranded DNA break once it is delivered into a cell. Table 1 summarizes the three gene-editing technologies and offers examples of their use for off-the-shelf CAR T cell generation.

Delivery systems for gene-editing tools

Delivery of gene-editing tools is challenging because these tools are macromolecules. Unlike small molecule reagents that can easily be dissolved in an aqueous solution and enter the cells by diffusion, macromolecules need an active delivery system to get into the cells. Currently numerous delivery systems are available for *in vivo* and *in vitro* gene-editing applications. These methods include electroporation (EP) or nucleofection, lipid or polymer nanoparticles, cell-penetrating peptides, and viral vectors such as lentivirus, adenovirus, or adeno-associated virus (AAV). The methods are based on one of three basic principles that can facilitate gene-editing tool delivery into the cells:

1. **Charged-surface interaction**—a positively charged particle binds to the negatively charged cell membrane and the cell takes up the particle
2. **Application of an electrical current to the cells**—the electrical current destabilizes the cell membrane, creating pores that allow particles to pass through
3. **Viral vector systems**—modified viruses that are not harmful to humans are used to infect cells and deliver cargo of interest

Here, we will discuss the most used delivery system of each method, and they are lipid-based transfection, electrical pulse-based transfection, and viral transduction (Table 2).

Cationic lipid-based delivery system

This is the most economical and easiest method of delivery for gene-editing tools because this method does not require the use of special equipment or laboratory vessels. This approach relies on lipid molecules to form liposomes to encapsulate the gene-editing tool cargo, aiding transfection into cells. (e.g., **Invitrogen™ Lipofectamine™** reagents). In this method, the positively charged lipid nanoparticle complex fuses with the negatively charged cell membranes, facilitating the delivery of the constructs into the cells through a process called endocytosis. The gene-editing tools are then released into the cytoplasm and ultimately enter the nucleus for gene editing to begin.

Table 1. Summary of three commonly used gene-editing tools for allogeneic T cells.

Editing tool	Target recognition	Nucleases	Nucleotides target length	Nuclease design time	Target gene	Delivery methods	References
ZFN	Zinc finger binding to 3 base pairs	FokI	18–36	~10 weeks	TRAC/TRBC TRAC/TRBC HLA-A	EP Lentivirus EP	Torikai H, et al. [8] Provasi E, et al. [9] Torikai H, et al. [10]
TALEN	TALE protein binding to 1 base pair	FokI	30–35	~4 weeks	TRAC/TRBC TRAC/TRBC TRAC TRAC ß2M	EP EP EP EP AAV	Poirot L, et al. [11] Knipping F, et al. [12] Osborn M, et al. [13] Qasim W, et al. [14] Eyquem J, et al. [15]
CRISPR-Cas9	Hybridization of gRNA with DNA	Cas9	20–24	~1 week	TCR/ß2M TRAC and ß2M TRAC ß2M TCR TRAC/TRBC TRAC	EP AAV EP EP EP EP	Ren J, et al. [16] Eyquem J, et al. [15] Georgiadis C, et al. [17] Ren J, et al. [18] Knipping F, et al. [12] Osborn M, et al. [13]

Electrical pulse-based delivery system

This method can be costly because it requires special equipment and cuvettes. The electrical pulse-based approach involves placing the cells in a cuvette, suspending the cells in conductive buffer, and briefly applying high voltage electrical pulses. The electricity creates temporary pores in the cell and nuclear membranes, which allow the delivery of macromolecules.

There are two electrica pulse-based techniques, electroporation and nucleofection (Table 2). These two techniques differ based on the devices used, parameter controls, types of buffer, and where the macromolecules are delivered into the subcellular space. An electroporation device (e.g., the Invitrogen Neon Transfection System) is an "open system", allowing researchers to manually set the parameters for the optimization of each cell type. The gene-editing nuclease components are delivered into the cytoplasm and ultimately enter the nucleus of the cells. In contrast, a nucleofection device (e.g., the Nucleofector System) is a "closed system" because the electrical pulse parameters and propriety buffer solutions are preset for each cell type by the manufacturer. In this method, most of the gene-editing macromolecules are delivered directly into the nucleus.

Viral-based delivery system

Lentivirus, a single-stranded RNA virus, is a robust vector and one of the most used viral vectors for gene editing. Lentivirus is an excellent vector for both immunotherapy and gene therapy. Studies showed that lentiviral vectors can deliver various CAR constructs into immune cells, deliver gene-editing tools such as CRISPR-Cas 9 into target cells, or deliver a heathy gene to correct diseases such as sickle cell disease, severe combined immunodeficiency, and 18 β-thalassemia [19, 20]. Most noticeable, lentiviral vectors have been approved for the clinical application of delivering CAR constructs into T cells for CAR T therapies. The biggest advantage of lentiviral viral vectors is their high infection (or transduction) efficiency in both dividing and non-dividing cells. The vectors have good safety profiles, have a large carrying capacity, and can maintain long-term transgene expression [21]. The large carrying capacity of single-design lentiviral vectors can deliver Cas9, sgRNA, and a puromycin selective marker into a target cell [22].

Table 2. Delivery methods used for gene editing.

Methods	Description	Advantages	Disadvantages	Examples
Lentivirus vector	DNA or RNA is packaged into the infectious viral particles and introduced into cells	• High transduction efficiency • Suitable for primary immune cells • CAR T clinical usage precedent	• Special lab environment needed • Requires safety measures • Labor intensive	**CTS LV-MAX** LentiCRISPR
Electroporation	Electrical pulse creates pores in the cell membrane, allowing the entry of DNA, RNA, and RNP into the cell cytoplasm the nucleus	• Fast and easy • Large number of cells can be transfected in minutes	• Requires special equipment • Cytotoxic anions can form during the procedure, leading to cell death	**Neon Transfection System** MaxCyte GT (Flow electroporation)
Nucleofection	Similar to electroporation except the tools are delivered directly into the cell nucleus using very specific conditions provided by manufacturers	• Effective for non-dividing cells • High throughput potential–multiple samples can be transfected simultaneously	• Requires special equipment • Less flexible–special protocols and reagents can't be controlled by the user	Lonza Nucleofector™System
Cationic lipids	Positively charged liposomes encapsulate protein and RNP and interact with negatively charged cell membrane, facilitating entry into the cell cytoplasm via the endocytosis pathway	• Easy and versatile (no special equipment), not very toxic and can be a high throughput system	• Lower transfection efficiency • Not direct delivery into the nucleus • Not applicable for all cell types	**Lipofectamine CRISPRMAX Cas9 Transfection Reagent**

Overview of the gene-editing workflow

The complexity of generating engineered allogeneic T cells for CAR T therapy is quite formidable. With the help of gene editing, numerous immunological hurdles can be overcome to create an allogeneic T cell that will be accepted by the recipient patient without causing life threatening consequences. The workflow can be summarized as follows:

- Design and creation of chosen gene-editing nuclease
- Transfection of gene-editing tools into T cells
- Monitoring cleavage efficiency
- Analysis and validation of knock-ins and knockouts

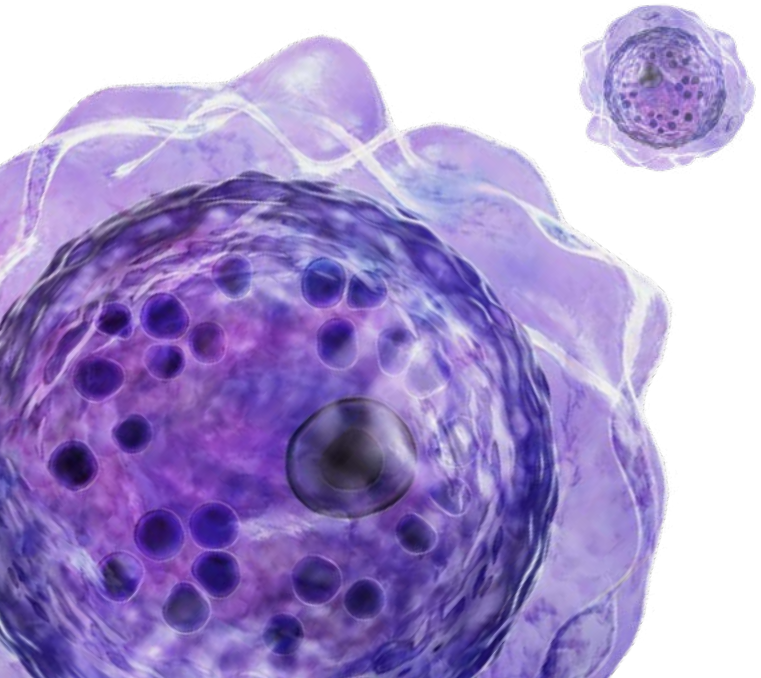
A number of preparatory steps need to be performed before the actual engineering of the T cells, beginning with the choice and design of the nuclease components. Each of these gene-editing tools has its own rules and criteria for the design of the DNA binding domain; however, the final design for each relies heavily on the use of sequence analysis software tools to determine best sequences surrounding the targeted cut sites that limit undesirable or "off-target" binding sites. Free online software tools specifically developed for use in gene editing are available (e.g., Invitrogen™ TruDesign™ Genome Editor), and many gene-editing nuclease vendors offer design help. Once the design is finalized, the gene-editing nuclease components can take from 1–10 weeks to produce (Table 1).

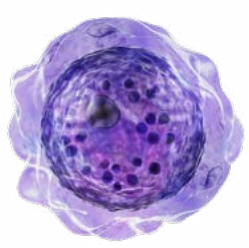
Once T cells are isolated and activated from donors, the cells can be transfected with the chosen gene-editing nuclease and engineered immediately, or if cell numbers are low, they can be expanded and engineered when sufficient cell numbers are reached. The cells can also be frozen and engineered at a later time.

Every step in the gene-editing process needs to be confirmed before moving onto the next step. Typically, these steps can include: determining the efficiency of cleavage at the desired site, checking the sequence of PCR products, monitoring gene and protein expression, and checking the impacts (e.g., toxicity and viability) on the model cell system. Numerous reagents and flow cytometry systems are available to quantify TCR knockout efficiency.

Other cell sources for allogeneic CAR cell therapies

Several other cell sources exist that are compatible with generation of off-the-shelf CAR T cells such as embryonic stem cells (ESCs) and iPSCs. Both ESCs and iPSCs have the ability to self-renew, allowing potentially limitless *in vitro* expansion. Human ESCs are derived from embryos and therefore can be problematic for clinical development due to regulatory issues and limited sources [23]. Human iPSCs, on the other hand, are derived from adult somatic cells [24]. For example, fibroblasts can be reprogrammed *in vitro*, can be transformed into iPSC cells, and behave like embryonic pluripotent stem cells that can be differentiated into red blood cells, T cells, B cells, and NK cells with unlimited proliferation capacity [25] (For additional information, see the **Pluripotent Stem Cell Resource Handbook** and **Manufacturing pluripotent stem cells**).





Another iPSC-derived immune cell subset that is gaining momentum are NK cells, which comprise an important part of the innate immune response. NK cells are responsible for immune surveillance by targeting viral-infected cells and cancer cells that downregulate HLA I presentation and upregulate stress ligands. NK cells are interesting for CAR therapy since they do not have T cell receptors and cannot interact with HLA-I complex, which is the critical contributor to **graft-vs.-host disease**. Unlike iPSC-derived CAR T cells, which only kill tumor cells that express the specific antigen that the CAR recognizes, iPSC-derived CAR NK cells can kill both tumor cells that express the CAR antigen as well as tumor cells that do not express the CAR antigen (Figure 4).

Another attractive feature of using iPSC-derived CAR NK cells is their cytokine profile; upon activation they secrete a restricted level of IFN-γ, IL-12, and GM-CSF. CAR T cells secrete IL-1 and IL-6 continuously upon activation, and the presence of IL-1 and IL-6 can lead to cytokine-release-syndrome (CRS), a serious adverse event seen in some patients receiving CAR T therapies [26]. To date, adoptive transfer of iPSC-derived NK cells is well tolerated, and neither GvHD nor cytokine toxicity have been induced in patients [27,28].

Summary

There are a variety of gene-editing tools and delivery systems available to facilitate the genetic changes necessary for T cells to be used in CAR T therapies. Each system has its own advantages and disadvantages, which should be carefully weighed for each CAR T cell therapy. Tradeoffs such as target specificity versus ease of use or cost versus speed will need to be made. As with many cutting-edge technologies, the tools available are rapidly being improved, and new approaches are appearing to address the current limitations of the field.

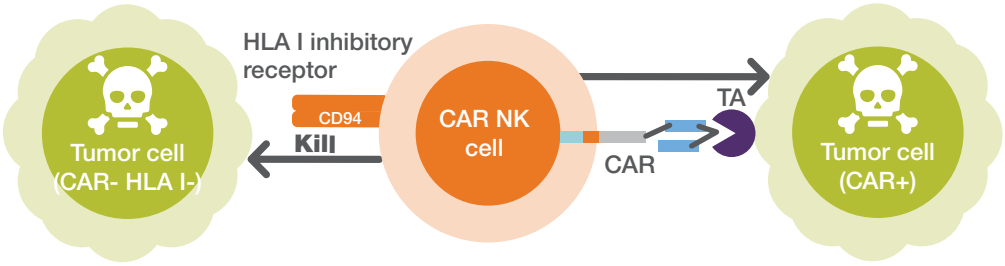
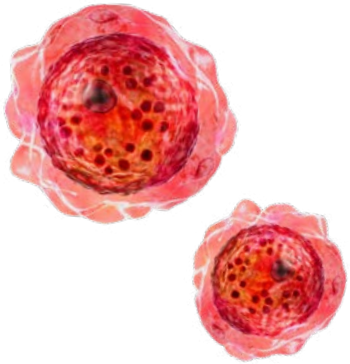


Figure 4. CAR NK cells can kill a broader range of tumor targets. On the left, CAR NK cells kill tumor cells that are missing HLA I expression without the engagement of CAR. On the right, CAR NK cell-mediated killing is shown to be dependent on the engagement of CAR and the tumor antigen (TA) expressed on the tumor cells.



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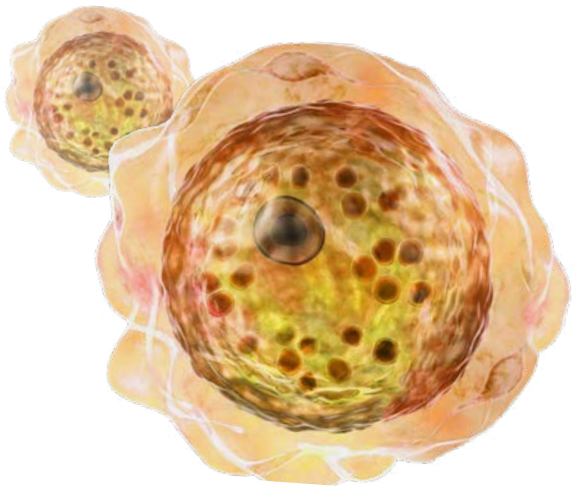


Section 6:
Cell expansion

Introduction

CAR T cell therapy has advanced into commercially available treatments that have driven an influx of companies into the immunotherapy landscape. In a standard workflow, a patient's (autologous) or donor's (allogeneic) genetically engineered T cells must be expanded *ex vivo* for clinical use. Typically, T cell expansion is the longest and one of the most critical phases in the CAR T workflow.

Engineered T cells are sensitive to their microenvironment and growth conditions. These responses can lead to potential undesirable cellular changes, which pose production and quality control challenges that could ultimately delay patient treatment.

One of the main objectives during the expansion phase is to maintain "younger", less differentiated memory cell phenotypes. Figure 1 illustrates the spectrum of T cell differentiation with the less differentiated, most therapeutically desirable central memory T cells (TCM) on the left, and the less desirable, more mature effector memory T cells (TEM) and effector T cells (TEFF) on the right. Surface markers such as CD62L, CCR7, and CD28 are not present when T cells differentiate and transition toward the TEFF cell populations and become less efficacious.

T cell viability and quality following expansion has a strong influence on treatment efficacy. Fortunately, relative factors such as media and supplement use, cell density, and the culture platform have been identified and improved upon to optimize the conditions needed for a successful and robust workflow. Sampling and analysis of CAR T cells is required during the expansion process to assess cell quality and ensure safety and potency of the product.

Allogeneic vs. autologous expansion

Early cell therapy work placed an emphasis on **autologous workflows**, where a diseased patient's own T cells are isolated, activated, genetically modified, expanded, and finally infused back into the patient. Key benefits to an autologous workflow are that it allows for personalized treatment and minimizes the risk of immunorejection and transfer of other diseases or viral infections. Autologous cell therapy poses several challenges, as the patient's cells often demonstrate slower growth profiles and display more mature T cell phenotypes, most likely due to the nature of the patient's illness and the *in vivo* cell environment [1]. According to a 2019 review article, research has demonstrated these inherent patient T cell deficiencies causally relate to the slower CAR T cell expansion, persistence, and lower cytotoxicity observed in autologous therapies [2]. These cell deficiencies directly correlate to autologous cell expansion and quality issues, and most importantly, to loss of therapeutic efficacy and slower turnaround time for patient treatment.

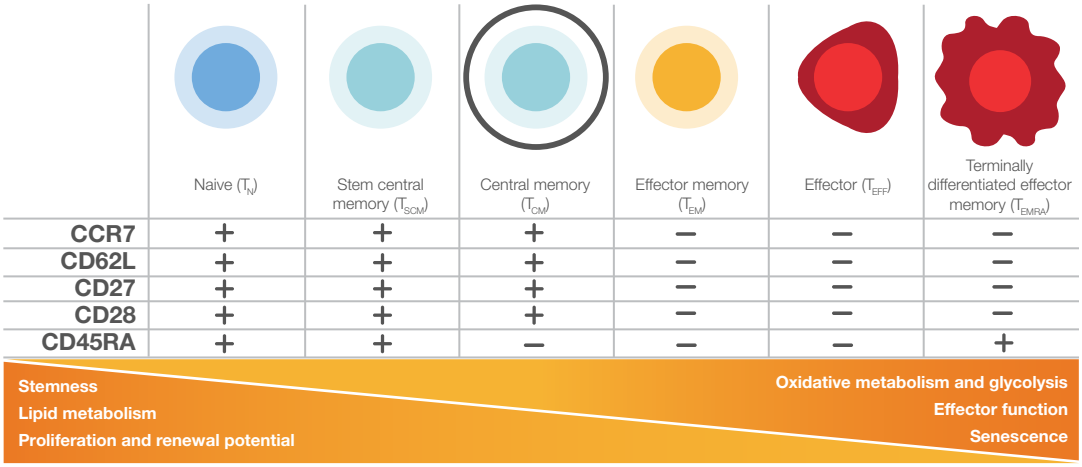


Figure 1. Younger is better. Maintenance of early TCM cells (left) is critical during *ex vivo* expansion as this phenotype is associated with higher efficacy treatment of patients due to its proliferation and renewal potential.

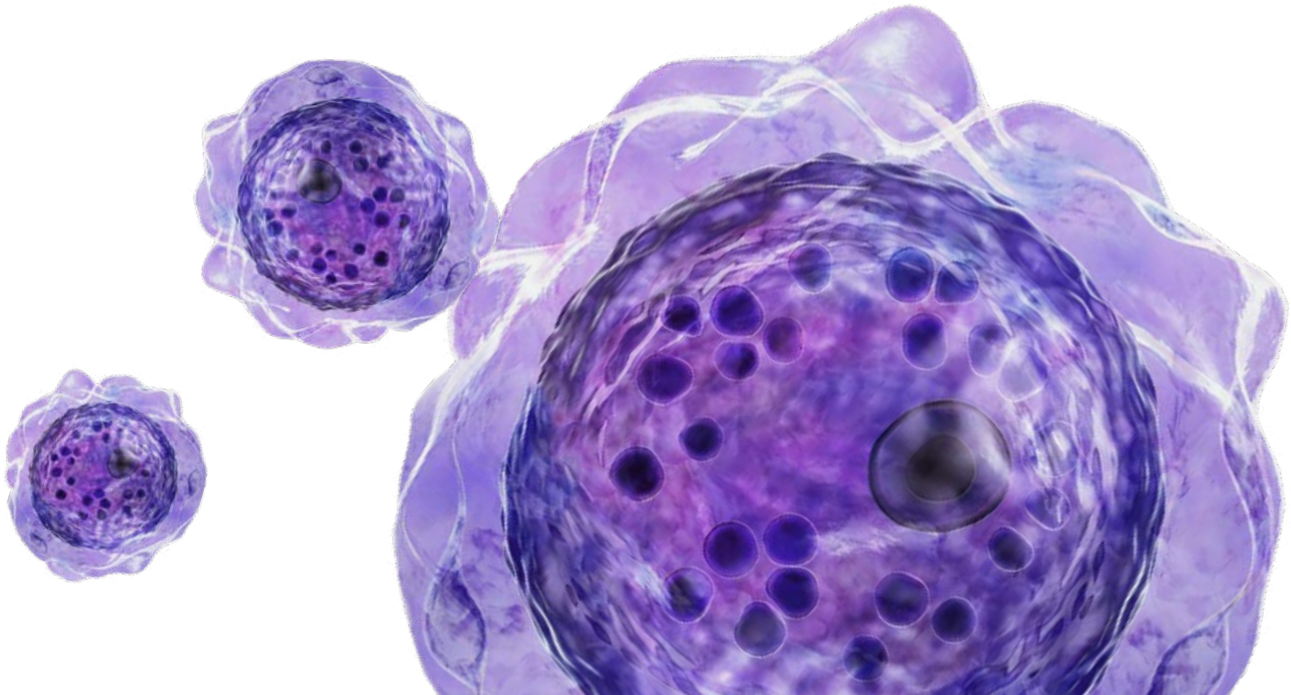
Allogeneic CAR T therapy has shown strong potential to change and improve the therapeutic landscape. An allogeneic workflow, which uses starting material derived from healthy donor cells, can provide more efficacious and timelier "off-the shelf" treatment options that offer standardized therapeutic product for multiple patients. Using healthy donor cells can help overcome many of the expansion and quality issues posed by patients' T cells [3,4]. It also provides the potential for re-dosing or delivering a combination of CAR T cells directed against different therapeutic targets. Despite the benefits, **allogeneic cell therapy can pose a significant risk to patients by causing life-threatening graft-versus-host disease or elimination by the host immune system**. Currently, these issues are a high priority, and research is underway to develop genetic CAR modifications that could mitigate host rejection risks [5].

Since allogeneic therapies require a greater quantity of cells for production of multiple doses, allogeneic workflows tend to be of larger scale than autologous workflows and require a longer timeline, with the expansion phase generally lasting 12–18 days. The additional culture time can negatively impact cell differentiation and function of the therapeutic product. As previously discussed, unwanted differentiation of T cells results in the loss of the younger TCM populations, which can lead to decreased patient therapeutic responses and treatment efficacy. However, successful mitigation strategies to address this include controlled isolation and activation of TCM cells, in conjunction with considerations towards cell density and supplementation throughout the expansion process. Ultimately, with successful mitigation, a potential scalable allogeneic workflow could reduce the overall cost of T cell therapy and provide greater treatment accessibility [6].

Activation to expansion

In cell therapy workflows, white blood cells are collected from donors in a process called leukapheresis. **Selected T cell phenotypes from the white blood cells** are isolated and activated to **support gene transfer and the reprogramming of T cells to express CARs**. With allogeneic workflows, the sourcing of T cells from healthy donors dramatically increases the probability of isolating a more desirable, early memory T cell population, which can result in higher cellular output and overall increased treatment efficacy.

Co-stimulation through CD3 and a secondary signaling receptor, such as CD28, provides the "wake-up" signal to activate naïve cells. CD3 signaling is indispensable for T cell growth, while agonistic ligation of CD28 contributes to T cell survival and plays a role in cytoskeletal remodeling, production of cytokines, differentiation, and transcription and post-translational changes during expansion [7].



Currently, T cell activation is primarily performed using an antibody-coated magnetic bead (Figure 2) or nanoparticle technology that imitates antigen-dependent signaling with anti-CD3 and anti-CD28 antibodies. These technologies replace traditional home-brew methods for generic activation that used antigen-presenting cells (APCs), mitogens, soluble or plate-bound antibodies, or chemical activators. Additionally, specific cytokines, such as interleukin-2 (IL-2) and interleukin-7 (IL-7) have been shown to support activation and maintenance of the desirable TCM phenotype with a greater expansion capability [8].

Products such as CTS Dynabeads CD3/C28 allow for isolation and activation in a one-step process for expansion of the desired T cell phenotype [9] (Figure 2). This covalently bonded antibody bead technology does not require feeder cells, antigens, or APCs. The beads can be removed following activation or prior to genetic modification and expansion. It is important to note, both the product and protocol selected for activating the T cells should conform to the application, process, and regulatory requirements, i.e., research use only (RUO) or clinical use application.

Following activation and engineering, buffer exchange is performed to transfer the desired T cells into the expansion medium. This step can be done using counterflow centrifugation in a closed an automated manner. During expansion, release of immunostimulatory cytokines, such as IL-2 and IFN-¥ at desired levels can allow CD8+ cells to survive as memory T cells during expansion.

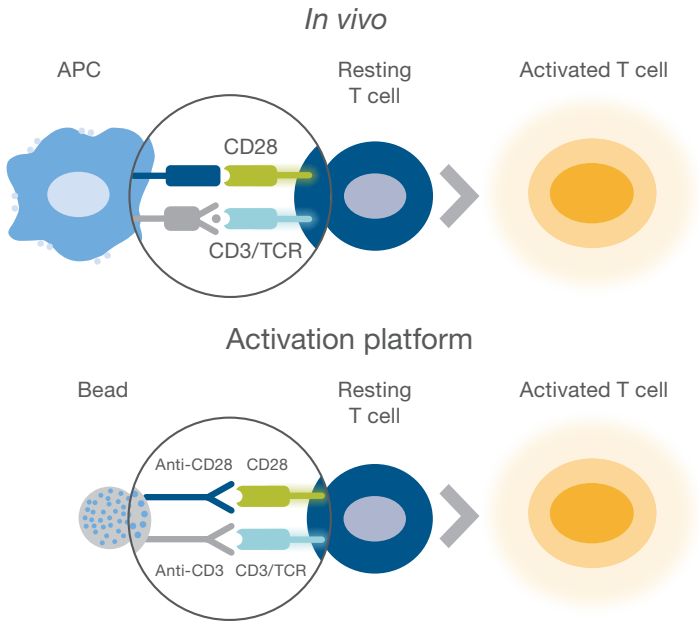
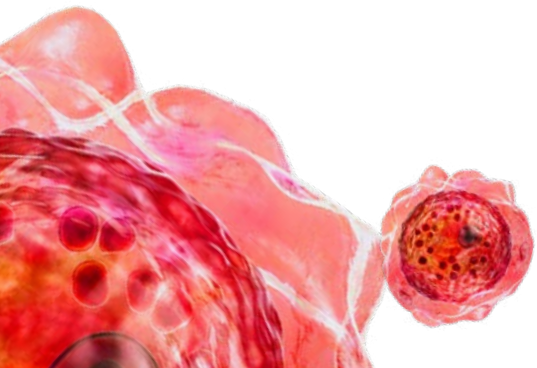


Figure 2. Magnetic bead approach to T cell isolation and activation. Bead technology products, such as **CTS Dynabeads CD3/CD28**, offer an *ex vivo* method for isolation, activation, and expansion of T cells. The uniform, inert, superparamagnetic beads are similar in size to antigen-presenting cells and are covalently coupled to anti-CD3 and anti-CD28 antibodies. These two antibodies provide primary and co-stimulatory signals, optimized for efficient T cell activation and expansion.



Conditions and factors impacting T cell expansion

Expansion platforms

Selecting a platform for T cell expansion is dependent on the end-user's application, working volume, workflow, and regulatory requirements. Table 1 provides a summary of the different platform options available along with their benefits and disadvantages. *Ex vivo* expansion can occur in static or dynamic culture systems. The processes of a static culture system can be performed in flasks or gas permeable static culture bags. To maintain a closed system, many static cell culture bags include sealed sterile tubing and connectors for sampling. The temperature and CO_2 levels can be controlled by placing static cultures in a controlled incubator that is set typically at 37°C and 5%, respectively. With this method, gas exchange occurs only through media exchange. The G-Rex™ device may be an alternative closed static culture system option, designed to enable gas transfer through a permeable membrane on the bottom of the device that can permit relatively larger medium to surface working volumes compared to flasks [10].

Due to limitations in volume, nutrient, and gas exchange, a static culture system may not be the ideal platform for allogeneic workflows. When scaling up T cell workflows, it is not a given that small-scale success will ensure the same with a larger system. For scaling, select an appropriate bioreactor and develop a quality by design strategy.

Closed and automated rocking bioreactor platform systems are applicable and robust for autologous and allogeneic workflows and scaling. The use of bioprocess single-use cell culture container technology along with automated rocking bioreactor systems, allow for monitoring and control of dissolved oxygen (DO), pH, glucose, and metabolites such as lactate and ammonium. The movement and angle of the rocking bioreactor allows for uniform mixing and gentle agitation.

Compatible presterilized, single-use culture bags are armed with multiple sensors, allowing for automated control and reporting. For fed-batch processing, these systems monitor the culture volume with continuous weight measurements. During expansion, typical conditions include pH of 6.6–7.0, a CO_2 range between 6.6–7.5%, and a DO range of 30–50% to ensure viability. The ideal agitation speed and angle is dependent on the working volume and bag size. For a rocking system such as the **Thermo Scientific™ HyPerforma™ Rocker Bioreactor**, a volume of 1.5 L in a 10 L bioreactor (bag) will typically have an agitation of 8 rpm and a rocking angle of 6 degrees. An increase in the agitation and angle is expected to increase with the use of larger T cell expansion volumes [11].

Recently, using a low-shear force benchtop stirred bioreactor with a fed-batch or perfusion process has been considered as a T cell expansion platform. If shown successful, this may have a significant impact, particularly on allogeneic therapies, that require high T cell yields. A modular stirred benchtop bioreactor system could potentially help reduce costs since these systems are highly automated and require less handling. In addition, they can be used across a greater range of volumes, from research to commercialization scale (Table 1). This capability reduces or eliminates the need to re-engineer and transition to an entirely different scale-up process, which can be costly and introduce transition error risks. This can be especially important when processes are locked in at clinical stage 2 or 3 trials. Additionally, stirred benchtop bioreactors can reach a higher volumetric mass transfer coefficient (KLa), which supports effective and homogenous oxygen delivery inside the bioreactor.

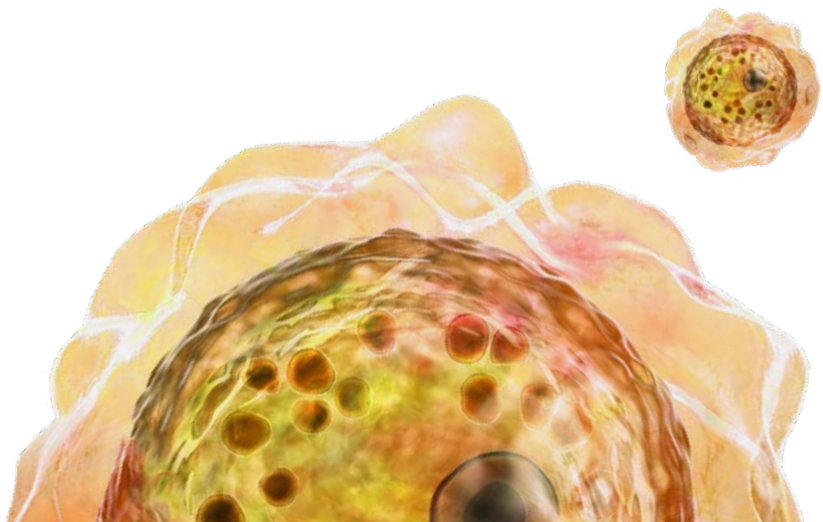


Table 1. Comparison of T cell expansion platforms.

Options	Typical working volume	Clinical or RUO	Advantages	Disadvantages
Static bags	5 mL–3 L	RUO or Clinical	• Closed system	• Limited gas transfer and working volume
T-flasks and static plates	100 µL–370 mL	RUO	• Economical and great for screening multiple conditions	• Open system • Small scale
Rocking motion bioreactor	300 mL–50 L	Clinical	• Closed, automated, scalable • Gas, liquid, DO, and pH control and sensing • Perfusion capable • Digital integration	• Large footprint • High cost
G-Rex	8 mL–5 L	RUO or Clinical	• Supports scaling • Automated • DO and pH control • Supports KLa value compared to static culture	• Difficult to close system • Requires training • High cost
Stirred tank bioreactor	250 mL–2,000 L	Clinical	• Closed, automated, scalable • Gas, liquid, DO, and pH control and sensing • Perfusion capable • Digital integration • Supports higher KLa values	• Large footprint • High cost

The intended use of the products in this table varies. For specific intended use statements, please refer to the Instructions for Use (IFU).

Media

The choice of media and supplements can significantly influence the growth of the T cell population, differentiation, viability, and the CD8:CD4 ratio during expansion. It is important to select a flexible expansion medium that is compatible with other workflow processes such as T cell isolation and activation, while being amendable to various platforms ranging from static cell culture systems to larger scale dynamic bioreactors.

As variation in workflows and protocols increases, manufacturers have investigated and developed optimized media and conditions to support a flexible, seamless, and scalable workflow. The media source can be a bottleneck in T cell expansion by presenting challenges including batch-to-batch variability, which can negatively impact the consistency and quality of the product output. Utilizing a chemically defined and serum-free medium from a dependable supplier with strong quality control processes can help reduce this variability. These product attributes will also minimize downstream purification and regulatory risks, as well as lower overall production costs.

The significant influence of media on T cell expansion was demonstrated in a recent study conducted using a novel culture medium that was developed specifically for expansion of human T cells in allogeneic cell therapy workflows [12]. A major challenge in CAR T workflows is the need for a larger number of cells with the preferred younger central memory T cell phenotype that results in more functionality and effective therapeutic outcomes. Modest increases in central memory cells early in the expansion phase result in larger cell yields at harvest. In this study, healthy donor T cells were tested in an 18-day allogeneic type workflow, with the results demonstrating approximately 20% higher cell proliferation by day 10 and nearly a 100% increase by day 17 using the newly formulated medium, when compared to the control medium (Figure 3). A 10–20% increase in the size of the desired central memory T cell subset was also demonstrated when normalized to the control medium (Figure 4) [12]. In addition, this boost in cells displaying an early memory phenotype coincided with a higher level of interferon gamma (IFNγ) release when healthy donor cells are used. An average 187% increase in IFNγ production across 6 patients was demonstrated when normalized to the control medium (Figure 5). The increase observed

will likely act as a catalyst to enhance the overall immune response and provide more efficacious patient therapies by stimulation of macrophages, neutrophils, and natural killer cells [12].

Not only does the medium impact the expansion phase, but the benefits are dependent on and specific to the initial cell source used during the expansion phase. In these studies, a metabolic shift in the T cells allowed for a longer workflow [12].

Supplements

In addition to medium, supplements also play an important role in T cell expansion. L-glutamine is an essential carbon source for metabolism and differentiation that needs to be supplemented throughout the cell therapy manufacturing process.

Serum is commonly used in research and can aid in supporting basal activation of T cells as well as cell growth. **While serum use may be acceptable for research, it poses regulatory and supply concerns for use in clinical applications** and can demonstrate variability from batch to batch, which can impact T cell differentiation and the overall product output. The availability of serum replacement alternatives, which are defined supplements shown to support comparable T cell $CD8^+/CD4^+$ ratios while maintaining high cell expansion and viability [13,14], has addressed these issues.

Perfusion

Perfusion is a bioprocessing technique that involves continuous exchange of spent media with fresh media, while retaining cells within the culture vessel. This method refreshes nutrients while preventing the buildup of toxic metabolic waste products that could negatively impact culture performance and impact product quality. This technology allows the culture to reach much higher cell densities and fold expansion within a smaller footprint and can lead to increased productivity compared with traditional batch or fed-batch processes. Perfusion has been applied to both autologous and allogeneic T cell expansion, but it is essential for most allogeneic workflows, which require a higher quantity of cells. Achieving greater density and expansion of cells in a shorter time frame can reduce the population of exhausted cells and increase the population of younger TCM cells, which are known to support more efficacious treatments.

A side-by-side T cell expansion experiment comparing fed-batch and perfusion workflows in rocking motion bioreactors was used to evaluate the impact of perfusion on cell growth, cell viability, key metabolites, and growth factors [15]. Perfusion supported a higher density of viable T cells by reducing the accumulation of lactate and ammonia during expansion. As with other cell types, these metabolites are toxic to T cells and can induce arrest of cell growth or apoptosis, which directly impacts cell viability and expansion.

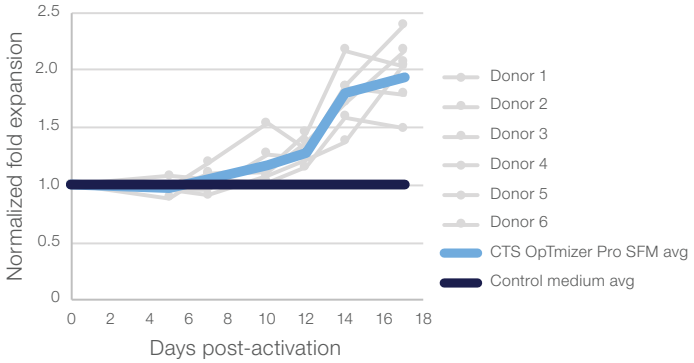


Figure 3. Expansion of T cells in specialized medium. Normalized proliferation with CTS OpTmizer Pro SFM in an 18-day workflow with 6 healthy donors shows approximately 20% higher fold expansion by day 10 and nearly a 100% increase by day 17, when compared to the control medium.

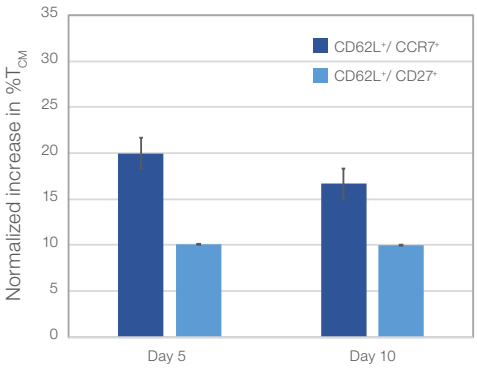
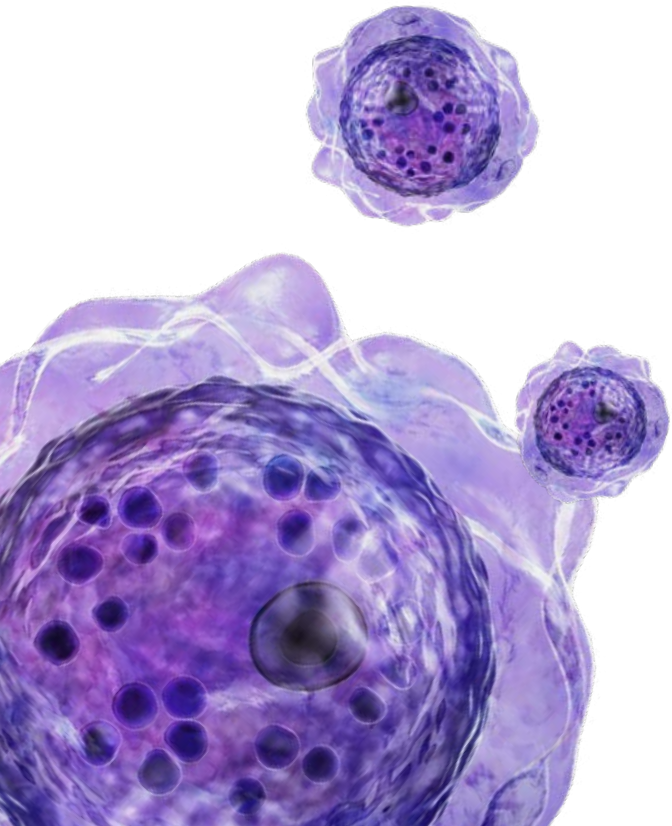


Figure 4. Expression of TCM markers in cells grown in specialized medium. In an allogeneic workflow, 6 healthy donor cells grown with Gibco™ CTS™ OpTmizer™ Pro SFM showed a 10–20% increase in the size of desired TCM population based on analysis of central memory markers CD62L, CCR7, and CD27.

Another key to allogeneic workflows is having a sufficient IL-2 concentration to support T cell survival and proliferation. Through perfusion, a sufficient level of IL-2 was maintained during expansion. On the contrary, the non-perfused culture showed the IL-2 concentration dropping to undetectable levels by day 12, which prevented cells from reaching comparable cell viability [15]. To fully understand the role of perfusion and IL-2, an additional experiment was setup to analyze and compare cell growth and viability with perfusion and non-perfusion setups. The amount of IL-2 injected into the test non-perfusion bioreactor was equivalent to what was delivered to the perfusion control bioreactor. Despite the daily equivalent IL-2 injections, by the end of the T cell expansion phase (day 14), the non-perfusion culture expansion was only 63% of the perfusion control [15].

While perfusion has its advantages, the high volume of perfusion medium required can be costly, and it is imperative that manufacturers optimize their process and perfusion rates to avoid medium waste. That said, the benefits of perfusion technology are undeniable for T cell expansion; it is a readily scalable solution that can help maintain a favorable culture environment for longer, more productive workflows specifically important for allogeneic cell therapy.



Maintaining cell density on expansion

Additional cell culture parameters, such as maintenance cell density, have been shown to influence T cell expansion. The results of a recent evaluation of the impacts of maintaining different T cell densities demonstrated several direct effects on the quantity and quality of the cellular products. Many classical T cell expansion protocols call for the maintenance of cells at 0.5 x 10^6 cells/mL; however, the results of this study demonstrated that a lower cell density of 0.25 x 10^6 cells/mL correlated with higher-fold cell expansion and improved viability. Conversely, increasing the maintenance cell density to 0.75 x 10^6 cells/mL demonstrated lower-fold expansion and slightly lower cell viability. These results suggest that maintaining a lower T cell density can positively impact the quantity and quality of the cellular output by exposing the T cells to more nutrients and help maintain a larger central memory phenotype [6].

Restimulation

An ongoing shift toward supporting viability of the desired T cell phenotypes has created interest in understanding the durability of the response elicited by activation and reactivation in the expansion phase. In a recent study, secondary “restimulation” during the expansion phase was evaluated using CTS Dynabeads CD3/CD28 [6]. The goal was to better understand the effects of restimulation of the T cells, how it affected the therapeutic cell output, and whether it had any impact on the T cell manufacturing process.

This study revealed that a single round of activation with the beads was not only sufficient to induce robust cell proliferation and high viability over the entire 20-day workflow, but also provided evidence that restimulation can cause a temporary growth lag and plunge in viability during the following days (Figure 6A and 6B). In addition, cells subjected to secondary activation displayed a lower CD8:CD4 ratio and a sharp downregulation of central memory cell biomarkers. In both cases, with and without restimulation, there is a clear decrease in the central memory population ($CD62L^+$ $CCR7^+$) and an increase in the double-negative effector population (CD62L- CCR7-) in the later stages of expansion, which is much more pronounced within the restimulated group (Figure 6C) [6].

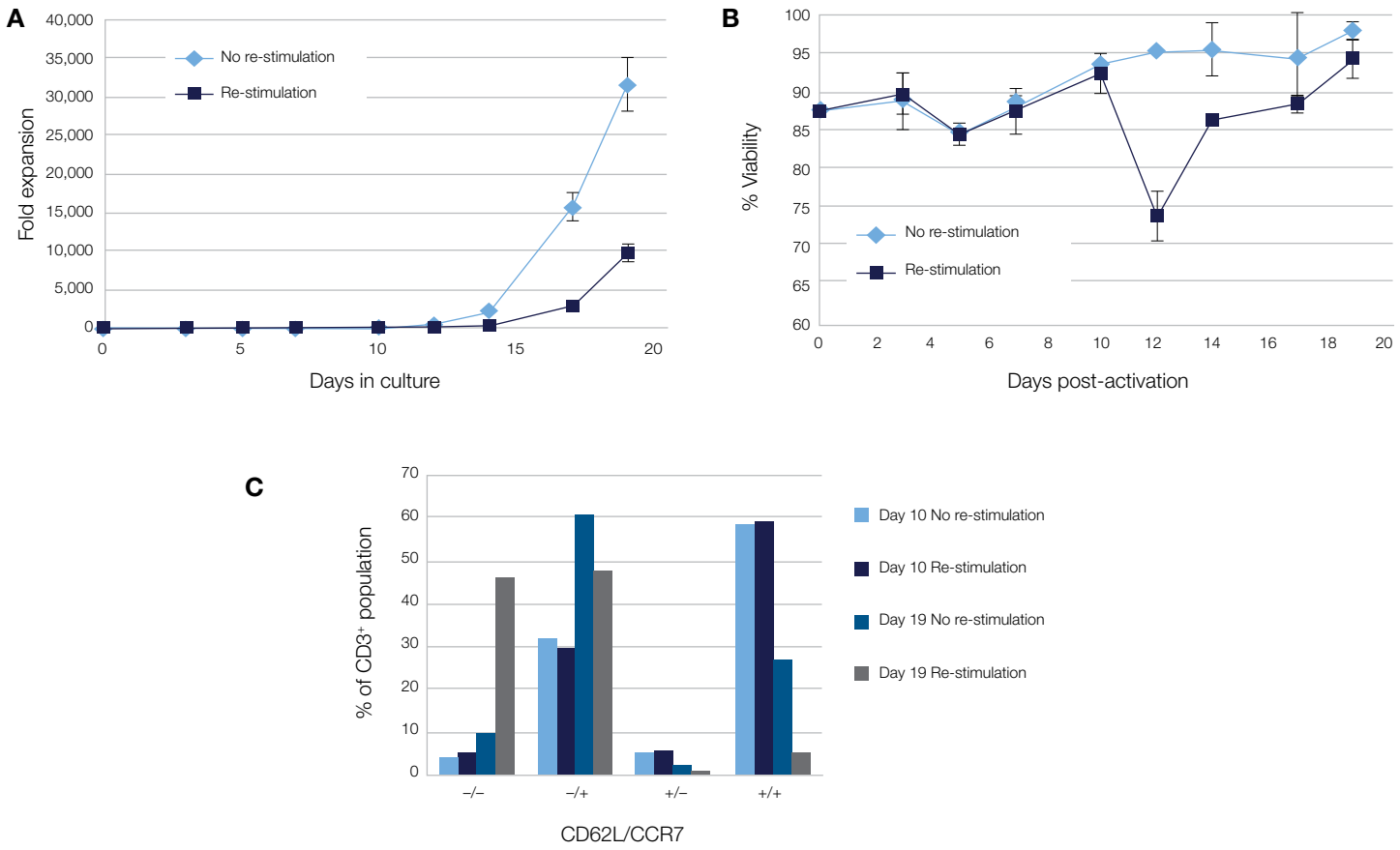


Figure 6. Effect of restimulation on T cell growth and phenotype. A second round of stimulation with CTS Dynabeads CD3/CD28 during a longer workflow negatively impacts: **(A)** cell expansion, **(B)** cell viability, and **(C)** cell differentiation. (Error bars represent the standard deviation of three donors performed in triplicate).

These results suggest that restimulation catalyzes the transition of early memory T cells into effector T cells. Slowing this transition is important to developing and fine-tuning T cell manufacturing processes.

Overall study results show the critical importance of choosing the optimal media, supplements, platform, and process parameters that will support robust T cell expansion, while maintaining the desired early central memory T cell phenotype which is key for efficacious T cell therapies.

Regulatory and analytical testing

Knowledge of the regulatory requirements is critical to maintaining clinical approval at each stage to avoid critical issues and delays in progressing to later clinical phases. In addition, regulatory requirements influence many of the analytical quality testing requirements of cell therapy products.

During the expansion phase, robust and reliable analytical tools are required to accurately measure and monitor various cellular characteristics such as proliferation, viability, differentiation status, and other cell phenotype attributes. During and after the expansion process, testing is performed for a range of required quality attributes with regulatory body-approved or in-house validated assays. These tests evaluate quality attributes related to safety, purity, and potency of the cellular product. Safety testing

for microbial and fungal sterility, and the absence of mycoplasma, as well as testing for replication competent viruses and vector copy number are usually required when viral vectors are used in the manufacturing process. Purity testing is often conducted with flow cytometry techniques to assess the proportion of positive cells for respective T cell- and CAR-associated surface markers, as well as safety testing to make sure undesirable cell types are not present. Potency testing is conducted to assess the CAR T cell content with flow cytometry or PCR methods, while cytotoxicity and cytokine secretion is assessed with various *in vitro* assays [16].

There are several different ways to analytically test any given cell characteristic, therefore, variability in testing presents a known source of variation during the manufacturing process [16].

Vision and concluding remarks

Over the last decade, many advances have been made in the field of cell therapy and there are many more to come. As time is of the essence for patient therapy solutions, efforts to reduce production and product quality release timelines are a critical focus of research and development efforts. The T cell manufacturing process is complex and currently many opportunities for variability exist at each step. Further research is underway to identify and address manufacturing variables that can affect the critical quality attributes of the final product [16]. Implementation of greater automation, closed system manufacturing, and real-time characterization will likely be important future developments [17].

Achieving “off-the-shelf” allogeneic, and improving upon autologous, therapeutic solutions for patients requires substantial investment in product and process development. Current and developing work for T cell expansion involves mitigating risk and developing a robust more standardized and transferable process. Key to making the journey from initial research and development to commercial manufacture as simple and as seamless as possible is a detailed understanding of the product and process development to enable successful scale-up [17]. Equally important will be innovation within the field to explore new approaches and expand upon the currently available technologies.

A note about CAR NK cell therapies

While CAR T therapies have demonstrated success in treatment of circulating blood-related cancers, utilizing engineered T cells for other cancers, such as solid tumors, has proven challenging. An approach under extensive development involves the use of NK cells, a cell subset of the innate immune system, which have been shown to be effective in a number of clinical trials for various cancer treatments. Allogeneic NK cells exert their cytotoxic effect in an antigen-and HLA-independent manner.

Engineering of CAR NKs has facilitated specific targeting to tumor-specific antigens enabling them in a solid tumor microenvironment. This aspect combined with iPSC-derived CAR NK technologies could lead to an unlimited supply of cells as an off-the-shelf solution, bypassing long lead times for patients. Recent studies with CAR-NK-19 for lymphoid tumors (CD19) [18, 19] and CAR-NK-GPC3 for solid tumors of hepatocellular carcinoma (HCC) and ovarian cancers [20] demonstrate the promise of this approach.

The workflow to generate CAR NK cells is similar to that of CAR T cells but involves sufficient *ex vivo* expansion of NK cell cultures to meet dose and lot size requirements for allogeneic therapies. Typically, each dose requires approximately 5×10^6 cells/kg of body weight, or nearly 500×10^6 cells per person. The cell culture platforms that are used for *ex vivo* expansion of CAR-NK cells include T-flasks, G-Rex vessels, bioreactors, or culture bags depending on scale of final clinical product. Recommended culture conditions include use of a xeno-free medium supplemented with IL-2, IL-15, and IL-21. As is the case with CAR T cells, extensive QC requirements precede the use of CAR NK cells in a clinical setting, and often include testing the cell product for its cytotoxic killing abilities on various tumor cell lines *in vivo* animal tumor models.

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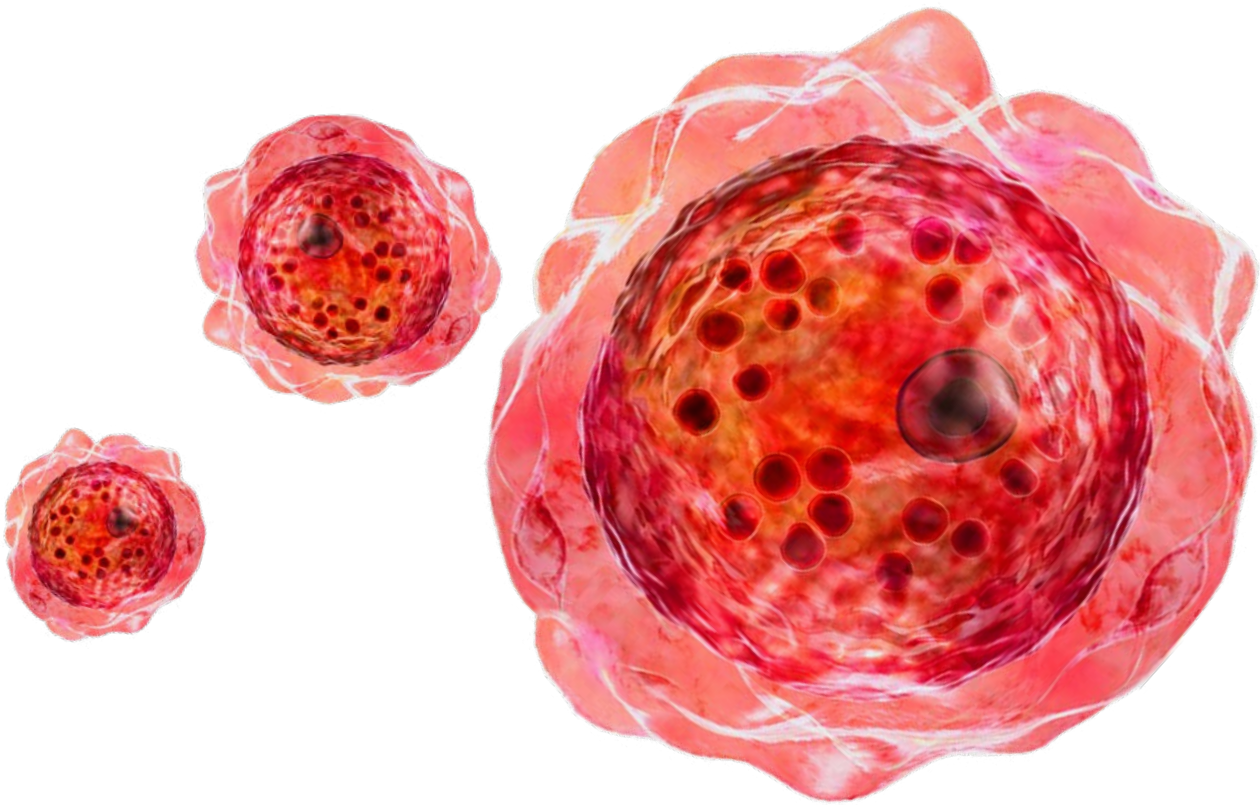


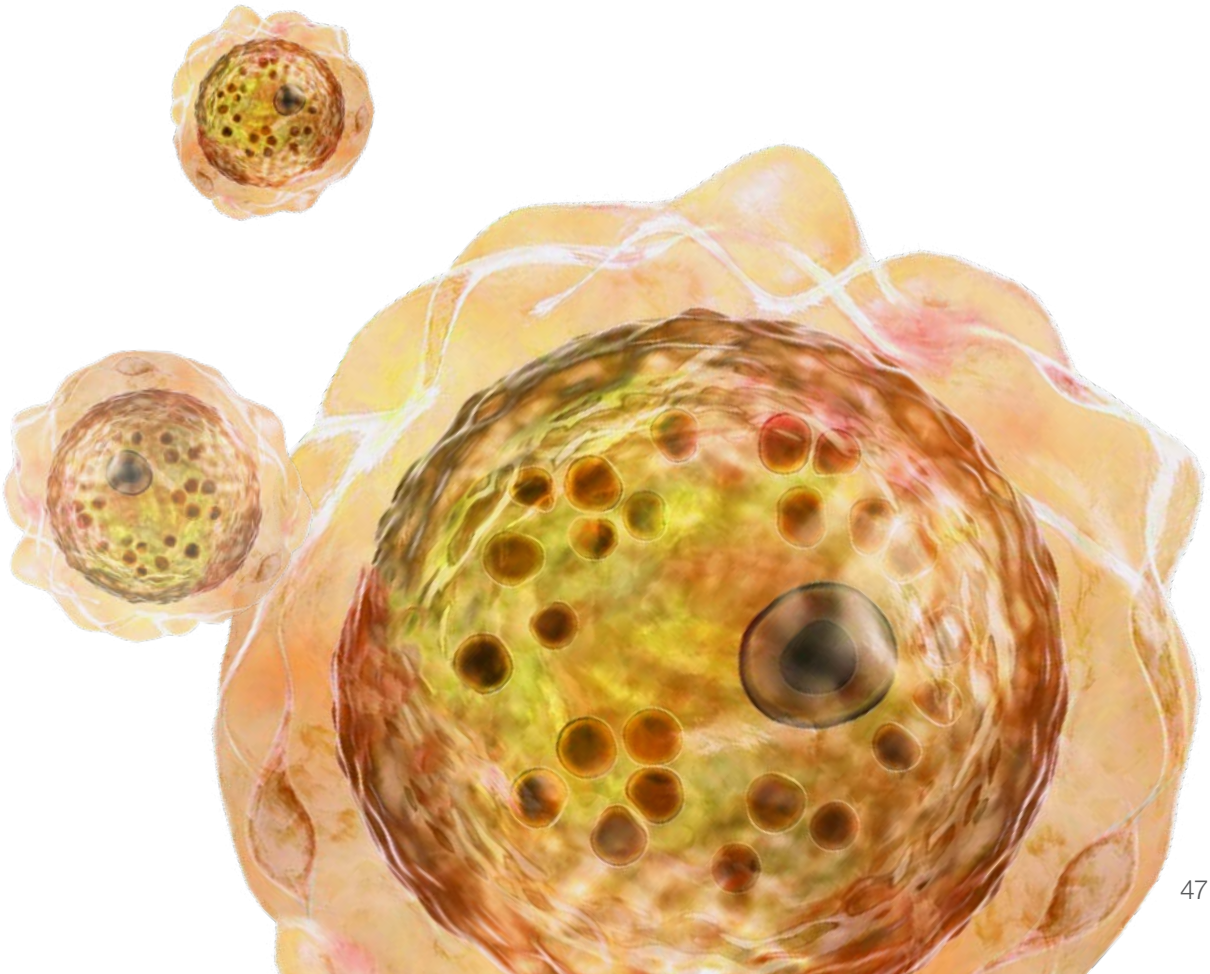
Section 7:
Closed system automation for cell therapy

Introduction

Initial research into CAR T technology relied on manual, open systems for development. As the technology matures, CAR T cell manufacturers are continually looking for process improvements that can decrease overall costs. They are also looking for improvements that will decrease contamination, improve batch-to-batch consistency, and allow for monitoring and capture of critical information, which will help with the ever-changing regulatory environment. To this end, CAR T cell therapy manufacturers are turning to automated, closed systems with integrated software controls to achieve lower manufacturing costs, maintain product consistency, and meet regulatory requirements.

Closed versus open systems for manufacturing CAR T cell therapies

Numerous steps in the methods for isolation and expansion of CAR T cell therapies have options that can be performed using an open or closed system. For example, density gradient centrifugation for the isolation of PBMCs is typically performed using an open system and T flasks commonly used for research-scale cell growth are an open system. However, open systems can expose the cell therapy product to a room's environment and require increased user interaction, such as working under a laminar air flow hood [1]. These open processes tend to be more labor intensive and can take up larger footprints, especially when trying to reach a larger manufacturing scale. Another consideration in using open systems is the need to utilize a grade A or grade B manufacturing facility, whereas a closed system can be implemented in a grade C facility. The difference in manufacturing conditions required for a closed system can considerably decrease costs, labor, and space requirements. In research settings, manual open processes may be the route chosen. However, these methods should be avoided in clinical applications and final cell therapy manufacturing because of the increased risk of contamination and batch-to-batch product variability, which can hinder regulatory approval and possibly result in product failure.



According to the European Medicines Agency (EMA), a closed system is "A process system designed and operated so as to avoid exposure of the product or material to the room environment. *Materials may be introduced to a closed system, but the addition must be done in such a way so as to avoid exposure of the product to the room environment* (e.g., by means of sterile connectors or fusion systems)" (italics added) [2]. Systems can be closed using a variety of devices and techniques (e.g., sterile barrier filters, disposable sterile doc connectors). The use of single-use technologies (SUTs) further assists closure of the system by providing enhanced protection outside of a clean room or biosafety cabinet. **SUTs include plastic single-use bags, bioreactors, tubing, filter capsules, and connectors, making them compatible with closed system techniques** [3]. An important consideration when using SUTs is compatibility of all parts, particularly the connections between bags and tubing. If tubing sizes and bags are mismatched, connections can still be made, but will require aseptic joining in a laminar flow hood.

The importance of closed processes and SUT

Contamination in bioprocessing is costly. It results not only in product loss, but can also lead to facility shutdown for cleaning and validation. According to the FDA, for aseptic processing of cell therapy products, "Cellular therapy… represents a subset of the products that cannot be filter-sterilized… Where possible, closed systems should be used during manufacturing" [2]. Closing the system significantly reduces risk of contamination by viral, bacterial, or other adventitious agents. In addition, closed systems may be placed in a controlled but non-classified environment [4], which could improve manufacturing flexibility (e.g., reducing the facility footprint or reducing the amount of segregation in a facility). In addition, equipment and personnel can be moved around more easily to meet production needs. These simpler designs enable manufacturing suites that are easily duplicated at multiple sites. Combined with SUT, closed systems can greatly reduce processing time including cleaning, setup, and batch turnaround times. Moreover, cost of goods will be significantly lower due to reduced operating costs, which can include labor costs, energy costs for environmental monitoring and air handling, costs to grow the material, as well as facility costs [5].

The importance of automation in GMP manufacturing

To further improve manufacturing costs, cell therapy manufacturers will look into automating most of the manual steps in the process. Automation is not restricted to production; it can also be expanded to include analytical steps such as offline or inline process analytical technology (PAT).

Implementation of automation is critical for large-scale cGMP manufacturing [6]. The EMA suggests that "The use of automated equipment may ease compliance with certain GMP requirements and may also bring certain advantages in respect to product's quality"[2]. Automation would improve operator safety, reduce human errors, and enable processing robustness and reproducibility. Automated systems can simplify operations overall. Manual procedures that have multiple steps or require multiple operators can be combined within a single machine with a single operator, reducing the product turnover time and the number of personnel required in the operation space. As a result, facility production capacity will increase. The overall cost of goods for similar quantities of cell therapy products will significantly decrease.

Existing closed automation systems in cell therapy manufacturing

Several steps in patient-specific cell-based therapy development (e.g., CAR T) can be implemented using an automated, closed system: cell isolation, expansion, processing, and formulation. Two categories exist based on the degree of automation [7]:

1. Integrated closed system
2. Modular closed system

Integrated closed systems are fully automated. They are all-in-one, easy-to-use, and designed as an end-to-end, one-patient-at-a-time solution. Once employed, the integrated closed instruments will be dedicated to producing a specific patient's cell product for a certain period of time (usually 1-2 weeks). This approach refers to the automation and closure of a single machine for a specific patient or purpose and integrates several steps into a complete workflow.

Modular closed systems are more versatile, with each instrument primarily optimized for a single step. This approach does not restrict bioprocessing companies to a single supplier—they can choose instruments that are best suited for individual steps in the process. More importantly, manufacturers have the flexibility to develop new processes using existing instrumentation as needed. For example, one can use the Rotea™ (a closed counter-elutriation system offered by Thermo Fisher Scientific) to isolate PBMC/CD3 T cells, and the G-Rex system to expand the T/CAR T cells (a Wilson Wolf culture expansion system) **(see the white paper on this topic)**. Table 2 summarizes some current cell processing automated systems and their parameters.

Digital integration of the CAR T cell therapy manufacturing workflow

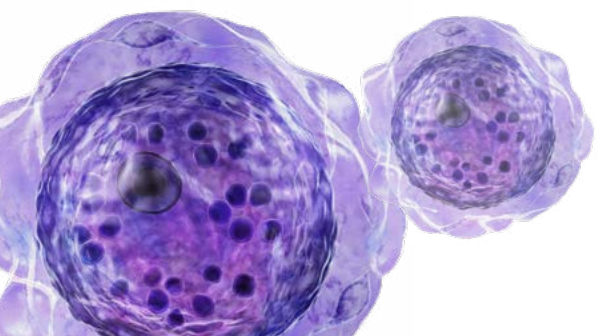
Software-driven, digital integration plays an essential role to support full automation across the entire cell therapy manufacturing workflow. Digital integration can improve manufacturing productivity and process control by monitoring the entire workflow starting from sourcing of raw materials through product delivery to the clinic. This tracking can ensure data integrity, traceability and regulatory compliance, plus aid in the scale up of the process. Ideally, a mature manufacturing environment would connect production (hardware and controllers), control layers (e.g., supervisory controls), and manufacturing execution systems (below Figure 1). Software tools can offer the ability to mine and analyze data from upstream and downstream batch records across batches for real-time optimization and troubleshooting.

In current digital solutions for cell therapy manufacturing, the workflow is managed by connecting the instrument to a distributed control system (DCS). The DCS layer allows for scalable process optimization, workflow management, and data transferring across the entire workflow. Some software systems are more easily configured to DCS and manufacturing execution system (MES) than others.

Table 2. Comparison of common cell processing systems.

	Modular system				Integrated system
Core technology	Counterflow centrifugation[1]	Electric centrifugation motor and pneumatic circuitry for piston drive[2]	Spinning membrane filtration[3]	Acoustic cell processing[4]	Magnetic separation[5]
Cell recovery	95%	70%	70%	89%	85%
Input volume	30 mL–20 L	30 mL–3 L	100 mL–22 L	1–2L	1–2 L
Input cell capacity	10×10^9	$10–15 \times 10^9$	3×10^9	1.6×10^9	3×10^9
Cell processing time	45 min	90 min	60 min	40 min	NA

1. Rotea system; 2. Sepax™; 3. LOVO®; 4. ekko™; 5. CliniMACS Prodigy®



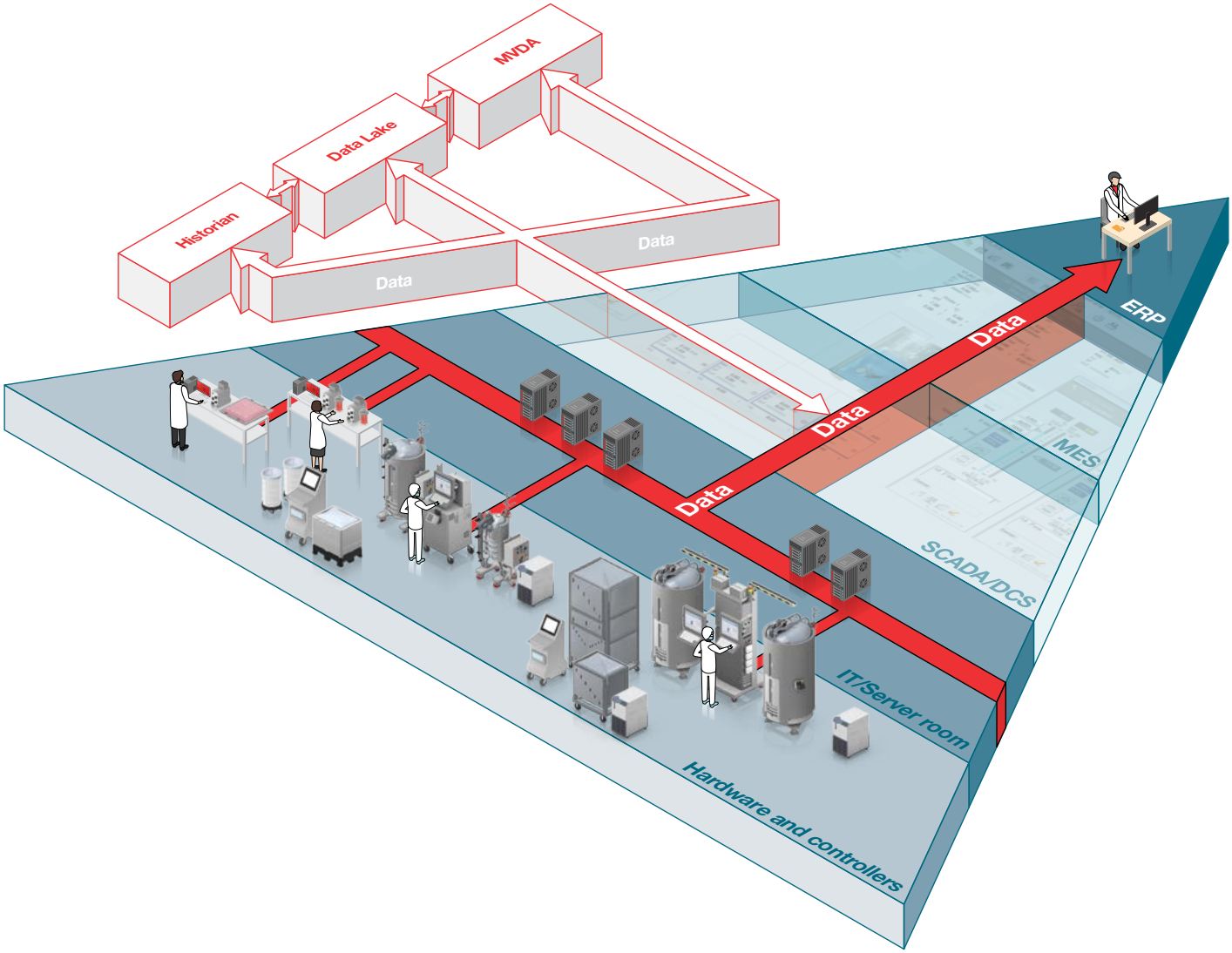


Figure 1. A comprehensive process control, digital connection, and data stream of a mature manufacturer for commercialized products. To easily scale up the process and accelerate time to market, it is essential to manage the workflow through a distributed controlling system (DCS) to control the process and ensure traceable, reproducible, and secure data connectivity through a manufacturing execution system (MES), which can be further integrated into an enterprise resource planning (ERP) system.

Summary

Tremendous efforts have been made to make CAR T cell therapy more effective, safe, and persistent in treating patients. Yet, the manufacture of CAR T cells has been prone to errors, lot-to-lot variation, and contamination. These errors commonly result from the use of open processes with manual handling. Using closed automated systems that integrate the complicated, multistep CAR T workflow can easily overcome these challenges. The use of closed integrated systems improves consistency, purity, and safety while helping to lower overall manufacturing costs. These benefits can contribute to making cell therapies more affordable and accessible to patients in the future.

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Section 8:
Characterization and analysis of cell therapies

Introduction

The goal of cell therapy developers is to produce safe, efficacious, and consistent products that help patients, many with intractable diseases. Analytical methods are critical to achieving this aim and to the overall development process. These methods form the basis under which all critical development decisions are made, including what manufacturing equipment to use, which genetic engineering methods produce the best combination of performance and viability, what media system and feeding schedules perform the best, and what patient populations to target.

Cell therapy manufacturers face several challenges in developing the appropriate tests to analyze and accurately characterize their biological products. This section will discuss these challenges, the current characterization strategies and tools that manufacturers use, and some future trends to overcome existing hurdles.

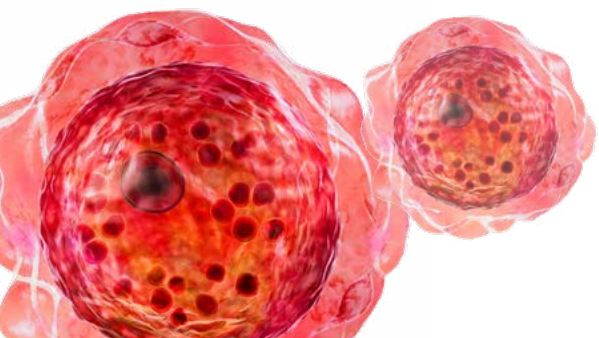
Defining product specifications

As defined in *ICH Q6B, Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products*, characterization of a biological product "includes determination of physiochemical properties, biological activity, immunochemical properties, purity and impurities" [1]. A comprehensive understanding of the biological product allows the appropriate specifications to be established. At a higher level, each specification contributes to a set of "criteria to which a new drug substance or new drug product should conform to be considered acceptable for its intended use" [1]. By definition, a specification consists of the assay or test, the protocol for performing it, and the numerical limits, ranges, or other observations that define a product's acceptance criteria. These acceptance criteria can be divided into several categories—identity, purity, potency, safety and other—which are defined in Table 1.

While the concepts of full product characterization and release specifications are easy to comprehend, in practice they are very challenging to implement.

Table 1. Categories of acceptance criteria for new cell therapies.

Category	Definition
Identity	• Assays that identify the product for proper labeling and will distinguish the product from other products manufactured in the same facility (21CFR610.14) • Examples include cell surface or intracellular markers, gene expression, secreted molecules, and peptide sequences
Purity	• Assays covering residual testing for materials used during the manufacturing process (e.g., isolation or activation beads, digestion enzymes, or genetic engineering reagents) • Can refer to contaminating cell types that may have an adverse effect on final product safety and efficacy
Potency	• A measure of biological activity that demonstrates the capacity of a cell therapy product to affect a given result (21CFR600.3) • A matrix of assays is recommended because of difficulty selecting a single assay that assesses product quality and consistency while predicting clinical efficacy
Safety	• Assays that test mainly for adventitious agents (e.g., sterility, mycoplasma, or endotoxin levels) • Can also include other areas of concern (e.g., immunogenicity or tumorigenicity)
Other	• Includes tests for appearance, dose, viability, and cell counts



Challenges in characterizing cell therapies

In 1982, a new era of pharmaceuticals began with approval of the very first biologic—recombinant insulin. In the time since, more complex recombinant proteins and monoclonal antibody therapeutics have been developed, many of which have become best-selling drugs. While seen as more challenging to develop, manufacture, and distribute when compared to small-molecule medicines, these therapeutic modalities pale in comparison to the complexities associated with "living" cell therapy development, such as CAR T cell therapy.

To begin, human cells are large, each consisting of millions of protein molecules. Human cells also have a complex structure consisting of a membrane, cytoskeleton, and organelles that carry out specific functions. In addition to their large size and diverse biochemical makeup, cell therapy products are heterogenous and dynamic, continually interacting with and responding to their environment. This dynamism makes it impossible to fully characterize all properties and functions of a heterogenous mixture of cell types that could change depending on how they are manufactured, stored, and administered. Each characterization assay only reveals a single or limited number of attributes at a point in time. The selection of the most appropriate cellular attributes to test coupled with the inherent limitations of each assay method produce a formidable challenge that the industry will need to address to realize the field of cell therapy's full potential.

Nearly all cell therapies start with an initial cell source derived from donor tissue. Donor-to-donor variability presents a tremendous challenge when trying to achieve a consistent, predictable manufacturing output. For autologous therapies, additional variability occurs because patients present varying severity of illness or have undergone several treatments prior to providing tissue. These factors make it challenging to set meaningful specifications that ensure consistent production of high-quality therapeutic products.

Quality by design approach

More recently, cell therapy developers have been implementing quality by design (QbD) principles in their product development process, with the goal of providing the highest quality products to patients. The concept was first described by engineer Joseph M. Juran [2] and later the FDA highlighted its utility in pharmaceutical development in their 2007 report "Pharmaceutical Quality for the 21st Century: A Risk-Based Approach" [3]. The overriding principle of QbD is "quality should be built into a product" via "a thorough understanding of the product and process" [3] and process control. The QbD systematic approach starts with predefined objectives and is based in sound science. Moreover, this approach seeks to understand the risks "involved in manufacturing the product and how best to mitigate those risks [4]."

As an example, the FDA presented a general QbD scheme [4] (see Figure 1). Generally, the QbD process starts by defining the end goal or product, with developers formulating a hypothesis on a product's mechanism of action (MoA). The MoA describes the specific action(s) a cell product will produce to achieve a desired therapeutic effect. The MoA then informs the target product profile (TPP), which describes the desired attributes of a product, including safety and efficacy-related characteristics. Next, the developer must establish which critical properties or critical quality attributes (CQAs) must be controlled to achieve the desired clinical outcome. CQAs are defined as "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to assure the desired product quality" [5]. A CQA is identified by the risk of harm to a patient if that CQA is not met. In cell therapy, the "product is the process" is commonly heard; the direct corollary in QbD are critical process parameters (CPPs), which are a key part of a manufacturing control strategy. A CPP, per ICHQ8(R2), is "a process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality" [5].

Figure 1. Quality by Design (QbD) principles. This general scheme provides a decision framework that cell therapy developers must make to define and control the quality of a cell therapeutic. Based on a scheme presented by Dr. Finn from the FDA [4].

According to the FDA presentation, “CQAs and CPPs are used together to ensure <product> quality and manufacturing consistency” [4]. Specific examples are provided in Table 2.

Table 2. Categories of acceptance criteria for new cell therapies.

Critical quality attributes	Critical process parameters
Acceptance criteria for source material	Action limits for specific steps
Criteria for intermediates	Equipment performance
In-process and release criteria	Process limits

The CQAs then become a product’s in-process and release specifications, with the latter determining a product’s suitability for its intended use (i.e., to treat a patient). However, these criteria are not fixed and are continually evaluated in an iterative process. Over time, a developer will gain additional product characterization data, accumulate more insight into a product during clinical trials, and gain more experience manufacturing the product. Such new data and its findings can justify revising, adding, or removing a specification (Figure 2).

In summary, the QbD approach is a way to link the patient, therapeutic product, and manufacturing process together by monitoring clinical outcome (safety and efficacy), CQAs, and CPPs. In order for the approach to work, developers must choose fit-for-purpose assays that assess the essential attributes that predict a product’s quality with appropriate performance.

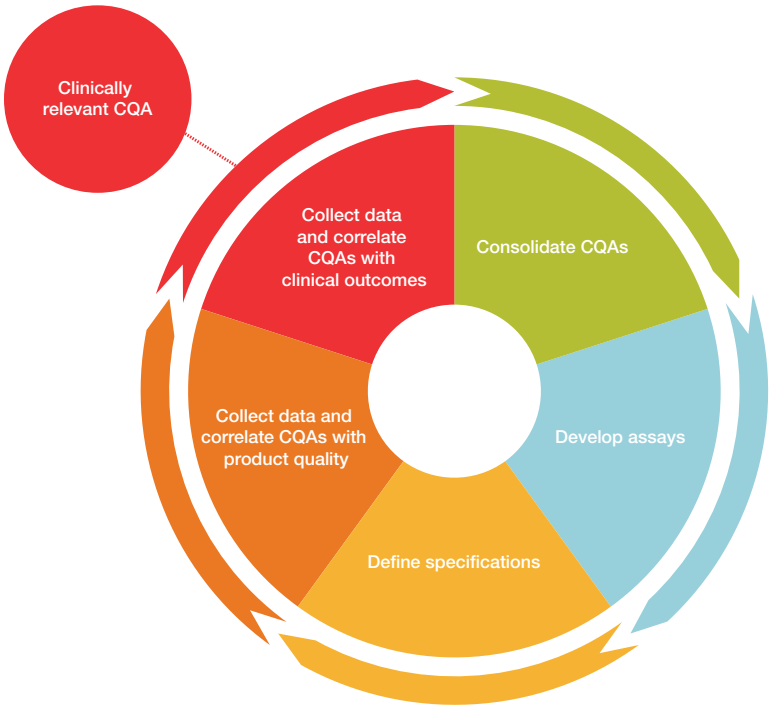


Figure 2. Systematic and iterative approach to identifying CQAs that are clinically relevant. As cell therapy manufacturers gain more information on their product from a variety of sources, they will revise or add specifications.

Regulatory perspective and development stages

While investment in cell therapy development has grown exponentially recently, it is still a young industry. Consequently, the regulatory landscape is evolving as the industry and regulators learn more about how the various types of living cell products are manufactured and how they behave when administered to patients. Given the complexity of these products and the diversity of indications they are meant to treat, no single framework exists to govern how these therapies are evaluated. Instead, each characterization scheme must be tailored to a particular product. Several guidelines are available for characterization and analytical methods, but only offer overarching principles and recommendations to help developers (Table 3).

Table 3. Examples of regulatory requirements and corresponding methodologies.

Attribute	Requirements and parameters	Method	MCB*	Vector	Cell
Identity 21CFR610.14	Phenotype	Flow cytometry, PCR, methylation-PCR, microarray	●		●
	Authentication	Short tandem repeat (STR), HLA-PCR or NGS	●		●
Purity 21CFR600.3	Impurity profile (Cas9, host cell DNA, host cell protein, residual vector DNA)	qPCR, ELISA	●	●	●
	Raw material residual (e.g., Benzonase, BSA, antibiotic resistance gene, transfection reagent, column leachables etc.)	qPCR, ELISA, HPLC, mass spectrometry		●	
	Residual bead	Flow cytometry, cell counting			●
	Empty:Full capsid ratio	qPCR/ELISA, HPLC, electron microscopy, ultracentrifugation		●	
	Contaminating cells	Flow, qPCR, sequencing	●		●
Potency 21CFR600.3	Functional assay	Cell-based assay	●	●	●
	Surrogate assay	Cell type-specific	●	●	●
Safety	RCR/RCL	Cell-based assay, qPCR		●	
	VCN	PCR, ddPCR	●		●
	Genomic stability	Cell-based assay, microarray, NGS	●		
	Tumorigenecity	Teratoma	●		
	Vector aggregates	Dynamic light scattering (DLS)		●	
Sterility & Adventitious agents 21CFR610.12 USP<71>	Sterility, endotoxin, mycoplasma, virus	Growth promotion test/bacteriostatis & fungistasis tests, LAL, PCR	●	●	●
Dose USP<1046>	Viability	Live/dead	●		●
	Cell counting	Cell counts	●		●
	Vector genome titer (VG)	PCR, ddPCR		●	
	Infectious genome titer (IG)	$TCID_{50}$		●	
Other	Product and process specific		●	●	●

In general, specific product assays are evaluated based on whether they are fit-for-purpose and performance. Developers generally take an incremental approach (Figure 3). For early-stage trials, regulators appear more interested in the rationale for the developer's choices, specifically demonstrating suitability of the method, justifying the choice of an attribute (CQA) to measure, and providing evidence and interpretation of results over the entirety of a product's developmental history. In these early stages, acceptance limits may have wider ranges. In later stages, the assays and acceptance criteria become more defined and the focus turns to method validation and a statistical approach to method capability. Table 4 describes the eight essential steps of method validation–accuracy, precision, specificity, detection limit, limit of quantitation, linearity, range and robustness–as outlined in *ICH Q2/R1 Validation of Analytical Procedures* [6].

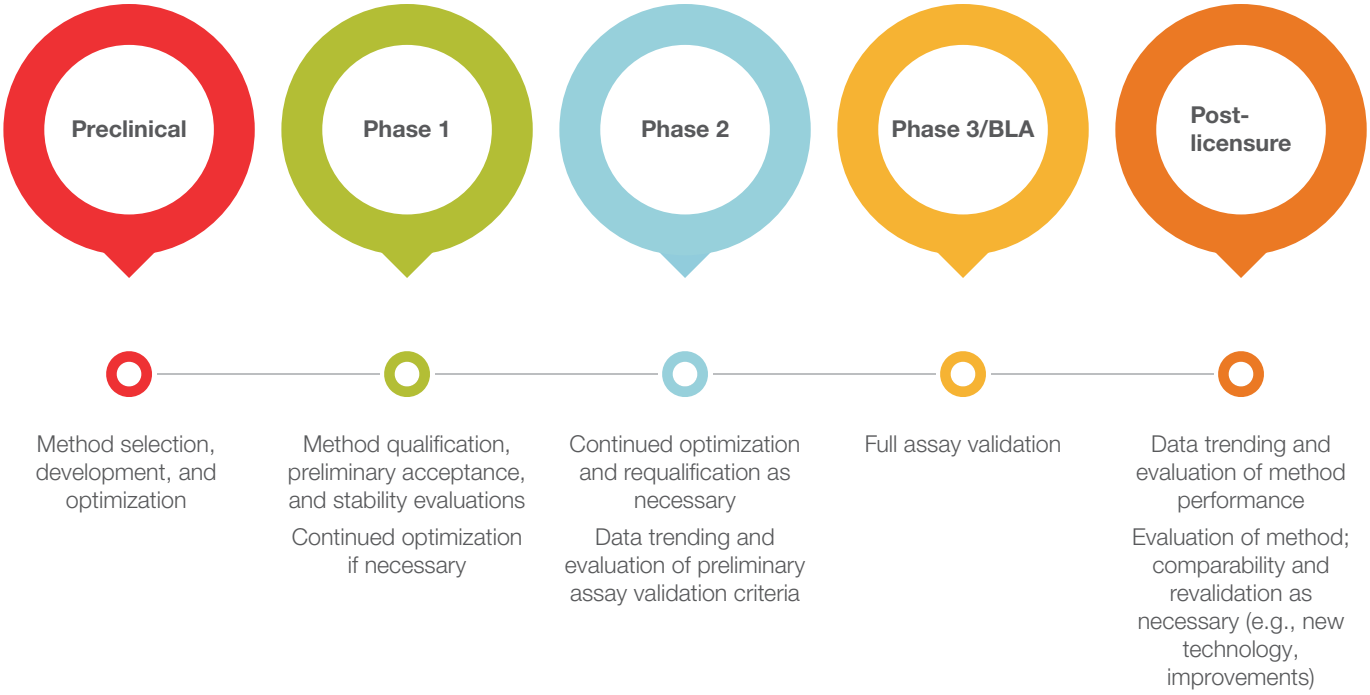


Figure 3. Stage-specific assay development for cell therapy characterization.

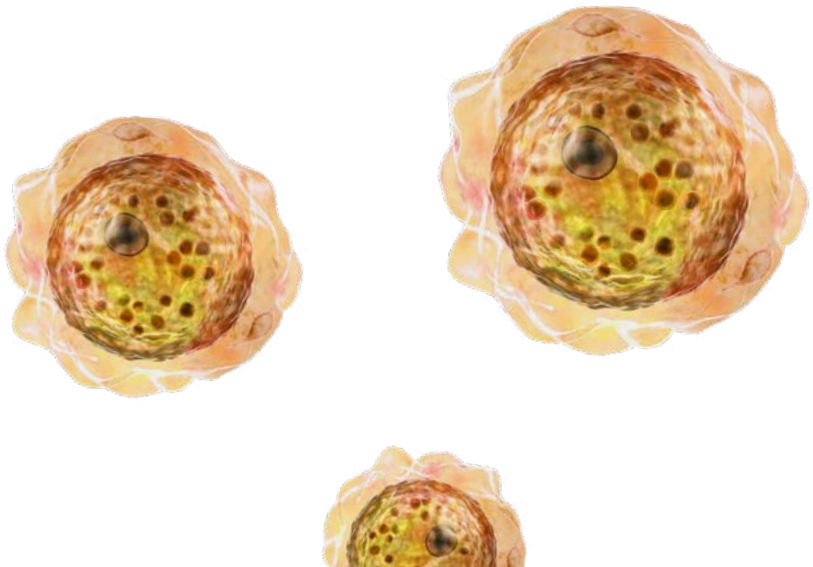


Table 4. Method validation steps of an analytical procedure as defined in *ICH Q2/R1 Validation of Analytical Procedures* [6].

Accuracy	"The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found."
Precision	"The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions."
Specificity	"Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present."
Detection limit	"The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value."
Limit of quantitation	"The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy."
Linearity	"The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample."
Range	"The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity."
Robustness	"The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage."

Trends and future

Most cell therapy developers will affirm that product characterization and analytics are some of the biggest challenges facing the industry. Until progress is made on those fronts, cell therapies will be relegated to a last line of therapy and never reach their full potential. What then are some of the trends and opportunities for improvement, and where is the field headed?

Flow cytometry has become an indispensable tool in cell biology, immunology, and cell therapy given its versatility. With a single platform, a developer can perform a wide variety of tasks, including for example, cell counts, cell viability tests, transduction for genome editing efficiency assays, and phenotyping. Additionally, flow cytometry can quantify the subpopulations of cells with a particular set of attributes, which is especially important in CAR T therapy. Despite its utility in cell therapy research, flow cytometry is not easily transferable into the GMP manufacturing environment primarily because of the variability inherent in the method. Multiple sources contribute to this variability including operator, reagents, instrument setup, sample preparation, and data acquisition and analysis. However, flow cytometry will never be completely replaced, challenging suppliers to introduce solutions to improve its compatibility with GMP environments (e.g., utilizing automated flow cytometry gating for analysis).

Wherever possible, the cell therapy field is moving away from cell-based methods to molecular-based methods because the variability of human cells makes cell-based methods more difficult to implement. Molecular methods offer numerous advantages over cell-based ones. First, molecular methods tend be to more sensitive. This sensitivity is important for such cases as identifying potentially harmful contaminating cell types such as undifferentiated induced pluripotent stem cells in a differentiated cell product or contaminating B cells in a CAR T product for B cell malignancies. Next, molecular assays are more readily standardized, ensuring that an assay run on different instruments, by different operators, at different sites, and at different times yields consistent results. Last, molecular assays typically require less material for the test. Some autologous cell therapy developers use up 50% of the dose for quality control testing. Under these conditions, if patient's starting material is limited, a developer may not have enough cells for a full therapeutic dose. In the future, developers will migrate to assay platforms that require less input material, most of which would be molecular based.

Single-cell analysis is becoming increasingly prevalent and important in cell therapy, especially after recent events with lentivirus-transduced stem cell products in clinical trials [7,8] .This event highlighted the need to understand exactly where transgenes integrate in the genome and if possible, determine if any endogenous genes have been disrupted. Overall, the industry is pivoting towards gene-modified cell therapies (e.g., CAR T), and as a result, the FDA is requesting that developers identify the location of a transgene on a single-cell level. With the advent of genome editing technologies and ever more complex engineering steps, it will also be important to quantify efficiency and demonstrate specificity and safety of the edits.

Many clinical and commercial stage developers are working on next-generation manufacturing processes to shorten the duration of manufacturing to decrease overall labor and facility costs and improve throughput. Some studies have suggested that in-process and release testing contribute up to 25–30% of production costs. These high costs are particularly burdensome in autologous cell therapy, because the entire cost of manufacture is allocated to a single drug for a single patient. The ability to multiplex compatible assays is one way to reduce these testing costs. Likewise, more rapid testing methods will be key to shortening vein-to-vein time. For example, commercially available rapid qPCR-based mycoplasma assays could be used in place of the 28-day USP <63> culture method. Another area of opportunity would be rapid sterility testing that several groups are working on, including The Standards Coordinating Body.

Over the last decade, several companies have partnered on applying digital technology solutions to healthcare manufacturing [9,10]. Scientists from the National Institutes of Health and National Institutes of Standards and Technology published a groundbreaking paper on the implementation of artificial intelligence (AI)-based quality control of their stem cell-derived product for treatment of age-related macular degeneration. The algorithm they developed analyzes images obtained by quantitative bright-field absorbance microscopy and are able to determine the maturation level of retinal pigmented epithelial cells. Such AI-enabled, image-based quality control methods allow for non-destructive monitoring of cell products and could enable adaptive manufacturing and real-time release. With digital methods like these, the potential exists to revolutionize the way cell therapeutics are tested and released, improving manufacturing throughput and reducing the time for delivery to patients.

As the field starts developing commercial-ready manufacturing processes, closed automated manufacturing systems will be deployed. Key to the implementation of these systems will be process analytical technology (PAT). The FDA defines PAT as a “system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes” [11]. The ability to make real-time, sensor-based measurements of critical process parameters such as metabolite production or nutrient consumption could improve a developer’s understanding and allow finer control of the manufacturing process. While PAT has been successfully implemented in large molecule biologics production, it is still in the early days of implementation in the cell therapy field. PAT designed specifically for cell therapy will be key to developing robust manufacturing operations that provide a steady supply of life-changing medicines to patients in need.

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Section 9:
Factors that impact cryopreservation of cell-based therapies

Introduction

Where traditional pharmaceutical drugs have failed, cell-based therapies can offer promise and solutions for treatment of diseases, which could revolutionize medicine and human health. Cell-based therapies are referred to as "living pharmaceuticals" because they use viable cells as the final drug product. However, manufacturing processes for these cell therapies are complex and pose unique challenges in comparison with traditional pharmaceutical drug manufacturing. In particular, cell-based therapies typically require a cryopreservation step to ensure that the cell therapy product is preserved without compromising sterility and efficacy. This cryopreservation step, however, increases the complexity of cold-chain management for late-stage clinical or commercial products. Cryopreservation of the final product allows for the product to be shipped at times that are most convenient and timely for the patient and clinical staff. It also obviates the need to maintain cells in culture for an extended period, minimizing the chances of senescence, genetic drift, and epigenetic changes that could alter the cells' beneficial characteristics. Cryopreservation of the final product ensures that cell-based therapies are accessible and available on a global scale.

Cryopreservation

Cryopreservation is the process of lowering the temperature of biological systems (e.g., cells) in order to preserve their structural and functional integrity. The goal of an optimal cryopreservation strategy is to lower the temperature below –130°C without intracellular ice formation during the transition from aqueous phase to ice phase. Successful cryopreservation will ensure that cells achieve the glass transition temperature (i.e., when liquid begins to behave as a solid), arrest molecular transport, and remain in the state of "suspended animation" without compromising the number and quality of cells [1]. Cryopreservation is preceded by manufacturing steps that include cell wash, cell harvesting, and formulation. The formulation step that involves the addition of cryoprotectant and ancillary materials or excipients to the cell suspension, is a time-sensitive and temperature-sensitive step and even minor execution errors in this step can have a negative impact on the final product [2].

Successful cryopreservation strategies are influenced by many factors including cell size, morphology, cell membrane permeability, and composition of organelles. Success is also significantly influenced by external factors such as composition and density of cell culture medium, choice of cyroprotectant, and cooling rates (Figure 1) [3]. To complicate things further, these factors need to be tailored specifically for each final cellular product. Suboptimal cryopreservation can result in loss of viability, insufficient cell number per dose, and dose-to-dose variability that may affect the overall efficacy of the therapy.

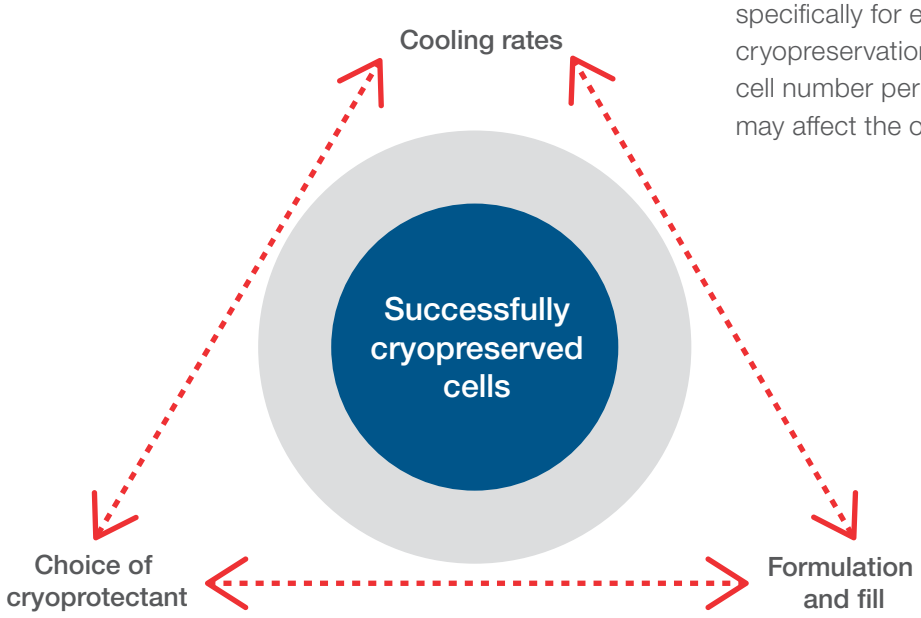
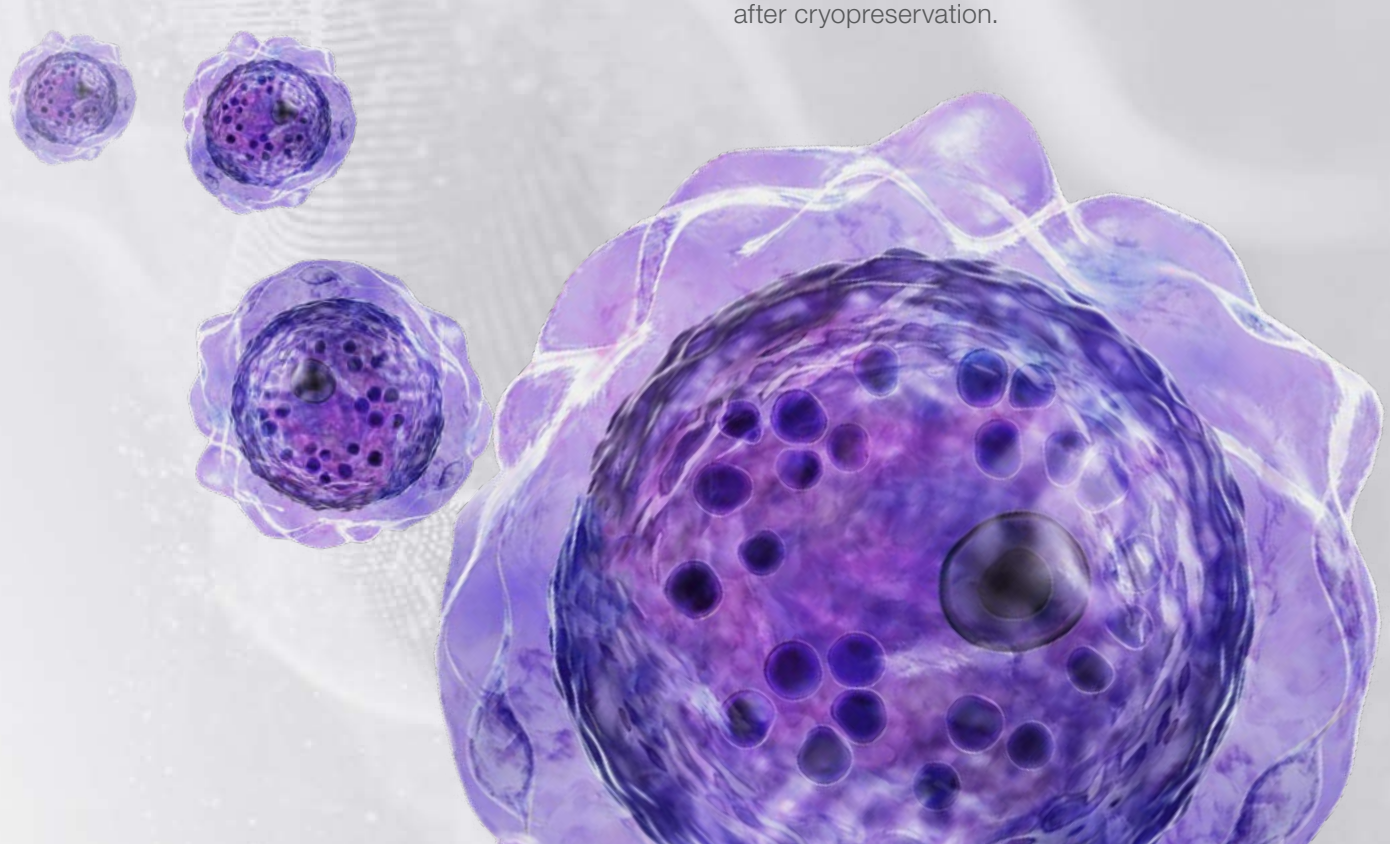


Figure 1. Optimal cryopreservation of cell therapeutics is impacted by several external factors, which must be optimized for individual cellular products.

A major challenge in cryopreservation is ensuring that cells not only survive the freezing process, but also maintain safety, efficacy, and potency profiles post-thaw. It is critical to optimize the process in order to avoid osmotic shock and membrane damage, which may lead to post-thaw cell death. Inadequate and non-uniform use of freezing parameters can lead to the artificial selection of subpopulations with phenotypic characteristics that are different from the desired population. Cryopreservation-induced stresses arising from various factors, including cryoprotectant toxicity, intra- and extracellular ice crystallization, altered intracellular pH, osmotic imbalance, and suboptimal rates of cooling and post-thaw warming, also represent a major hurdle and contribute to significant loss of cell viability and cellular function. Cryopreservation-induced stress can result in two types of cell death: apoptosis and necrosis. Following cryopreservation, apoptosis and necrosis are normally observed 6 to 24 hours into post-thaw culture [4,5]. Necrosis, characterized by swelling and disintegration of cellular organelles, is fast acting, caused by external stressors, and results in massively significant cell loss. In contrast, apoptosis, commonly referred to as programmed cell death, is characterized by cell shrinking and formation of apoptotic blebs, affecting single cells or small populations of cells [4,5].

Measuring cell viability post-thaw has its challenges. Immediate post-thaw viability measured by membrane integrity tests such as trypan blue dye exclusion or fluorescent cell imaging is not an accurate measure of cryopreservation process quality [6], highlighting the need for other assays to obtain a realistic viability profile. Preferably, post-thaw assessment of cell viability and cell number should be carried out beyond the 24-hour period [6]. Long-term testing (e.g., over 3–5 years) at multiple post-thaw intervals would be extremely beneficial in evaluating the robustness and stability of the cryopreservation process.

Cryopreservation-induced delayed-onset cell death (DOCD) is another form of post-thaw cell death that has been observed and appears to arise from a combination of necrotic and apoptotic stresses. However, unlike necrosis and apoptosis events, cryopreservation-induced DOCD may not be obvious through one time-point analysis of viable cells during the first few hours in the post-thaw process [7]. Instead cryopreservation-induced DOCD is usually characterized by a significant decrease in viability 12–24 hours post-thaw. DOCD results from permanent damage to cells when the level of oxidative stress is beyond the cells' ability to sustain or repair [7]. The choice of cryoprotectant and freezing medium formulation is critical to minimize DOCD and improve cell survival after cryopreservation.



Choice of cryoprotectant

Cryoprotectants preserve cells and tissues by minimizing physical and chemical damage during cryopreservation and promoting cell survival and cellular structural integrity. Effective cryoprotectants have a low molecular weight, are nontoxic, and do not influence the behavior of post-thaw cells. Cryoprotectants can be divided into two main classes: intracellular agents and extracellular agents (Table 1). Intracellular cryoprotectants work by penetrating the cell membrane and discouraging ice crystal formation. Extracellular cryoprotectants work by improving osmotic imbalances that can arise during the freezing process. While intracellular cryoprotectants are commonly used in cell-based therapies, interest is growing in the use of a combination of cryoprotectants to reduce toxicity while maintaining structural and functional integrity.

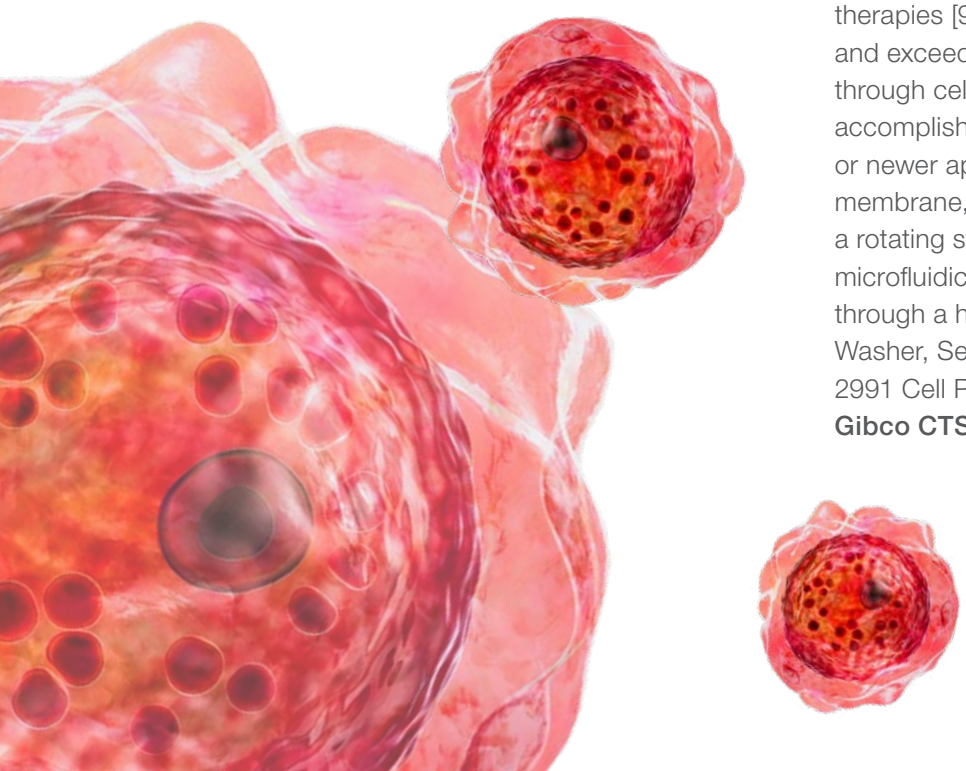
Table 1. Types of cryoprotectants.

Type	Characteristics	Examples
Intracellular agents (cell membrane-permeating)	Penetrate the cell membrane and prevent the formation of ice crystals that could result in rupture	DMSO, glycerol, ethylene glycol, and propylene glycol
Extracellular agents (nonmembrane-permeating)	Act to improve the osmotic imbalance that occurs during freezing	Sucrose, trehalose, dextrose, methylcellulose, and polyvinylpyrrolidone (PVP)

Intracellular cryoprotectants

The most commonly used cryoprotectant in pharmaceutical manufacturing is the intracellular cryoprotectant dimethyl sulfoxide (DMSO, Me_2SO) (Figure 2) because it offers enhanced penetration, provides long-term stability, and maintains safety and potency of the cells in final formulation [8]. DMSO has been used as an ancillary agent and as an excipient in final formulations.

While DMSO is the most commonly used cryoprotectant, it does have disadvantages. DMSO can adversely affect genomic and proteomic profiles of the cells and cause damage to cellular structures including mitochondria, the nucleus, and the cell membrane. DMSO can also cause a variety of adverse reactions in patients. When used as an excipient, the toxicity associated with DMSO requires that it be used at very low concentrations. Intake of DMSO at <50 mg/day is acceptable, and intravenous administration of up to 1 g/kg/day is common practice in transplantation therapies [9,10]. If DMSO is used as an ancillary material and exceeds ICH and FDA guidelines, it must be removed through cell washes. These additional wash steps are accomplished using traditional centrifugation methods or newer approaches such as filtration by spinning membrane, stepwise dilution and centrifugation using a rotating syringe, diffusion-based DMSO extraction in microfluidic channels, or controlled dilution and filtration through a hollow-fiber dialyzer [11,12] (e.g., CytoMate™ Cell Washer, Sepax™ S-100 Cell Separation System, COBE™ 2991 Cell Processor, Lovo™ Cell Processing System, or **Gibco CTS Rotea Counterflow Centrifugation System**).



Extracellular cryoprotectants

Recent advancements using non-DMSO agents with a combination of osmolytes like sugar, sugar alcohol, amino acids, and proteins show promise by improving post-thaw recovery [13,14]. DMSO-free cryoprotectants are the preferred option because of their lower risk profile, better tolerance by patients, better compatibility with bags and weldable tubing, and the potential of eliminating wash steps prior to infusion.

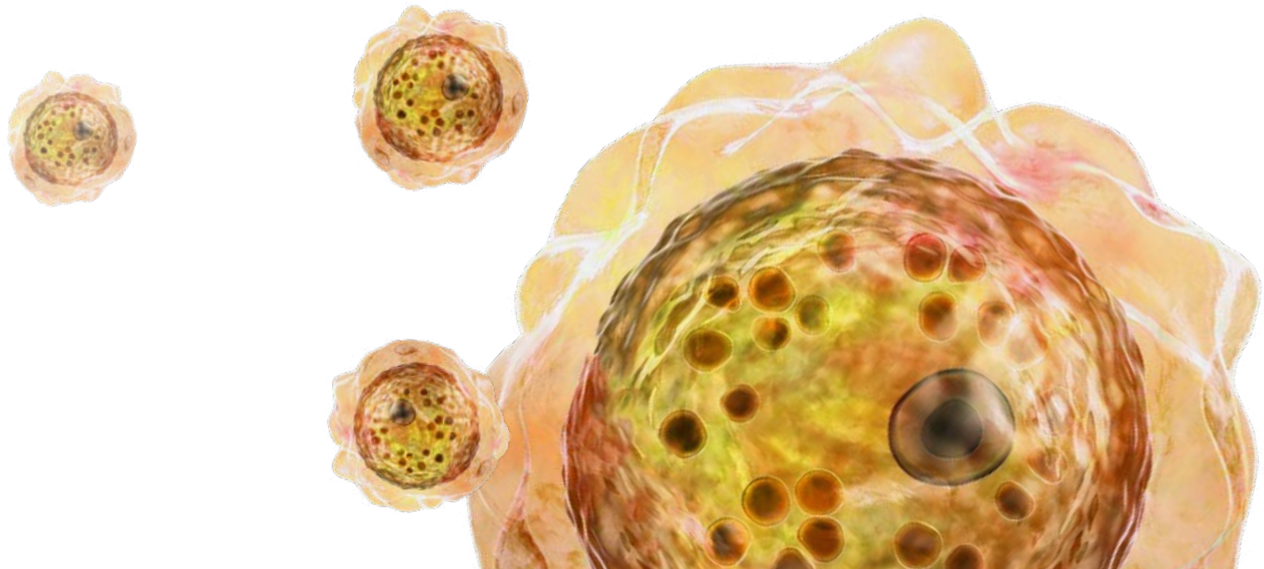
Currently, extracellular cryoprotectants have limited use in cell-based therapies because their addition typically results in suboptimal cryopreservation performance with poor cell viability post-thaw. However, recent research has shown some promise with trehalose, a nonreducing disaccharide of glucose. Trehalose demonstrates an exceptional ability to stabilize and preserve cells and cellular structures during freezing. Research has shown that the low penetration issue with trehalose can be overcome by addition of P2X7 (an ATP-activated receptor that opens transmembrane pores of the cells) [14]. Some other initial studies suggest the use of PVP in cryopreservation of human adipose tissue–derived adult stem cells resulted in recovery of cells that was comparable to that of DMSO with animal serum [15]. Methylcellulose either alone or combined with low concentrations of DMSO and human serum albumin (HSA) also demonstrated some promise [16]. While these studies are encouraging, further research is needed to evaluate extracellular cryoprotectants for cell-based therapies.

Formulation and fill

Formulation

Formulation is the process of combining cells, buffers, proteins, ancillary materials, and cryoprotectants and is carried out immediately after the cells are washed and harvested and following the cell expansion step. Formulation is a temperature-dependent and time-sensitive step because the harvested cells are held in suboptimal conditions without nutrition. Appropriate formulation is needed to stabilize the cells so they can withstand stress factors such as temperature excursions, pH changes, and mechanical stress caused by handling, storage, shipment, and bedside preparation. Because formulation precedes the actual cryopreservation step, optimal formulation is critical to produce a final cryopreserved cellular product that is stable, safe, efficacious, and meets regulatory requirements.

Formulation and final fill strategies involve selection of the appropriate cryoprotectant and other excipients and the final containers (see below). The selection of excipients plays a key role in the maintenance of critical quality attributes (CQAs) of the final product. Human serum albumin is one of the most popular excipients in cell therapy because it is the most ubiquitous protein in blood and is known to create an optimal microenvironment for sustained cell viability. It acts as a scavenger of toxins and other reactive oxygen species, maintains pH, provides insulation, and maintains cell viability during cryopreservation [17]. Additional components of the final formulation include dextran, which serves as an osmotically neutral volume expander and as parenteral nutrition, sodium chloride as a normal saline diluent, and stabilizers such as sodium caprylate and N-acetyltryptophanate that protect proteins such as HSA from oxidative stress [18].



Container choice

The choice of container can greatly impact the success of the overall therapy. Containers provide physical protection and are responsible for the stability over the entire lifecycle of the final product. The design and manufacturing of the containers must adhere to specific standards for storage and shipment reproducibility. Containers must also feature characteristics such as ease-of-use, stability at below-freezing temperatures, the absence of leachables and extractables, resistance to cryoprotectants (e.g., DMSO), and an optimal labeling surface [19]. The types of final containers most commonly used for cell-based therapies are screw-cap cryovials, bags, and plastic or glass vials (Table 2).

Screw-cap cryovials have been extensively used to store many cell-based products, especially for banking of GMP-grade master cell banks. Screw-cap vials are convenient and cost-effective; have a long-standing cryopreservation record; and work well for analytical and stability testing. However, they pose several regulatory challenges. Screw-cap cryovials require open steps for product filling that need to be carried out in a biosafety cabinet (BSC), making the process labor intensive, subject to human error, and more prone to contamination. They are also limited in volume per dose, have a limited labeling surface, and require extensive manipulation at the receiving site prior to delivery into patients.

Use of single-use bags is preferred by manufacturers of cell-based therapies. Single-use bags and kits along with a combination of ports and accessory tubing are available in standard and custom sizes based on their usage for various unit operations (e.g., cell washing, cell expansion, volume reduction cell harvest, cryopreservation). Single-use bags offer the advantages of optimal labeling surfaces, multiple sampling ports and minimal bedside manipulation (for final dose delivery). Use of these bags, however, requires investment in specialty instruments such as welders and sealers, specialized training for operators, and carefully planned processes for air removal and specialized packaging to ensure that the bags do not develop cracks and cause leakage of product after thawing. Though multiple bags can be filled using kits or automated systems, scale-up is challenging and lot sizes for a single manufacturing run are typically capped at 150–200 product bags [3].

The use of “ready-to-use” containers such as vials made of cyclic olefin copolymer and a pierceable septum that acts as a sterile barrier offer the advantages and flexibility of a closed system and scale-up for commercial needs [19]. However, they are expensive, require specialized training, and may require filling operations to be conducted inside the BSC unless a substantial financial investment is made in purchasing large and complex multifunctional automated systems or ISO 5 GMP manufacturing suites.

Table 2. Advantages and disadvantages of common container types for cell-based therapeutics.

Container type	Advantages	Disadvantages
Screw-cap cryovials	• Cost-effective • Convenient • Long history of use in cryopreservation	• Open system • Increased potential for contamination • Labor intensive • Increased fill error rates • Limited volume per dose
Bags	• Closed system • Customizable options • Good labeling surface • Minimal bedside tasks	• Increased costs for specialty equipment • Specialized operator training required • Scale-up issues limiting lot sizes
Plastic or glass vials	• Closed system • Improved manufacturing scale-up capabilities	• Very expensive • Specialized training required

Cooling process and rates

The process of lowering the temperature of cells to a frozen state requires a series of steps, which are individualized to cell type. First, in sequential steps, the cryopreservation formulation medium is added to the cells at a controlled rate to prevent cell loss resulting from osmotic stress. It is common practice to prechill the cryopreservation medium and to keep the cell suspension and the admixture chilled using cold packs, a frozen blanket, or a chilled work surface to prevent heat-related cell damage when adding DMSO. After adding cryopreservation medium, the cell suspension is transferred to the precooled chamber of a controlled-rate freezer. During the freezing process, product temperatures are recorded using a probe to generate a freeze curve.

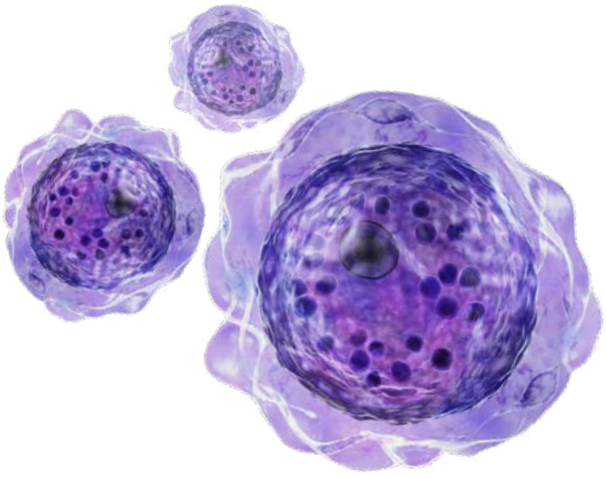
The rate of cooling during cryopreservation has a dramatic impact on cell viability of the final product. Cooling rates control the formation and size of both intracellular and extracellular ice crystals and can impact solution effects during the freezing process. While rapid cooling maximizes intracellular ice formation and minimizes solute concentration effects, slow cooling has the opposite impact. Currently, slow cooling is the most frequently used method of cryopreservation for a variety of cell types [5]. Furthermore, rapid cooling methodologies require a much higher concentration of cryoprotectant, resulting in toxicity-induced cell loss or addition of a washing and reformulation step at the clinical site [5].

Figure 1. Controlled rate freezers, such as the Thermo Scientific™ CryoMed™ freezer, help manage the cryopreservation process to ensure optimal parameters are met. For more details on the use of the CryoMed freezer, please read this **Smart Note**

Summary

The process of cryopreserving final product for cell-based therapies is critical, as sub-optimal cryopreservation processes can lead to failed product lots and ultimately failure to treat patients. While some point-of-care facilities for early stage clinical trials continue to deliver non-cryopreserved or “fresh” final product to the patient’s bedside, this is not a sustainable option. As the field of cell-based therapies matures, delivery of cryopreserved final product that is standardized, scalable, reproducible, in compliance with global regulatory agencies, and has a maximized shelf life for an “on-demand distribution” will prove to be the best option.

For more details on the cryopreservation process, please check out this **white paper**



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Section 10:
Cell therapy supply chain logistics

Introduction

The journey that a cell or gene therapy takes as it goes from development, to manufacturing, to patient bedside requires intricate logistics to maintain product integrity at ultracold or cryogenic temperatures. This takes on an additional level of complexity when it comes to cell therapies. Logistical strategy requirements can vary according to the specific type of cell therapy and requires a deep understanding of the complete cell therapy production workflow. This multistep manufacturing process broadly involves:

1. Cell collection from a patient or donor
2. Transportation to a manufacturing facility for manipulation
3. Processing into the drug product
4. Return of the final product to a clinical environment where it is administered to a patient

As a patient's cells are transformed into a living drug product, they move through a range of storage conditions varying from refrigeration (2°C to 8°C) to cryo-storage (–150°C to –196°C) (Figure 1). This chapter will outline some of the broad topics to consider when developing a cell therapy supply chain or distribution strategy, and highlight nuances that may apply differently when considering autologous cell therapies versus allogeneic cell therapies.

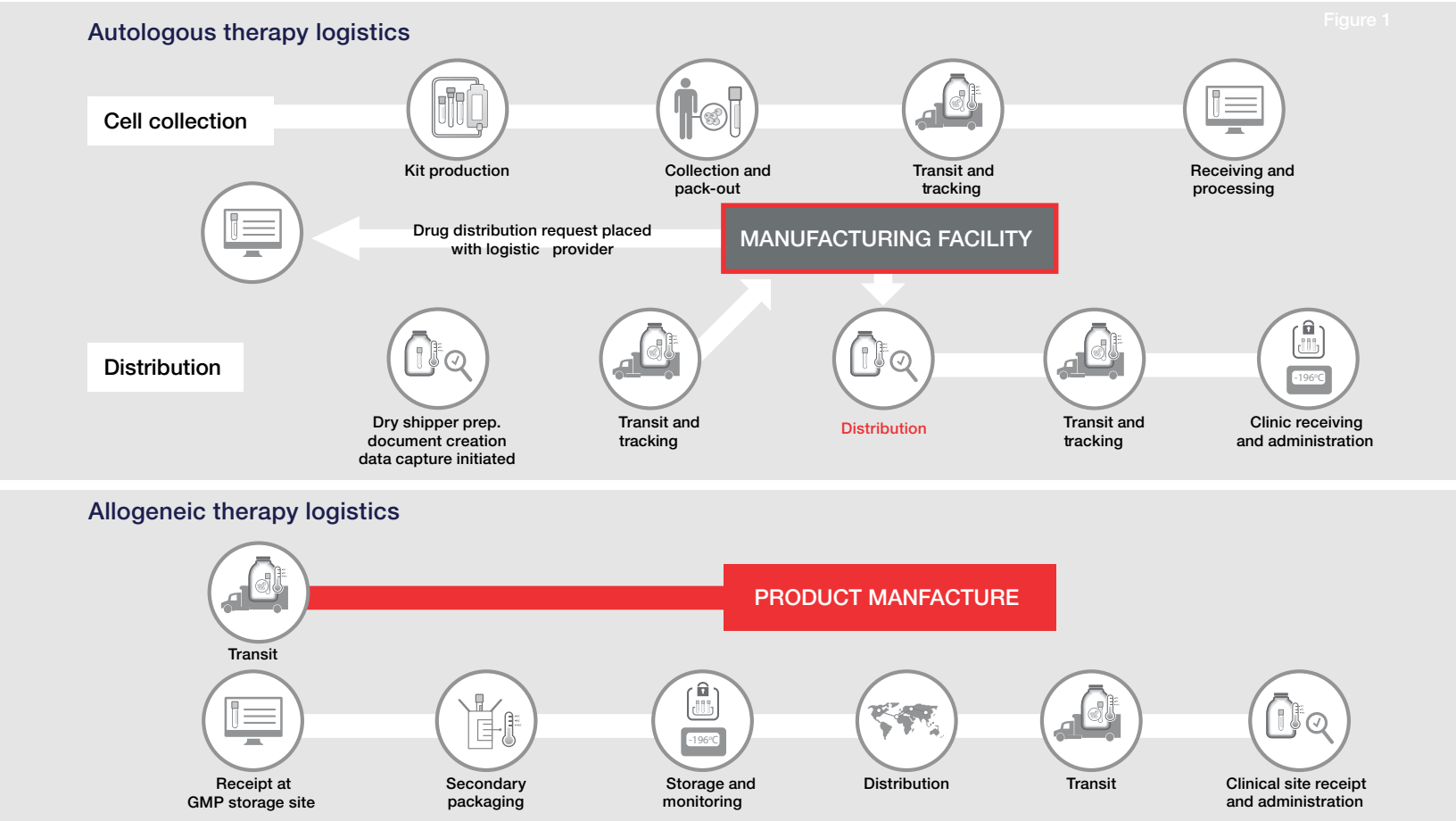


Figure 1. Overview of cell therapy logistics.

Autologous versus allogeneic cell therapy logistics

Autologous cell therapy is a vein-to-vein supply chain process where the starting point is material collection directly from a specific patient. This specimen serves as the raw material input for the manufacturing process. Vein-to-vein supply chains begin with the collection and transportation of patient starting material to the cell manufacturing site, and ends with the distribution of a final drug product from the manufacturing site to the hospital. Because of the critical temperature requirements at each step of the manufacturing process, and the fact that the therapy is personalized to a single patient, it is of utmost importance to mitigate risk within the supply chain as much as possible to ensure final product quality and chain of identity. Critical drivers for maintaining this supply chain include:

1. **Dose availability**—there may only be one dose available for a particular patient.
2. **Patient condition**—the receiving patient may be very sick, which means that any delay in the supply chain could put the receiving patient at risk.
3. **Therapy identification**—ensuring chain of identity and chain of custody throughout the logistics process is essential to delivering the right drug to the right patient.

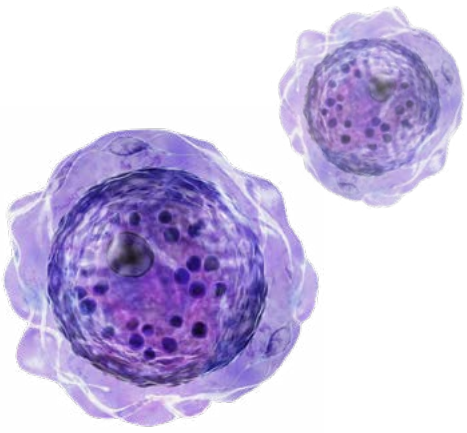
The logistical strategy begins with **leukapheresis** (collection of the patient's blood) or other patient specific samples. To maintain consistency and ensure high cell quality, standardized apheresis (or sample) collection kits can be used to ensure conformance and adherence to standard operating procedures (SOPs), and promotes a method for standardized collection of the raw material input. Kit usage ensures consistent starting material quality for the manufacturing process and can minimize operator-induced effects on efficacy. Coordination of kit assembly; shipper preparation and arrival at the collection site; and apheresis collection need to occur simultaneously. Following harvest from the patient, cells can either be shipped fresh (2–8°C) or cryopreserved and stored for later use. While cryopreservation allows for more flexibility in the manufacturing timeline, most manufacturing protocols still require fresh cells.

Allogeneic therapy logistics have similar requirements to autologous therapy, but the overall workflow allows for some flexibility of supply. An immediately apparent difference in the supply chain is the sourcing of donor material. Once the HLA type or other cell characteristics have been identified, a donor must be identified to provide the starting material. While autologous therapies rely on cell harvest from individual patients, repeating the supply chain per each individual treatment, **allogeneic products** make use of pooled healthy donor cells as the input starting material. Cells must be sourced according to GMP guidelines, and must also adhere to strict chain of custody and identity requirements. Until truly allogeneic products are developed, the characteristics of the starting material will determine whether a patient is a good match for the final drug product.

Regardless of the treatment type, once any cells enter into the manufacturing process, they are packaged and labeled in adherence to strict considerations and guidelines. Following completion of the manufacturing process, the drug product is **cryopreserved**, placed inside a dry vapor shipper, and shipped via next-flight out specialty courier services to the clinical site for patient dosing.

Storage

Storage conditions of the cells throughout the entire process are critical to ensure the integrity of the drug is not compromised during any stage. Gene therapies require a minimum storage temperature of –80°C, and cell therapies are primarily stored at –196°C or below. These ultra-cold temperatures make it essential to consider some key factors when selecting a storage facility to ensure proper ultra-cold or cryogenic storage conditions.



Ultra-cold and cryogenic storage

Whether you are selecting a cold storage service provider or establishing an internal storage facility, there are several important considerations to note, as described in Table 1.

One important way to mitigate risk is by utilizing decentralized storage facilities and storing samples at multiple sites. This is particularly important for master cell banks (MCBs) and drug products. Utilizing an organization that has multiple sites can also influence the ability to perform and accommodate just-in-time (JIT) deliveries for patient procedures.

Table 1. Considerations for cold storage in cell therapy logistics.

Redundancy	• Is there more than one storage system in place to protect your specific material? • Are there redundancies within the systems in case one path fails to ensure continuous protection of your materials?
Risk mitigation	• What is the backup capacity to transfer materials in case of storage failure? • Are there backup generators to maintain storage conditions in the event of power failure? • How secure is the facility? Is it access controlled? Monitored 24/7?
GMP compliance	• Does the facility have the appropriate licensing and permits needed to meet regional, state, and national requirements? • Are regular audits performed by regulatory organizations to ensure the facility's compliance?
Scale-up ability	• Can the provider accommodate a future transition from clinical to commercial-scale material volumes? Considering this particular feature will allow the early establishment of more universally applicable SOPs and facilitate a future transition from clinical to commercial processes seamlessly.
Cell and gene therapy experience	• This factor is very important due to the small volume packaging nature of many final drug products. • Storage considerations and SOPs require cognizance of minimizing the time spent "out of temperature" whenever a sample is removed from storage.
Storage model	• Is the site a centralized single-site model or a decentralized (multi-site) model? It may be important to have storage facilities across different geographies and various regions.

Packaging and labeling considerations for cryogenic drug products

For both allogeneic and autologous therapies, many of the considerations for packaging and labeling of the products are similar. In either case, the final drug product is typically stored and transferred under **cryogenic storage conditions**. This requires specific considerations when deciding on the packaging and labeling of these products. An important difference arises in handling autologous therapies, because these therapies require an ability to trace the treatment products through the entire process—from start (patient cell or tissue harvest) to finish (therapy administration to the patient). Unlike allogeneic therapies, autologous therapies will require an additional chain of identity (COI) tracing capability to ensure that the right therapy is administered to the right patient, and the therapy has not been compromised during any stage in the development process.

The first consideration is the type of container to use. Both the choice of whether to use a cryovial or cryobag and the volume of the storage container are important. These initial selections will impact your options for the label type, which then affects the kind of information that can be included on the primary label. Once the type and size of your packaging is determined, the process by which the label is applied to the container must be addressed. Options include application of the labels manually or mechanically by a machine. This has further implications for the type of environment where the labeling activities will take place—specifically the temperature conditions (ambient, cold, dry ice or cryocart). Machine labeling often leads to more consistent labeling results, but may not be part of the process when manufacturing a single dose. Importantly, how application of a label at ambient temperatures relates to the temperature of the product itself are important to understand for quality and consistency of label adherence. It is critical to choose labels that are appropriate for the final storage temperature conditions, ensuring that the label will maintain adherence throughout the lifetime of the product.

Finally, choosing the type of label can also affect several downstream decisions. A label can be a single-panel label or a booklet label. While booklet labels allow more information to be included, several components are involved in manufacturing a booklet label, and not every component is optimized to handle cryogenic conditions (e.g., the type of paper used and the hot melt used to bind pages together). Cell and gene therapy products are generally stored and shipped in cartons, and it is important to anticipate whether the label connections and the final labeled material can fit inside the chosen storage boxes.

The actual content printed on the label can vary depending on different country regulatory requirements. While these requirements can vary from region to region, basic required information typically includes:

- Dosing instructions
- Translations into local languages
- Final storage temperatures
- Details related to the sponsor and manufacturer

For autologous therapies, because of the highly individualized nature of the therapy, there may be additional requirements for label information. This can include the listing of unique tracing information such as the chain of identity and the use of specific barcodes or identification tags to minimize the risk of an incorrect treatment delivery to a patient.

Transportation considerations for high-value materials

The top priority for cell and gene therapy transportation is maintaining the integrity of the drug while ensuring it arrives on time. With autologous therapies, there is a high value placed on individual components of the supply chain given the highly personalized nature of the drug product itself. The impact of failure during any part of the supply chain, whether it affects the initial patient-harvested sample or the final drug product, has significant impact on the final patient outcome. It is essential to move materials quickly and safely to meet the needs of often extremely ill patient populations. This includes ensuring sufficient supply and storage conditions for raw materials; collection and

transportation of apheresis or other patient-derived starting material; and final distribution back to the patient. To achieve flawless operation, qualified shipping technologies and data loggers can be employed to monitor and track temperature fluctuations and maintenance of the cold chain. The task of maintaining speed, temperature, and integrity becomes complicated with these drugs because many gene therapies are transported at a minimum of –65°C, while many cell therapies are moved at cryogenic temperatures, which can be as low as –150°C. Important characteristics to look for when evaluating qualification protocols include but are not limited to:

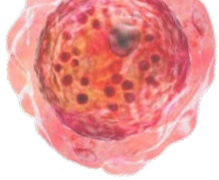
- Ambient profile—custom or industry standard
- Seasonality—e.g., summer and winter
- Payload used (minimum and maximum)
- Repeatability—triplicate tests
- International Safe Transit Association (ISTA)-certified packaging testing lab
- Industry standard-calibrated equipment
- Design qualification (DQ), operational qualification (OQ), and performance qualification (PQ)

Equally important when making decisions and establishing SOPs is the shipping lane validation. This is a complex process that requires consideration of many different attributes that could affect product integrity in transit. The origin, destination, and transport route can all impact the stress temperatures through which a product passes. Seasonality can also add an additional dimension in establishing protocols. Placement of temperature monitoring devices within the container is important to ensure an accurate representation of actual product temperature maintenance is recorded rather than fluctuations in the outside environment. All shipping protocols should be validated using a minimum of triplicate shipments and importantly, should undergo periodic re-evaluation to ensure that any subtle changes in any part of the supply chain are constantly monitored and quality controlled. An often underestimated aspect to logistics and supply chain is the level of regulatory compliance needed for the final step of the supply chain, the actual delivery of the drug product. Maintaining 21 CFR part 11 compliance in the ever-evolving regulatory landscape requires constant evaluation of product integrity over the course of the product's shelf life. This is often addressed by choosing trusted service providers. These partnerships typically provide a range of services, including offering validated SOPs and different levels of service such as just in time (JIT) service (e.g., Patheon logistics next-flight out).

Using an experienced and trusted partner that has the ability to navigate trade compliance, regulatory compliance, and risk management is critical. Processes need to take into consideration potential issues arising from customs holds or delays that could affect product integrity. When shipments are made at –65°C conditions, re-icing can be performed to maintain temperature continuity. However, for liquid nitrogen (LN2) shipments, recharging is not possible with current packaging technology, which limits the maximum hold time to 8–14 days depending on shipper type. To minimize this limitation, multiple layers of risk mitigation can be employed:

- Prepare appropriate documents ahead of time to avoid possible delays (e.g., custom holds)
- Partner with experienced custom brokerage services
- Consider free-trade zone options
- Develop decentralized supply networks to reduce the risk

In order to orchestrate a flawless operation, many clients or service providers have found utilization of a control tower approach to be an effective way to manage cell and gene therapy transportation and logistics. The control tower is a centralized hub that consolidates all of the monitoring systems and data gathering tools that are employed across all stages of the supply chain. This gives the user complete visibility over the entire process. The control tower can either be developed by a client to encompass their chosen tools and components, or can be developed by the logistics service providers.

A major advantage of using a control tower approach is the ability to identify points of failure and strengths of the supply chain, enabling implementation of predictive analytics to maximize efficiency and improve processes. Additionally, full visibility allows for real-time monitoring to ensure execution of specific instructions to individual logistics service providers. Ultimately the data feed into effective management of incidents or exceptions, allow the customer to generate pre-developed escalation plans and contingency procedures, and drive effective customer service.

Chain of custody and identity

As mentioned, the supply chain for cell therapies, especially for patient-specific autologous therapies, requires a strict level of compliance and chain-of-custody establishment. Complete end-to-end visibility and documentation is required and includes location monitoring, temperature tracking, and documentation of all handoffs between providers (i.e., ground transportation to air transportation to customs inspections, etc.). During transit, individual tracking from the point of pickup through final delivery requires identifying authorized individuals (shipper and consignee) and associated chain-of-custody documents including initials, dates, and times. Additionally, real-time temperature and GPS location monitoring and confirmation of location throughout transit allows for the ability to alert customers of potential delays.

In the case of cell and gene therapies, information must be maintained at a per-dose level, and this documentation must be readily available in case of a temperature excursion or other issue. Allogeneic therapies can use bulk storage solutions and be stored in vapor phase LN2. Because not all companies are able to support vapor phase LN2 transportation, partnering with companies (e.g., Thermo Fisher Scientific) that do have an ability to support this type of supply chain can ensure quality of the final drug product.

For autologous therapies, chain of identity becomes a critical driver of logistics to ensure that the correct drug product makes it to the right patient on time. When establishing a chain of identity process, it is important to consider the following:

1. List patient unique identification number and lot information on product primary and secondary packaging
2. Routinely evaluate chain of identity (COI) controls in a risk management cycle: define plan and stakeholders; draft and approve process maps for applicable areas; draft failure-mode exception analysis (FMEA); develop deviation and risk assessment; review and approve in document control; training and implementation
3. Ensure SOPs are in place and regularly reviewed
4. Minimize risks of mix-ups with appropriate segregation, line clearance, and changeover within the plant
5. Implement verification steps (manual or electronic) throughout the process to ensure the appropriate link is maintained from cell collection to drug product infusion at the treatment site
6. Balance COI with Health Insurance Portability and Accountability Act (HIPPA) and other privacy laws

Distribution—pick/pack/ship

The next phase is selecting a partner to distribute the product. When selecting a service provider, some questions to ask are:

1. Does this service provider have appropriate proper licenses to operate both for clinical samples and for commercial products?
2. What are the lead-time requirements for the service? Do you need to request shipping materials in advance? Can they distribute next-day or same-day? Make sure to establish service-level agreements so that you understand the logistics, including what information your provider needs in order to get the shipment out on time.
3. Is the partner able to accommodate JIT requests? (Note that JIT scheduling needs to account for the health of the patient receiving treatment).
4. Does the provider have serialization capabilities to support my future commercial launch ?

Transitioning from clinical to commercial supply volumes

Initially, most decisions about supply chain and logistics will be made when working at a clinical level, where the sample size and volume may be smaller and less likely to be in bulk. However, whether the current supply chain will be able to transition and accommodate commercial supply requirements is critical to success. While clinical supply chains may operate on a regional or domestic level, commercial supply chains often require transportation across borders and involve multiple points of contact between the initial pickup and final point of delivery. As the manufacturing process becomes more global, additional compliance, transportation, and supply issues need to be addressed (see Table 2 for summary).

With commercialization, these highly sensitive materials may need to travel greater distances and encounter a greater number of transitions based on different world destinations. Different countries have different regulations and what may comply with one country's requirements may not work for another. Processes may need to change to ensure global trade compliance. Additionally, different countries may have tax requirements that add tax at different points in the manufacturing-distribution-sales process. This could affect the ability to operate in or provide a product to certain countries such as countries within the European Union.

Within the United States, there are important FDA compliance considerations as a product transitions from clinical use to commercial use. Compliance with the Drug Supply Chain Security Act allows the ability to trace prescription drugs as they are distributed around the country. In particular, the ability to implement an electronic interoperable system is critical to comply with Title II of the Drug Quality and Security Act.

When a product transitions from a clinical trial setting into the commercial realm, many strict guidelines covering final product label artwork and information have an impact. These requirements are governed by the FDA in the United States, and by equivalent agencies in other

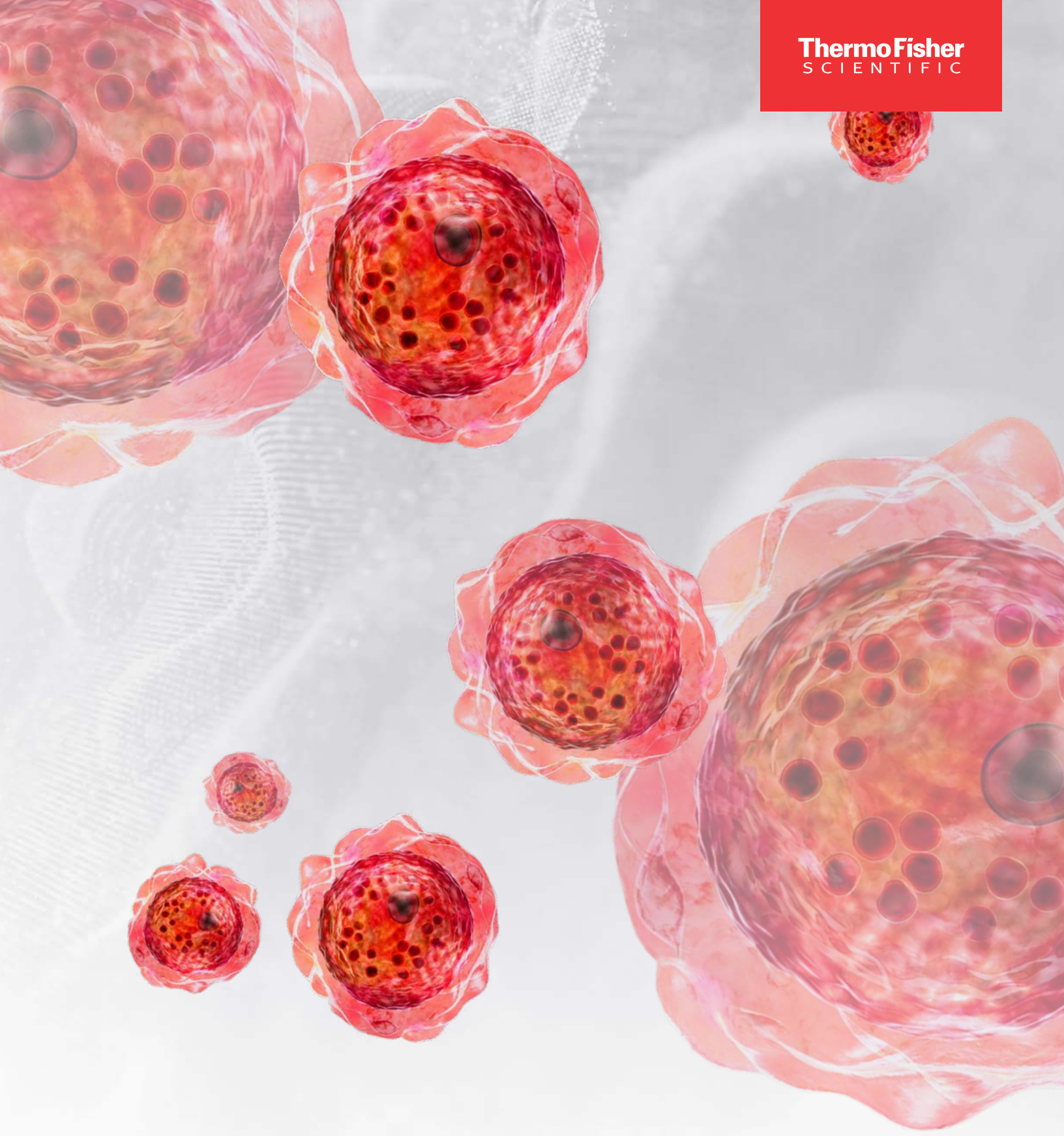
Table 2. Questions to consider in transition towards a commercial-scale operation.

Issue	Questions
Scalability of supply chain	• Can the current supply chain handle a scale-up and scale-out to accommodate commercial level needs? • Are the partners you selected for the different phases of storage, transportation, and distribution able to accommodate a higher volume as therapy requirements increase?
Global trade compliance and value-added tax (VAT)	• Are there any adjustments or modifications required to your current processes to ensure global trade compliance? • What are the tax requirements of each region?
Regulatory compliance	• Are there providers with ATMP QPs on staff that can issue a QP declaration for my product? • What documentation does the QP require to issue a QP declaration? • What is the lead time for QP batch or product release? • How far in advance do I need to engage with a QP prior to applying for EMA approval?
Serialization	• How will my product be required to comply with the Drug Supply Chain Security Act? • What EPCIS system should we select to integrate with our commercial packaging and distribution partner?
Label and artwork design	• Does the final commercial product label comply with the appropriate region's requirements? • What labels or components do I select in order to withstand storage and distribution at cryogenic temperatures?
European Union "Blue Box"	• Does the label comply with guidelines issued by European Medicines Agency (EMA) authorization?

countries around the world. Every country has their own requirements, so approval from the United States or the EMA does not mean the label will comply with other countries' standards, leading to a label re-design to comply with each individual target market. For the European Union, label guidelines are issued by the EMA. Their authorization for a single drug application outlines guidelines dictating what information must appear on a label; the languages in which the text must appear depending upon the country where the product will be marketed; additional text requirements that may be member-country specific; colors, logos, color schemes, and other features of the label; and the inclusion of a marketing authorization number on the label, among other specifics.

Summary

Although the specific details for each supply chain and logistics plan will differ depending on the specific clinical and commercial needs of the product, key areas exist where forethought and planning can decrease risk and decrease the potential loss of materials and expensive solutions. For cell therapies, these special considerations include storage temperatures, transportation modes, chain of custody and identity, and labeling, and the regional regulations and differences that impact them. Choosing a trusted materials and service supplier experienced in working with cell therapies can alleviate some of these challenges, helping cell therapy manufacturers deliver their precious drug in a timely and safe manner to extremely ill patients.



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