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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

Cell therapy analytics and CMC

GUEST EDITOR

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CELL & GENE THERAPY INSIGHTS

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Cell & Gene Therapy Insights—cell therapy analytics and CMC edition

Ashish Saksule

FOREWORD

"Through collaboration between scientists, industry pioneers, and health authorities, clearer frameworks have emerged, bringing more predictability and rigor to the field."

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Dear readers,

Welcome to the November edition of *Cell & Gene Therapy Insights*. This issue brings together a series of scientific and commentary articles that reflect both the progress and growing maturity of our field. Together, they explore how we are refining science, strengthening analytical and regulatory frameworks, and preparing for the next generation of therapies that can truly transform patient care.

Among the highlights are thoughtful long-form pieces from leaders in quality, regulatory, and analytical development. One contribution explores CMC strategy and regulatory considerations in cell therapy, including approaches for qualifying cellular starting materials and implementing rapid microbiological methods in clinical manufacturing. Another focuses on AI-driven quantitative imaging of iPSCs, using reporter cell lines to better

understand pluripotency and differentiation. Additional articles from experienced analytical and CMC experts discuss evolving strategies in potency assay design, flow cytometry–based analytics, and new testing paradigms that are helping shape a more consistent, data-driven future for cell and gene therapy manufacturing.

As a community, we have traveled far from the early days when even basic definitions such as which assays truly mattered or what regulators would consider acceptable were uncertain. Through collaboration between scientists, industry pioneers, and health authorities, clearer frameworks have emerged, bringing more predictability and rigor to the field.

The workforce has evolved alongside this progress. A decade ago, skilled professionals with hands-on cell and gene therapy experience were rare, and organizations invested heavily in training and mentoring to build internal expertise. Today, we are fortunate to have a talented and experienced community of scientists and engineers. However, with several companies scaling back or closing programs, many skilled professionals now face instability. Preserving this expertise and supporting the workforce that built this industry will be essential to sustain future innovation.

The challenge of cost remains equally important. Despite remarkable scientific progress, the COGs for advanced therapies continues to limit global accessibility. The next phase of advancement must focus on improving efficiency, simplifying manufacturing, adopting smarter automation, and enhancing reproducibility to make these therapies more affordable and widely available.

The current landscape is undoubtedly complex. Expanding or sustaining a CGT program requires significant investment, regulatory navigation, and operational resilience. Yet this is also a moment of tremendous opportunity. Over the next two to three decades, cell and gene therapy is expected to become one of the central pillars of medicine, shifting healthcare from treatment to cure. In the near term, biologics and small molecules particularly in metabolic, CNS, and immunology and inflammation areas will continue to dominate. However, with advances in AI-driven analytics, multi-omic characterization, adaptive clinical trials, and improved safety frameworks, the path toward safer, faster, and scalable living medicines is becoming clearer.

This edition captures that balance the optimism of innovation and the discipline of execution. It reflects a field learning from experience, adapting to challenges, and steadily moving toward global impact.

To all our readers, scientists, engineers, clinicians, and leaders, thank you for your continued dedication. May this issue inspire collaboration, reflection, and renewed purpose as we work together to make cell and gene therapies that are safe, scalable, and accessible to patients everywhere.

Warm regards,
Ashish Saksule

BIOGRAPHY

Ashish Saksule is an accomplished cell and gene therapy process development expert, specializing in the bioprocessing of viral vectors. With over 10 years of experience, he has worked across various domains, including academic research, clinical stage vaccine development, and process optimization for cell and gene therapies. His expertise spans

both upstream and downstream processing for viral vectors, including lentiviruses, AAV, live viruses, and proteins.

Ashish holds a Master's degree in Biotechnology from Harvard University, Cambridge, MA, USA and a further degree in Chemical Engineering from Michigan Technological University, Houghton, MI, USA. Currently, he serves as a Principal Scientist and Core Lead at Vertex Pharmaceuticals, where he oversees the Vector Core facility and the Cell Therapy Core facility. Prior to this, Ashish contributed to the Rare Disease Gene Therapy program at Takeda and worked at MilliporeSigma on virus and gene therapy process development for AAV and LV. His passion lies in developing cutting-edge bioprocessing tools for cell and gene therapy, with the goal of making these life-changing treatments more accessible and affordable for all patients.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY ANALYTICS AND CMC

SPOTLIGHT

EXPERT INSIGHT

Options for using optical microscopy in the development and manufacturing of iPSCs

Anthony J Asmar and Anne L Plant

The development and manufacturing of cell therapies using induced pluripotent stem cells (iPSCs) require effective methods for monitoring and characterizing cell cultures. Optical imaging is a valuable tool for assessing cell cultures during the manufacturing process by providing spatial and temporal information about various cell characteristics. Widely available imaging modalities, including brightfield and phase contrast imaging, can be used to monitor different aspects of cell cultures, such as confluency, morphology, and cell state. However, other forms of imaging are becoming more developed and accessible. The choice of imaging modality depends on the specific application and desired metrics. Here, we discuss some of the advantages and limitations of different optical imaging modalities for live cell monitoring, highlighting their potential applications and challenges in cell therapy manufacturing. By understanding the capabilities and limitations of these imaging modalities, one can better design and implement imaging-based monitoring strategies for cell therapy production.

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APPLICATIONS OF OPTICAL IMAGING

Quantitative characterization of cell products is still one of the most difficult challenges in the development and manufacturing of advanced therapies. Because of the expense and time involved in manufacturing, it is of great interest to monitor the characteristics of a cell population throughout the manufacturing process [1]. It is preferable to identify a population that is likely to fail early rather than at the end of an expensive manufacturing process. Imaging of cells is often part of the development, scale-up, and production process, and the most common imaging applications are performed on isolated cell samples at discrete time points. One common application is to monitor the confluency and growth rate of a 2D cell culture. More recently, imaging of live cell populations over time is

providing more unique information that is both spatial and temporal without requiring cell processing or destruction of the cell sample. Data on individual cells over time can provide quantitative analysis of temporal characteristics such as cell mobility, division rates, morphology changes, and parent-daughter lineage relationships for each individual cell, allowing analysis of the diversity of characteristics within the cell population [2,3]. These kinds of analyses require methods that allow the identification of individual cells, which need appropriate imaging modalities and analytical methods. Changes in those characteristics over time and in response to changes in culture conditions can be sensitive and rich attributes of cell populations and potential indicators of cell state, and likely can provide predictive value when paired with appropriate models [4–6]. This classification of different states of individual cells, colonies, and the overall population allows the monitoring of cell health, pluripotency, and differentiation. These metrics can allow the progression of cultures over time to be assessed and compared to help guide the development of manufacturing protocols and necessary interventions. Examples of such deployments show that quantitative imaging data produced in a continuous manner can be used as inputs to provide feedback for controlling automated processes such as passaging, expansion, colony picking, differentiation, and other protocols [7–11]. Some commercial solutions of image-based automated culture systems already exist (e.g. CellXpress.ai, Celligent, Nebula, C.STATION).

IMAGING MODALITIES

There are a number of types of imaging that are appropriate for use with live cells in traditional tissue culture containers, and here we will discuss a few of the most common modalities (Figure 1). In-depth information on optical microscopy modalities can be found elsewhere [12–15]. We focus on characterizing induced pluripotent stem cells (iPSCs), but these ideas and concepts can be applied to other cell types. Selecting which imaging modality to use for cell monitoring will depend on what one needs to monitor and measure, how rapidly one needs to take and process data, and limitations associated with the workflow.

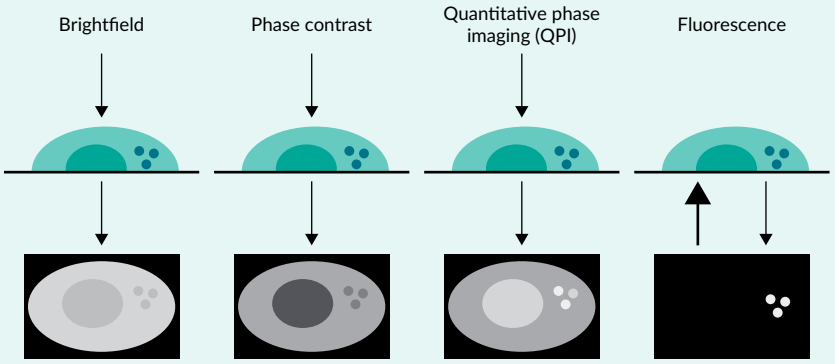
Different imaging modalities provide different types of information and make different features about the culture and cells accessible. The differences between imaging modalities include: the optical contrast between the cell and the background, the dosage of light required to generate an image without perturbing or damaging the cells, the time required to optically sample a sufficient number of cells, the commercial availability and system cost, and compatibility with the imaging vessels (tissue culture dishes or plates) and incubation environment.

BRIGHTFIELD IMAGING

Brightfield microscopy is a type of transmitted illumination imaging, meaning that light passes through the cell sample, and an image on the opposite side of the specimen is captured by a detector. This form of imaging uses low dosages of light, which makes it non-destructive and minimally perturbing. The images can be generated quickly, allowing for high volume sampling and high sampling rate. This method is label-free but provides relatively low optical contrast, which limits the morphological information that can be collected. This modality is minimally sensitive to aberrations caused by the meniscus that is present at the edges of the wells of a multi-well plate. This type of microscopy is low-cost and is readily available in multiple formats, from large benchtop microscopes to automated compact imaging systems, which can be placed within a tissue culture incubator. Optical contrast can be enhanced by

FIGURE 1

Summarized overview of common imaging modalities.



Aquisition time	Short	Short	Short	Long
Meniscus aberration	Minimal	High	High	Minimal
Commercial availability	Widely available	Widely available	Limited availability	Widely available
Cell perturbing	Minimal	Minimal	Minimal	Significant

The direction and dose of light are shown relative to the imaged sample by the arrows. The diagram of the generated image represents the relative contrast for each modality. A summary of features for each modality is shown.

collecting a stack of bright field images at different focal planes (i.e., a z-stack) at each field of view [16]. This operation increases the acquisition time, which can reduce the imaging throughput and sampling time. However, the enhanced contrast that is afforded by the collection of z-stacks makes it possible to use brightfield imaging effectively for individual cell segmentation, cell state classification, and even spatiotemporal tracking of cells [3,17–19].

PHASE CONTRAST IMAGING

Like bright field microscopy, phase contrast microscopy relies on transmitted illumination but provides enhanced contrast based on phase shifts in the light as it interacts with the specimen. This form of imaging also uses low dosages of light, which makes it non-destructive and minimally perturbing. The images can be generated quickly, allowing for high volume sampling and high sampling rate. This method is label-free, with high optical contrast, meaning rich morphological information can be collected. This type of microscopy is low-cost and is also readily available in multiple formats, from large benchtop microscopes to automated compact imaging systems that can be placed within a tissue culture incubator. One important note is that proper alignment of phase rings in the optical path is required to assure maximum contrast and prevent imaging artefacts. Major distortions occur when imaging near the edges of wells in typical well plates, but new methods can help reduce or correct this meniscus effect [20,21]. Some commercially available multi-well plates are designed to minimize the formation of a meniscus. These advances are making it feasible to sample every cell within a 2D culture plate using single focal plane images. Similar to brightfield imaging, applications in stem cell culture include stem cell colony

evaluation, individual cell segmentation, cell state classification, and spatiotemporal tracking [2,7,22–26].

QUANTITATIVE PHASE IMAGING

Quantitative phase imaging (QPI) microscopy is another type of transmitted illumination imaging based on phase shifts as the illumination light interacts with the sample. The term QPI refers to a group of different technologies, each with its own strengths and weaknesses. As with phase contrast and bright field imaging, this imaging modality also uses low dosages of light and is label-free, which makes it non-destructive and minimally perturbing. QPI provides the high contrast associated with phase imaging, but through the deconvolution of the signal provides quantification of the absolute phase shift of the light. The strength of the signal is dependent on the refractive index and the thickness of the sample, producing a measure of the dry mass of the sample. As a result, the appearance of morphological and internal cellular features is not identical to those in brightfield and phase contrast images. Depending on the QPI technology, image generation may be as fast as brightfield and phase contrast imaging, while other technologies are slower, which reduces the volume of sampling and sampling rate [27]. QPI microscopes have a higher cost, with only a limited number of commercial or turn-key solutions available as an automated compact imaging system that can be placed in a tissue culture incubator. In addition, QPI is sensitive to meniscus effects, but there are image acquisition strategies to correct this effect [28]. Since QPI is a quantitative measurement, reference materials can be used to benchmark instrument performance and for calibration [29], providing better reproducibility, comparability, and transferability of measurements. Applications of QPI in stem cells are not widespread, but this modality has the advantages of reference materials and quantitative measurements of intracellular features, providing additional orthogonal information about cells.

FLUORESCENCE IMAGING

Fluorescence microscopy imaging is based on the signal emitted by fluorescent molecules when they are excited at a specific wavelength of light. To collect enough signal, this form of imaging uses higher dosages of light and longer periods of illumination compared to phase contrast and brightfield imaging. The dosage of light can be on multiple orders of magnitude higher than that used for transmitted modalities [2]. This light exposure can be destructive to cells. The increased time required to generate an image reduces the sampling rate. The advantage is that fluorescent protein molecules can be expressed within the cell in association with the expression of a specific cellular protein, providing nearly real-time semiquantitative determination of expression of specific genes. Genetic engineering of a cell line of choice can produce stable cell lines that express a fluorescent protein associated with the activation of a gene of interest. The commonly used fluorescent proteins are not toxic to cells, which allows imaging of living cells over very long times with intermittent exposure to excitation light. Depending on the cellular protein the fluorescent protein is associated with, the expression of the fluorophore can indicate cell state, for example, pluripotency, lineage commitment, or differentiation. Such cell lines can provide a continuous source of experimental material for live cell imaging to enable uncovering signalling pathways, or to enable the design of manufacturing protocols [30]. Two or three fluorescent probes could theoretically be employed in a single cell line, although in practice this has been difficult to achieve with high fidelity. Alternatively, a fluorescent tag can be added to the cell from the outside to probe a specific molecular target. The cell processing

steps to label the cells with an external fluorophore are not generally compatible with long-term cell survival. However, the molecular details that fluorescence imaging can provide make it a useful technique to augment label-free methods. The information associated with the fluorescence from specific molecular entities can be used in conjunction with phase contrast imaging. Especially when used to train AI models, fluorescence data for molecular targets of pluripotency, differentiation or cell nuclei can enable valuable information to be inferred from brightfield and phase contrast images with a high level of confidence [2,3,19,25]. Another application of fluorescence microscopy is to measure the autofluorescence in a cell that can be sensitive to changes in metabolism and other internal features to assess the state of the cells [31,32].

Fluorescence microscopy equipment is readily available in different configurations. In general, the cost of the modality is greater than transmission modalities. There are technical challenges to quantification and data interpretation, including the presence of background signals and uneven illumination, which causes a gradient of excitation light across the image. While fluorescence imaging can easily perturb living cells, optimized optical configurations, sensitive detectors, and the use of brighter fluorophores can reduce the light dosage required and minimize the destructive effects. A common method nowadays in commercial instruments is the use of AI to computationally enhance the image contrast. This may provide nicer images, but the user should be careful when interpreting the results and be advised that this renders even relative quantification of fluorescent signals unreliable [33].

CHALLENGES & FUTURE PROSPECTS

While imaging modalities have the potential to provide continuous, real-time monitoring of living cells with single-cell resolution, there are several challenges that need to be addressed when considering their use in biomanufacturing. These include:

Use of AI models

Artificial intelligence models are powerful tools which have enabled the analysis of stem cell cultures by assessing and selecting colonies, segmenting cells, classifying cell states, and tracking individual cells [2,7,18,19,22–26]. However, the development of these models requires annotated data for training and validation, which will continue to be a challenge for some time. Annotation is often a human manual process which is subject to variability, fatigue, bias, and a training data set that may be smaller than desired. The generation of reference data through automated analyses [2,3] makes it possible to produce large amounts of annotated data without relying on manual, human-generated training data, but its relative accuracy must be evaluated for the potential to introduce errors. Validating such methods is challenging given the large amount of data generated, and strategies that involve comparing models and assessing the robustness of the results need to be thoughtfully developed and deployed. The development and application of AI models have become more accessible with multiple open-source tools available [34,35], AI tools packaged with microscope control software, stand-alone commercial tools, and commercial services focusing on image analysis. Maintaining consistent image instrument operation through calibration and benchmarking will be critical to achieve feature reproducibility of the AI model.

Data infrastructure

Continuous imaging over long periods of time can generate large amounts of

data, with storage, processing, analysis, and visualization being major hurdles. Storage and computational capabilities have become more affordable in recent years, but specialized expertise is required to create hardware and software systems that combine large-scale data storage with computational capabilities that provide an analytical output with useful visualizations. With the complexities of data processing and analysis steps, especially when AI model development is involved, metadata is also crucial to help in providing data provenance. For example, in our lab, we have developed pipelines in which metadata with details about the sample, data collection, image processing, image analysis, AI models, software, and hardware are appended to every image as it is produced, processed, and saved.

Reproducibility, comparability, and data sharing

Imaging protocols, hardware, and data analysis methods need to be well documented to ensure reproducibility and comparability across different biomanufacturing sites. Microscopes using the same imaging modality can still produce differences in the image features generated due to differences in their optical components, imaging protocols, and hardware configurations. Benchmarking and calibration of imaging instruments are crucial to ensuring that there is comparability in image data.

CONCLUSION

Using imaging to provide cell data in a continuous fashion can enable predictive outcomes and adaptation to deviations in a manufacturing process. Imaging of live cells provides access to rich, spatiotemporal information to provide quantifiable measurements. There are various imaging modalities and instrumentation which come in different shapes and sizes. There is no single best imaging system, as the choice is highly dependent on the specific application and compatibility within a designated workflow. With the advancement of instrumentation and computational capabilities, imaging is becoming more easily implementable as a measurement tool for biomanufacturing.

Disclaimer: Commercial products are identified in this document in order to foster understanding. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the products identified are necessarily the best available for the purpose.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY ANALYTICS AND CMC

SPOTLIGHT

INNOVATOR INSIGHT

Genetic integrity testing of genome-edited pluripotent stem cell lines used for cell therapy applications

Philip Hublitz, Vipul Patel, Heloise Philippon, Lukas Gebauer, Alexander Vogt, Felix Hermann, Manuel Landesfeind, and Matthias Austen

Human pluripotent stem cells (PSCs) can be the starting point for powerful tailored allogeneic cell therapies. Genetically engineered clonal lines constitute the basis for custom production of defined effector lineages, which enable targeting of otherwise difficult-to-treat diseases, e.g. in the fields of immune-oncology, neuronal and metabolic disorders. Fully characterized starting material is essential for advanced therapy medicinal product (ATMP) manufacturing, and therefore it is also essential to perform a comprehensive and thorough genetic integrity validation of PSC starting material during and after genetic engineering.

Genome editing of PSCs for clinical applications is usually multifactorial. Therapeutic edits include amelioration of cell function by inactivation of suppressive factors, enhancing disease-relevant functions by e.g. expression of chimeric antigen receptors (CARs), or introduction of immune shielding modifications for long-term persistence of therapeutic cells. Besides generation of the desired edit, genome engineering can lead to accidental acquisition of genetic aberrations or unwanted editing-related changes. In this article, we discuss the rationale of genetic integrity testing approaches for gene-edited PSCs, and how these procedures allow identification of thoroughly characterized, ON-target genome-edited PSC clones serving as starting material for safe and effective cell therapy products.

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INTRODUCTION

Autologous cell therapies using patient-derived starting material can be a highly efficacious way to treat leukemia, lymphoma, multiple myeloma or B-cell mediated autoimmune diseases [1,2]. However, cells for this treatment approach are difficult to manufacture and commercialization is challenging due to limited patient cell

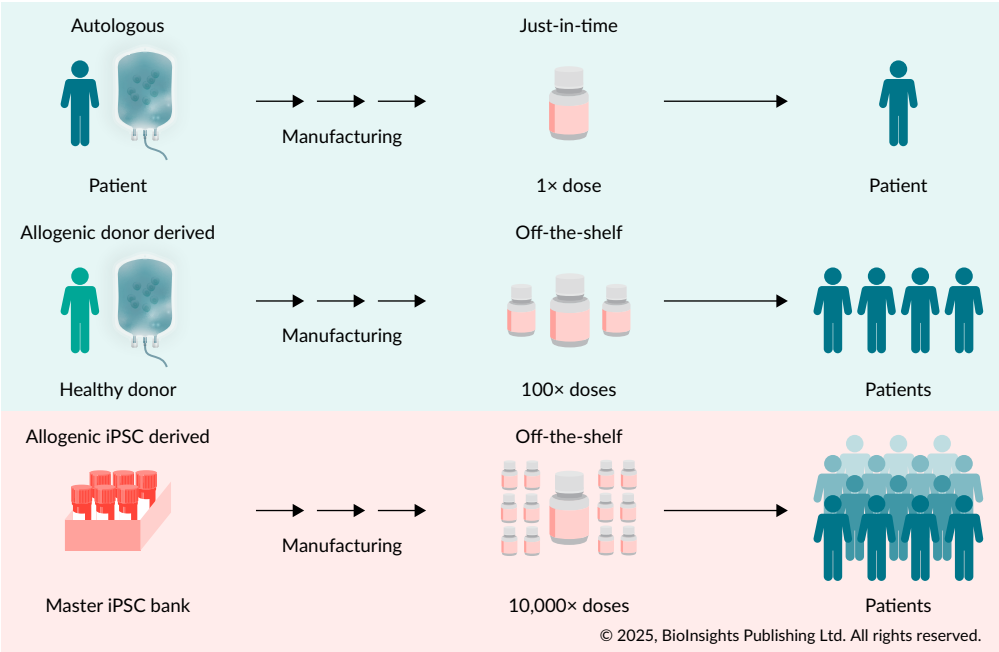
availability, limited scalability and cumbersome logistics resulting in very high manufacturing costs. Allogeneic, healthy donor-derived cell therapy approaches can overcome many of these problems and can be prepared off-the-shelf. However, they face challenges due to overall limited cell yields and immune incompatibilities between donor and recipients (Figure 1).

The development of directed and scalable differentiation procedures for the generation of therapeutic cell types from pluripotent stem cells (PSCs: embryonic stem cells or induced pluripotent stem cells) has opened a wide array of potential therapeutic avenues [3–5]. Due to their unlimited expansion potential, allogeneic PSC-derived products help to overcome above-mentioned bottlenecks. Well-characterized master cell banks allow mass-manufacturing of homogeneous cell therapy products and can provide off-the-shelf doses for many patients (Figure 1). Major benefits of allogeneic PSC-derived cell therapy products lie in reduced manufacturing complexity (no dedicated patient material required for the manufacturing) and in its versatility, in which a single platform is suitable for manufacturing multiple cell types for various diseases. As a testimony for the significance of cell therapy approaches, by December 2024 a total of 116 approved clinical trials using allogeneic PSC-derived cell products have been registered [6].

PSC-derived cell therapy products have applicability in many disease areas [7]. Unmodified PSC products, however, often have limited applicability due to

FIGURE 1

Different cell therapy applications.



(Top) Autologous approaches use patient cells with their inherent perfect immune-match. Manufacturing is personalized and serves one patient at a time. (Middle) Allogeneic healthy donor derived cells serve as off-the-shelf products. However, due to limited expandability and due to donor immune signatures, they have a limited range of potential patients. (Bottom) Allogeneic PSC-derived cell therapy products can be expanded as validated master cell banks, can be genetically engineered to e.g., enhance effector function or to avoid host immune responses, can be off-the-shelf differentiated into required effector cells, making them available to a vast number of patients and disease applications.

immune-restrictions (allograft rejection) or limited functionality. Most applications require starting material with genetic modifications adapted to the target disease. For example, in immune oncology-based applications using e.g. PSC-derived macrophages, T cells or NK cells, differentiated effector cells have a strongly improved therapeutic effect when expressing chimeric antigen receptors (CARs), which drive specific recognition and killing of target cells [8–10]. Also, PSC-derived immune cells have been shown to benefit from overexpression of accessory factors adapted to the respective therapeutic program, e.g. by overexpression of receptors, chemokines or cytokines to enhance iNK-based tumour therapies [11]. Importantly, if repeat-dosing or long-term engraftment is the envisioned clinical regime, recognition and clearance of grafted cells can be reduced (or eliminated). This is particularly important if PSC-derived effector cells are meant to persist in the patient's body (for instance in cell therapy targeting type 1 diabetes), in which grafted PSC-derived islet cells not only need to persist and function over many years despite alloimmunity, but in which especially beta cells also must be protected from persistent autoimmunity [12,13].

Designated PSC starting material must be well-characterized and its genetic stability validated since PSCs are known to accumulate genetic aberrations in culture. This has been previously reviewed in-depth in *Cell & Gene Therapy Insights* [14]. Our article aims to highlight the specific challenges and considerations for genetic integrity testing of PSCs after genome engineering. We address how to ensure high quality gene-edited PSC starting material for allogeneic advanced therapy medicinal products (ATMPs).

GENETIC ENGINEERING OF PSCs

Our insight article restricts the discussion to cut-and-repair approaches and will not cover base or prime editing considerations. Most genome engineering approaches use Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based systems to generate targeted DNA double strand breaks (DSBs) [15]. Such DSBs will be repaired by cell endogenous repair pathways. In absence of a homologous donor template, break points will be re-joined by one of the predominant and fast-acting end-joining pathways: non-homologous end joining (NHEJ), microhomology mediated end joining (MMEJ) or polymerase theta mediated end joining [16]. End joining pathways predominantly result in a precise repair outcome. However, due to continued CRISPR ON-target activity, insertions or deletions (indels) can arise when the repair machinery gets exhausted, with their size depending on the respective end-joining pathway (in most cases less than 10 nucleotides) This is a useful end-result if functional disruption of a target gene is desired (knock-out, KO) since any exon-based indel non-divisible by three will invariably translate to a functionally inactivated protein.

If a homologous donor is presented at the DSB, homology dependent repair (HDR) can occur, leading to precise insertion of the custom transgene repair cassette (knock-in, KI) [17]. This procedure allows generation of cells with the potential to (over-) express any kind of beneficial factor to help effector cells better tackling its respective disease type, or to establish immune evasion if required. Unlike with, for example, largely random lentiviral vector insertions, overexpression from a pre-defined locus will result in a better controlled outcome.

Before starting the editing procedure, reagents for generation of the DSBs should be carefully pre-selected. This, in particular, applies to the target specific single guide RNAs (sgRNAs) that are responsible to target-specifically recruit the nuclease and to ultimately generate a single specific DSB. Several bioinformatic tools exist to select 'good' over 'bad' sgRNA choices [18]. The output is driven by potential off-targeting events based on sequence similarity

and gaps in the protospacer-target pairing. Those algorithms currently do not provide extensive information for ON-target efficiency or *in cellulo* effects, however, reliably identify guides recognizing almost identical regions with a high risk for OFF-targeting.

It is important to define two phases when dealing with potential OFF-targeting. After Bioinformatic selection, the first step is experimental OFF-target nomination to evaluate the precision of the editing spectrum of selected sgRNAs. The second step applies the nomination and applies this knowledge to validate absence of potential off-targeting post edit. As such, sgRNAs are initially selected using *in silico* approaches (CRISPOR, Cas-OFFinder or similar) to ensure minimal collateral editing propensity [19]. In the subsequent experimental step, proper OFF-target nomination can be performed *in vitro,* where isolated genomic DNA is treated with RNA-complexed CRISPR effector nucleases, and any cut (ON- or OFF-target) can be identified by short read WGS (e.g. Digenome-SEQ) [20] or targeted sequencing (e.g. BreakTag) [21]. The isolated genomic DNA is digested *in vitro* in absence of chromatin or cellular compartments, and any digestion pattern most likely represents over-digestion due to over-accessibility of target regions. De-risking a worst-case scenario of potential OFF-targeting and starting from a 'maximum damage' scenario can be judged preferable and less prone to miss events in later analysis of isolated clonal lines. *In cellulo* nomination methods treat cells with the selected RNPs, and ON- and OFF-target DSBs are mapped *in situ* by ligation of indexing adapters (recent approaches include CHANGE-Seq, GUIDE-Seq, CAST-Seq or Uncover-Seq) [19]. The above-mentioned *in cellulo* assays investigate nucleases cutting the actual chromatinized template and possibly are not prone to over-digestion, better mimicking the actual situation in the cells. The enzymatic ligation of index-adaptors could, however, present a limiting factor to nomination of the full set of potential OFF-targets. The FDA does not specify a preferred method for OFF-target discovery, however, emphasizes the importance of OFF-target analysis/nomination to ensure safety and efficacy of CRISPR-based therapies. Once the nomination procedure has been accomplished, the dataset will serve as the basis for the subsequent OFF-target validation procedure. Post edit, any nominated potential OFF-target will be validated, i.e. absence thereof ensured in the final clonal line. This procedure preferentially uses deep targeted sequencing of the nominated sites (see section 4 of 'Genetic integrity and quality assurance').

After genome engineering, PSC clonal lines with the desired ON-target edit need to be identified and characterized. Although straightforward to screen for, it is important to emphasise that perfect ON-target editing might not be the predominant engineering outcome. It is important to screen for potential accompanying OFF-targeting events. Precise genetic engineering of PSCs with CRISPR is a well-established and reliable technology platform. However, also due to the action of cellular DNA repair pathways, cell-inherent events can add a certain unpredictability to the outcome of gene editing. For example, evidence has accumulated over the past years that DNA repair pathways can create genomic rearrangements, globally and as well in the inserted homology dependent repair templates (HDRTs) [22]. Hence, a key element when gene-editing PSCs is to identify clonal lines with the desired ON-target edit and that are free of unwanted editing events.

POTENTIAL GENETIC ABERRATIONS POST GENE EDITING

OFF-targeting and formation of indels

Depending on the nuclease, most CRISPR-based target recognition occurs via a

18–25 nucleotide protospacer sequence. Perfect matching, coupled to presence of a defined nuclease-specific protospacer adjacent motif (PAM) is required for DNA cleavage. However, certain mismatch conditions in the protospacer-target base pairing can be tolerated by the nuclease and unwanted genomic loci can be affected (OFF-targets). Therefore, it is critically important to select the best suited guide with minimum predicted OFF-targeting potential. Noteworthy, any potential OFF-target site with >4 mismatches or with larger gaps in the protospacer-target pairing is considered having only a limited risk for OFF-targeting. Bioinformatic tools can nominate potential genome-wide off-target indels for each selected sgRNA, however, overall final editing outcomes with the chosen sgRNA in the cellular context cannot be predicted with absolute accuracy [23].

Random insertion of HDR templates

In case of KI experiments with HDRT-containing donor plasmids, the donor can insert by NHEJ into genomic regions having random DSBs, as well as integrate into any OFF-target-created DSB [22]. The overall frequency of OFF-target DSBs is determined mostly by the specificity of the sgRNA but can also be influenced by the HDRT architecture and the cellular environment.

ON-target HDR template rearrangements

Complex ON-target HDRT rearrangements have been reported, with MMEJ-driven repair based on micro-repeats in dsDNA donor templates as likely mechanism of action. Additionally, complex vector insertions mediated not only by MMEJ but also by NHEJ and HDR have been detected in almost 20% of clones analysed [24]. Potential HDRT ON-target rearrangements are not easy to detect by simple PCR-based screens and need to be factored in as possibilities in the detailed QC of clonal lines.

Larger insertions

Unintended larger insertions after nuclease-generated DSB have been uncovered in recent systematic analyses. Insertions mainly consisted of retro-transposable elements, host genomic coding and regulatory sequences and can occur at an allelic frequency of 0.7% [25]. When double stranded HDRTs are used for the edit, concatemerized or tandem integrated repair templates are among unwanted larger insertions [26]. ON-target rearrangements have been described in zygotes and cell lines, especially when using more than one sgRNA in the editing strategy. Dual or multi-guide strategies can trigger rearrangements ranging from inversions, tandem insertions, head-to-tail insertions and duplications in combination with larger deletions [27,28]. HDRT concatemerization and backbone insertion was observed using adeno-associated virus vectors as HDRTs in the generation of transgenic animals [29].

Larger deletions

Error-prone repair of nuclease induced DSBs can drive generation of larger ON- and OFF-target deletions. A thorough characterization of indel spectra after CRISPR editing in mouse zygotes detected deletion spectra of up to 600 bp and identified asymmetric deletions using single sgRNAs [30]. Also, larger deletions can be triggered by surrounding microhomologies, which should be considered in an ON-target context dependent manner [31]. A comprehensive analysis of unintended chromosomal modifications in primary hematopoietic stem and progenitor cells reports that large deletions occur in higher frequency at all selected and investigated ON-target sites [32]. Truncation

of one of the ends of a DSB during repair was reported to yield >100kb deletions in mouse zygotes [33]. Additionally, deleterious ON-target effects and larger mono-allelic deletions via HDR or NHEJ were reported to happen in up to 40% of gene-edited hiPSC lines [34], however, the range most likely is line- and edit-specific. Here it is important to note that larger deletions are inherently difficult to identify in simple primary PCR screens since they are usually linked to loss of primer or probe binding sites.

Chromosomal rearrangements

Large chromosomal aberrations triggered by nuclease mediated genome engineering are rare, but when observed, the spectrum can cover deletions, duplications, inversions, balanced or unbalanced reciprocal as well as nonreciprocal translocations [35]. Gene editing of targets close to telomeres has been associated with arm truncation and loss of heterozygosity [36,37]. Engineering pipelines using more than one guide (and studies affected by pseudogene-based multi-targeting) report megabase rearrangements and multi-lesion alleles [38,39]. In a CRISPR screen to address the effects of sgRNAs on primary T cell genetic integrity and comparing single guide/template conditions with conditions using multiple guides, both approaches resulted in the observation of chromosomal deletions [40]. Supporting this, genome engineering approaches using one sgRNA without intentional secondary targeting purpose also report chromosomal truncations [41]. A recent study employing improved high-sensitivity screening uncovered previously undetected levels of chromosomal translocations in the range between 0.15 and 1.5% [42], and use of single cell genomic DNA sequencing techniques confirmed above findings describing unique OFF-editing patterns in single clones [43]. Those results highlight the potential for unwanted aberrations following gene-editing and stress the importance to thoroughly characterize isolated edited clonal lines.

REGULATORY GUIDELINES

The EMA and the US FDA have released recommendations concerning the use of highly expanded allogeneic PSC-derived products for cell therapy [44,45]. An EMA guideline on the risk-based approach for evaluation of ATMPs is in place. The key point is to exclude cells that have acquired unwanted mutations or growth-advantageous chromosomal aberrations during the editing process from becoming therapeutic material [46].

FDA recommendations particularly recommend the use of whole genome sequencing with more than 50× coverage as a method of choice. 'Justification should be provided for the sequencing method, read depth, and for conclusions related to the safety of the product' [45]. Results need to be analyzed by cross-comparison to databases that reference proto-oncogenes and cancer-associated mutations (such as K-Ras, c-MYC, Tp53, BRCA). Recommendations also cover identification of ON- and OFF-target genome editing and cytogenetic testing with defined acceptance criteria. Validation using orthogonal methodologies is considered best practice. The genetic integrity testing strategies outlined in the following sections take those recommendations into consideration. Additionally, we discuss recent assay developments that are currently not included in the official guidelines but might have the potential to add important extra information in the future.

GENETIC INTEGRITY TESTING

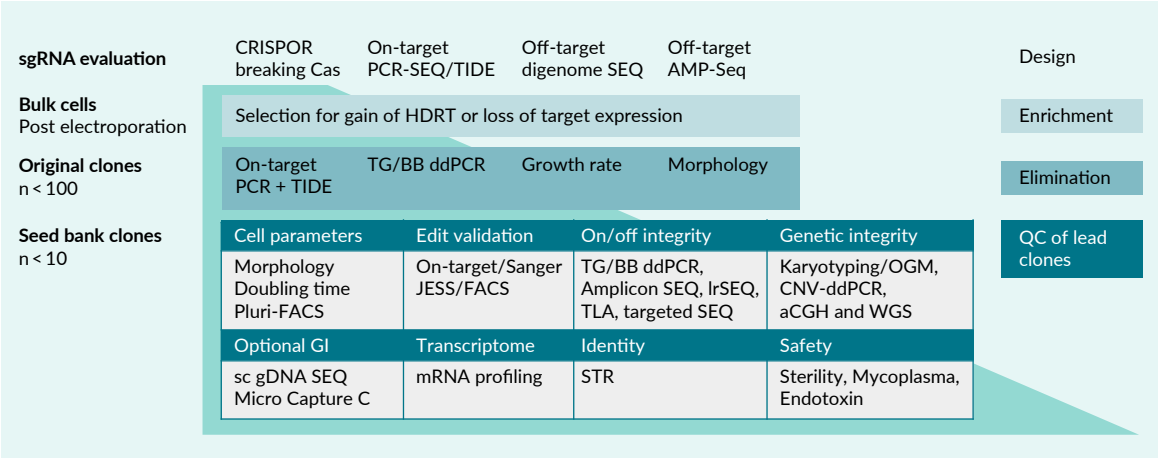
An ATMP must be accompanied by comprehensive genetic integrity testing panels

that can uncover critical point mutations and genetic aberrations that might have emerged in the establishment of genome engineered PSC clonal lines, and that may pose a significant risk to patient safety. The screening pipeline ideally directly starts at the initial design phase, in which safe design of reagents as well as experimental evaluation of potential sgRNA mediated OFF-targeting is established for downstream use (Figure 2, 'Design' phase). A second level will ideally allow enrichment of gene-edited bulk cells, already ensuring expression of the introduced transgene, or proving absence of gene expression in case of a targeted KO (Figure 2, 'Enrichment' phase, light green).

A third level can be added at an early, potentially in-process stage. When many clonal lines are generated, a pre-screen procedure can help reducing clone numbers at an early level of the process. Such a pre-screen must be robust and provide clear decision criteria to be able to *a priori* discard clonal lines ('elimination phase'). Several aspects can be screened, ranging from cell morphology and pluripotency marker expression, cell division kinetics, ON-target editing up to copy counting of HDRTs and remnant plasmid DNA backbones (Figure 2, 'Elimination' phase, green). The time-consuming full-integrity test ideally is performed once seed banks of pre-selected clones have been safely frozen (Figure 2, 'QC of lead clones' phase, dark green). Once best clonal lines are identified and validated, release and safety assays need to be performed. It is important to stress that only safest-possible starting material can be used for downstream differentiation and manufacturing procedures. At every stage, regulatory guidelines

FIGURE 2

Suggested schematic representation of validation pipeline for cell therapy medicinal products.



(Top) The design phase sets the stage before any cell engineering can start. sgRNAs are designed to best possible bioinformatic evaluation, tested in target cells for ON-target activity and potential OFF-targeting events are assessed and documented. Middle, light blue): this affects only HDR experiments, in which a potential selection marker can be used for enrichment of gene-edited cell pools. (Middle, darker blue): it is important to have a quick and reliable downgrading procedure from isolated primary clonal lines. ON-target assays, HDRT copy counting, screen for ON-target versus potential OFF-target integration and cell-based assays are applied. (Bottom, table): full QC is performed at frozen seed bank status. All potential pitfalls as discussed in this article are addressed according to regulatory authority guidelines. aCGH: arrayed comparative genome hybridization. AMP-Seq: targeted amplicon sequencing platform. BB: backbone. ddPCR: digital droplet PCR. FACS: fluorescence activated cell sorting. HDRT: homology directed repair template. JESS: automated Western blot system. lrSEQ: whole genome long read sequencing. OGM: optical genome mapping. sc gDNA SEQ: single cell genomic DNA sequencing. STR: short tandem repeat analyses. TG: Transgene. TIDE: tracking of indels by decomposition. TLA: targeted locus amplification. WGS: whole genome short read sequencing.

and rigorous scientific judgement must be applied to ensure maximum patient safety.

METHODOLOGIES FOR GENETIC INTEGRITY & QUALITY ASSURANCE

Genome engineering processes deliver bulk cells that, in best-case, can be enriched for presence of the desired edit (in case of KI) or by absence of expression of targeted endogenous genes (in case of KO). Clones are isolated, triaged, and full genetic integrity testing is performed at frozen seed bank stage. Many powerful technologies have been established to address individual questions concerning potential erroneous editing outcomes. We are discussing current methodologies and address the following questions: 'What can be detected with which methodology', 'Where are limitations of the approaches', 'Where is room and applicability for orthogonal approaches', and 'Which selected combination of assays could deliver the most comprehensive evaluation in an efficient manner'. We address different levels of genetic integrity testing in the following paragraphs, schematically summarized in Table 1.

Cellular fitness and pluripotency

Morphology assessment of PSC cultures requires expert supervision and is hard to automate. However, for a trained team, cell morphology rapidly and with essentially no cost provides information about unwanted spontaneous differentiation in cultured cells. Differentiated clones with spindle-like cells and undefined edges should generally be discarded. Verification of expression of key pluripotency markers by validated flow cytometry to >98–99% is an important measure to demonstrate and ensure cell pluripotency. Monitoring of maintained pluripotency level is a constant assay to be repeated in regular intervals during editing and during routine maintenance cell culture.

ON-target edit validation

The first step in ON-target validation is typically performed by a locus specific PCR. The logic of the assay differs between screening for KO or for precise integration of HDRTs (KI). Validation of a KO involves PCR amplification of the target site. The amplicon size can already reveal presence or absence of larger indels. Determination of biallelic vs. monoallelic KO as well as the nature of the indels can be analysed using the TIDE algorithm [47]. TIDE can estimate indel classes in a population average but cannot unequivocally identify mono- vs. biallelic homozygous events. Results can be either substantiated by subcloning of amplicons with subsequent clonal sequencing, or zygosity at the ON-target locus can be confirmed with targeted amplicon deep-sequencing (ideally with >1000x fold coverage). As a caveat, the initial PCR-based detection of only one allele can be caused by either predominant MMEJ repair outcomes (resulting in the biallelic same indel) or could result from a larger-than-expected deletion that removed one of the primer binding sites, potentially masking the actual genotype [31]. Amplification of larger ON-target fragments has the potential to resolve this issue, but ultimately joint efforts of short- and long-read WGS can confirm the allelic status of the ON-target event.

Confirmation of ON-target insertion (KI) can use external/internal PCRs for both arms of the HDRT to ensure 5′ and 3′ site-specific integration. Presence of both expected amplicon sizes is indicative for proper insertion, which on top can be confirmed by amplicon Sanger sequencing. This initial screen should be accompanied by a PCR to amplify the remaining, non-HDRed allele as proof of the allelic status. If the WT allele can be amplified, sequencing will determine the indel and confirms monoallelic HDR. If amplification fails, this can be indicative of either bi-allelic HDR or of a larger ON-target deletion.

TABLE 1

QC panel for gene-edited PSC products during establishment and screening of clonal lines.

Step	Evaluation	QC	Limitations	Alternative	Comment
Cell	Cells fit for purpose	Morphology; doubling time; pluripotency	No validation		In process QC
Edit validation	ON-target editing	PCR and Sanger (KO and KI); WGS; TLA	Complicated multi-allele insertions cannot easily be resolved		HDR more complicated
	TG expression	FACS; qRT-PCR	TG expression or absence of target expression required	JESS/ICC	PoC differentiation as functional proof
ON integrity	HDRT rearrangement	Amplicon Seq; WGS, lrSEQ	Large amplicons; tandem insertions difficult to resolve; resolution	TLA; Hi-C	WGS as ultimate proof
OFF targeting	HDRT copy number	TG/BB ddPCR, PCR-SEQ; WGS; lrSEQ	Resolution and HDRT integrity assembly	TLA	WGS as ultimate proof
	Small indels	WGS; targeted sequencing (TS); StemSeq	Sequencing depth of 50× in WGS; analytical pipeline	TS >10k coverage; StemSeq panel	Orthogonal QC required
Genetic integrity	Duplications	Karyotyping; WGS; lrSEQ	Low resolution	Hi-C; OGM	
	Inversions	WGS; lrSEQ	Difficult detection by WGS	Hi-C	
	Large deletions	WGS; lrSEQ; aCGH		Hi-C	
	Large Insertions	WGS; lrSEQ; aCGH		Hi-C	
	Loss of chromosome arms	CNV-ddPCR; Karyotyping; lrSEQ	Selected regions only; low resolution	Hi-C; OGM	
	Translocations	Karyotyping; WGS; lrSEQ	Balanced, unbalanced, non- and reciprocal hard to detect	Hi-C; OGM	Specialized pipeline needed
Transcriptome	Fitness post edit	Microarray			Not mandatory
Identity/ safety	Release of validated material	STR, MP, sterility, endotoxin			Part of release

General cell fitness is monitored as an in-process control. Doubling time and cell morphology are initial indicators for proper PSC behaviour, the Evotec proprietary Pluri-FACS ensures expression of all relevant pluripotency marker. ON-target edit is validated initially by ON-target PCR with Sanger sequencing accompanied by ddPCR HDRT copy counting. Detailed clonal testing (darker blue section) comprises evaluation of ON-target integrity, ruling out deleterious OFF-target effects and ensuring full genetic integrity. Transcriptome analyses are optional and can be used to corroborate other results. Release assays concern questions on cellular identity and sterility. All potential pitfalls are addressed in the accompanying text according to regulatory authority guidelines. aCGH: arrayed comparative genome hybridization. BB: backbone. ddPCR: digital droplet PCR. HDR: homology directed repair. HDRT: HDR template. Hi-C: micro C proximity capture for detection of chromosome interactions. ICC: immunocytochemistry. JESS: automated Western blot system. KI: knock in. KO: knock out. lrSEQ: whole genome long-read sequencing. MP: mycoplasma testing. OGM: optical genome mapping. PoC: proof of concept. QC: quality control. STR: short tandem repeat analyses. TG: transgene. TLA: targeted locus amplification. TS: targeted sequencing. WGS: short read whole genome sequencing.

In this context, full HDRT integrity and allelic status will be further validated using complementary methods (e.g. long read sequencing as detailed below).

For KI validation, expression of the payload, or for KO, absence of expression of the target gene, can be analysed on protein level using immune assays (flow cytometry, JESS, ICC, IHC), given availability of target antibodies. Any applicable immune assay can give an important functional read-out and is already possible at early stages. Protein data can uncover expression strength in PSCs, can reveal functional protein trafficking and can validate correct subcellular localization.

ON-target HDRT integrity assessment

This paragraph is applicable only to HDR events, where transgene cassettes need to be precisely repaired into the genome. When analysing homology directed repair outcomes, a ddPCR assay can detect payload copy numbers (TG, transgene assay) as well as remnant plasmid DNA (BB, backbone assay, which would be indicative of random insertion events). Additionally, target specific ddPCR assays can be used to investigate the overall structure of the HDRT. Primers and probes can be designed to detect potential boundaries of head-to-tail or tail-to-head concatemeric insertions, which are generally undetectable by simple ON-target PCR screening. If short homology arms are used or if NHEJ based insertion mechanisms are employed, junction ddPCR assays can help determining precise HDRT insertions. Also, tiling ddPCR across the entire length of the HDRT can be applied to map potential truncations or rearrangements, however, a follow up with base-pair resolution is highly recommended for final validation (e.g. by one or several of the below listed assays).

HDRT ddPCR can uncover potential multi-copy integrations (in concert with the ON-target PCR screen). Having a fast turnaround time, the assay allows de-selection of clones during the initial clonal selection phase, with TG copy numbers exceeding 2 or with any detectable backbone plasmid being indicative of random genomic insertions. As examples for HDRT based ddPCR, TG n=1 can result from proper monoallelic targeting (in combination with ON-target PCR results. TG n=2 could be either a biallelic targeting, a monoallelic ON-target event combined with a single copy random insertion or by a concatemerized insertion (all result need correlation with HDRT and remaining WT allele ON-target PCRs). TG n>3 is a clear identifier that at least one copy of the HDRT has been randomly or erroneously inserted or episomal plasmid DNA is unexpectedly maintained after prolonged culture after editing and clonal isolation. The HDRT assay does not give information on overall HDRT-integrity and is applicable only for clonal populations, however, clear advantages of this assay lie in its easy quantification and its short turnaround time. Combined transgene and backbone ddPCR assays (in correlation with ON-target PCR assays) establish a robust foundation for subsequent analyses and have the potential to eliminate improperly edited clonal lines as a robust pre-screening effort as discussed above.

ON-target long-range amplicon sequencing (i.e. PCR over the entire integration site using primers placed outside the HDRT sequence, and analysis of the amplicon via NGS) is a useful process-development tool and can even work in bulk populations. Although potentially cumbersome to establish and custom for each locus and HDRT, the assay has the potential to be validated for ON-target integrity assessment. As a beneficial side effect, tandem or multi-copy ON-site insertions would naturally impede amplification due to the larger payload and would produce a non-amplification, in which case aberrant clones would be automatically eliminated.

Long-read whole genome sequencing (lrSEQ) with sufficient coverage has the potential to identify HDRT rearrangements (deletions, translocations, tandem insertions, inversions) and can detect general HDRT OFF-target insertions. Since lrSEQ generally delivers a shallow read depth, interpretation of bulk results, in which ON-target editing happens in less than 1–5% of total cells, is impaired by the low coverage. lrSEQ, however, is very well suited for clonal populations with defined mono- or biallelic insertions. The read-depth of current lrSEQ does not allow precise base-calling, nevertheless, lrSEQ can reliably uncover structural HDRT aberrations.

A simple approach with 5× coverage will suffice as a primary screening methodology. For reliable detection of structural aberrations using currently available platforms (e.g. Oxford Nanopore or PacBio HiFi), a >15–20× coverage for clonal lines when screening for structural variations, and a >30× coverage when aiming to detect larger structural and smaller variants at high precision is recommended. lrSEQ data can give a comprehensive overview not only on HDRT integrity but also on larger chromosomal rearrangements (see below). To corroborate the findings, lrSEQ ideally is combined with assays providing higher resolution (e.g. short read WGS or targeted sequencing assays), combining their analytic powers.

Alternatively, targeted locus amplification (TLA) is a proximity ligation-based assay with NGS evaluation. It can uncover payload integrity ON-target and, in the same run, can identify OFF-targeting of additional copy insertions with their precise location [48]. TLA, due to its targeted sequencing approach, however, is naturally biased and therefore usually requires complementation with orthogonal methodologies.

Short read WGS, even while delivering a high coverage and read depth, cannot fully verify HDRT integrity as a standalone methodology. HDRT homology arms are usually larger than 500bp and as such short read approaches are inherently not suitable for precise determination of the insertion site. An additional complication can arise from HDRTs containing non-codon-optimized human genes, since those templates generate unmatchable reads. However, once long-read WGS in conjunction with TG ddPCR and ON-target PCR has established a bona fide single copy insertion, WGS validation on a single base resolution can be feasible. As for most integrity testing areas, not a single methodology will give all answers, but when combining several assays with TG expression data and WGS ON-target data, proper HDRT-integrity can be ensured.

Assessment of OFF-target indels

Genome wide short OFF target indels (insertion or deletion of in average less than 10 nucleotides) as a byproduct of genome engineering are the most common deleterious outcome. Analysis of potential harmful mutations is mandatory. Due to genome size and abundance of data that needs to be analysed in sufficient coverage, orthogonal approaches and well-defined analysis pipelines are required.

Genome editing OFF-target indels are typically present in much less than 1% in bulk populations. The generation of an ATMP usually does not end with a bulk population but requires isolation and characterization of clonal lines. The previously mentioned nomination and validation procedure (see paragraph Genetic Engineering of hiPSC) is used for bulk cells but, most importantly, also for isolated clonal lines. Well-selected sgRNAs generate mostly ON-target DSBs, but erroneous OFF-targeting cannot be excluded. Nominated loci can be addressed using targeted high-depth validation to quantify potential OFF-target indels. The amplification method of choice (e.g. using custom amplicon panels such as rhAmpSeq or duplex-UMI amplicon sequencing) is sufficient with 100x coverage per target in clonal populations but should exceed 1000x (preferably 10.000x) coverage to ensure sufficient resolution in bulk populations. Additional targeted approaches using custom designed panels are based on prior knowledge. Targeted sequencing is the most sensitive detection assay for rare OFF targets concerning indels, SNPs, CNVs affecting known risk loci associated with frequent aberrations in PSCs [49]. Targeted amplification, however, should best be combined with unbiased NGS approaches. As recommended by the FDA (Safety Testing of Human

Allogeneic Cells Expanded for Use in Cell-Based Medical Products), in particular screening for mutations in the p53 pathway post genome engineering [50] should be an integral part. It is important to stress that all above-mentioned targeted panels are site-limited and should be paired with unbiased WGS as an orthogonal method.

WGS at >50× coverage is considered the gold standard recommended by regulatory authorities. It is important to define which strategies will be applied for the evaluation and assessment of CNVs, indels and SNPs. Analyses require comparison to a public database reference genome, creating a donor-specific (an option in the case of iPSCs) or cell-line-specific baseline genome which can serve as starting point for all analyses of subsequent gene edits. Any new WGS-build at different steps of cell culture and cloning can be co-referenced for genetic integrity monitoring over time. The analysis pipeline should investigate well-curated lists of genes and variants with impact on the fitness of the edited clone. Those lists can contain genes from relevant pathways, lineage specific genes, oncogenes and tumour suppressors, genes affecting PSC pluripotency, as well as ClinVAR/COSMIC/OMIM disease-relevant variants. A defined strategy (following EMA recommendations) is required for interpretation of short WGS reads to have enable decisions based on pre-defined criteria and arguments on the use of the final selected clone, requiring expert review of the results. This is a labour-intensive approach, however, delivers highest coverage of most genetic aberrations that can occur. In a best possible scenario, a fully annotated new genome build is assembled for the clone selected for manufacturing. Importantly, assembly of repeat structures, tandem insertions, unbalanced and balanced translocations or reciprocal translocations is not always unambiguous since large structural genome variations are not fully covered in the process of classical short read NGS (see section on genetic integrity and stability below). Further methodologies (as outlined in the sections below) need to be considered to have a comprehensive understanding of the overall genetic integrity of the derived clonal lines. It is important to stress that short-read WGS at this sequencing depth can provide sensitive discovery of low-frequency OFF-targeting events only in clonal but rather not in bulk populations.

Genetic integrity and stability

ddPCR services to detect CNVs commonly observed in PSCs are commercially available. Custom probes typically cover about 20 published known high frequency chromosomal breakpoints in PSCs [51]. This type of selective assay is relatively cheap and has a fast turnaround time. While it cannot be used as a stand-alone genetic integrity assay due to the limited range of targets, it nevertheless is a useful tool for pre-exclusion screening whose results need to be later substantiated by other methods.

One methodology suggested by the FDA for analysis of global rearrangements is karyotyping by G-banding [52], with at least 40 metaphase spreads to gain statistical significance. G-banding is an established assay, and certified clinical diagnostic service providers are available. Polyploidies, duplications, large deletions and larger translocations can be identified, potentially even larger inversions. Disadvantages of the methodology are mostly the limit of its statistical power due to low sample numbers, as well as a poor detection potential for smaller rearrangements. A technical improvement in this area could come from optical genome mapping (OGM) [53]. Herein, high molecular weight DNA is stained with intercalating dyes and subjected to imaging on chip-nanochannel arrays. Changes in the patterning in comparison to a reference genome reflect copy number and structural anomalies, including detection of balanced

translocations. This is a recently developed technology, and future research needs to demonstrate its full range of applicability.

lrSEQ can give a wholistic overview about most common larger chromosomal aberrations and at the same time informs about HDRT copy numbers and about general integrity of the HDRT ON-target. With a typical read depth <5–10 and with higher per-base error rates, it cannot reliably deliver SNV calls and should be confirmed by higher coverage methodologies. Importantly, however, bioinformatic assembly of the average 10kb long-read fragments allows assembly of the co-linear sequence of larger fragments, especially for larger HDRTs. WGS can ensure trustworthy sequence information, however, in turn has only limited capability in calling rearrangements (as discussed above). Potential caveats could come from biallelic modification of the same target, where precise calling might be impossible. Therefore, combined long- and short-read WGS to date seems the best methodology for verifying HDRT integrity, and for detection of most potential genetic aberrations that can be accounted for.

Arrayed comparative genome hybridization (aCGH) is an orthogonal approach to WGS with a lower resolution, but capable to detect larger insertions or deletions [54]. Comparative hybridization with differentially labelled fluorescent probes results in equal, over- or under-representation in the test sample and indicates genomic aberrations that can be mapped according to the probe sets, which are placed at regular intervals along the genome. aCGH is a genome-wide, unbiased assay that several approved clinical laboratories can provide, comes at a relatively low cost and with a relatively fast turnaround time. Limitations of the assay is resolution (standard assays have a 50kb lower limit) is that CNVs can only be uncovered in regions where probes are hybridizing, and that no information can be obtained at the SNP level. As such, aCGH can be considered a robust first test, giving an idea of the overall genetic integrity post edit, however, should be accompanied with deeper coverage methodologies.

Two recently developed methodologies have the potential to add additional information into the genetic integrity evaluation pipeline. Tapestri single cell genomic DNA sequencing is a technology designed for in-depth analysis of editing outcomes that aims to allow simultaneous sequencing of several 100 loci in up to 20k of single cells, adding a high level of resolution [43]. Tapestri uses custom defined panels with high sensitivity, potentially most orthologous to targeted sequencing panels, however, with single cell output. Its key strength could lie in the evaluation of bulk editing outcomes and could also be used for confirmation of genetic integrity in isolated clonal lines. It is a custom approach with a limited target size and does not give full genome coverage, however, in combination with non-biased WGS could be a useful tool, especially for process development purposes.

Micro capture C [55,56] is a cross-linking positional approach evaluated by NGS. DNA fragments next to each other within cell nuclei are cross-linked, and co-linear as well as rearranged interactions can be revealed using a proximity interaction matrix, similar to the establishment of 3D chromosome interaction matrices [55]. The principle of the methodology is analogous to TLA; however, it is unbiased, not payload-centred and covers the whole genome. The methodology has the potential to reveal breakpoints, inversions, translocations, fusions, duplications, tandem inversions, smaller and larger deletions all in one in one assay, all depending on the sequencing depth [57]. Being a very recent technology development, future research needs to demonstrate how reliably Micro capture C or similar adaptations can complement or potentially even surpass the previously described combinations of genetic integrity testing approaches.

Transcriptome effects

Transcriptome analyses are indirectly assessing genetic integrity, however, can reveal insights into perturbations that potentially could be undetectable using above listed methodologies. This assay can uncover changes in preference of isoform expression as well as altered lncRNA or miRNA profiles. It is an inherently unbiased approach and has the potential to add a different viewpoint on global changes resulting from aberrant genome engineering.

Cell identity

Cell identity determination is a mandatory release assay. Short tandem repeats (STRs) are short repetitive DNA repeats that are used to classify the origin of the cell line and are detected by a PCR-based length polymorphism. STR profiling is used to validate origin of cells [58]. Several clinically accredited providers are available.

Safety and release assays beyond genetic integrity and identity testing

Mandatory safety and release assays include sterility tests, mycoplasma testing, endotoxin release testing, adventitious agents and viral safety testing, and absence of genome engineering reagents post edit in the final medicinal product.

TRANSLATIONAL IMPLICATIONS

Genetic integrity monitoring is a fundamental aspect when producing ATMPs. PSCs are an ideal starting point for allogeneic cell therapies, but long-term culture has shown to predispose cells to acquisition of genetic alterations. Continuous monitoring of genetic integrity is key during routine maintenance, expansion and banking, but is equally important during and after genetic engineering. Based on rigid assessment of potential aberrations and on detailed analysis of the coverage potential and inherent gaps of available testing methods, a comprehensive testing portfolio needs to be in place to cover both, general and editing-related risks.

We summarized potential 'mishaps' that occur during genetic engineering of cells and have presented a variety of different analytical assays to address significant issues. Not all types of events necessarily occur in the same cell, in the same cell line or even at the same time, however, knowing what can potentially go wrong raises awareness which points to address with relevant genetic integrity testing assays. For long term culture and post genome edit, as previously discussed [14], not a single assay can cover all potential genetic rearrangements. A thoughtful combination of different assays is required to gain access to the full picture. We would like to highlight two scenarios that are particularly important in the generation of genome-edited PSCs and present two possible minimalistic assay scenarios (Table 2).

When genetically engineering a loss-of-function allele (Table 2 upper part: KO), an ON-target edit can be confirmed by PCR amplification of the edit area with subsequent Sanger sequencing. Indel composition in both alleles can be determined by TIDE, and only biallelic out-of-frame mutations are considered successful KO edit outcomes. Short range WGS can assess any engineering-based off-targeting, preferably supported by targeted sequencing informed by initial sgRNA pre-evaluation. General genetic integrity can be validated using a panel of orthogonal assays as discussed in the sections above.

When aiming to validate a gain of function allele (Table 2 lower part: KI), initial ON-target integration can be validated by outside-in and inside-out PCRs to validate proper HDR of both arms of the donor. The PCR spanning the unedited WT locus

TABLE 2

Minimal QC procedures for KO and KI lines.

	Step	Assay	Purpose
KO	1	ON-target PCR	Validation of biallelic KO
	2	Targeted sequencing	Assessment of engineering-based OFF-targeting
	3	WGS; lrSEQ; aCGH; CNV-ddPCR; Karyotyping	General genetic integrity
KI	1	ON-target PCR	Validation of correct 5′ and 3′ HDRT integration
	2	ON-target WT PCR	Allelic discrimination
	3	TG ddPCR copy count	n=1: ON-target PCR OK → indicative of monoallelic targeting n=2: ON-target PCR OK/WT PCR positive → potential biallelic targeting n=2: ON-target and WT PCR OK → ON + OFF-target or tandem ON-targeting n>3: irrespective of ON-PCR → random insertion ON or OFF
	4	BB ddPCR copy count	n>0 indicative of random targeting
	5	ON-target lrSEQ	Determination of HDRT integrity Proof of absence of duplications, tandem insertions, inversions, rearrangements which otherwise would be hard to detect by WGS alone
	6	ON-target WGS	Requires proof of absence of ON-target duplications by lrSEQ If TG ddPCR n=2, validation of biallelic or ON-target + random insertion Full sequence confirmation of HDRT
	7	Targeted sequencing	Assessment of engineering-based OFF-targeting
	8	WGS; lrSEQ; aCGH; CNV-ddPCR; Karyotyping	General genetic integrity

KO: the 3 typical successive steps of validation of the genetic model are depicted. A 'KO only' edit is usually not the main ATMP outcome. KI: 8 steps for full validation of ON- and OFF-targeting including HDR integrity are depicted. In both cases, the choice of orthogonal methodologies critically depends on the editing pipeline. aCGH: arrayed comparative genome hybridization. BB: backbone. ddPCR: digital droplet PCR. KO: knock out. lrSEQ: whole genome long-read sequencing. TG: transgene. WGS: short read whole genome sequencing.

serves as conformation of the allelic status. TG and (artefactual plasmid DNA remnant) BB ddPCR copy counting, in correlation with the ON-target PCR, can inform on potential mono- or biallelic HDR. Likewise, the same assay can also uncover potential random insertion events (BB > 0 and TG > 2). Whole genome long range sequencing can be applied to validate ON-target HDRT integrity and to support TG ddPCR results on total copy counting. Additionally, lrSEQ will give an overview on general genetic integrity. Absence of ON-target inversions, deletions or tandem insertions can help selecting clones for subsequent complimentary short read WGS, with which also any indels accidentally caused by the editing procedure can be identified. If single-copy ON-site HDRT integration can be assured, WGS can inform on HDRT integrity on the base-pair level as well as confirm the allelic ON-target status. Indel prevalence as well as genetic integrity of cells can be further addressed by targeted sequencing.

CONCLUDING REMARKS

Diverse editing strategies require different combinatorial sets to uncover potential genetic aberrations that may have occurred as a by-product of the editing procedure. Many independent studies have elucidated individual aberrations with varied frequencies in various cell lines, all analyzing different editing strategies.

Ultimately, when generating ATMPs it needs to be ensured on a clonal level that the cell therapy product does not pose a risk. Many different methodologies are available, and their use needs to be adapted to the question of choice. The use of combined long- or short-read WGS data sets seems the currently most appropriate starting point. In line with EMA and FDA recommendations, the evaluation thereof mainly relies on curated gene lists. Any detected *de novo* mutation requires a science-based decision, and an expert-based de-risking approach. If implemented properly, this can be performed by pre-defined algorithms and lists and applying them in an automated fashion once data become available, thereby eliminating the human factor. In the coding genome, mutations in exons (out of frame or codon changing), mutations that create *de novo* STOP codons or deleterious missense mutations, that alter splice sites or change regulatory regions are obvious red flags, while effects of SNPs in intergenic and intronic regions usually are less impactful and therefore are typically not applied for ATMP release. Regardless, it is of utmost importance to take all available information on validation of genetic integrity into consideration to ensure the safest-possible starting material for cell therapies.

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SPOTLIGHT

REGULATORY PERSPECTIVE

Understanding GLP and cGMP regulatory framework for cell-based products to support early-phase clinical trials

Aaron Jankelow, Steven R Bauer, Graça Almeida-Porada, Anthony Atala, Christopher D Porada, and Stephen W Sawyer

The development of cell-based products is a growing field which is receiving a great deal of attention due to the potential of regenerative medicine. When developing new cellular products for clinical trials, it is important to understand the regulatory guidelines that must be followed to ensure that translation from bench to bedside is in accordance with regulations. The path for bringing a new cell-based drug to market in the USA usually falls under the IND pathway laid out by the US Food and Drug Administration, and generally requires preliminary nonclinical studies performed according to GLP guidelines followed by clinical trials and an eventual license application. Additionally, the manufacturing of these products must follow relevant cGMP guidelines. In this work, we summarize relevant GLP guidelines for nonclinical studies and outline cGMP manufacturing requirements needed to bring cell-based INDs to a Phase 1 clinical trial.

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INTRODUCTION

In recent years there has been increasing interest in the potential of cell-based products as regenerative medicine therapies [1–3]. These potential products range from somatic cell therapies, such as those used to replace damaged skin [4,5], to stem cell therapies that aim to repair damaged tissue [1] or treat autoimmune diseases [6] to natural killer cell therapies to help patients combat cancer [7]. These products are regulated by the US Food and Drug Administration (FDA) as HCT/Ps (human cells, tissues, or cellular or tissue-based products) and apply to any human cells or tissues that are intended for delivery to a human recipient and that are not vascularized organs, do not contain blood vessels for transplantation, are not whole blood,

and do not have any blood components, or blood derivative products [8].

In the USA, the process for translating nonclinical research into clinical trials is usually governed by the Investigational New Drug (IND) pathway to a Biologics License Application (BLA) (Figure 1) through the US FDA Center for Biologics Evaluation and Research (CBER) under section 351 of the Public Health Service (PHS) Act, which governs biological products. This Act governs the testing of new drugs in clinical investigations and ensures the safety and efficacy of the potential drugs prior to reaching the market [9,10]. Some exceptions apply in which simpler regulatory frameworks are in place under the PHS act, specifically section 361 for some HCT/Ps that are minimally manipulated and intended for homologous use [8]. An FDA guidance gives further information into the considerations for what qualifies for this pathway [11], however this topic is beyond the scope of this paper. Additionally, some HCT/Ps are instead classified as medical devices and governed under the Investigation Device Exemption (IDE) pathway instead of the IND pathway [8,12], however these will largely fall outside the scope of cell-based products and are not relevant to this discussion. The IND pathway involves a submission process where the Sponsor submits details including the name and purpose of the drug, planned protocols, and data from relevant studies to CBER and the Sponsor can begin their clinical trials 30 days after the FDA has received the application unless they are put on clinical hold, or earlier if explicitly notified by the FDA. A Phase 1 clinical trial is an introductory trial that focuses mainly on safety with a small number of subjects. Studies with fewer than 20 subjects are relatively common for cell-based products during Phase 1 trials [13]. Subsequent Phase 2 clinical trials are larger and focus on evaluating the effectiveness of the drug as well as determining common short-term risks and side effects with an expanded subject pool. Finally, Phase 3 clinical trials are intended to demonstrate the effectiveness and safety of the drug with an appropriately larger number of subjects. It is worth noting that many cell-based drugs are designed to address unmet medical needs and orphan indications and thus may qualify for an expedited testing pathway that does not strictly follow the 3-phase structure [14]. Regulations that are applicable to testing differ between Phase 1 and later phase trials, due to certain requirements not being as relevant during the early development and manufacturing of the products used in early-phase trials [9].

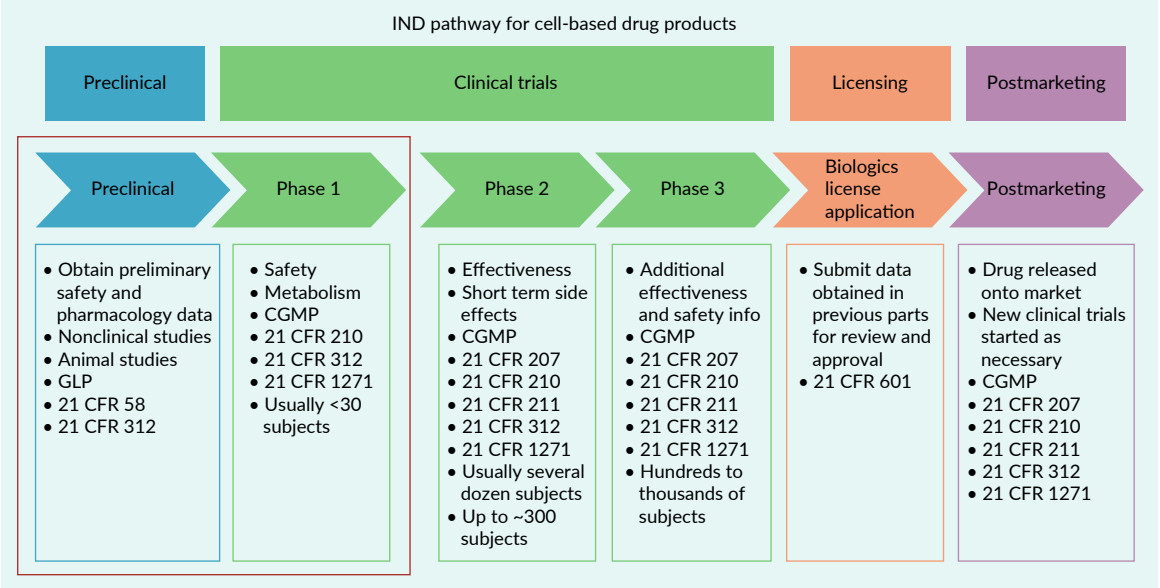
Regulations surrounding nonclinical studies that are used to support clinical trials are governed by Good Laboratory Practice (GLP) and are intended to support IND applications by ensuring the quality and integrity of the safety data. This data is then used to support clinical trials where the manufacture of cell-based therapies is governed cGMP regulations that ensure the quality and safety of the manufacturing of drugs. This paper seeks to give phase-appropriate guidance on how to translate work in cell-based therapeutics to the early-stage clinical trials by discussing the relevant regulations for non-clinical and Phase 1 clinical trials set by the FDA for the IND pathway. We will also briefly discuss how these guidelines compare to regulatory standards in the EU and UK.

GOOD LABORATORY PRACTICE

GLP are a set of requirements put forth in order to ensure the quality and integrity of data collected under nonclinical laboratory studies that are intended to support product applications [15,16]. These practices are standardized internationally by the Organization for Economic Co-operation and Development (OECD) guidelines to ensure studies done in one member country will be accepted in another, though

FIGURE 1

Overview of IND pathway for cell-based drug products highlighting stages of process alongside focus of each stage and most relevant regulations for manufacturing and testing of cell-based therapies at each stage.



Red box denotes focus area for this paper.

some aspects such as archival duration may be dictated by the authorities within individual member nations [17]. GLP exists to support future clinical trials by providing guidelines for nonclinical studies to ensure the quality of research as well as to aid the Sponsor in risk assessment prior to field tests in human or animal subjects [15,16]. Nonclinical studies are intended to detail toxicology and pharmacology information that intend to support the safety and potential of a cell-based product and are necessary requirements of the IND pathway. While some nonclinical studies such as those intended for screening, basic research, and discovery fall outside the scope of GLP, nonclinical studies intended to ensure the safety of drugs to support research or marketing applications are required to follow GLP guidelines. For cell-based therapies, toxicity can arise via multiple mechanisms so it is important to perform a variety of studies to establish safety of the therapy which can include biodistribution, immunogenicity, and dose selection. These studies should match the intended clinical product and formulation where feasible in order to best support any planned clinical trials. Ensuring such studies are GLP compliant is usually necessary to gain approval to move into clinical trials, although this can be waived on a case-by-case basis if sufficient justification is provided as to why they were not conducted in compliance with the regulations [10].

There are a number of personnel requirements in GLP studies [15]. First and foremost, all personnel must have sufficient qualifications and training for their assigned roles. Personnel must take proper precautions to avoid contamination, such as ensuring proper sanitation, wearing appropriate clothing, and avoiding direct contact with test systems when ill. GLP standards also require testing facility management to ensure that studies are appropriately staffed and equipped to carry out their work as scheduled and

assure that personnel understand their assigned roles. Additionally, GLP guidelines require the assignment of a study director in charge of the technical conduct of the study. This director must ensure that protocols are maintained, data is properly documented, unforeseen issues are documented and addressed, and that all experiments are compliant with GLP standards. Furthermore, according to GLP guidelines, the testing facility will have a QA unit independent from the teams conducting and directing the studies who will monitor each study to ensure compliance with regulations. QA's duties include maintaining a master schedule sheet of all studies conducted at the testing facility, maintaining copies of all protocol for these studies, performing regular inspections to assure the studies' integrity, informing study directors and management of any issues and corrective actions to be taken, and assuring any deviations from approved protocols and procedures are properly documented and approved [15].

Laboratory facilities are required to have separate areas for receipt and storage of test and control articles, as well as separate areas for the mixing of said articles, and the storage of mixtures. Additionally, there must be separate areas dedicated to the housing of test systems and for the performance of routine and specialized procedures required by the study. When doing animal studies, there must be separate rooms in animal care facilities to assure proper separation of species, isolation of distinct projects, the quarantine of specific animals, and the housing of animals, as well as additional separate rooms to ensure isolation of studies involving biohazardous agents such as volatile substances, aerosols, radioactive materials, and infectious agents. There should also be separate spaces for the diagnosis, treatment, and quarantine of potentially diseased lab animals to prevent the spread of disease to other animals in the facility. Storage areas for feed and bedding should also be kept separate from test systems and should be protected from contamination and infestation. When working with animals, independent facilities must exist to collect, store, and dispose of animal waste in a way that minimizes odor, infestation, disease, and environmental contamination [15].

Equipment used in GLP studies must be adequately maintained and calibrated to ensure proper functionality, with written records of all inspections, maintenance, testing, and calibration that describe what tasks were performed and when. It must also be noted whether these operations were routine or the result of a failure or malfunction. If the latter, the nature of the defect, how said defect was spotted, and remedial actions taken must be recorded. Equipment must have written standard operating procedures (SOP) detailing the methods, materials, and schedules for routine tests, calibrations, and maintenance, and specify actions to be taken in the event of equipment failure. These SOPs should also designate who is responsible for performing each operation [15].

Any reagents and solutions used in the manufacturing of drug products or test articles must be labeled to indicate identity, concentration, storage requirements, and expiration dates, and deteriorated and outdated reagents are not to be used in the studies. Test and control articles should be properly documented for key characteristics like identity, strength, purity and composition relevant to the study and the methods of creation of these articles should be documented. However, if pre-marketed products are used, for instance as control articles, these products will be characterized by their labeling. The stability of these articles should be determined either before the study begins or during periodic analysis as designated by an SOP. For studies that run over 4 weeks, reserve samples from each batch of test and control articles

should be maintained for archival, though samples that degrade in storage should not be kept past the period in which their quality would remain sufficient to allow for proper evaluation. For mixtures of test articles with carriers, tests should be conducted to determine the uniformity of the mixture and to periodically ensure the stability and concentration of the mixture. Finally, mixtures should not be used past the earliest expiration date of any component in the mixture [15].

SOPs should exist in writing for all laboratory methods for all aspects of the study. Studies should also contain written protocols describing the method and objective of any given study including a descriptive title, statement of purpose, identification of control and test articles used, descriptions of the materials used, details on the test system used, experimental design, the nature and frequency of tests performed, and statistical methods used to analyze the results. These SOPs and protocols should be clearly written to ensure standardization of practice and any deviations, changes, and revision of the SOPs and protocols must be recorded alongside the reason for these changes [15].

In order to confirm the quality of the study findings, a final report must be prepared including details on when and where the study was conducted, personnel details, objectives and procedures in the approved protocol including any revisions made, the identity of test and control articles including relevant characteristics, an assessment of the stability of these articles under the conditions of administration, details on the test system, the study methodology, how data analysis was performed, and the locations of where data and specimens are archived. All data, documentation, and specimens must be archived except for specimens from mutagenicity tests and wet specimens of biological fluids. Archives should allow for orderly storage and expedient retrieval of data, documentation, and specimens, and should minimize the deterioration of specimens. Storage may be outsourced as long as information on where everything is being stored is retained by the testing facility. Records and specimens for studies supporting INDs should be retained for at least 5 years following submission of results or until they have deteriorated past the point where they can be properly evaluated [15].

CURRENT GOOD MANUFACTURING PRACTICES FOR PHASE 1 CLINICAL TRIALS

Once work is ready to be moved into clinical trials and beyond, the guidelines surrounding the production of cell-based drug products are governed by cGMP [18]. As the name suggests, cGMP are requirements for the manufacturing of medical products that are intended to ensure their safety and efficacy and are updated as necessary to keep them in line with the current best practices. Cell-based drug products are regulated as HCT/Ps and are thus governed by 21 CFR 1271 as well as other relevant regulations [8]. Cell-based drug products that fall under the '361 HCT/P' pathway are only governed by 21 CFR 1271 and section 361 of the PHS Act and are deemed lower risk, while products regulated as INDs are subject to sections 351 and 361 of the PHS Act, the Federal Food, Drug, and Cosmetic Act (FD&C Act), and need to follow other relevant regulations including, but not limited to, 21 CFR Parts 1271, 600s, 200s, 312, 800s, and 812 [8].

HCT/Ps regulated under the 351 IND pathway are not exempt from other regulations and thus must go through clinical trials prior to a BLA. While HCT/Ps regulated under INDs are subject to 21 CFR 211's cGMP guidelines, these were written for the commercial manufacture of drugs and may not all be applicable for the manufacture of a drug product used in a Phase 1 IND [18]. Phase 1 INDs for cell-based products are

however still subject to the cGMP requirements of 21 CFR 1271, and the FDA has also published a guidance discussing how cGMP can be applied to Phase 1 INDs [9]. Furthermore, while these regulations are not strictly binding until a Phase 2 clinical trial and beyond [9], it is still worth keeping the regulations in mind since relevant aspects not covered by 21 CFR 1271 may still be required by the FDA, and planning for them where feasible in Phase 1 trials will make the transition to a Phase 2 trial and beyond more seamless.

Basic personnel requirements such as having properly trained staff remain the same for cGMP as with GLP, however unlike GLP, cGMP does not require the assignment of a study director [8,19]. Under 21 CFR 1271, cGMP guidelines for quality control requires a quality program, which goes further than simply relying on a quality assurance unit like with GLP, to ensure compliance with current good tissue practice (cGTP), a set of guidelines that intends to prevent the spread of disease through the manufacture and use of the HCT/Ps [8]. The quality program must create procedures relating to the core cGTP requirements, share any information pertaining to the possible contamination of HCT/Ps or the potential for the spread of diseases by the HCT/Ps with recipients and other establishments using HCT/Ps from the same donor or with similar methods, investigate and document deviations and ensure corrective action is taken as necessary, and establish monitoring systems to ensure compliance. Additionally, quality audits must be periodically performed and the performance of all cGTP-critical computer software must be validated [8]. Establishing a Corrective and Preventative Action (CAPA) system as part of the quality management system is crucial for cGMP compliance in order to properly and systematically prevent and address potential deviations and issues in the manufacturing of drugs [20].

The manufacturers of drugs in Phase 1 trials should establish a written plan that describes the roles and requirements of quality control including, at a minimum, who is responsible for: examining the materials used in Phase 1 to ensure they are appropriate and meet relevant standards, reviewing and approving protocol and acceptance criteria, releasing or rejecting batches of Phase 1 investigational drugs based on the relevant data and records, and investigating unexpected results or errors and taking corrective action as needed [9]. While all personnel must work to ensure quality, the FDA recommends assigning at least one individual to perform quality control functions independent of manufacturing responsibilities for Phase 1 drugs [9]. Additionally, 21 CFR 211 requires the existence of a quality control unit beyond Phase 1 who performs the quality control duties previously mentioned with the added responsibility of approving or rejecting all procedures [19]. While these guidelines are not strictly necessary for a Phase 1 trial, incorporating this during Phase 1 trials could allow for a smoother transition to Phase 1 and beyond.

cGMP manufacturing facilities must be of proper size and construction with appropriate lighting, ventilation, plumbing, cooling, heating, and sanitation [8,9,19]. To prevent mistakes and contamination, each operation should be performed in distinct and defined areas [8,19]. To help prevent the spread of disease, standard procedures must be established and maintained for facility cleaning and sanitation, and all cleaning and sanitation activities to prevent the contamination of HCT/Ps must be recorded and maintained for at least 3 years after their creation [8]. In order to prevent contamination of HCT/Ps or equipment, proper environmental controls such as temperature and humidity controls and air filtration must be installed where appropriate, and aseptic operations must be ensured through proper cleaning and

disinfection [8,9]. Environmental controls should be periodically inspected to verify they are functioning properly, and corrective action must be taken as necessary. Environmental monitoring systems should also be implemented where such conditions could be expected to cause contamination, including monitoring for sterility where appropriate. Records should be documented and maintained for environmental control and monitoring activities.

cGMP requirements for equipment in Phase 1 trials largely overlap with GLP requirements, with the need for equipment to be properly maintained and calibrated with written SOPs for equipment maintenance and calibration, routine inspections, and proper record keeping [8]. However, in addition to these requirements records should be kept of equipment usage including each HCT/P made with each individual piece of equipment [8]. Furthermore, appropriate equipment should be used such that it won't react with or contaminate the drug being manufactured [9].

Under cGMP, one must follow donor eligibility guidelines to reduce risk of disease transmission unless they are for autologous use and properly labeled as such in accordance with cGTP requirements [8,21]. Donors must be tested with FDA-licensed, approved, or cleared tests for relevant communicable diseases. Donors should be determined ineligible if they test positive for any of the diseases screened for, show risk factors associated with them, or if factors such as dilution render the test results unreliable. HCT/Ps stored from a donor who has not yet been determined eligible must be kept in quarantine until eligibility is confirmed. Donor specimens for testing should be collected within one week before or after recovery of the cells or tissues from the donor, or up to 30 days before the recovery for donors of peripheral blood stem/progenitor cells, bone marrow, or oocytes. Records of eligibility tests should be kept (without donor personal identifiers) including what tests were performed, what establishment performed them, test results, test interpretations, and final eligibility assessments. The FDA has also produced a guidance further detailing their recommendations for eligibility screening that provides a more comprehensive insight into their requirements [21].

Materials and reagents used in the manufacture of HCT/Ps must be verified by either the facility using the material or reagent or by the vendor to meet specifications designed to minimize risk of disease. The processes used to produce in-house reagents must be validated and verified. These reagents must be sterile, barring justification for why they cannot be. Records should be kept pertaining to materials and reagents including the type, quantity, manufacturer, lot number, date of receipt, expiration dates, records of verification or certification of verification from the vendor, and records of the lot used in the manufacture of each HCT/P. Storage areas should be controlled to prevent contamination, and materials, reagents, and HCT/Ps should be stored at an appropriate temperature. HCT/Ps should be assigned an expiration date based on their type, processing, storage conditions, and packaging [8]. Materials should be examined and verified upon receipt for appropriate labeling and lack of damage and stored appropriately with proper labeling until verified [9,19].

Procedures must be created to meet the core cGTP requirements and should be readily available to personnel in the area where they would be performed. Standard procedures from other facilities must be verified for the appropriateness of the operation, all procedures should be approved by the relevant quality control personnel, and any changes to procedures and protocols must be approved with proper rationales to ensure the overall integrity of the facility is maintained. Any departures from standard procedures must be recorded and

justified [8,9]. HCT/Ps must be processed in a way that does not cause contamination or increase risk of disease from using the HCT/P, and there should be in-process controls to ensure processes remain consistent with this goal. Human cells or tissues from multiple donors must not be pooled during manufacturing. Additionally, if the facility is working with dura mater, published validated processes that are available that reduce the risk of transmissible spongiform encephalopathy must be used unless those processes would adversely affect the clinical utility of the dura mater [8].

Laboratory tests in Phase 1 trials should also evaluate critical quality attributes of the HCT/P, including those that define identity, strength, purity, and potency as appropriate since these may not be understood yet on initiation of Phase 1 trials. The FDA also recommends initiating a stability study of representative samples of Phase 1 drugs during the clinical trial [9].

There are also cGMP requirements relating to the shipping and distribution of HCT/Ps in the USA which overlap with Good Distribution Practice (GDP). When shipping HCT/Ps within or between facilities prior to distribution, the HCT/Ps should be kept isolated and steps to implement pre-established criteria needed to prevent contamination and the spread of disease must be followed. Before distribution, records pertaining to the HCT/Ps must be reviewed and verified and documentation needs to be established that it has met the proper criteria and is suitable for release. Packaging and shipping containers must be designed and constructed to protect the HCT/Ps from contamination and appropriate shipping conditions, such as temperature controls, should be established and maintained during transit. These activities should be performed according to an SOP, and all relevant activities should be properly documented. There must also be protocols to determine if an HCT/P that is returned can be brought back to inventory or properly disposed. To facilitate the investigation of transmissible disease it is also required to have a system of tracking all HCT/Ps from donor to consignee or final disposition and back. If multiple facilities are involved in the manufacture of an HCT/P at different steps of the process, these establishments may share a tracking system so long as it complies with cGMP requirements. HCT/Ps must also each have a distinct identification code tying them to the donor and relevant records, and this code must be used during tracking alongside the HCT/P type. The information maintained must allow any consignees to be promptly identified for each HCT/P. Additionally, appropriate specimens from donors must be archived to enable testing if necessary.

Complete records should be kept on all aspects of the manufacturing process. These records must be retained as electronic records (which should be backed up), original paper records, or true copies such as photocopies, microfilm, or microfiche. Electronic records should be compliant with the regulations put forth in 21 CFR Part 11 [22]. Records should be retained for 10 years after creation, or for ten years after the administration of the HCT/P. If the administration date for an HCT/P is unknown, then they should be kept for 10 years after the latest of distribution, disposition, or expiration. Complaints received must be recorded and evaluated according to an SOP. Adverse reactions and deviations that could lead to a risk of disease transmission or HCT/P contamination must be reported within 15 for adverse reactions and 45 days for deviations. A record management system must be established and maintained to facilitate review of the records before distribution as well as to aid in follow-up investigations and evaluations. This system should maintain and organize the records appropriately to ensure the prompt identification, location, and retrieval of all records.

COMPARISON TO EU & UK REGULATORY FRAMEWORKS

The regulations previously discussed were those set out by the FDA for the USA, but other countries have their own regulations and regulatory bodies that handle GLP and cGMP within their borders. The European Union regulations are governed and harmonized by the EMA, and details on cGMP regulations are found in the EudraLex Volume 4 GMP guidelines [23], although specific functions and details may be overseen by local regulatory authorities for the individual member nations [24]. While the UK left the EU in 2020 and has its own regulatory agency, the Medicines and Healthcare products Regulatory Agency (MHRA), they continue to largely comply and harmonize with EU regulatory standards on drug manufacturing, though some minor bureaucratic differences exist as a result of the split [25]. Cell-based therapies are governed as advanced therapy medicinal products (ATMP) and face additional guidelines compared to other drugs, similar to the treatment of HCT/Ps in the USA [26]. Due to the existence of Mutual Recognition Agreements between the USA, EU, and UK, cGMP compliance results approved by one of their regulatory agencies are accepted as valid by the others. Additionally, international standards for cGMP are set by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) and are used as the basis of cGMP regulations for members including the USA, EU, and UK [27]. However, there do exist some key differences between the FDA regulations and the European counterparts such as the responsibility for batch release falling to a Qualified Person (QP) in Europe whereas it is the responsibility of the Quality Control Unit in the USA. Another key difference is that the EU guidelines for ATMPs delineate a graded system for clean areas with explicit requirements for each grade such as explicit guidelines on appropriate clothing for each grade, while FDA regulations on HTC/Ps are more flexible and allow more case-by-case discretion on what requirements are applicable to comply with guidelines.

CONCLUSIONS & DISCUSSION

Understanding the regulatory frameworks behind GLP and cGMP, including where they overlap and differ, will help allow researchers to transition their work from nonclinical studies to Phase 1 clinical trials and beyond. This article provides an overview of the FDA regulations that should help in adopting a phase-appropriate, risk-based approach to Phase 1 trials consistent with ICH Q9 Quality Risk Management and Q10 Pharmaceutical Quality System guidelines [27]. The information in this article should help the reader better understand the differences between GLP and cGMP, where each are applied, and what the expectations and regulations surrounding them are during Phase 1 trials. As work moves beyond Phase 1 trials, additional considerations such as the full requirements of 21 CFR 211 will become relevant in addition to what has been discussed here. In this manuscript we focused on FDA regulations and touched on how these compare to those in the EU and UK, but other countries will of course have their own regulations which may differ from these in key ways. The information gained from GLP-compliant nonclinical studies and clinical trials, which should follow cGMP requirements, is vital to obtaining a license to bring cell-based products to market [28], so understanding the requirements is crucial in developing new cell-based products for patient use. Properly understanding and following the FDA's GLP and cGMP regulations ensures a higher quality product and is the foundation for successful clinical trials.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY ANALYTICS AND CMC

SPOTLIGHT

INNOVATOR INSIGHT

Unlocking advanced strategies in cell therapy analytics

Q&A

(From left to right) **Stephan Krause**, **Kato Shum**, **Anoop Chandran**, and **Seth Peterson**

In order to effectively characterize cell therapy heterogeneity and improve product safety, purity, and potency, it is crucial to advance analytical tools and maintain quality controls from early development through commercial manufacturing.

Cell & Gene Therapy Insights and Thermo Fisher Scientific assembled a panel of leading experts, including **Stephan Krause** (Executive Director for Analytical Strategy, Bristol Myers Squibb), **Kato Shum** (Global Product Quality Leader, Iovance Biotherapeutics), **Anoop Chandran** (Associate Director, Analytical Development, Adaptimmune), and **Seth Peterson** (Senior Manager of Application Scientists, Thermo Fisher Scientific), to discuss best practices for developing and implementing analytical assays in cell and gene therapy (CGT), highlighting challenges in early assay design, scalability, and regulatory expectations. They also explore the role of advanced technologies including rapid sterility testing, next-generation sequencing (NGS), and multiomics in improving product characterization, quality control, and correlation with clinical outcomes.

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Q Tell us a bit about yourselves

SP Currently, I oversee the North American Application Scientists team at Thermo Fisher Scientific, which is focused entirely on analytics for the biopharma market. We are a support team driven by the shared success in assisting with the implementation of analytical solutions, starting from suitability studies through

phase-appropriate validations. We provide comprehensive support for the entire workflow, from sample to answer, including platform and assay qualification, and we engage in all phases of the process—drug discovery, preclinical, clinical, and commercial stages. Our role is to help ensure methods are fit-for-purpose and that we achieve phase-appropriate validations, whether it is utilizing new technologies or ensuring those technologies align with regulatory expectations. We work with all existing and emerging modalities, including cell therapies.

KS I am Global Product Quality Leader at Iovance Therapeutics, responsible for the analytical lifecycle strategy for both commercial and clinical products. Before Iovance, I served as Head of Quality Control (QC) at CARGO Therapeutics and Instil Bio. Prior to that, I spent over 6 years at Kite Pharma, where I was one of the early members helping advance Yescarta® and Tecartus® to the commercial stage. I am passionate about advancing technologies to improve patient access.

AC I am Associate Director for Analytical Development at Adaptimmune. I studied biochemistry and biotechnology, and hold a PhD in molecular immunology. After my PhD, I began developing analytical methods, focusing on creating and validating T cell functional and phenotypic assays to monitor patient responses. Over the past 8 years at Adaptimmune, I have been focusing on developing assays to test various T cell-receptor (TCR)-based cell therapy products both in-process and for product release. My work also includes lifecycle management of critical analytical procedures, transferring them to our QC laboratories and external partners, and supporting a wide range of regulatory submissions including Investigational New Drug (IND) applications, Investigational Medicinal Product Dossiers (IMPDs), and Biologics License Applications (BLAs).

SK I have been a long-term industry practitioner as well as an advocate for best practice implementation. Early in my career, I recognized that one of the best ways to advance was by actively participating in and/or leading external workstreams aimed at improving industry practices. This has been especially important in the new field of cell and gene therapy (CGT), where many gray areas are still being defined. I first published an article in 2002, and since then, I have continued to publish best industry practice extensively. I have remained very active here.

Q What are the key considerations around selecting analytical assays in early development?

KS In my opinion, it falls into two categories: assays performed internally and externally.

For internal methods, it is crucial to consider their future usage—for instance, whether the method will end up being used for QC purposes. Additionally, for each method, factors such as multiplexing or automation come into play, depending on the attributes.

Regarding assays developed and performed by external organizations, one key consideration is the ownership of the method. If a method used on your product is developed by a CDMO, then who ultimately owns it? This often comes down to negotiations between the

> "One of the biggest challenges for assays in cell therapy products is balancing such practical considerations with the complexity of functional phenotypic readouts."
> Anoop Chandran

parties involved, especially when transferring the method either internally, or to another external group in the future.

AC When we talk about considerations in early development, I would also include the preclinical stages before products even reach CMC.

In early development, the assays and what they measure should be closely tied to the product itself. The selection of assays should always begin with a clear definition of the product's critical quality attributes (CQAs), since these determine what truly needs to be measured, rather than just what is technically possible at the time.

Assay selection is not always driven strictly by a 'textbook' ICH guideline-based approach (i.e., Quality Target Product Profile (QTPP), CQAs, Analytical Target Profiles (ATPs), etc.). More often, it is influenced by practicality, timelines, prior experience, and available resources. From my experience, it is still possible to take a balanced, practical, and systematic long-term approach if you design a risk-based control strategy. In other words, selecting an analytical assay in the early stages—no matter how small or how ambitious your company's vision for the product may be—is the true start of a control strategy for that product.

It's the beginning of what may be a long and winding journey and the key to future-proofing is anticipating changes along the way. Developers must perform a risk assessment, which forms the cornerstone of any control strategy, and address potential failure modes within it. This becomes most effective when done collaboratively with other functions across the organization—R&D, process development, quality, and regulatory.

It is also crucial to build flexibility into the analytical strategy. Even if that flexibility only comes through paper-based risk assessments—when time and money do not allow for full testing—it still brings in the practical consideration of what can realistically be transitioned from development to QC or release testing.

One of the biggest challenges for assays in cell therapy products is balancing such practical considerations with the complexity of functional phenotypic readouts.

SK Other than being able to deliver suitable test method performance that is ideally Analytical Target Profile (ATP)-based, which is already challenging in our space, it is important to consider the assay's capacity to handle as many samples as possible.

From a commercial perspective, the gap I see in current industry practice is around capacity validation. Typically, analytical method validation is done relatively early in relation to capacity extensions, which mostly happen later, closer to BLA approval as well as post-BLA approval in the autologous cell therapy space. When capacity expansions take place in the QC laboratory, meaning we increase the sample load on a single test and/or run more tests within a given time period, the QC team must find ways to compensate for the increased volume as the demand ramps up quickly.

This can create a problem in that multiple QC laboratories being used to test pre- and/or post-BLA for the quickly increasing manufactured product lots can diverge procedurally

from each other. As they often must compensate for the much-increased sample loads, small or larger differences in executing procedures can occur. This can lead to some drifting and/or increased variation in product test results. Furthermore, capacity validation is generally not as performance-controlled for QC operations versus manufacturing capacity ramp-ups. This is currently a gap in our industry as it is somewhat unique to autologous cell therapies, and I expect we may eventually see more regulatory guidance here.

Alongside that, we also work on getting ready to use platform technologies, even trying to establish platform analytical procedures within the context of ICH Q14 as well as established conditions as recognized in Q12 for manufacturing process conditions. The goal there is to ensure we can bring other products onto these analytical platforms with a high degree of success, using established platforms and conditions.

SP From an early development standpoint, it is essential to build upon your CQAs and ensure you are not moving too quickly, neglecting robustness and sensitivity. Early assays should anticipate the CQAs that regulators will examine later because redesigning assays at a later stage takes a significant amount of time, especially for impurity testing. Additionally, early-stage testing involves smaller test populations, so developers should consider robustness beyond that limited sample size.

I would also plan for success regarding scalability. It is important to consider methods that can 'grow' along with the product, either through automation or hierarchical platforms. Those approaches will ultimately result in less variability overall, especially during method transfer. If you cannot adopt automation early, look for solutions that can be automated at a later stage when it becomes viable.

Regarding transferability, I like to borrow a strategy from the semiconductor industry, primarily Intel. They created a strategy called 'Copy Exactly!', which allowed them to expand their fabrication business globally. It involved replicating the manufacturing process down to the exact blueprint and building from there. For our industry, that means ensuring solutions and equipment are as exact as possible, or at least broadly available.

Getting something 'exact' in CGT is rarely the case in practice, of course, but I would make it a consideration. At the very least, look for platforms or solutions that are available globally, or—if you are intending to scale—ones that are supported consistently across your countries or regions of operation.

Q What is your advice around bringing novel analytical technologies into the QC framework—how to identify the right balance between using advanced technologies and regulatory acceptance/compliance?

SK I am one of the pioneers of bringing rapid technologies for microbial testing into QC. Back in the early 2000s, when I was at Bayer Biologics, I validated the BACTEC™ system for commercial product testing, making it the first-ever rapid sterility test approved for use in the industry. Since then, the BACTEC has been used frequently throughout life sciences, and it has been successfully applied for CGT products with typical turnaround testing times of only 3–5 days.

There are now 1-day rapid sterility tests available, but thus far, they have not seen widespread adoption across the CGT industry. Given the nature of CGTs, the metrics and

sample sizes are not always ideal, so further work is still required—we will have to see how these faster tests fare in future.

Regarding mycoplasma testing, at Bayer Biologics, we were also the first biologics manufacturer to get a mycoplasma sequencing test approved for commercial product(s) more than 20 years ago. Since then, much progress has been made. However, as one would expect when it comes to critical patient safety test methods, any new or not-yet-approved microbial contamination testing system encounters a very high bar from regulatory assessors—and for good reason. Extensive data and clear evidence are required to show that new technologies are not inferior—or ideally, superior—when compared to existing approved methods. The earlier new technologies can be implemented in product development and testing, the easier it becomes to get full approval at the time of licensure.

SP I think there is a bit of a misconception that the US FDA and other regulatory bodies are hesitant to embrace novel technologies. In reality, they are open to collaborating on innovative approaches that push the analytical state-of-the-art forward, especially for cell therapy, as long as these approaches provide value to the market.

It is crucial to engage with the FDA early and proactively. For novel analytical technologies, such as rapid sterility testing, or AI/ML-based tools, it is important to leverage the FDA's Emerging Technology Program, which supports the development and validation of these process control and analytical methods. For broader CMC strategies, it is possible to utilize the INTERACT program to gain guidance on integrating these technologies into your plan.

Lastly, it is essential to leverage informational chapters from the United States Pharmacopeia (USP) and other standard-setting organizations as they provide insight into novel technologies currently under their consideration. Earlier this year, USP continued updating guidance on rapid sterility testing, so it is worth reviewing that regularly to see the latest progress.

KS Since the early stages of my career as a scientist, I have been heavily involved in developing alternative methods to replace traditional compendial methods, such as mycoplasma testing, sterility testing, or replication-competent lentivirus testing. I think the general principle is that these alternative approaches are more patient-need-driven and technology-enabled.

The reason is that every day is vital for the patient, especially in autologous cell therapy in the oncology space. When I worked in QC, sometimes the patient could not wait even a few days to receive their dose, and we had to release the batch on time. Therefore, having a shorter turnaround time is critical in the CGT space.

AC Regarding any new technologies, one challenge is that it can take years to truly understand the pros and cons of each method. If you are one of the pioneers, you may be at a disadvantage because you will learn the 'hard way'. I would recommend introducing internal procedures to track and trend data as early as possible.

"...having a shorter turnaround time is critical in the CGT space."
Kato Shum

> "All things considered, planning ahead to accommodate scale-up demands is essential."
> Seth Peterson

Developers should check whether these novel technology readouts are product stability-indicating, which is especially important for some of our bespoke cellular products. Additionally, it is essential to maintain good governance with version control, change control, and training. It is also an option to consider orthogonal methods or fallback options when implementing new technologies in case issues arise in the future.

Q Can you share any experiences that illustrate the risks in deferring the design and implementation of analytical strategy until later in development?

KS In many cases, analytical strategies are not planned in advance, which can create the risk of the regulatory agency requesting additional data during late-stage review or even at the BLA stage. As a result, this can potentially delay both regulatory submission and corporate timelines. My advice is to be upfront and transparent with the agency about your analytical plan and seek their feedback as early as possible.

In the early stages, we often ask ourselves what is enough for the IND or for the current stage of development. However, I think having a long-term perspective is also very important. For example, retaining sufficient samples throughout development can be crucial. That way, if the agency requests additional testing or analysis during the review, there are samples available to support it.

SP From a risk perspective, there are real-world examples, such as potency and impurities in cell therapy, where inferred expression led to challenges later on in filings with the FDA. In this case, the timelines were extended by well over a year. There are even recent instances where data integrity issues have caused failed filings.

Therefore, ensuring robust safety and impurity testing from the outset is essential. As sample sizes grow, so does variability—especially with autologous cell therapies. In that field, developers may encounter inconsistent performance due to starting material variability and the small-scale processes, which hinders standardization. As mentioned earlier, varying robustness can lead to regulatory holds if consistency across development phases is not demonstrated.

Regarding scalability, it is not just about testing volume. You also need to account for equipment and laboratory layout to handle increased testing volumes or prepare for decentralized manufacturing. Cost–effectiveness is a challenge because simply adding equipment or space is not always feasible. You need solutions that can scale at each phase and consider automation or automation-ready assays. All things considered, planning ahead to accommodate scale-up demands is essential.

AC Some risks arise if you defer the design and implementation of an analytical strategy. Firstly, it may cause insufficient product understanding because

you did not collect the required amount of data. This can lead to an inability to support trending investigations, root cause analysis, and many regulatory questions that may arise at later stages. These gaps can then force late-stage revalidation or bridging studies, which are time-consuming, especially if getting to market quickly is a priority for your organization. Analytical procedures can become a bottleneck in this scenario.

For example, potency is very difficult to introduce, fit in, or change at late stages of development—particularly if your earlier methods lack strong biological or clinical relevance. This can result in insufficient data to set acceptance criteria for the commercial stages. Not having a clear strategy can also lead to inadequate system suitability controls and reference standards, which can affect comparability and bridging when moving from site to site—an important consideration over a product's long lifecycle.

Regarding scalability, developers should always have a vision for the scale they may need at later stages and begin building toward that early in their strategy.

SK The more time you spend in the commercial stages, the more you see these challenges first-hand. Rather than just viewing them as lessons learned, we should be mindful and try to convince management that the industry needs some fundamental changes in how we plan and execute our work.

An ideal analytical strategy execution should follow an ATP and an enhanced development approach, as outlined in ICH Q14. Interpreting Q14 may not be easy, because it leaves many questions open. But in essence, once the testing method has been selected, two principles should guide strategy and execution.

Firstly, it is important to be disciplined about Total Analytical Error—it should be budgeted from product specifications throughout the entire method lifecycle, with further allocation for all analytical control strategy elements.

Secondly, typical changes and optimization opportunities during product development and commercialization should be used to adjust budgets for test method performance, especially as specifications typically become tighter.

Q What methodologies are most suitable for characterizing autologous cell therapy products?

AC Characterization is quite different from the QC and release testing done for cell therapy products. It is essential for generating a deep understanding of your product—its starting materials, mode of action, and establishing correlations with manufacturing successes and failures. Characterization also supports process improvements/changes, and ultimately, it helps us understand why the product does or doesn't work. This requires a balanced toolkit (which can vary depending on the specific product) that can comprehensively reveal the identity and functional relevance of highly variable products.

My experience is mostly in immunotherapies and in particular, T cell therapy products. I consider phenotypic assays, particularly multiparametric flow cytometry, as a key tool for characterizing starting materials from patients as well as final products. These assays can define lineage, functionality, activation, exhaustion, memory subpopulations, and, when panels are carefully designed, provide robust insights into product composition.

Functional assays, such as cytotoxicity, cytokine secretion, and proliferation tests, are also key for immune cells. They link the cellular phenotypes identified earlier to potency and mechanism of action.

For safety, especially with genetically modified autologous cell therapy products, it is important to assess copy numbers, genetic stability, insertion size, and the ability of cells to proliferate and transform. If your budget allows you to use state-of-the-art technologies that analyze at the single-cell level, this can be extremely useful for understanding your product.

Finally, whatever methodology is used for characterization, it is essential to use the right references and controls, particularly if you plan to link your findings to current or future release methods.

SP Rather than focusing on a 'best methodology', I would frame it as a lifecycle approach. Early on, we should analyze the systems in as much detail as possible, using tools such as single-cell analytics, which are particularly valuable for understanding the variability we are dealing with. Sensitivity is crucial, especially when working with very limited sample material, and this emphasis should continue as we move through the process.

KS It is also important to interrogate what we do with the characterization data and understand how it can help us. There may be future technologies or data analytics, including AI, that could help predict patient outcomes based on characterization data.

Q What is your vision for the use of multiomics analytics to integrate genomic/transcriptomic/proteomic/epigenomic data, and link product attributes directly to clinical outcomes? How could this data be used for CGT product development?

AC Multiomics analysis is a powerful way to understand complex and variable cell therapy products and their starting materials. It can help bridge the gap between product biology and clinical outcomes, which is the 'holy grail'. Its value really lies in how we apply it pragmatically. Whenever possible, start early, and if feasible, go down to the single-cell level. By integrating genomic, transcriptomic, proteomic, and epigenetic data, we can begin to understand which cellular features or cell types drive persistence, potency, and safety in patients.

Although success is not always guaranteed, ignoring the potential of these approaches could leave your product falling short and limit opportunities for improvement later on. In early development, these tools can generate hypotheses and identify novel biomarkers. These hypotheses and biomarkers can then be tested experimentally and evaluated against clinical outcomes.

One challenge with multiomics data is that there is a lot of it, and it can be quite complex. During product development, the focus should be on reducing complexity and filtering the data into something that can be linked to product CQAs. For instance, transcriptomic signatures and T cell exhaustion could inform potency assays, while proteomic and epigenetic profiles could help predict long-term persistence. In essence, these insights should

> "Some correlation modelling has been attempted in the industry, but it has not been particularly useful in driving significant progress. However, this does not mean we shouldn't be working on it."
> Stephan Krause

be testable, which is often the challenge. From my experience, success relies on data from a sufficient number of representative samples and establishing standardized procedures, even for multiomics methods. Additionally, it is crucial to use reference materials, harmonized sample processing, and effective ways to analyze and filter the large amount of metadata generated.

These insights can help refine release specifications, inform stability studies, guide product improvement strategies, and sometimes even inform patient selection. Again, I would encourage developers to start saving samples early, even if they do not yet have the budget or methods to analyze them immediately. Preserving that potential allows you to assess starting materials and final products later.

KS I think it is a viable vision to link multiomics data—genomic, proteomic, and other datasets—to clinical outcomes. One challenge, especially for smaller companies, is that the patient database is very limited. For example, at the IND stage, there are often only a handful of patients with limited data, which makes it extremely challenging to draw meaningful conclusions or correlations between the data and clinical outcomes.

SK From my experience, it has been challenging to directly and quantitatively link clinical safety and efficacy readouts to specific drug product CQAs. Some correlation modelling has been attempted in the industry, but it has not been particularly useful in driving significant progress. However, this does not mean we shouldn't be working on it. Once we can work more extensively with big data and AI tools, I think there will be opportunities to operate very differently.

SP The clinical space is an area we could potentially learn from, since it has been adopting a multi-level approach for some clinical oncology applications, often using just a single platform.

I am particularly interested in electric sequencing technologies, which traditionally cover genomic and transcription data very well. These platforms have recently expanded into the proteomics space, using methods such as proximity extension assays, which can profile thousands of proteins at once. This really expands the capability of these platforms.

How could this be used for product development? From a genomic perspective, you can assess genetic stability in engineered cells, as well as analyze vector integration sites and formal diversity patterns. Looking at the transcriptome, RNA expression profiles can confirm the functional state of cells, identify markers that help develop potency assays, and track exhaustion signatures. Incorporating proteomics can help monitor surface activation markers. Although I have not seen as much from epigenomic approaches as yet, I did recently observe an approach for mesenchymal stromal cells (MSCs) using senescence markers that correlated them with efficacy.

Q What are some key takeaways in terms of how to successfully embed the requisite mindset in cell therapy analytical development teams from the earliest stages of development?

SP Firstly, it is fundamental to embed the requisite mindset across all development teams from the earliest stages to prevent working in isolation, and ensure that process development and analytical development are aligned with the QC department. I would focus on fostering that proactive and collaborative approach so that robustness is not sacrificed. I think every analytical method should connect to either safety or efficacy early on.

Secondly, I would encourage embracing new technologies, especially those that meet this market's demands in terms of speed and turnaround time. Regulatory agencies are generally collaborative with these new approaches and open to reviewing comparability studies. Along those lines, in order to improve turnaround times and reduce the need to release risk, focus on platforms that can scale. When planning development phases, it is important to envision a strategy for a multi-year lifecycle and look for partners who can support that journey, potentially at a global scale.

SK Analytical development scientists should be as transparent as possible, and management should encourage this. We all know the challenges that arise when something is not working but this is only reported lightly. The mindset should be to report failures and challenges early—perhaps even more so than successes. Lastly, the guiding principles I mentioned earlier should be fully understood and embedded in everything development does.

AC Analytical development is a very important function in any organization. It is not just a supporting function—analytics can really make or break your product. If your organization is not approaching analytical development this way, it is time to change that by focusing on the product, adopting a CQA-driven approach, and bringing in a culture of phase-appropriate development.

This prevents over-engineering assays early on and under-preparing for later regulatory expectations. Developers should focus on data integrity from day 1 by standardizing metadata, reference materials, and controls as early as possible. The organization should view analytical assays similarly to products, with lifecycles, evolution, required revisions, and re-qualifications all being important considerations. I would encourage building change control procedures into the analytical methods just as you do for other processes in the organization.

Furthermore, built-in tracking and trending are crucial. The analytical development function must work cross-functionally and collaboratively with the process development, research, QC, and regulatory teams. If we can foster this culture and mindset, the journey to regulatory filings from an analytical perspective will be much smoother.

KS Always consider the future stage of the assays, especially their commercial use in QC. In early development, think about the fact that you will be running this assay every day in QC. Therefore, design or develop assays with flexibility and a plan for final-stage use.

BIOGRAPHIES

Stephan O Krause is a biopharmaceutical quality and analytical science leader with over two decades of experience advancing global standards across biologics, cell therapies, and analytical control strategies. As Executive Director for Analytical Strategy at Bristol Myers Squibb, he leads analytical strategy development and lifecycle execution for cell therapy products across global sites. Stephan brings a rare blend of deep technical skills and strategic standard-setting experience, having held leadership roles at AstraZeneca, MedImmune, Bayer, and other companies. He is the recipient of over a dozen publication and industry recognition awards and is known for his PDA leadership. He currently serves on several advisory boards and chairs several high-impact task forces, including PDA TR for Phase-Appropriate QMS for ATMPs and ANS-07 Analytical Standard Development. He earned his PhD in Analytical Chemistry and Biochemistry from the University of Southern California, Los Angeles, CA, USA, where his research explored enzymatic mechanisms through isotope-labeled ATP hydrolysis studies.

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Kato Shum is an experienced and strategic Quality leader with over 12 years of experience across gene and cell therapy (i.e., RNA therapeutics, TIL cell therapy, allogenic and autologous CAR-T). Kato is currently the Product Quality Leader at Iovance Biotherapeutics. Most recently, Kato was Head of QC at CARGO Therapeutics, where he built the QC organization in both UK and US clinical manufacturing sites to advance the development of TIL therapy. Prior to Instil Bio, he was Associate Director of QC Technical Services at Kite Pharma, where he played an instrumental role in submission of both Yescarta® and Tecartus® BLAs as well as INDs of numerous investigational allogenic and autologous CAR-T products. In particular, he supported the QC method development, validation, transfer, product quality and oversight of external partners. Kato earned his BS and PhD degree in Biochemistry from the University of Hong Kong, Hong Kong, and completed post-doctoral fellowship in RNA therapeutics at City of Hope National Medical Center, Los Angeles.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY ANALYTICS AND CMC

SPOTLIGHT

COMMENTARY

Cell therapy analytics and CMC: balancing innovation with standardization

Jay Tanna

Cell and gene therapies (CGTs) are reshaping modern medicine, with more than 45 FDA-approved products to date [1–3]. Yet while clinical impact has grown, the analytical and CMC frameworks that support these therapies lag behind. Manufacturing variability, donor-to-donor differences, lengthy assay turnaround times, high reagent costs, and reliance on manual workflows continue to challenge reproducibility and scalability. Emerging solutions—process analytical technology (PAT), AI/ML, and integrated manufacturing systems—offer hope, but regulatory confidence and harmonized standards remain essential. This commentary reflects on current bottlenecks, highlights promising innovations, and calls for collaborative strategies to align scientific advances with practical realities.

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MANUFACTURING VARIABILITY: THE CENTRAL CHALLENGE

One of the defining features of CGT manufacturing is variability. Autologous therapies depend on patient-derived starting material, which can differ dramatically in composition and yield. Allogeneic therapies, while more scalable, must balance expansion with potency and quality. Across both approaches, the lack of standardized analytical tools creates uncertainty. Even subtle process changes, such as culture duration, oxygen exposure, or cytokine supplementation, can alter memory phenotype, exhaustion markers, or *in vivo* function [4–7]. Without consistent analytics, comparability between platforms or across sites is difficult to establish.

ANALYTICS AS A BOTTLENECK

Many assays remain labor-intensive, slow, and reliant on highly trained operators. Sterility testing can take up to 14 days, which is longer than the usable life of some fresh products. Conventional mycoplasma testing relies on a compendial 28-day culture-based assay, and although PCR-based methods accelerate results, they still require

multiple days. Potency assays are often bespoke, product-specific, and poorly standardized. These limitations directly delay product release, complicate multi-center trials, and hinder regulatory confidence [8].

EMERGING SOLUTIONS

Efforts to modernize analytics are underway (Table 1). Rapid sterility and mycoplasma assays are approaching regulatory readiness, but adoption remains uneven. Process analytical technology (PAT) tools, such as sensors capable of tracking oxygen, metabolites, and morphology, could provide continuous in-line data rather than endpoint snapshots. Automated, robotics-driven platforms promise closed, end-to-end manufacturing with embedded analytics, which may reduce variability and reliance on manual labor. The challenge is validation since regulators will need robust evidence before accepting in-line analytics as surrogates for conventional assays.

AI/ML

AI and machine learning are increasingly touted as game changers for cell therapy analytics. Potential applications include predictive modeling of T cell growth, automated image analysis, and streamlined interpretation of high-dimensional flow cytometry data [10,11]. However, adoption remains cautious. Algorithms that work like 'black boxes'—where the decision-making process is not easily understood—raise concerns about transparency and reproducibility, which regulators cannot overlook when patient safety is at stake [2,12,13]. A measured approach is needed, in which AI/ML supplements validated assays rather than replaces them. Clear differentiation between true AI/

TABLE 1

Current assay challenges and emerging solutions in cell therapy.

Assay type	Current challenges	Emerging/advanced solutions
Sterility	Sterility testing requires a 14-day culture, which exceeds the shelf life of many fresh products	Advanced rapid microbiological methods and automated real-time monitoring are already used to shorten assay times relative to traditional compendial methods;emerging approaches, including biocalorimetry-based systems and next-generation qPCR assays, aim to further reduce turnaround while providing high sensitivity
Mycoplasma	PCR-based testing reduces time but still requires several days	Ultra-rapid molecular assays are in development and may reduce turnaround to hours
Potency	Potency assays are product-specific, highly variable, and difficult to standardize	Functional surrogate assays, AI-assisted flow cytometry, and reference standards are being explored
Phenotype	Flow cytometry is operator-dependent and subject to variability	Automated cytometry and AI/ML-based image analytics can improve consistency
Viability/Count	Manual counting methods are error-prone and subjective	Automated counters and in-line viability sensors provide more reliable measurements
Endotoxin	Reliable assays but may add time to batch release workflows in rapid-release processes	Recombinant Factor C assays, which are rapid and animal-free, are commercially available; emerging microfluidic platforms integrating endotoxin detection offer near real-time monitoring and represent faster alternatives
Stability	Cryopreserved products require long stability testing timelines	Real-time monitoring and predictive AI/ML modeling are under investigation [9]

ML approaches and conventional predictive tools is essential to avoid overstated claims and ensure regulatory confidence. Confidence will grow gradually, through benchmarking against established methods, building robust datasets, and developing reference standards [14,15].

COST OF GOODS

The economics of cell therapy are shaped not only by manufacturing but also by analytics. GMP-grade cytokines, growth factors, and ancillary materials are exponentially more expensive than their research-grade equivalents. Transitioning from discovery-stage reagents to compliant versions increases costs and complexity. For example, IL-7, IL-15, and IL-21 are widely available in GMP-grade formulations, but each comes in multiple formats, all requiring qualification. These transitions add cost, time, and validation burdens. Collaborative solutions, such as pooled purchasing agreements, shared reference materials, and selective acceptance of well-characterized non-GMP reagents, could help reduce the financial weight of analytics [8]. Labor costs remain the most significant driver of QC cost, as many workflows remain manual and highly resource-intensive [16]. Automation offers a solution to reduce variability and cost per test, yet upfront investment can be challenging, particularly for smaller sponsors transitioning into GMP operations.

STARTING MATERIAL: THE FIRST CRITICAL QUALITY ATTRIBUTE

The quality of leukapheresis material is one of the most decisive factors in autologous manufacturing [4,7]. Donor age, prior therapies, and disease burden influence cell yield and phenotype. Poor-quality input material cannot be salvaged downstream, regardless of the sophistication of analytics. For allogeneic therapies, donor screening adds further complexity. FDA exemptions for autologous products contrast with stricter international requirements [17]. Harmonizing donor eligibility standards across geographies would reduce inconsistencies and improve global trial conduct.

ANALYTICS ACROSS DEVELOPMENT

Analytical expectations evolve alongside manufacturing. Pilot runs are exploratory, flexible, but must be carefully documented. Engineering runs are closer to the final process, using GMP-grade reagents and qualified assays. Qualification runs are the final step before clinical manufacture and require validated release assays. Early planning for analytics is critical since retrofitting validation late in development almost always leads to delays.

REGULATORY CMC STRATEGY

The science of CGT is advancing faster than regulatory frameworks. IND reviewers increasingly expect rigor, even for early-phase programs, yet guidance for novel assays and AI/ML tools remains limited. This creates a patchwork environment where expectations vary case by case. Three key gaps persist: regulators now expect reproducibility, stability, and comparability data up front [18]; the field continues to explore the need for enhanced analytical standardization, particularly for multi-site studies; and novel analytics such as AI/ML and PAT remain unproven in regulatory eyes until validated transparently. Engaging regulators early, sharing data openly, and participating in pre-competitive consortia will be essential to close these gaps [19,20].

THE WAY FORWARD

To ensure therapies are reproducible, accessible, and sustainable, the field should prioritize collaborative standards, in which reference materials and validation

frameworks are co-developed across industry, academia, and regulators. It should support the development of integrated, automated platforms with embedded analytics, which will reduce variability and reliance on artisanal methods. Finally, regulators should adapt by piloting pathways that allow for controlled introduction of novel analytics under defined conditions.

CONCLUSION

Analytics and CMC strategy are the backbone of successful cell therapy development. Emerging technologies such as PAT, AI/ML, and automated platforms offer transformative potential, but adoption will depend on validation, cost management, and regulatory confidence. At the same time, foundational issues such as starting material variability, high reagent costs, and lack of harmonized standards must be addressed. If the field can align scientific innovation with practical standardization, cell therapies will not only expand their reach but will do so with the safety, reproducibility, and affordability required to realize their promise.

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AFFILIATION

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CELL & GENE THERAPY INSIGHTS

CHARACTERIZATION AND VALIDATION

What do product-specific CQAs tell us about rAAV-based *in vivo* gene therapy products?

Yan Zhi

VIEWPOINT

"Due to the limitation of analytical methods, product-specific CQAs currently included in the release testing panel may not be able to paint a complete picture of a complicated rAAV-based *in vivo* gene therapy product."

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INTRODUCTION

Since AAV was first discovered as a contaminant of adenovirus cultures [1,2] back in the 1960s, AAV has gradually become the most popular viral vector for *in vivo* gene therapy application. Wild-type AAVs (wtAAVs) are generally not considered human pathogens, and no viral genome except the inverted terminal repeats (ITRs) remain in recombinant AAV (rAAV) as gene therapy product. Furthermore, without a viral gene (Rep), the integration of rAAV genome is greatly reduced. In addition to these attractive safety features, rAAVs, either derived from different serotypes discovered in human and non-human primates or based on novel capsids through bioengineering, can transduce both dividing and non-dividing cells with

broader tissue tropism and possess the capacity to bypass the blood–brain barrier. Nevertheless, the unique challenges for rAAV are the limited package capacity and the requirement of helper virus for its replication.

PRODUCT-SPECIFIC CRITICAL QUALITY ATTRIBUTES (CQAS) FOR RAAV-BASED *IN VIVO* GENE THERAPY PRODUCTS

It is encouraging that numerous clinical trials with rAAV manufactured using different production systems to target liver, eye, or brain for the treatment of ultra rare/rare genetic, aging-related, or neurodegenerative diseases have resulted in impressive clinical efficacies with tolerable safety profiles. As a result, eight rAAV-based *in vivo* gene therapy products have been approved by the US FDA and/or EMA:

- Glybera, approved in 2012 for lipoprotein lipase deficiency (LPLD)
- Luxturna, approved in 2017 for inherited retinal dystrophy caused by mutations in the RPE65 gene
- Zolgensma, approved in 2019 for spinal muscular atrophy (SMA)
- Upstaza, approved in 2022 for aromatic l-amino acid decarboxylase (AADC) deficiency
- Roctavian, approved in 2022 for hemophilia A
- Hemgenix, approved in 2022 for hemophilia B
- Elevidys, approved in 2023 for Duchenne muscular dystrophy (DMD)
- Beqvez, approved in 2024 for hemophilia B

More importantly, there is a clear regulatory pathway, including an expected CMC data package, for health agencies to approve rAAV-based *in vivo* gene therapy products. BioPhorum has recently published a summary of cell and gene therapy method validation controls to be used in establishing release methods for cell and gene therapy products based on current practices and considerations [3]. A typical release testing panel for a rAAV-based *in vivo* gene therapy product would consist of analytical methods for safety, strength/content, biological activities, purity/impurity, and identity.

rAAV-based *in vivo* gene therapy products are designed to provide a blueprint for patients to make functional therapeutic proteins using their own cellular machineries. Consequently, the efficacy or potency of such gene therapy product is determined by a matrix of product-specific CQAs, which typically consist of the total number of vector genome copies (i.e., GC) for the dose of administration in patients, the infectious particles normalized to vector genome copies (i.e., IP/GC) for the ability to transduce the targeted tissues/cells in patients; and *in vitro* potency measured by function of the intended therapeutic protein to indicate the desired clinical efficacy in patients.

As summarized by BioPhorum [3], there are expected analytical methods with appropriate standards and controls for product-specific CQAs (i.e., vector genome copies, infectious titer, and *in vitro* potency), which have been accepted by health agencies world-wide to support rAAV-based *in vivo* gene therapy products regulatory approval for commercialization.

First, a real-time PCR (qPCR) or digital PCR (dPCR) assay targeted to a unique sequence within gene of interest expression cassette after DNase treatment of rAAV particles is developed and validated to quantify vector genome copies (i.e., GC). Next, a tissue culture infectious dose 50 (TCID50) assay using HeLaRC32 cells

containing the integrated copy of AAV2 Rep and Cap to support rAAV replication in the presence of human Adenovirus type 5 (Ad5) as the helper virus with target-specific qPCR or dPCR as the read-out is developed and validated to measure infectious titer (i.e., IP). Finally, a cell-based assay, ideally using a clinical relevant cell type, is developed and validated to measure the intended therapeutic effect based on the mechanism of action to measure *in vitro* potency.

The genome copy (GC) assay is required to be validated prior to Investigational New Drug (IND) submission to ensure the accuracy of clinical dosing, while infectious titer assay and *in vitro* functional potency assay must be validated prior to Biologics License Application (BLA) submission. It is recommended to have both assays available and qualified as soon as possible in order to collect valuable data during product development, including clinical studies. The specification for product-specific CQA should be established based on analytical variability, manufacturing capability, clinical experience, and product knowledge.

LIMITATION OF PRODUCT-SPECIFIC CQAs FOR rAAV-BASED *IN VIVO* GENE THERAPY PRODUCTS

Because AAV is a single-stranded non-lytic DNA virus, either helper virus or essential helper functions derived from helper virus would be required for AAV replication, which inevitably increases the complexity of the manufacturing process for rAAV. Hence, even with clear health agency expectation and increased industry experience, what do these product-specific CQAs tell us about rAAV-based *in vivo* gene therapy product?

A perfectly designed GC assay by qPCR or dPCR could only quantify the copy number of a couple of hundred base pairs of rAAV genome spanning between forward and reverse PCR primers in a given sample. Even though the genome integrity is critical to ensure the expression of therapeutic protein delivered by rAAV, GC measured by qPCR or dPCR assay could not directly monitor rAAV genome integrity within any given product lot or among different product lots. It is encouraging that single molecule, real-time (SMRT) sequencing of rAAV genome based on long-read next-generation sequencing (NGS) technology platform such as PacBio has been developed to characterize the rAAV genome from ITR to ITR [4], while duplex dPCR assay has been developed to quantify rAAV genome integrity in addition to GC [5,6]. Even though neither assay is ready to be validated as a QC release method yet, the information of rAAV genome gathered by these new technologies would facilitate the better understanding and, more importantly, tighter control of complex rAAV-based *in vivo* gene therapy products.

rAAV infectivity assay would require an engineered cell line, which not only needs to express Rep/Cap but also is permissive to rAAV transduction and helper virus infection. Vastly different *ex vivo*/*in vitro* transduction efficiency of mammalian primary cells and cell lines has been observed by nine natural AAV serotypes 1–9 and one engineered AAV serotype [7]. Therefore, it is not surprising that a 'standard' rAAV infectivity assay using HeLa RC32 in the presence of Ad5 could not truly reflect the transduction efficacy of rAAV in the targeted tissue/cell of the intended patients. At best, such 'standard' rAAV infectivity assay would demonstrate the consistency of manufacturing process within the limitation of high intrinsic TCID50-based assay variability.

The FDA guidance [8] provides the agency's recommendations of potency tests for cell and gene therapy products. A fully validated potency assay per ICH guidance and reflecting mechanism of action is expected for a BLA submission. The intrinsic

challenges associated with *in vivo* potency assay, such as high variability, challenging validation, and high cost, in combination with 3Rs – Replacement, Reduction, and Refinement – compliance have driven the industry to develop and validate *in vitro* potency assay. A human-derived cell line, ideally mimicking the targeted tissue type in patients, will be selected to develop a cell-based *in vitro* potency assay, in which a range of selected genome copies of rAAV would be used for transduction in order to measure the desired biological activity (i.e., potency), considering GC has been exclusively used for clinical dosing of rAAV-based *in vivo* gene therapy products.

In such experimental settings, empty rAAV particles could directly compete with full rAAV particles by binding to the same cellular receptor during transduction. As a consequence, rAAV containing higher percentage of empty particles per the fixed amount of GCs could result in a reduced *in vitro* potency. Interestingly, it has been reported that empty particles could absorb the pre-existing neutralizing antibodies to AAV in order to improve transduction efficacy in both mouse and non-human primate models [9]. In contrast, empty particles in rAAV8 preparation have been reported to reduce transduction efficiency and cause total viral particle dose-limiting side effects in mouse models [10]. Taken together, *in vitro* potency may not directly translate to *in vivo* potency for a rAAV-based gene therapy product, considering the reported controversial effect of empty particles in animal models.

CONCLUSION

Due to the limitation of analytical methods, product-specific CQAs (i.e., GC, infectivity, and *in vitro* potency) currently included in the release testing panel may not be able to paint a complete picture of a complicated rAAV-based *in vivo* gene therapy product. Advanced analytical tool for empowering multi-attribute methods would be welcomed to directly and adequately evaluate and quantify different types of rAAV particles (i.e., empty, partial, full, etc.) together with the exact DNA payload packaged within each individual rAAV particle (i.e., full or partial expression cassette of gene of interest, residual host cell DNA, residual plasmid / helper viral DNA, etc.). Continuous technology innovation will fuel the next breakthrough of rAAV-based *in vivo* gene therapy products.

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BIOGRAPHY

Yan Zhi has spent over 15 years in global contract testing organizations (WuXi AppTec and Charles River Laboratories) following postdoctoral research training with Dr James M Wilson at Institute of Human Gene Therapy, University of Pennsylvania. Yan provides scientific leadership to biologics testing services with a strong focus on cell and gene therapy industry as well as a global contract development and manufacturing organization (Fujifilm Diosynth Biotechnologies) to design cell and gene therapy product development programs from clinical to commercial manufacturing. In 2020, Yan joined Spirovant Sciences, Inc. to lead the analytical development and develop CMC strategy of a novel serotype AAV based *in vivo* gene therapy product for IND submission. Since 2022, Yan has been a core team member for technical transfer and the CMC lead working with regulatory, quality, and supply teams at CSL Behring to ensure the commercial supply of Hemgenix as well as regulatory submissions to additional countries. Yan received a PhD in Microbiology and Molecular Genetics from University of California, Irvine, and a BA in Molecular Biology from University of Science & Technology of China. She is a leading author and co-author of numerous publications in peer-reviewed scientific journals and a patent holder.

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CELL & GENE THERAPY INSIGHTS

TRANSLATIONAL R&D

Bringing gene therapy to the brain: Cardiff's role in the landmark Huntington's trial

INTERVIEW

"What the AMT-130 trial shows us is that lowering mutant huntingtin is a feasible and effective way to progress in the treatment of Huntington's disease, and that progression of the disease can potentially be slowed."

Abi Pinchbeck (Editor, *Cell & Gene Therapy Insights*) speaks with **Anne Rosser** (Professor of Clinical Neuroscience, Cardiff University) about Cardiff's leading role in the recent landmark Huntington's disease trial of UniQure's AMT-130 gene therapy trial in collaboration with UCL in the UK and sites in Poland and the US. This is the first gene therapy trial to report statistically significant slowing of Huntington's progression [1,2]. They discuss the scientific breakthroughs behind the study, the challenges of neurosurgical delivery, possible implications for other neurodegenerative conditions, and the evolving relationship between gene and cell therapy approaches for Huntington's disease.

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Q Cardiff played a pivotal role in this landmark Huntington's disease trial. Can you reflect on your team's contribution to the study?

AR In Cardiff, we were involved in both recruiting patients and delivering the gene therapy for all UK participants, including those from UCL. My co-investigator, Professor William Gray, and I co-lead the Advanced NeuroTherapies Center (ANTC) in Cardiff, which aims to become a global center of excellence for delivering gene and cell therapies to the brain.

Professor Gray was instrumental in acquiring and training a highly skilled team at the University of Wales Hospital in Cardiff and ensuring the right infrastructure was in place. This involved significant preparation, traveling to the US for training, collaborating closely with US teams, and working with our imaging center—Cardiff University Brain Research Imaging Centre (CUBRIC)—so that this complex surgery could be carried out safely and effectively. Establishing this capacity took at least a couple of years. Each therapeutic delivery required several days of preparation and multidisciplinary coordination. We were also responsible for the ongoing follow-up of our patients after surgery.

Q This study represents the first time a drug trial has demonstrated a statistically significant slowing of Huntington's progression. From your perspective, what do you see as the key scientific and translational breakthroughs that enabled this success?

AR It is genuinely exciting, but I do want to emphasize that these are preliminary, top-line results released by the company, and the data have not yet been peer-reviewed. That said, the data looks promising.

This is the first attempt at delivering a gene therapy directly into the brain for Huntington's disease. Other studies are using different approaches to lower mutant huntingtin, and together this represents the culmination of decades of research leading to multiple concurrent trials.

What the AMT-130 trial shows us is that lowering mutant huntingtin is a feasible and effective way to progress in the treatment of Huntington's disease, and that progression of the disease can potentially be slowed. For those two reasons, this is very big news for Huntington's disease.

Q The trial used direct delivery of a gene therapy vector into the brain—a technically and logistically demanding neurosurgery approach. What were the main challenges in implementing this surgery-led delivery model, and how did your team overcome them?

AR This type of surgery requires assembling a highly trained team that understands the entire process. Consistent, high-quality training is critical. In multi-site trials, ensuring that every procedure is done in exactly the same way across

"People tend to fear neurosurgery, but when it is done properly and carefully with the required monitoring, the neurosurgery has been quite safe."

centers is a real challenge. It may sound trivial, but maintaining perfect consistency is difficult, and even small variations can affect outcomes.

There are also important considerations around patient consent. Participants must understand that this is an experimental treatment that cannot be removed. Unlike a drug trial, where you can stop dosing if there are adverse events, you cannot take the gene back out of the brain. Patients who receive gene therapy are also unlikely to be eligible for other advanced therapy trials later.

Trial design poses another layer of complexity. For instance, the US arm of this study used a placebo group, but the European regulators did not permit a surgical placebo at this stage of development, so the placebo arm was limited to the US. The placebo involved the patient undergoing anesthesia for 14–16 hours, and partial burr holes in the skull–this mirrored the therapeutic surgery but without a needle penetrating the brain and without the therapeutic vector being delivered. There was also ethical pressure to offer those patients the therapeutic after a year, which meant that the placebo group was only informative for the first 12 months. The significant efficacy differences, however, appeared after 2–3 years, so the placebo data were of limited long-term value. I am not suggesting that placebo controls should never be used in gene therapy trials, but they are complex and carry risks that must be carefully considered.

Finally, some drug trials can involve hundreds or thousands of patients, but for a gene therapy requiring significant neurosurgical intervention, such large-scale trials are unlikely ever to be feasible.

Q Many in the gene therapy field are watching this result closely as a proof-of-concept for CNS-targeted AAV therapies. What do these findings tell us about the potential, and the limits of, gene therapy for complex neurodegenerative diseases beyond Huntington's?

AR I think this trial gives us valuable lessons both for Huntington's and for delivering gene therapies in general. For neurological conditions, we may eventually be able to deliver gene therapies through an infusion into a vein, but at the moment, that is very difficult to do. The genes need to be delivered directly into the brain.

This trial demonstrates that such delivery can be done safely. People tend to fear neurosurgery, but when it is done properly and carefully with the required monitoring, the neurosurgery has been quite safe. Any adverse events in this study seemed to have been related either to the capsid itself, potentially allergic responses to the capsid, which resolved, or to the immunosuppression that was given to some patients to mitigate those responses.

It tells us that neurosurgery can be done safely and provides important information about what we need to consider going forward. For example, while the surgery was safe, it was also quite slow and took a long time. Neurosurgeons are now coming together

"The key questions are how to deliver effectively, how to make delivery safer and more efficient, and how the delivery process itself affects clinical trial design."

internationally to think innovatively about how this kind of procedure could eventually be done more quickly and simply so that it becomes accessible to more people. That will take time, but the lessons from this study will be applicable more widely.

Q Are there other conditions that could benefit next from these lessons, particularly around the neurosurgery approach?

AR Yes, there are lessons for conditions with a similar underlying genetic basis. In Huntington's, the gene mutation is an expanded tandem CAG repeat. There are many other neurological diseases with similar repeat expansions in different genes, where we may also want to use gene therapy to lower specific protein products. Lessons from Huntington's could be translated to those conditions.

In terms of neurosurgery more broadly, gene therapy is already being investigated in Parkinson's disease and Frontotemporal dementia (FTD). The key questions are how to deliver effectively, how to make delivery safer and more efficient, and how the delivery process itself affects clinical trial design. Addressing these challenges in one condition is very likely to accelerate progress in other diseases.

Within the Huntington's field, there are already international working groups addressing such issues: for example, in the European HD network there is a working group focused on improving the speed and efficiency of neurosurgery, and another on trial design and how to make advanced therapy trials more efficient. These discussions will be crucial as the field progresses.

Q You've previously worked with stem cell transplantation and other cellular therapy strategies in Huntington's research. How does the success of a gene therapy intervention reshape your thinking about where cell-based strategies now fit in the therapeutic landscape?

AR In many ways, I find it encouraging. Cell and gene therapies do quite different things. The cell therapy that my group is developing, which we are working to take into a first-in-human trial, is designed to replace the cells that are lost in the brain due to disease. By contrast, gene therapies and other therapeutic approaches aim to slow or prevent further loss.

A cell therapy would be compatible with a successful gene therapy. Ultimately, we may want to combine them: put cells into the brain to replace those that have been lost and deliver a gene therapy or other disease-modifying treatment to slow progression. These approaches are entirely compatible.

This trial has also significantly advanced thinking around neurosurgical delivery, which will be essential for any future cell therapy. Such therapies will also require direct delivery into the brain through a similar procedure, and the experience gained here will be invaluable.

Q What do you see as the next frontiers in Huntington's research?

AR I think combination approaches are likely to be the next major step, though we are not quite there yet. The appropriate combinations will become clear as more therapies become available.

It is important to emphasize that the AMT-130 trial, although high-profile, is not the only one. Many Huntington's trials are underway, and some are already approaching regulatory discussions. Ideally, we will reach a point where we have multiple therapeutics in our toolbox, each suited to different disease stages, patient profiles, and comorbidities, that can be used alone or in combination.

Q Looking forward, what are the next scientific or clinical questions your group hopes to address?

AR My own group is particularly focused on cell therapies. We are pursuing two main avenues: advancing our cell therapy product toward a first-in-human trial, and conducting basic research to better understand how to guide cells to differentiate into the exact neural phenotypes we need.

In parallel, I am also involved in clinical research that looks at the behavioral and cognitive aspects of Huntington's and how to develop better management strategies for these symptoms. It is a multifaceted effort, but together these lines of work aim to bring us closer to effective, long-term treatment options for patients.

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BIOGRAPHY

Anne Rosser is Professor of Clinical Neuroscience at Cardiff University and Honorary Consultant Neurologist at the University Hospital of Wales. She undertook her medical training and PhD at Cambridge University, Cambridge, UK and completed specialist training at the National Hospital for Neurology in London and Addenbrookes hospital in

Cambridge. She moved to Cardiff in 2001, where she directs the Cardiff University Brain Repair Group and is deputy director of the Wales Advanced Neurotherapeutics Centre, which aims to deliver therapeutics into the brain in neurological conditions. She has a long-standing interest in Huntington's disease (HD) and was Chair of the European HD network until October 2024. She has a special interest in regenerative medicine and the delivery of advanced therapy medicinal products (ATMPs) for neurodegenerative conditions, in particular cell therapies for HD. Her group are currently working to take a stem cell therapeutic for HD towards a first-in-man clinical trial.

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CELL & GENE THERAPY INSIGHTS

TRANSLATIONAL R&D

CRISPR in action: inside the first genome-wide NK cell screen and what it means for immunotherapy

INTERVIEW

"We wanted to uncover what lies 'beneath the iceberg' by taking an unbiased approach to identify novel targets that may have previously been unrecognized"

In a recent landmark study, researchers at MD Anderson Cancer Center reported the development of the first genome-wide CRISPR screening platform for primary human natural killer (NK) cells [1]. The platform, termed PreCiSE, was pioneered under the mentorship of Dr Katy Rezvani, Vice President & Head, Institute for Cell Therapy Discovery & Innovation, MD Anderson Cancer Center, and co-led by **Alexander Biederstädt**, now Junior Group Leader, Technical University of Munich, and Dr Rafet Basar, MD Anderson Cancer Center. In this interview, **Abigail Pinchbeck**, Editor, BioInsights, speaks with Dr Biederstädt about how PreCiSE enabled the identification and validation of novel genetic targets that enhance NK cell persistence, proliferation, and antitumor activity, and how these discoveries could be translated to inform the design of next-generation NK cell therapies.

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" Until now, no systematic approach had been applied to interrogate thousands of genes in NK cells in a massively parallel manner."

Q First, can you briefly summarize for us the key findings of your recent study published in *Cancer Cell*?

AB We successfully performed the first genome-wide CRISPR screen in primary human NK cells to systematically interrogate gene targets that, when deleted, enhance NK cell function. We identified a comprehensive landscape of actionable gene targets whose modulation can improve NK cell persistence, proliferation, and antitumor potency.

Specifically, we validated three targets: *MED12*, *ARIH2*, and *CCNC*. When targeted using CRISPR precision editing, these genes significantly enhanced antitumor potency by inducing favorable metabolic changes in NK cells. This editing also promoted the development of cytotoxic phenotypes and cytotoxic subsets, as revealed by large-scale multi-omics assays.

In particular, we observed that a dual knockout of *ARIH2* and *CCNC* synergistically enhanced CAR-NK cell potency. We tested this approach in both unmodified NK cells and in CD70-CAR/IL-15-NK and TROP2-CAR/IL-15-NK cells. The dual knockout consistently produced profound improvements in NK cell antitumor function, driven by metabolic reprogramming, induction of cytotoxic subsets, and transcriptional modulation. We observed these effects across multiple tumor types, including both hematologic and solid cancers, using both *in vitro* and in various mouse models.

Q What motivated your team to focus on developing a genome-wide CRISPR screening tool specifically for primary human NK cells, and how does this address existing gaps in CAR-NK cell therapy research?

AB There is significant clinical interest in NK cells, particularly as CAR-NK cells have demonstrated highly promising responses in heavily pretreated patients with lymphoid malignancies, and the Institute for Cell Therapy Discovery & Innovation at MD Anderson Cancer Center, led by Dr Katy Rezvani, has been at the forefront of leading this effort [2,3,4]. The CRISPR toolkit now provides the ability to further modulate immune cells, improving their function and overcoming barriers in existing NK cell therapy approaches.

However, the key challenge has been that most efforts to date have been driven by a hypothesis-driven approach, where certain aspects of immune cell biology are targeted one by one. Until now, no systematic approach had been applied to interrogate thousands of genes in NK cells in a massively parallel manner. We wanted to uncover what lies 'beneath the iceberg' by taking an unbiased approach to identify novel targets that may have previously been unrecognized. This strategy allowed us to decipher fundamental

drivers of NK cell biology in an unbiased manner which can be harnessed to guide cellular engineering and precision gene editing strategies for next-generation NK cell therapies.

Q Can you walk us through how PreCiSE was optimized for NK cells and what makes it unique compared to previous CRISPR screening approaches in immune cells?

AB CRISPR screening was first established in 2013, with seminal studies in primary human T cells beginning in 2018 that demonstrated the feasibility of the large-scale functional assays in primary immune cells. However, NK cells are far more challenging to edit genetically and more difficult to culture *ex vivo*, which presented numerous technical barriers that we needed to overcome in order to make this work.

Essentially, our strategy required a multi-pronged approach, where we first optimized *ex vivo* expansion methods, then genetically edited large numbers of cells in a pooled manner using a combination of retroviral vector delivery and electroporation of recombinant Cas9.

Notably, primary human NK cells are highly delicate. Editing efficiency must therefore be carefully balanced with NK cell recovery and viability. We had to be very systematic and thorough in optimizing different technical parameters to develop a safe method that enables efficient large-scale modification of NK cells, while maintaining cellular viability and proliferative capacity.

Q How did you prioritize the *MED12*, *ARIH2*, and *CCNC* targets from your genome-wide screen, and what made these particular genes stand out as the most promising candidates for therapeutic intervention?

AB A key challenge in any CRISPR screen is interpreting the long list of candidate genes, some of which score more highly than others due to their level of enrichment. In essence, enriched hits represent genes that confer a competitive fitness advantage in NK cells when deleted. To prioritize these hits, our first step was to validate the plausibility of our findings. We cross-referenced our findings against established NK cell biology, and we were encouraged to see that our screen recapitulated many well-characterized regulators of NK cell function, giving us strong confidence in the results.

We also identified several novel hits that had not previously been described—these were of particular interest. To prioritize them, we went beyond conducting a single genome-wide CRISPR screen. Instead, we performed multiple screens across different donors and applied diverse selective pressures. For example, we tested tumor co-culture using aggressive solid tumor models, such as pancreatic cancer, as well as under various tumor microenvironment (TME) immunosuppressive conditions, including lactic acid, hypoxia, and transforming growth factor-beta (TGF-β).

We then focused on hits that consistently emerged across these different screening modalities. Although improved NK cell 'fitness' could manifest in several ways, including

enhanced viability, proliferation, cytotoxicity, or resistance to immunosuppressive cues, our goal was to identify the targets most strongly associated with improved antitumor function.

To do this, we took a weighted scoring approach, assigning greater weight to hits identified in tumor co-culture screens, while also requiring consistent appearance across other screening modalities. We also interrogated gene expression patterns of these hits in publicly available single-cell RNA sequencing datasets to determine which genes were consistently upregulated in dysfunctional NK cells from cancer patients. This allowed us to identify clinically meaningful checkpoints that increased in NK cells that lost their function.

Lastly, and of particular importance, several of our key findings overlapped with those reported by Nicholas Huntington's group at Monash University, Melbourne, Australia who conducted genome-wide CRISPR screens in an immortalized NK cell line and subsequently validated them in human NK cells. Seeing many of the same results come out of their study, discovered completely independently on the other side of the world, really underscore the strength and robustness of both studies.

Q As you mentioned, dual knockout of *ARIH2* and *CCNC* works much better than targeting either gene alone. As multiplex gene-editing technologies advance, how do you identify these powerful gene combinations, and how do you decide between targeting single genes versus multiple genes?

AB In our case, all the individual targets we identified were independently relevant for improving NK cell fitness. A systematic multi-editing discovery approach is a logical next step, as it would allow us to assess whether different combinations of targets synergize to enhance NK cell function. Technically, there are CRISPR screening platforms available that enable combinatorial screening, but these have not yet been applied to NK cells, yet. Going forward, it will be interesting to see how these next-generation screening platforms can be applied to study actionable immune cell checkpoint combinations to guide rational multiplexed genome-engineering strategies.

Q A key finding was that edited NK cells showed improved function under TME pressures that typically suppress immune activity. How did you model the immunosuppressive conditions of the TME, and which aspects of NK cell function showed the most dramatic improvements after target knockout?

AB Any CRISPR screen is only as meaningful as its readout. It is therefore essential to put findings into context by selecting clinically relevant endpoints and selective pressures. In our case, tumor co-culture was an obvious model, but we also sought to capture additional factors of the solid tumor microenvironment.

Solid tumors are notoriously difficult to penetrate and impose multiple barriers, including immunosuppressive metabolites such as TGF-β, as well as hypoxia, nutrient deprivation, and lactic acid accumulation. Because NK cells are particularly sensitive to TGF-β signaling, this became a critical focus. Hypoxia and lactic acid were also key considerations.

"The real richness of our work is that many of the hits we have not yet validated are relevant for translational purposes."

To address these factors, we developed models that exposed NK cells to these pressures *in vitro*. This required a delicate balance: the stress needed to be strong enough to reveal subtle competitive advantages over time, but not so extreme that it would suffocate the NK cells, necessitating extensive titration efforts to determine appropriate exposure levels.

Our screens identified several enriched genes, including programs linked to apoptotic pathways such as Fas. Knocking out certain targets markedly improved NK cell persistence. However, a critical question we asked was whether enhanced NK cell persistence necessarily translates into improved antitumor killing. This is precisely why our prioritization approach, integrating findings across multiple screens, was so important.

Q How might these findings be used in a translational manner in the development of next-generation NK cell therapies with improved efficacy against both hematologic and solid tumors? How might PreCiSE be used further in this regard?

AB The Institute for Cell Therapy Discovery & Innovation at MD Anderson Cancer Center, led by Dr Katy Rezvani, is a highly translational-oriented NK cell development program, and much of the work we do in the lab can be rapidly moved into Phase 1 clinical trials. In fact, there are already several ongoing trials testing allogeneic CAR-NK cells that have been edited using CRISPR precision gene editing, for instance with *CISH* knockout, to improve NK cell fitness and antitumor function.

The logical next step will be to advance the validated targets from our genome-wide CRISPR screen toward Phase 1 clinical testing. Naturally, this will require additional steps, including comprehensive preclinical safety assessments, before translation to the clinic can be realized.

The real richness of our work is that many of the hits we have not yet validated are relevant for translational purposes. Thus far, our efforts have mainly focused on checkpoints, i.e., the brakes that hold NK cells back, that we can overcome. However, there are additional hits that can be validated and potentially moved into clinical testing. The findings that we did not pursue in this study are equally interesting: for example, we identified several NK cell-specific depleted gene programs, indicating they are critical for NK cell proliferation and survival. These gene programs could be rationally augmented in engineered NK cells including by tools such as CRISPR activation and CRISPR knock-in to augment NK cell fitness and enhance antitumor function.

In fact, there is exciting research in the T cell field showing how transcription factors can be artificially augmented to drive improved T cell function and mitigate challenges such as tonic signaling and functional exhaustion. That is an exciting approach that could similarly be applied to NK cells.

Regarding PreCiSE, our effort was already very ambitious and as stated, this is the first report of genome-wide CRISPR screening in primary human NK cells to date. However, there are a number of important research questions that remain unanswered.

Specifically, while we tested critical immunosuppressive pressures of the TME, there are additional pressures that could be modeled and exploring these immunosuppressive challenges and how they can be circumnavigated will be essential for engineering NK cells that can effectively combat solid tumors. Another intriguing direction is to perform *in vivo* screens to identify gene targets that specifically improve tumor penetration and NK cell trafficking.

Lastly, PreCiSE itself can be further modified. As I mentioned, there are additional CRISPR modalities including CRISPR activation and CRISPR knock-in as well as non-heritable gene editors including base and prime editing. Essentially, PreCiSE could serve as the foundation to translate other CRISPR modalities to primary human NK cells that allow us to systematically uncover transcriptional bottlenecks or rewrite the NK cell genome using novel synthetic gene programs not physiologically expressed in NK cells.

Q Are there other areas of NK cell research or applications that you find particularly exciting right now?

AB While not the focus of our recent study, I think the combination of NK cell engager molecules with adoptive NK cell therapy is very promising. Currently, we are in an exciting time where multiple immuno-oncology modalities are rapidly advancing in parallel, including adoptive cell therapy approaches and NK cell engager molecules. We recently published a study showing that combining adoptive NK cell transfer with NK cell engagers can elicit remarkable responses in heavily pretreated cancer patients [5].

This opens up another field, where CRISPR-edited adoptive NK cells could be combined with NK cell engagers, making the enhanced function and antitumor potency available to broader patient populations, potentially decreasing cost. This represents a versatile and fundamental scientific approach that can be applied in many different directions.

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BIOGRAPHY

Alexander Biederstädt, MD is a Physician-Scientist and Junior Group Leader (Principal Investigator) with the Department of Internal Medicine III (Hematology and Medical Oncology) at TUM University Hospital. Dr Biederstädt focuses on the treatment of patients with advanced hematologic malignancies using novel adoptive cell therapy approaches within the context of our CAR-T cell therapy service. During his postdoctoral training at the Institute for Cell Therapy Discovery & Innovation, MD Anderson Cancer Center, under the mentorship of Dr Katy Rezvani, Biederstädt pioneered new methods using CRISPR precision gene editing to engineer next-generation NK cell therapeutics with enhanced anti-cancer potency. Building on this foundation, Biederstädt aims to devise novel strategies for synthetic biology-engineered immune cells with increased therapeutic efficacy. Ultimately, Biederstädt aims to develop powerful multi-engineered immune cell therapeutics positioned for rapid Phase 1 clinical translation to combat advanced hematologic malignancies and aggressive solid tumors.

Dr Biederstädt completed his medical degree at Ludwig-Maximilians University of Munich (LMU Munich), Munich, Germany with clinical rotations at Harvard Medical School, Weill Cornell Medical College and the University hospital systems of Tokyo, Sydney and Zurich. He completed his medical dissertation (Dr med.) at the Department of Internal Medicine I, LMU Munich, focusing on molecular cardiology and stem cell research. Biederstädt attained his clinical training in Internal Medicine and Hematology & Medical Oncology at the Technical University of Munich (TUM). He is a recipient of several notable scientific grants and awards including the prestigious DFG Walter Benjamin Fellowship.

Prior to joining our department, Biederstädt worked as Junior Engagement Manager/ Senior Consultant at a premier global management consulting firm (McKinsey & Company) advising board-level clients from diverse stakeholders in the healthcare sphere, including payors, providers and leading global pharmaceutical innovators, focusing on clinical drug development, organizational design and mergers and acquisitions.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

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CELL & GENE THERAPY INSIGHTS

INDUSTRY INSIGHTS • NOVEMBER 2025

Unpacking the FDA's plausible mechanism pathway, regulatory turbulence, and progress in autoimmune cell therapy

Abi Pinchbeck

As a commissioning editor with extensive experience in advanced therapy journal publishing, Abi's focus is on progressing the field by facilitating and disseminating high-impact, open access content covering novel and existing cell and gene therapies.Abi works closely with academic scientists and industry professionals to publish cutting-edge original research, expert reviews, and multimedia content with a translational and interdisciplinary focus. Abi's key aim is to explore the latest advances in cell and gene therapy R&D, clinical development, manufacturing, and commercialization. In addition to Abi's editorial responsibilities, she maintains a strong network of experts across the biotech and pharma industries, staying up to date with emerging trends and breakthroughs in advanced therapies.

Cell & Gene Therapy Insights 2025; 11(10), 1337–1342 · DOI: 10.18609/cgti.2025.155

SUMMARY

Cell & Gene Therapy Insights presents a comprehensive overview of the latest developments shaping the cell and gene therapy landscape in October/November. This Industry Insights update reviews significant regulatory actions, including the US FDA's recent stance on key investigational products, alongside major financing rounds, strategic acquisitions, and emerging clinical data across oncology, rare disease, and autoimmune indications.

COMPANY START-UPS

Azalea Therapeutics launches with US $82 million to pioneer in vivo gene editing [1]

Azalea Therapeutics has launched with Jennifer Doudna as a scientific co-founder, backed by an initial financing round of US $82 million, to push forward its vision of engineering therapeutic cells *in vivo*. For the advanced-therapies field, this launch underscores the growing pivot from manufacturing cells *ex vivo* toward *in vivo* interventions, but also heightens scrutiny around delivery specificity, off-target risk, and regulatory readiness.

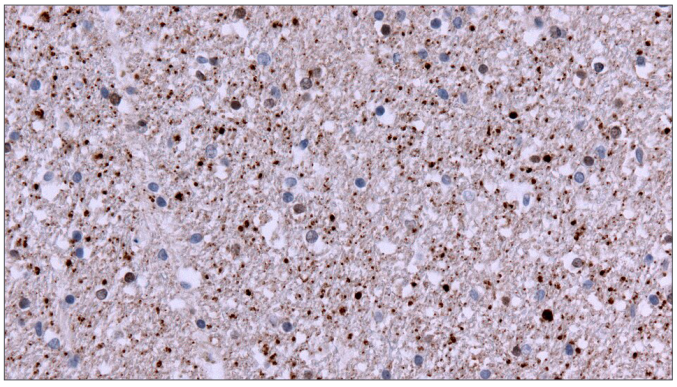
REGULATORY UPDATES

FDA outlines regulatory pathway to support bespoke treatments for ultra-rare disease [2]

The FDA has introduced a new 'plausible mechanism' regulatory pathway to accelerate bespoke therapies for ultra-rare and single-patient conditions. Detailed in a *New England Journal of Medicine* article by Makary and Prasad, the framework enables approval without traditional trials when a scientifically credible mechanism of action is demonstrated.

US FDA roadblock for uniQure's promising AMT-130 gene therapy for Huntington's [3]

The FDA has advised that existing Phase 1/2 data for uniQure's AMT-130 gene therapy in Huntington's disease are insufficient to support a marketing submission. Despite this regulatory pause, long-term data continue to show encouraging durability, and the company is engaging with US, UK, and EU regulators to define next steps.



FDA places clinical hold on Intellia's CRISPR trials following liver event [4,5]

The US FDA has issued a clinical hold on Intellia Therapeutics' two Phase 3 MAGNITUDE trials of its in vivo CRISPR candidate, nexiguran ziclumeran (nex-z), following a serious liver-related adverse event. The company had paused dosing and screening days earlier after a patient experienced Grade 4 liver transaminases and increased total bilirubin. Intellia is working with regulators and investigators to identify the cause and implement safety measures.

FDA guidance update for drug development [6]

The US FDA has issued the final guidance report entitled *Patient-Focused Drug Development: Selecting, Developing, or*

Modifying Fit-for-Purpose Clinical Outcome Assessments, the third in its PFDD series. The document provides a structured framework for ensuring that clinical outcome assessments capture aspects of health that are meaningful to patients and are scientifically robust for regulatory decision-making.

FINANCING AND MARKET TRENDS

AAVantgarde Bio secures US $141 million Series B for retinal gene therapy trials [7]

AAVantgarde Bio has raised US $141 million in Series B financing to advance its dual-AAV gene therapy programs for inherited retinal diseases. Funds will progress two lead candidates into planned clinical proof-of-concept trials: AAVB-039 for Stargardt disease (CELESTE trial) and AAVB-081 for Usher 1B syndrome (LUCE trial).

MERGERS AND ACQUISITIONS

Bristol Myers Squibb acquires Orbital Therapeutics for US $1.5 billion [8]

Bristol Myers Squibb has agreed to acquire Orbital Therapeutics for US $1.5 billion, expanding its cell therapy portfolio into *in vivo* CAR-T and RNA-based platforms. Orbital's lead program, OTX-201, a next-generation CAR-T cell therapy approach, uses circular RNA to drive CD19 CAR expression *in vivo*. The deal underscores major pharma interest in RNA delivery technologies.

Lilly to acquire Adverum Biotechnologies [9]

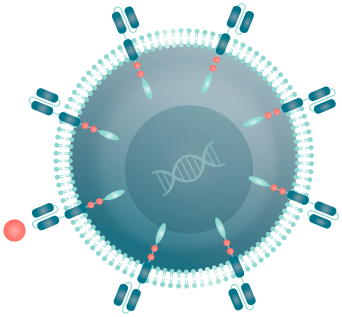
Eli Lilly & Co. has agreed to acquire Adverum Biotechnologies, developer of the intravitreal gene therapy Ixoberogene soroparvovec (Ixo-vec) for wet age-related macular degeneration (AMD), in a transaction valued at up to US $1.5 billion.

The acquisition, expected to close in Q4 2025, underscores pharma's growing interest in one-time AAV treatments that could replace frequent anti-VEGF injections.

CLINICAL TRIALS AND RESEARCH

Caribou Biosciences reports positive Phase 1 CaMMouflage trial data for CB-011 [9]

Caribou Biosciences reported compelling early data from its Phase 1 CaMMouflage trial of the allogeneic anti-BCMA CAR-T therapy CB-011 in relapsed/refractory multiple myeloma. Among 12 patients treated at the recommended dose, the overall response rate was 92%, with 75% achieving complete response and 91% minimal residual disease negativity.



Lyell Immunopharma secures global rights to next-generation CAR-T for metastatic colorectal cancer [10]

Lyell Immunopharma has acquired exclusive global rights to a next-gen CAR-T candidate targeting metastatic colorectal cancer, LYL273. The product, currently in clinical development, strengthens Lyell's expansion into solid tumor cell therapies. LYL273 has demonstrated a 67% overall response rate, an 83% disease control rate, and a manageable safety profile in patients with refractory metastatic colorectal cancer enrolled in an ongoing US Phase 1 clinical trial.

Cell therapy momentum builds in autoimmune disease [12–16]

Multiple developers reported encouraging clinical progress for cell-based autoimmune therapies. Sonoma Biotherapeutics' Phase 1 REGULATE-RA trial of SBT-77-7101 showed early efficacy in rheumatoid arthritis. Kyverna Therapeutics' KYSA-6 Phase 2 data demonstrated 100% clinical response in myasthenia gravis. Bristol Myers Squibb's Breakfree-1 study of CD19 NEX-T™ CAR-T therapy showed signs of early clinical activity across lupus and systemic sclerosis. Cabaletta Bio presented first rese-cel data without preconditioning in pemphigus vulgaris, while Cartesian Therapeutics advanced mRNA-engineered CAR-T programs toward late-stage development.

Regeneron reports encouraging gene therapy outcomes in pediatric hearing loss [17]

Regeneron Pharmaceuticals reported that its investigational gene therapy DB-OTO achieved meaningful and sustained improvements in hearing and speech perception in children with otoferlin-related profound genetic hearing loss.

Multiple sclerosis: Tr1X IND clearance [18]

The FDA has cleared an Investigational New Drug (IND) application for Tr1X's first-in-class allogeneic CAR-engineered Treg cell therapy TRX319 for progressive multiple sclerosis (MS) [18].

Hear how Tr1X navigated IND-enabling studies under FDA scrutiny and avoided a projected 6-month setback—gaining IND clearance in only 30 days in this **on-demand *Cell and Gene Therapy Insights* webinar**.

COLLABORATIONS AND PARTNERSHIPS

Nona Biosciences expands collaboration with Umoja Biopharma to advance *in vivo* CAR-T therapies [19]

Nona Biosciences has expanded its partnership with Umoja Biopharma, building on a multi-target antibody-discovery collaboration to develop novel *in vivo* CAR-T cell therapies. The agreement highlights the trend toward off-the-shelf and direct *in vivo* engineering approaches in CAR-T.

MeiraGTx in ophthalmology gene therapy collaboration with Eli Lilly

MeiraGTx Holdings plc has entered into a strategic partnership with Eli Lilly and Company, granting exclusive global rights to its AAV-AIPL1 program and other ophthalmology gene therapy technologies. The deal includes a US $75 million upfront payment and potential milestone payments exceeding US $400 million, with tiered royalties on future sales.

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Cryo-processed leukapheresis using an automated closed system: a high-quality starting material for autologous and allogeneic CAR-T cell therapy manufacturing

Alexandre Michaux, Senior Scientist, Process Development & MSAT Manager, Cryoport Systems

Cell & Gene Therapy Insights 2025; 11(10), 1267 · DOI: 10.18609/cgti.2025.147 ·

CAR-T manufacturing relies on high-quality leukapheresis starting material, but manual cryopreservation introduces variability that can impact product consistency. This poster presents data evaluating the IntegriCell™ automated closed cryoprocess (ACP), designed to standardize leukapheresis cryopreservation while preserving cell viability and phenotype. The ACP workflow supports reproducible, cGMP-compliant processing for autologous and allogeneic CAR-T applications.

INTEGRICELL™ AUTOMATED CLOSED PROCESS MAINTAINS CELL QUALITY & VIABILITY

The IntegriCell platform addresses manufacturing flexibility challenges by providing standardized cryoprocessing of fresh leukapheresis. The automated closed process (ACP) follows a structured workflow consisting of cell dilution, washing, pre-formulation, formulation, and cryo-processing steps designed to maintain cellular integrity.

A comparative study evaluated leukapheresis from healthy donors, with samples split between fresh storage at 4 °C and ACP cryoprocessing. The ACP demonstrated high cell recovery rates and post-thaw viability (Figure 1).

Cell phenotype analysis revealed maintenance of cell populations with <10% variation in lymphocyte ratios compared to fresh material (Figure 1C). Additionally, the automated system achieved superior contaminant removal, with significantly higher red blood cell and platelet clearance than manual centrifugation.

The closed system approach, which utilizes spinning membrane filtration technology, provides consistent results across different donors while minimizing contamination risks. Additionally, temperature-controlled processing maintains cellular integrity during the cryopreservation workflow.

CAR-T MANUFACTURING DEMONSTRATES COMPARABLE PERFORMANCE WITH ENHANCED FUNCTIONALITY

Following cryoprocessing, fresh and ACP-processed materials underwent standardized 12-day CAR-T manufacturing process. The study evaluated both autologous and allogeneic anti-CD19 CAR-T cells, with allogeneic constructs incorporating shRNA-mediated TCR knock-down technology to enable universal applicability.

Culture parameters monitored throughout manufacturing revealed no significant differences in cell viability, expansion kinetics, or transduction efficiency between fresh and cryopreserved starting materials (Figure 2). Cell expansion showed comparable fold increases across conditions (Figure 2B), with maintained CD4+/CD8+ ratios (Figure 2C) and consistent transduction efficiency (Figure 2D) using truncated CD34 as a purification tag. Phenotypic characterization demonstrated similar expression patterns for activation markers (CD25, CD69), exhaustion markers (PD-1, LAG-3, TIM-3, TIGIT), and memory phenotypes throughout manufacturing.

Functional assessment revealed comparable interferon gamma secretion upon CD19 antigen recognition across all conditions (Figure 3A). Notably, CAR-T cells manufactured from ACP cryopreserved material demonstrated significantly enhanced killing activity against CD19-expressing target cells (Figure 3B,C), suggesting potential functional advantages of the cryoprocessing approach.

SUMMARY

The IntegriCell automated closed cryoprocess validates cryopreserved leukapheresis as a robust starting material for CAR-T manufacturing, preserving cell viability and phenotype comparable to fresh material while enabling greater manufacturing flexibility and scheduling efficiency. Enhanced cytotoxic function, reduced operator variability, and improved process reproducibility support its potential for global CAR-T therapy deployment. This standardized approach offers a GMP-compliant solution for autologous and allogeneic manufacturing pipelines.

Figure 1. Cell viability and recovery data demonstrating maintained viability throughout ACP processing steps from fresh collection through post-thaw recovery.

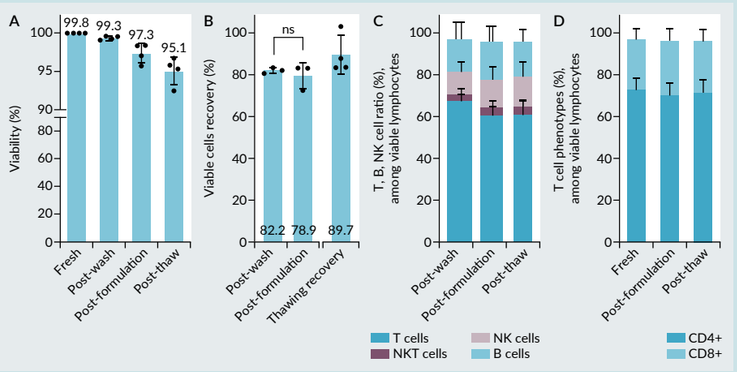


Figure 2. Analysis of cellular characteristics at various timepoints along the CAR-T manufacturing process compare (A) cell viability, (B) expansion kinetics, (C) CD4 to CD8 T cell ratios, and (D) transduction efficiency of fresh and ACP-processed starting materials.

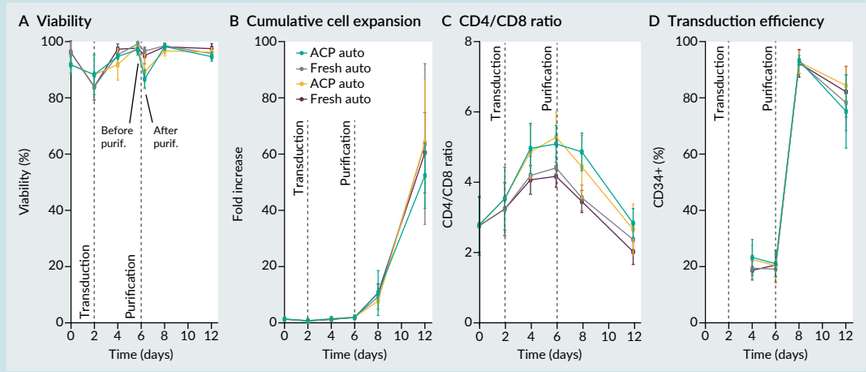
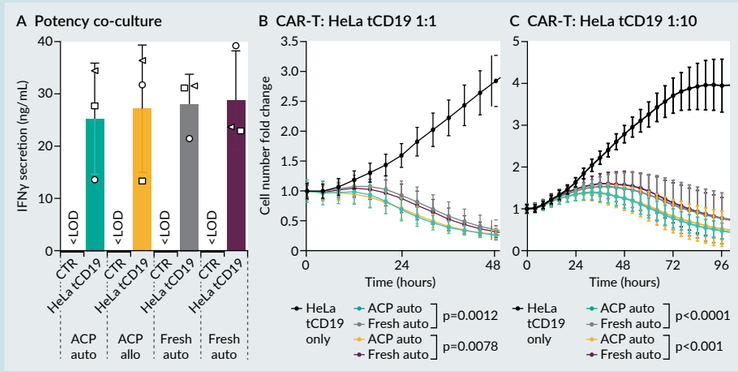


Figure 3. Functional analysis demonstrating interferon gamma secretion and enhanced cytotoxic activity of CAR-T cells derived from ACP-processed material.



Alexandre Michaux, PhD is a Process Development & MSAT Manager at Cryoport Systems. He has an MSc in Molecular and Cellular Biology from the University of Namur, Belgium, and his PhD from the Ludwig Institute for Cancer Research, Brussels. Before joining Cryoport Systems as Manager, Process Development and MSAT, he worked at Celyad Oncology as a Senior Scientist, R&D. His current focus at Cryoport Systems is on the IntegriCell™ platform to support cryo-process development for advanced cell and gene therapies.

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

De-risking clinical trials and enabling innovation: key strategies for empowering emerging biotechs

Amanda Moore, Luke Timmerman, Jenny Gordon, Debra Schaumberg, and Kanya Rajangam

INTERVIEW

"Knowing what you want in the long run, not just from first-in-human or dose escalation, but the big picture strategy for your program, and how you can de-risk that, is one of the biggest challenges that goes along with a Phase I/II trial."

Emerging biotechnology companies face a unique landscape of risk when transitioning from preclinical development to clinical trials, compounded by resource constraints and limited experience with regulatory processes.

We sat down with a panel of experts and asked them to share their practical insights and real-world experiences with common early-phase trial challenges that disproportionately impact emerging biotechs. They share their advice for recognizing and avoiding pitfalls that can derail Phase I/II studies, in order to avoid costly delays and improve the likelihood of clinical success.

Cell & Gene Therapy Insights 2025; 11(10), 1281–1289 · DOI: 10.18609/cgti.2025.150

Q What advice do you have for first-time biotech leaders who are starting their clinical journey?

AM For new leaders, it is most important to stay both curious and informed. When we start leading biotech clinical development, we have a responsibility to our company and to our colleagues, and we also have a duty to our patients to do our best.

One thing many of us find is that doing your best doesn't mean having all of the answers, but instead knowing where to go to find them. Most biotech companies have cross-functional teams full of great folks, and our strength is the strength of our team members and their areas of expertise. Fostering true teamwork and knowledge sharing is what brings success.

As a new leader, it is also important to realize that the skills that you have gained earlier in your career—maybe through doing research for your PhD, working in clinical operations, or perhaps working as a clinician—may not be the same skills you need now. You will need a whole new set of skills to allow you to lead in the dynamic and challenging environment of biotech.

Build deep therapeutic knowledge and build your understanding of your company's goals and strategies, as well as the competitive and regulatory landscape. You should aim to understand how all of that will translate into strong clinical development strategies.

LT What Amanda noted about teamwork is very important, as large and small companies are often collaborating either right around this stage or a little bit later. From my perch as a journalist, I've often seen these partnerships not go so well after a few years. When you interview people afterwards, they'll sometimes say the large company came in and said 'This is the way we've always done it. We know better.' This rankles people in the small company, who may feel like their ideas are not being fully valued.

However, small companies can also learn from the way large companies operate. There are processes that are there for a reason. If you stay curious, you can both learn from each other and get to the critical answers you need as fast as possible. We are in a fast-moving, dynamic environment. There is competition from all parts of the world, and speed is important. It's crucial to ask yourself how you can best collaborate in order to get to the answers you need as fast as you possibly can.

JG I would love to build on what Amanda and Luke just spoke about, which is to create partnerships with internal and external teams, and to also make sure that you are utilizing the information that has come before you, including everything that is in the public domain for your therapeutic area. The message I use as my foundation is to keep things as simple as possible. Overcomplicating things is where we run into trouble.

Engaging your stakeholders is a fundamental responsibility of everyone who is part of your team. Don't only include your internal team—your team includes your vendors and your sites too.

One thing I find very helpful for establishing partnerships is developing a sense of team psychological safety. We need to move quicky and do things we may not have prior experience in. In order to do that well, we need to ensure our team feels safe and comfortable with the possibility of making mistakes, learning from them, and going back and trying

again. Have face-to-face time with the people you work with, in order to build trust and understand their needs. Be open to their feedback.

Make sure you have a distinct vision for exactly how you want things to work. Establish the roles and responsibilities of who is doing what, along with your goals, so it's clear to your team. Establish how you are keeping track of progress and celebrate your wins. These are also really important steps to becoming a more cohesive team, and the closer you work together, the faster you can successfully execute your plans and projects.

DS From my perspective in a large global CRO, the best delivery we achieve with our biotech customers is always informed by a collaborative, respectful and positive relationship—that is critical.

Something that can be overlooked is ensuring the connectivity of the nonclinical and clinical programs. There are so many interdependencies and failing to connect those pieces together on the nonclinical side as well as in the clinical program is a common challenge we see with some early biotech leaders. As a first-time biotech leader, don't be afraid to tap into knowledge and experience from thought partners such as CROs, who can help you learn and work outside your deepest area of expertise. Aim to build your knowledge and understand dependencies to gain a broad understanding of the drug development process, including both anticipatable and unanticipated risks and mitigations.

KR Leaders starting their careers should be critical about the career opportunities they are considering. Very early on someone who I view as a mentor gave me a good way to evaluate companies—because you should of course be evaluating the company as much as they are evaluating you as a candidate.

You can look at it through the prism of the three 'P's. Firstly the product, i.e. the science. Are you excited by it? Secondly, the people, as you spend a lot of time with your colleagues at work. Are they people that you want to be spending all that time with? Finally, the pennies—this could be considering the financial stability of the company, or your own financial priorities.

Be clear-eyed about which of these 'P's is the big focus in the opportunity you are taking up. This may change over your career lifespan, and very rarely do you get all three at one company. If you are clear on what you are getting out of the opportunity, this will help you fully engage in what you are doing and what you are delivering.

Q From your experience, what are some of the biggest challenges small biotech companies face when initiating a Phase I or II trial?

KR If I narrow this down to the very first Phase I a small biotech is doing, there is a big step change from doing preclinical *in vitro* and *in vivo* work to then filing an IND and starting to evaluate new science in patients. Even if everyone around the table is very experienced, it's still the first time this particular team and company have put that work together.

In my younger days I used to backpack quite a bit. Someone wise once said that when you're ready to go on a trip, you should lay out everything you think you need and then take half the clothes and twice the money. It's similar when you file an IND.

Teams tend to overdo the preclinical evaluation and may not need as much as they think, as there is no real substitute for patient data. When you are putting together your pre-IND plan, consider that maybe you don't need as many experiments as you think, but you definitely need more money and time.

JG I love that analogy. It feeds into my answer for this question: firstly, we need to anticipate risks and make sure we have mitigation strategies for them.

One risk, which was just highlighted, is the fact that often we want to include everything under the sun in our first clinical programs, in order to learn more about our drug, our patient population, and so on. These things add a lot of inherent risk from a budget and timeline standpoint, and we need to make very careful considerations about what we actually require.

I'd therefore highlight the crucial role of your clinical operations team within the design of your Phase I/II study. You need an operational perspective in order to understand the time and budget implications for adding all of these additional components. They may actually fall more under 'nice to have' , versus the 'must haves' that you need to prove your objectives and end points for your clinical trial.

Make sure that you have a very clear picture of what you need for your clinical trial, and think very carefully about the additional time burden, operational challenges, and potential risks that come with adding more.

LT From a journalist's perspective I would be thinking ahead and asking what success looks like for your Phase I or II trial. Think about that in advance and communicate it accordingly when it comes. It is interesting to hear folks talk about just getting all this data loaded and ready to go for an IND, because from the outside point of view, we don't see everything happening behind the scenes.

What we do often see, especially now in a stressful market environment, is that when companies get the first glimmer of success, they start communicating this very early, almost drop by drop. I would advise them not to spike the football too early—it's not great for credibility in the long run, especially if you still have a lot of unanswered questions and need to follow the patients a bit longer.

Think about the proper follow-up and the statistical power that you need to have the confidence to make that go/no go decision at the end of your trial. It's tempting to run out there and declare success to the world early—but there's a time and a place for that, when you have the data you envisioned back at the start of the process.

AM What challenges you might face depend somewhat on whether you are a preclinical company moving into clinical for the first time, or a company already doing Phase I trials. For a small biotech, regardless of which side of that you are on, one of the challenges is how to do early-stage clinical trials.

If it's your first time ever, you might not know how to best work together as a team to put together a full clinical development strategy. To Jenny's point, make sure that you are baking in the right things, but maybe not baking in too much too early.

Then, Luke raised the point of understanding what you want in the end. That's the other piece of this challenge and sometimes companies can do this better once they have already run a Phase I or a Phase I/II program.

Knowing what you want in the long run, not just from first-in-human or dose escalation, but the big picture strategy for your program, and how you can de-risk that, is one of the biggest challenges that goes along with a Phase I/II trial.

"Aim to have as much clarity as possible around the strategy, the study design, and the value of information you are going to get out the other end."
Debra Schaumberg

DS On the strategy piece, I would look at maximizing the value of information at the end of the study. How do we design a program that's going to provide us with clarity of decision-making? Whatever that inflection point is in your journey, we all know that the drug development process is essentially a gated decision-making process, where we use what we have learned up until this point to make our decisions about where to go next.

It is critical in those early-phase trials to have clarity on what the data will show you, and what the design may still leave open as a question. Decide if you are okay with that, because that is where it gets tough. We see a lot of programs where the trials run smoothly, we are able to enroll the patients, and the data reads out, but there is still a lack of clarity on what to do next with the development program.

We encourage early dialogues and playing out scenarios—if it reads out a certain way, then what will that tell us? Aim to have as much clarity as possible around the strategy, the study design, and the value of information you are going to get out the other end.

A common challenge in competitive indications where there is a lot of development ongoing is finding the right partners to execute your trial, and ensuring you have the right study design to make the trial attractive to patients. You want to ensure patients are being provided with value for their participation to avoid challenges on the recruitment side. This enables you to design a study that not only gives you declarative information but allows you to enroll it effectively and meet your timelines and financial metrics.

Q Can you share an example of a small early mistake that became a major delay or cost later down the line?

DS The example that comes to my mind relates to my answer to the first question –connecting your nonclinical and clinical program. Sometimes a very different team within the biotech is responsible for CMC and nonclinical, versus the clinical team designing the trials and moving them forward.

I had experience with a company that had a mismatch between the amount of drug substance they were producing and the amount needed for their clinical trial. Everything was moving in parallel, but in disconnected teams. This resulted in expiration of the drug substance, and then the entire batch of drug substance production had to be repeated. This set the whole program back significantly.

Connecting those two pieces together is critical. We have also seen issues where there is a misread of what's needed from a toxicology supportive perspective early on in the process. This resulted in the program moving too quickly without the supportive toxicology, and they have to retrace their steps and go back to do more toxicology studies. Depending on what you need, this can add 6–12 months to your timeline.

> "It is important to ensure we balance our budgets and timelines, and factor in how they relate to vendor costs, CRO costs, and all the other things we have to juggle."
> **Jenny Gordon**

JG A couple of the things that we've talked about in answering these questions relate to timelines, as well as overall cost of clinical trials. Timeline can be a really tough thing for us to gauge.

It is important to ensure we balance our budgets and timelines, and factor in how they relate to vendor costs, CRO costs, and all the other things we have to juggle. While we want to fight for the most reasonable internal timelines, we may have some extremely aggressive external timelines to meet.

We also need to make sure that whatever we are forecasting has accounted for variability and risk, so that we are prepared and can plan how to mitigate potential issues. If you don't do this up front, you may find yourself having to scramble and do that in the middle of your trial.

Some of the other tactical and practical aspects of this are the components of your investigator site budgets. Make sure that they are aligned across the board and that you are looking at fair market value. Make sure that the variability of your sites and investigators is minimized so that if you do have robust enrollment, you can figure out really how to either control costs at sites, or have patients spread out across all of the sites that you have.

These more practical tips might seem like they are on the tactical and not on the strategic side, but setting your sites up in the right fashion with the right feasibility and country footprint is a huge undertaking. If you put time and effort into these considerations at the start, it can get you to where you need to go in a much more efficient manner.

KR I would emphasize the need to avoid siloing and make sure that all functions are talking to each other. This can be via one unified reporting structure as we do at Senti Bio, or if not, by making sure that everybody is talking to everybody else.

As a small company executive, capital is sometimes a big deciding factor in what you can do. There might be 20 things that you need to do, but you might have capital for only 10 or 15 of them. In those situations, you might deliberately decide to kick the can down the road, so to speak, because you simply don't have the resources to do everything.

Go into this with your eyes wide open. It might mean additional work or extra time further ahead, and that's okay. Especially when you come to something like cell and gene therapy, where the CMC aspect is simply so expensive, it really is a struggle. Sometimes decisions need to be made by capital constraints. On the other hand, it also creates an exciting intellectual challenge, and for me personally this makes my role more enjoyable.

LT To underscore what has already been said—those early manufacturing decisions about how you make the product, especially with some of these advanced cell and gene therapies, are crucial. If you go with version 1.0 or the 'quick and dirty' version in order to meet your timelines and your limited budget, you could find yourself much further down the road later on, without a viable product.

At that point, a lot of time and money has gone down the drain. I have seen too many companies reach an NDA submission, they're on the clock at the FDA for their PDUFA date, and then get the complete response letter that something has held it up in CMC. It's very unfortunate, and there are definitely ways to head that sort of thing off much earlier on in the process.

AM When it comes to heading off early mistakes, don't forget how much you can learn from vendor partners, who have seen so much across so many different companies.

Specifically for Phase I studies, when you are dealing with a protocol that's going to go into an established Phase I unit, design that study in collaboration with your vendor.

Q How do feasibility errors usually occur, and what are the warning signs?

JG The answer here is really a build of all the things that we have been discussing. Ensure the assumptions you are putting into place are as accurate as possible, put them in writing, and then create your risk mitigation strategy.

You want your partnership with your CRO to be extremely collaborative, strategic, and tactical. Utilizing their feedback and giving them as much of an input and stake in the game as you can is extremely beneficial. Make sure that you have all of the feedback that you need from your KOLs and your advisory boards.

Another thing that we often miss, and need to do a better job of building into our timeline, is utilizing our site study coordinators and also our patients. Patient advisors can take a look at our protocols and our designs and let us know what's important to them, and what's feasible or not, and why. They often provide perspectives we could never think of since we're not patients in that particular patient population.

Getting as much external feedback as you can will help establish whether or not your trial is truly feasible. It will help you in the long run with enrollment and retention, and with making sure that you've got robust data, because you have holistically included everyone that you need to provide input into your study.

DS There are multiple downstream stakeholders that depend on the execution of the protocol of your trial. There are the regulators, of course, because the drug won't get anywhere if it's not approved. Then there are the patients, the providers, and the payers, and those perspectives all need to be incorporated into the design of the protocol. Sitting in the CRO space, we typically see protocols from multiple companies in a given indication. Therefore, we often have a different point of view and a valuable perspective to contribute to a clinical program.

We might not have the same depth of information about a biotech's particular product, but we may have seen a lot of regulatory interaction for that particular indication. We may have seen a lot of different protocols and executed on those and seen what some of the operational and implementation challenges have been.

Incorporating stakeholder input from across the value chain will let you design a study that's going to be attractive for the PIs to execute, provide value for the patients, and provide you and the regulators with all the information needed to make an informed decision.

"Getting a clear answer, whether positive or negative, should be the driving force."
Kanya Rajangam

AM I've noticed some CROs now have site coordinator panels, so you can send your protocol design to a whole coordinator panel via your CRO and get their feedback. Having patient advisory panels to give feedback on your protocol is also not unusual. The biggest feasibility error you can make is to not take these steps—and the biggest warning sign is if you haven't gotten external guidance on how good your protocol is!

KR A protocol is pretty much the single most important document you work with when you are doing a clinical trial. Much of my career has been in oncology, an indication with very sick patients with high unmet need. So as a CMO, my outlook is patient-first. In these early phase trials you are answering a very simple question: is this a drug, or not?

The impetus is looking at how you can get to that answer as fast as possible while exposing as few patients as possible in the process. Designing a study oftentimes tends to be filled with whole bunch of competing needs, if you will. Research scientists want to ask some really good questions, and the regulators have certain things that they want to see.

All of these needs should be filtered through the question of whether their addition will help us to answer this principal question as quickly as possible. When we do these studies, they might meet their primary endpoint or not. I don't view that as success or failure, because that's science. Not all of our questions will have a positive answer. To me, the real failure is if we don't reach an answer, and we need to do another two or three studies. Getting a clear answer, whether positive or negative, should be the driving force.

A good approach for this is something called the grandma test. When you are designing a study, if your grandparent had that disease, would you recommend that trial for them? That is key to how I approach protocol design. If you start viewing it through that prism as you are making these design choices, it provides a lot of clarity.

Then in terms of how you tell if things are falling apart—if enrollment is lagging that is a very good indication. If that happens, look very closely at what you are doing and at that patient population. Perhaps there are other options available to them, or something is off.

LT Kanya put it beautifully, and the grandma test is a great way to frame the importance of focusing on the patients and hearing their voice, especially when they might not have a seat at the table in the same way as the scientists or the regulators. Ensuring their points of view are reflected in these protocols is so important to success.

For example, there might be a question of changing a product from an intramuscular injection to a subcutaneous, or perhaps dosing once a month versus once a quarter. A scientist or a regulator might not fully understand the importance of these aspects compared to how tremendously important it may be to a patient. In the investment world these questions risk being viewed as less important too, but ultimately these aspects will play a big part in the success or failure of a product. The patient point of view really should be there, right from the very early stages of development.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Advancing AAV genome integrity assessment with digital PCR

Gene therapy transforms how we approach genetic diseases, turning challenges in medicine into achievable goals. Among the most widely used tools in this field are adeno-associated virus (AAV) vectors, non-pathogenic carriers that enable stable delivery of therapeutic genes. Maintaining consistent viral vector quality across each production stage has become essential as more programs move toward clinical and commercial stages. One of the most critical aspects of AAV quality is assessing genome integrity, which directly influences potency and therapy efficacy.

When AAV genomes are incomplete or truncated, activity can be reduced. Conventional techniques such as gel electrophoresis, Southern blotting, or sequencing have provided valuable information, but they are often time-consuming and provide only limited quantitative precision for routine decision-making. Next-generation sequencing (NGS) has become a powerful tool for assessing AAV genome integrity, offering detailed insights into sequence composition and structural variations. While NGS excels in deep characterization, it is not optimized for rapid, high-throughput decisions, creating a need for complementary methods like digital PCR (dPCR). dPCR addresses these limitations by providing an accurate, sensitive, and reproducible way to determine whether AAV genomes are intact, fragmented, or likely to perform as intended.

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PRECISION TOOLS FOR A COMPLEX CHALLENGE

AAV manufacturing is inherently complex and variable. DNA molecules may break or rejoin incorrectly during replication and packaging, leading to fragments, rearrangements, or unwanted host DNA incorporation. While modern purification steps can effectively remove empty capsids, they cannot distinguish between full capsids carrying complete genomes and those with partial sequences. This is where dPCR makes a measurable difference. By dividing each sample into thousands of tiny reactions, dPCR counts target molecules directly using Poisson-based statistics to calculate absolute quantities. This approach eliminates the need for reference standards or calibration curves and tolerates common inhibitors found in viral vector preparations. Because of its partitioning power, dPCR can detect subtle differences between intact and fragmented molecules that other methods may overlook.

By partitioning samples, dPCR enables precise counting of target regions. When multiple regions are analyzed simultaneously, differences in copy numbers reveal whether genomes are intact or fragmented.

Modern dPCR systems can simultaneously analyze up to 12 targets in a single multiplexed experiment, covering critical areas such as promoter, transgene, and polyadenylation sequences at both ends. This saves time and sample material while providing a more complete picture of vector quality.

UNDERSTANDING THE FACTORS THAT SHAPE GENOME INTEGRITY

Several biological and process-related factors can influence AAV genome integrity. GC-rich promoter regions tend to form stable secondary structures that can stall replication, increasing the risk of incomplete genomes. Nicks that occur during replication may trigger unwanted end-joining events, resulting in rearranged or shortened sequences. The conditions used during capsid lysis also matter because thermal and enzymatic treatments can yield different integrity outcomes [1, 2, 3].

Another critical factor is storage. Experiments show that AAV lysates stored at 4 °C for a week retain around 60% integrity. Meanwhile, a single freeze–thaw cycle can reduce integrity to roughly 20% [1, 2].

These findings emphasize the need for optimized handling conditions and highlight how dPCR can be used to evaluate and refine the production process. By incorporating integrity analysis early in the workflow, potential issues can be detected early and adjustments can be made before product quality is affected.

BUILDING CONSISTENCY THROUGH STANDARDIZED WORKFLOWS

Accurate integrity assessment depends not only on the right instrument but also on the process behind it. Standardized workflows for AAV integrity testing can simplify preparation and analysis while reducing variability across operators and sites. A typical procedure includes DNase treatment to remove residual DNA, followed by controlled lysis and dilution to ensure the DNA concentration falls within the optimal range for dPCR analysis. Combining the CGT Viral Vector Lysis Kit and the QIAcuity® Digital PCR System provides this consistency. When used together, they enable integrity testing across various AAV types and sample purities with excellent reproducibility, typically within ±10% variation, regardless of operator or laboratory. The workflow works reliably across different AAV serotypes and with material from different production systems, ensuring that the resulting data can be compared across batches and facilities.

Additionally, the QIAcuity Software Suite automatically calculates the percentage of intact versus fragmented genomes and provides statistical outputs, including error estimates, removing the need for manual calculations. Updated algorithms minimize signal overlap between fluorescent channels in multiplex assays, improving precision. The platform also supports compliance with 21 CFR Part 11 requirements, helping ensure data integrity, traceability, and suitability for use in regulated GMP environments. These refinements make integrity analysis faster and more reliable.

Another vital aspect is well-defined reference materials, which have become increasingly important in ensuring comparability and reproducibility across laboratories. These standards contain multiple target sequences representing different genome regions, allowing users to validate assays and confirm accurate results across various instruments and workflows.

CONCLUSION

Ensuring AAV genome integrity is no longer optional; it is fundamental to helping

deliver safe, effective gene therapies. dPCR provides the precision and reproducibility needed to monitor this important quality attribute, while standardized workflows and reference materials make results comparable across batches and facilities. Together, these advances turn integrity testing into a practical, routine part of manufacturing rather than a specialized and complex task.

As the field grows, shifting from reactive troubleshooting to proactive quality control will help accelerate development timelines and build confidence in every therapeutic vector.

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Development and scale-up of a transient transfection rAAV process from bench scale to a next-generation, single-use bioreactor system

Kelly Cybulski and Kyle Burrell

The scale-up of transient transfection processes for recombinant AAV poses challenges in maintaining consistent productivity and product quality across volumes. This article presents case studies demonstrating the use of a HEK293-derived transient cell line to achieve reproducible rAAV9 titers (2–4 × 10^{11} vg/mL) with 23%–33% full capsids, while demonstrating low encapsidated host-cell DNA of 18 ng/10^{14} vg. Results confirm reproducibility from 15 mL bench-scale runs through 200 L single-use bioreactors, supported by defined strategies for agitation, pH, and dissolved oxygen control.

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BENCH-SCALE STUDIES ESTABLISH PRODUCTIVITY ACROSS SEROTYPES

Shake-flask experiments compared a HEK293-derived transient cell line (ELEVECTA™) with and without a transfection enhancer (RevIT™). Across four AAV serotypes (2, 5, 8, and 9), titers of 1–4 × 10^{11} vg/mL were achieved with 25%–60% full capsids, and higher titers were consistently observed in enhancer-supplemented conditions.

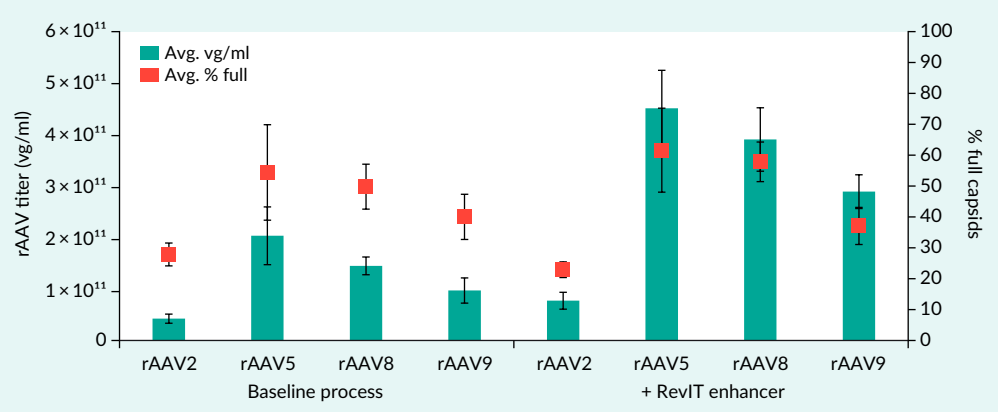
To ensure these results could be reproduced under controlled conditions, the process was transferred into an Ambr® 15 system (Figure 1). In this setting, agitation, gassing, and pH were actively controlled. The addition of online pH regulation improved reproducibility compared with flasks, providing early evidence that key parameters established at bench scale could inform subsequent scale-up.

AGITATION & OXYGEN STRATEGIES DEFINE OPERATING WINDOWS FOR SCALE-UP

Scale-up was supported by a ReadyToProcess WAVE™ 25 bioreactor n–1 expansion step, followed by production in Xcellerex™ X-platform stirred-tank reactors. Cultures were inoculated at 0.25 × 10^{6} cells/mL and incubated for 3 days

FIGURE 1

Titer and % full capsids measured in Ambr® 15.



before triple plasmid transfection with PEI MAX™ at a 1:1:1 molar ratio. Transfection was performed at a cell density of 3×10^6 cells/mL, and harvest occurred three days later using a Tween®-20 lysis step with endonuclease treatment.

Gas delivery was standardized across scales, with air sparging applied at constant rates and oxygen supplied under dissolved oxygen (DO) control. DO was maintained at 40%, deviating only briefly at the point of transfection.

To identify scalable mixing conditions, agitation was evaluated in Ambr 250 across a range of 10–60 $W \cdot m^{-3}$ (Figure 2). These studies established an acceptable window of 25–60 $W \cdot m^{-3}$, from which 50 $W \cdot m^{-3}$ was selected as the operating setpoint for 50 L and 200 L production runs.

STABLE CONTROL SUPPORTS REPRODUCIBILITY AT 50–200 L

Online monitoring during three 50 L runs and one 200 L run demonstrated consistent process control (Figure 3). The DO setpoint of 40% and the pH setpoint of 7.3 were sustained within a narrow deadband, with only brief deviations observed at the time of transfection. Metabolite profiles supported these findings: lactate accumulated and was subsequently consumed, while glucose utilization and pCO_2 trajectories remained aligned across scales.

PRODUCTIVITY & PRODUCT QUALITY ARE CONSERVED ACROSS SCALES

Across volumes from 15 mL to 200 L, titers of $2–4 \times 10^{11}$ vg/mL and 23%–33% full capsids were consistently achieved (Figure 4). These findings confirmed that productivity and vector quality attributes could be conserved during scale-up of transient AAV production.

FIGURE 2

rAAV titer and % full capsids at agitation power inputs of 10, 25, 45, and 60 $W \cdot m^{-3}$.

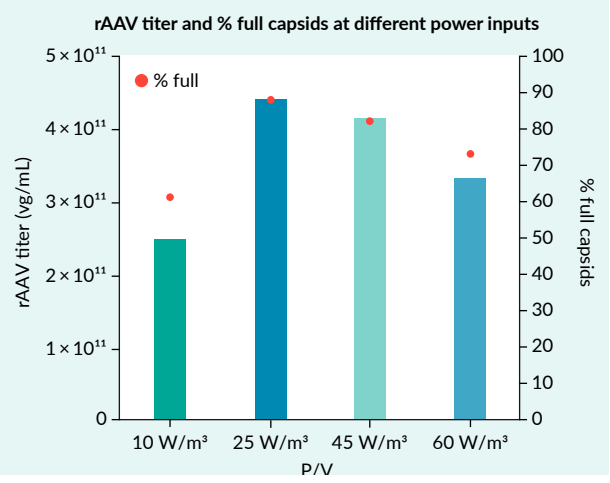
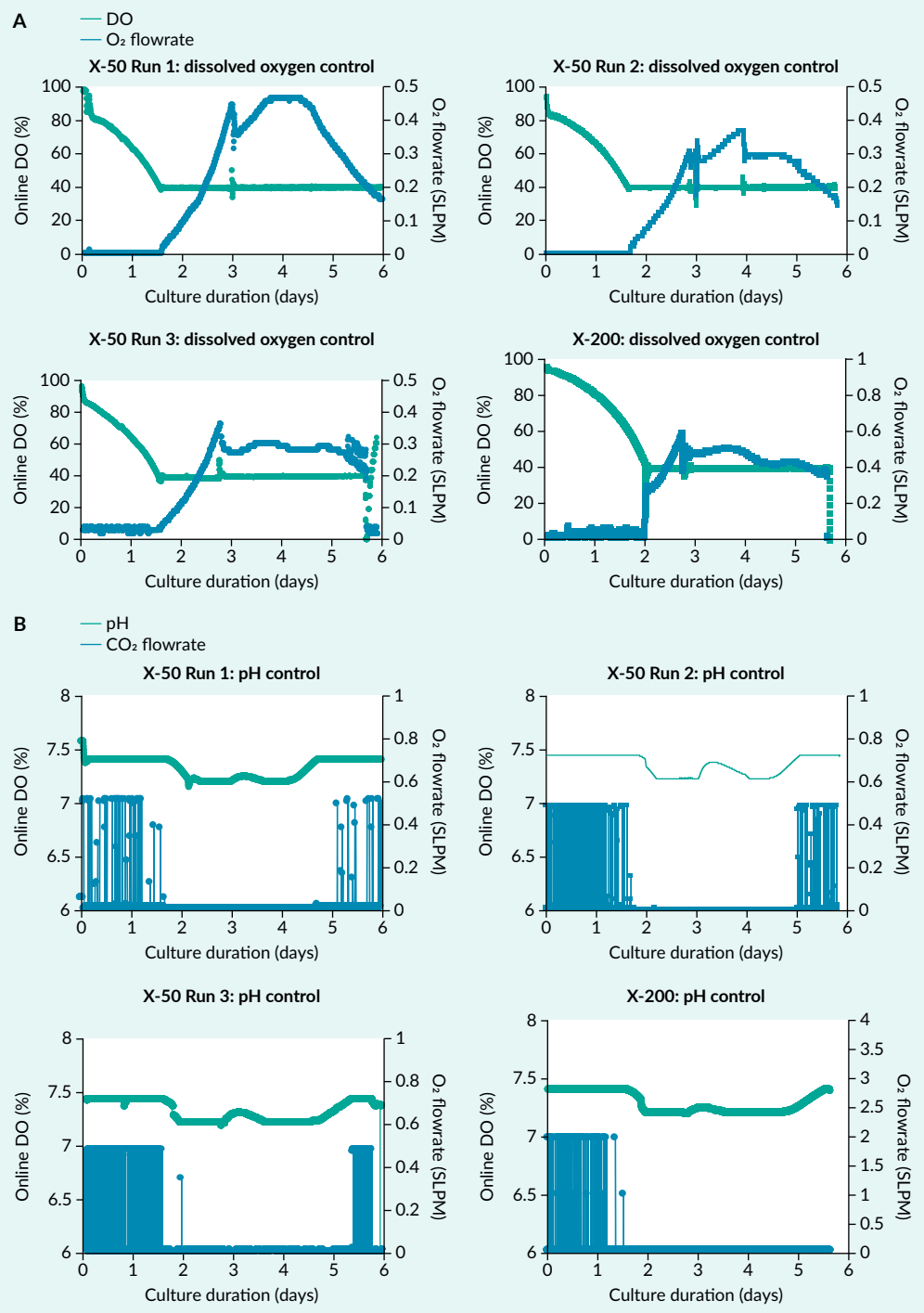


FIGURE 3

(A) DO traces at a setpoint of 40% in three 50 L runs and one 200 L run.
(B) pH traces at a setpoint of 7.3 in three 50 L runs and one 200 L run.



A specific focus was placed on encapsidated host-cell DNA (hcDNA), an impurity of regulatory concern. The ELEVECTA™ cell line demonstrated a ≈2-log reduction in encapsidated hcDNA compared with 293-F at 3 L scale. This effect was conserved at 3 L and 10 L scale for AAV8, and 50 L scale for AAV9, indicating that the benefit was

FIGURE 4

rAAV titer and % full capsids measured at 15 mL, 50 L, and 200 L scales.

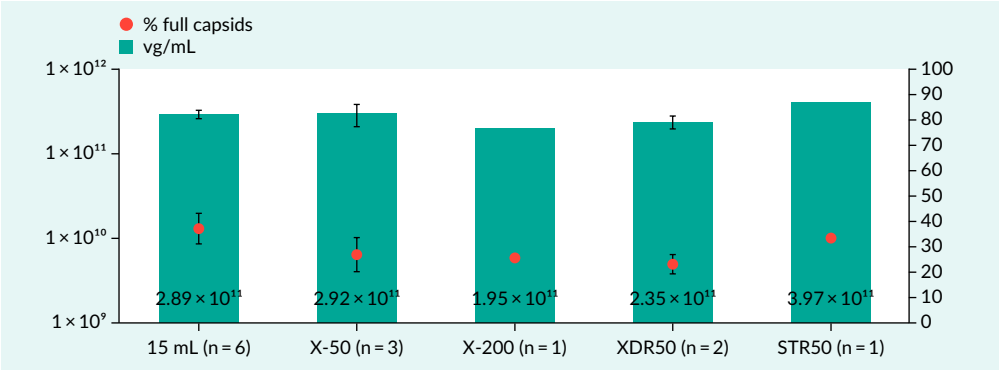
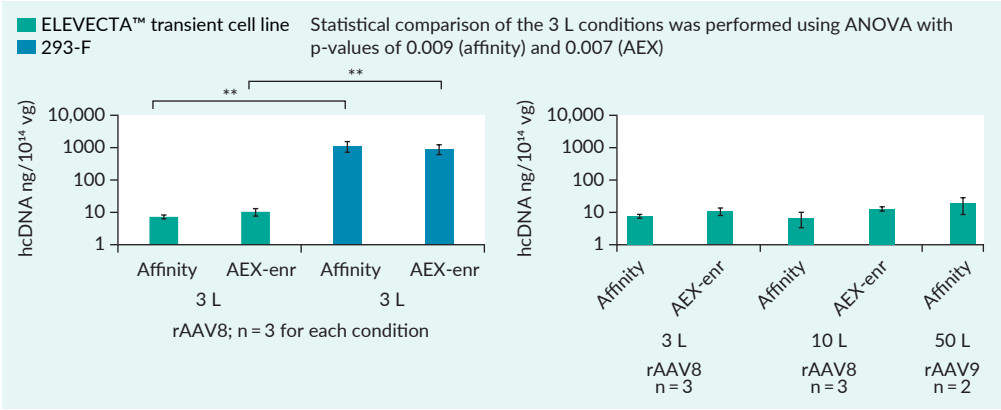


FIGURE 5

(A) hcDNA levels in ELEVECTA™ transient and 293-F cell lines at 3 L scale, measured after affinity purification and AEX-enrichment. (B) Encapsidated hcDNA levels in ELEVECTA™ transient cell line-produced rAAV8 and rAAV9 at 3 L, 10 L, and 50 L scales.



independent of scale and serotype (Figure 5).

Growth and viability were compared across bench-scale (Ambr 15) and production-scale Xcellerex™ X-platform stirred-tank systems (50–200 L). Performance was consistent, with only minor differences at 15 mL, likely reflecting higher seeding density or vessel geometry. The ability to maintain agitation and gas strategies across systems confirmed that the process could be reliably transferred between platforms.

SUMMARY

A transient transfection rAAV process was scaled from flasks through Ambr 15 to 200 L single-use bioreactors while maintaining titers of 2–4 × 10^{11} vg/mL and 23%–33% full capsids. Control strategies based on DO at 40%, pH at 7.3, and an agitation setpoint of 50 W·m^{-3} enabled reproducibility across scales.

The use of the HEK293-derived ELEVECTA™ transient cell line resulted in an approximately 2-log reduction in encapsidated host-cell DNA compared to 293-F, an effect consistent across various volumes and serotypes. These findings demonstrate that transient AAV production can be scaled reliably across volumes and serotypes, with consistent productivity and reduced encapsidated host-cell DNA.

Q&A

Kelly Cybulski (left), **Kyle Burrell** (right)

Q Was pH kept constant during production?

KC Yes—the pH was controlled at a set point of 7.3 with a narrow ±0.01 deadband, and this was maintained throughout the run.

Q What gene of interest was used in this work?

KC A GFP–luciferase construct was chosen. The rationale was to use a relatively large gene of interest so the process would better reflect the kinds of genes likely to be used in customer applications, making the case studies more representative.

Q Was plasmid size accounted for when applying the 1:1:1 ratio?

KC Yes. The ratio is molar, so the base-pair length of each plasmid is taken into account.

Q What challenges were encountered at larger-scale transfection?

KC The main challenge was delivering the full complex volume into the bioreactor quickly and efficiently. At the 50 L and 200 L scales, this was addressed by using large-diameter tubing and, in some cases, by pumping the complexes into the vessel. At those scales, gravity feed with large tubing was also sufficient. Looking ahead, one potential improvement would be to reduce the complexation volume, allowing for more concentrated complexes that would be easier to handle at very large scales.

Q What maximum cell density can be achieved in the Xcellerex™ X-platform bioreactor, and what was used here?

KB The platform can achieve Chinese hamster ovary cell densities up to ~5×10^7 cells/mL. However, viral vector processes, especially transient ones, typically run at much lower densities. For this study, the transfection density was ~3×10^6 cells/mL. The platform is also capable of oxygen transfer rates (k_La) up to 45 h^{-1}, while viral vector processes generally require only 2–5 h^{-1}.

Q How many impellers are used in the X-platform?

KB A single hybrid impeller is used. It is not a standard Rushton or marine impeller but a design tailored to the vessel geometry.

Q How was the transfection reagent selected?

KC Several reagents were evaluated. PEI MAX™ was chosen because it consistently gave the highest titers, was among the more cost-efficient options evaluated, and demonstrated low run-to-run variability.

Q Is the X-platform capable of microbial processes?

KB The system was not initially designed with microbial applications in scope. However, given ongoing work in plasmid DNA production, microbial processes are now being evaluated. At present, no data have been presented.

Q How were vector genomes and empty/full ratios quantified? Was potency tested?

KC Vector genomes and empty/full ratios were assessed using qPCR and ELISA. A potency assay is under development but was not available at the time of these studies.

BIOGRAPHIES

Kelly Cybulski is a Senior Scientist in R&D at Cytiva, where she has spent the past 6 years advancing viral vector process and product development. With a Master's degree in Cell and Molecular Biology from the University of Rhode Island, RI, USA and 19 years of experience spanning good manufacturing processes (GMP) production, process development, and research, Kelly brings deep technical expertise and a proven track record of innovation in biopharmaceutical manufacturing.

Kelly Cybulski, R&D Senior Scientist, Cytiva, Westborough, MA, USA

Kyle Burrell joined Cytiva in 2022 as a Bioreactor Application Specialist, bringing with him a strong background in bioreactor scale-up and process development. In 2023, he became part of the Genomic Medicine Operating Company, where he now focuses on viral vector technologies. Prior to joining Cytiva, Kyle worked in assay and analytical development for viral vectors. He holds a Master's degree in Veterinary Medical Sciences from the University of Calgary, AB, Canada, with a specialization in Comparative Biology, Experimental Medicine, and Stem Cell research.

Kyle Burrell, Upstream Field Application Specialist, Cytiva, Westborough, MA, USA

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Strategic human raw material selection for cell therapy manufacturing

Daniel Benítez Ribas

The use of human-derived raw materials plays a critical role in the success of cellular therapies, such as CAR-T cell manufacturing. In this article, we focus specifically on human serum albumin and male AB serum, exploring their evaluation and integration into production processes. Drawing on eight years of clinical-grade ARI-0001 manufacturing experience, we examine regulatory requirements, quality considerations, and practical implementation strategies.

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OVERVIEW OF CELL & GENE THERAPIES

CGT manufacturing relies heavily on strategically selecting raw materials to ensure product quality, safety, and regulatory compliance. While presenting unique regulatory challenges, human-derived components remain essential for achieving optimal cell viability and expansion in many therapeutic applications. The complex regulatory landscape surrounding these materials requires careful consideration of their classification, intended use, and documentation requirements.

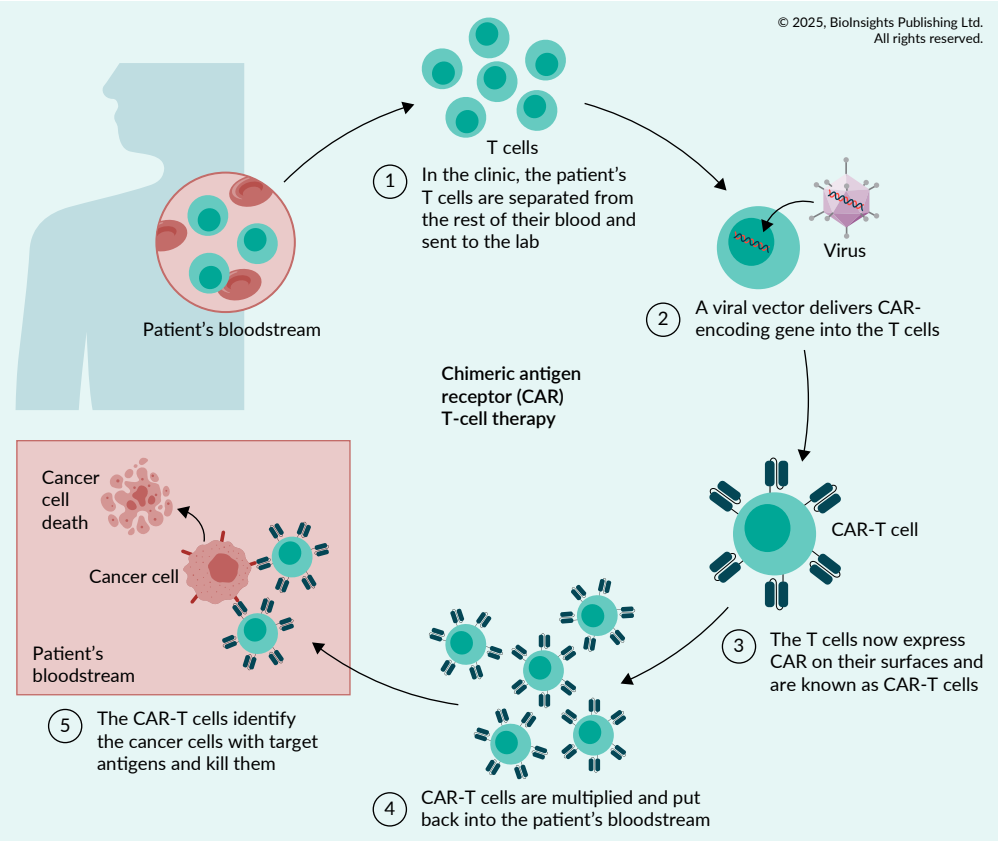
ATMPs encompass cell therapies, gene therapies, and tissue engineering products that contain substantially manipulated cells or tissues. These living medicines must be produced under GMP conditions, in our case following European (EU) GMP Annex 4 guidelines. The incorporation of human-derived ancillary materials introduces additional considerations into the manufacturing process, requiring a balance between therapeutic necessity, regulatory compliance, and supply chain reliability.

CAR-T CELL MANUFACTURING WORKFLOW & RAW MATERIAL INTEGRATION: ARI-0001

CAR-T cell production follows a well-established manufacturing sequence beginning with patient leukapheresis and progressing through T-cell isolation, lentiviral transduction, expansion, quality control testing, and final product release, as seen in Figure 1. Each manufacturing step requires specific raw materials to maintain cell viability, support expansion, and ensure product stability.

FIGURE 1

Manufacturing workflow of CAR-T cells.



CAR-T cells function by specifically targeting tumor-associated antigens expressed on malignant cells. Figure 2 shows an example of a target that is the CD19 molecule, a tumor-associated antigen expressed on B cells. CD19 is present not only on malignant B cells in leukemia and lymphoma but also on healthy B lymphocytes. Following genetic modification, the CAR construct is expressed on the T-cell membrane, and the extracellular domain of the CAR contains an antibody-derived recognition region capable of binding CD19.

Upon encountering a cell expressing CD19, CAR-T cells initiate intracellular signaling cascades that first induce target-cell lysis and subsequently promote T-cell proliferation and cytokine secretion to support cell survival. As living cellular therapies, CAR-T cells are capable of integrating multiple, complex signals to generate coordinated immune responses.

The ARI-0001 CAR-T cell product was initially developed at the Hospital Clínic de Barcelona. This second-generation CAR construct incorporates the extracellular domain of the monoclonal antibody A3B1, which functions as the antigen-recognition domain. The construct also contains a CD8 transmembrane domain, a 4-1BB costimulatory domain, and a CD3ζ T-cell receptor (TCR) signaling domain. The CAR transgene is introduced into T cells using a third-generation lentiviral vector, and the modified cells are expanded in the closed, automated bioreactor system CliniMACS® Prodigy (Miltenyi Biotec) to generate the final product for clinical administration.

The manufacturing workflow for ARI-0001 CAR-T cells is organized into defined steps, including timelines, specific

reagents, and materials required for production. Notably, human serum albumin and human AB serum are used as critical reagents throughout various stages of the process. These reagents are applied at specific manufacturing steps to ensure optimal cell viability, proliferation, and functionality of the final CAR-T cell product.

This approach reflects the dual nature of human-derived raw materials: their essential role in supporting cellular processes, and their regulatory complexity as human-sourced components that require additional documentation and safety validation.

HUMAN SERUM ALBUMIN: REGULATORY CLASSIFICATION & APPLICATIONS

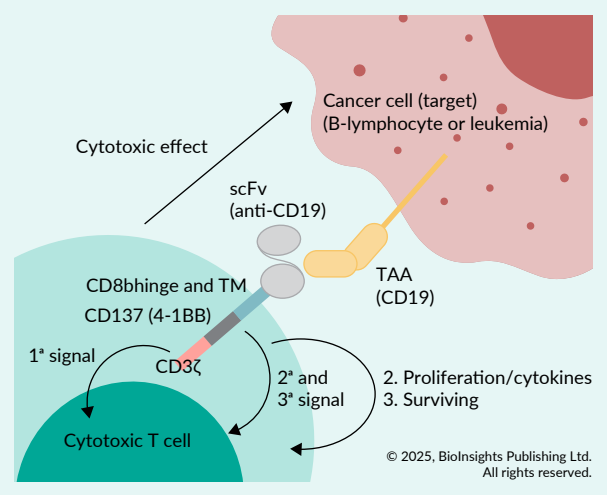
Human serum albumin functions in two distinct capacities within CAR-T cell manufacturing, each carrying different regulatory implications. As an ancillary material, albumin supports intermediate processing steps, including cell isolation, expansion, cryopreservation, and washing processes. The first application of albumin in ARI-0001 CAR-T cells is typically washed-out during processing and does not constitute an active ingredient in the final product; however, it is still present.

The second application positions albumin as an excipient in the final cryopreserved product, which remains present during patient administration. This dual functionality requires careful regulatory documentation to address both ancillary material and excipient requirements under relevant regulatory standards and guidelines.

Therapeutic-grade human albumin, meeting >96% purity specifications, qualifies as a licensed biological product under pharmacopoeia standards. This classification provides several regulatory advantages, including established safety profiles, reduced comparability requirements versus novel excipients, and clear documentation

FIGURE 2

Mechanism of action of CAR-T cells.



pathways. The material functions as a low-risk ancillary material while remaining eligible for final formulation use.

Process advantages of European Pharmacopoeia/US Pharmacopoeia (EP/USP) albumin include enhanced cell viability and stability during processing steps and reduced shear stress in bioreactors. Regulatory benefits encompass pre-qualified safety profiles, established pharmacopeial standards, and reduced comparability requirements versus novel excipients.

MALE AB SERUM: QUALITY CONSIDERATIONS & SUPPLIER EVALUATION

Male AB serum presents certain regulatory considerations due to the absence of therapeutically approved and registered products for this application. However, its continued use highlights its practical value and relevance in established cell therapy workflows. Unlike albumin, AB serum is completely washed-out during processing and does not appear in the final product.

Source and traceability requirements mandate that serum must originate from healthy donors screened according to

blood donation standards. Donors undergo testing for infectious diseases, including HIV-1/2, hepatitis B and C, and syphilis. Serum sourcing must occur through licensed blood establishments that maintain complete donor records and traceability documentation.

Quality control specifications require comprehensive Certificates of Analysis (CoA), including identity testing, sterility confirmation, mycoplasma testing, endotoxin levels typically <0.5 EU/mL, and viral marker testing using nucleic acid testing and serology. When applicable, documentation must include irradiation or virus inactivation status, ensuring these treatments do not compromise functional components such as complement or growth factors.

The preference for male serum derives from several practical considerations. Male donors typically exhibit less variability and provide better cell culture consistency due to hormonal stability. Additionally, male serum carries a lower risk of anti-HLA antibodies that may be present in female donors, particularly those with previous pregnancies. This reduced variability contributes to more uniform donor serum profiles and enhanced manufacturing reproducibility.

MALE AB SERUM: REGULATORY STRATEGY & RISK MITIGATION APPROACHES

Regulatory agencies express a preference for xeno-free and serum-free culture systems when feasible. When human serum AB is employed, manufacturers must provide scientific justification demonstrating the necessity for cell viability, activation, or expansion. This justification should include process comparability data supporting transitions between serum lots or potential conversion to serum-free conditions.

Batch qualification strategies become essential for mitigating inherent variability in human-derived materials. Effective approaches include pre-screening individual lots against defined performance criteria and strategically pooling qualified lots to reduce batch-to-batch variation. These strategies require robust analytical methods and clear acceptance criteria aligned with product specifications.

Viral safety risk assessment remains mandatory despite human origin. Nucleic acid testing for viruses and validated viral inactivation steps are strongly recommended when feasible without compromising functional integrity. Storage and handling procedures must follow GMP-compatible standard operating procedures, typically requiring controlled temperatures at −20 °C or −80 °C with validated thawing, aliquoting, and use procedures.

CLINICAL EXPERIENCE & MANUFACTURING CONSISTENCY

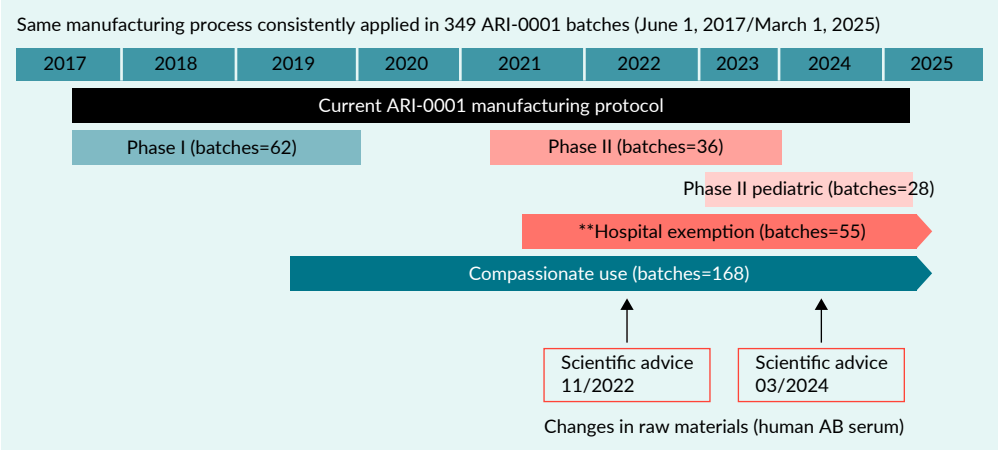
Eight years of ARI-0001 manufacturing experience from the Hospital Clínic de Barcelona team demonstrates the feasibility of maintaining consistent raw material strategies throughout clinical development, as seen in Figure 3. Since January 2017, nearly 350 ARI-0001 products have been manufactured using the same processing approach, spanning Phase 1 and 2 clinical trials, pediatric studies, compassionate use applications, and hospital exemption production.

This manufacturing consistency provided the foundation for ARI-0001's hospital exemption authorization from the Spanish Agency of Medicines and Medical Devices in 2021. The authorization represented the first advanced therapy medicinal product approved through hospital exemption for cancer treatment in Europe, specifically for patients over 25 years with relapsed/refractory acute lymphoblastic leukemia.

Sustained manufacturing success validates the importance of early raw material strategy development and consistent

FIGURE 3

Manufacturing process experience showing consistent application across 349 ARI-0001 batches from 2017–2025.



supplier relationships. Process robustness emerges from thorough material qualification, appropriate risk mitigation strategies, and comprehensive documentation supporting regulatory submissions throughout development phases.

STRATEGIC CONSIDERATIONS FOR RAW MATERIAL SELECTION

Organizations evaluating raw material suppliers should prioritize regulatory compliance and quality systems that support progression from early clinical trials through commercial manufacturing. Material consistency and comprehensive safety testing provide the foundation for reliable supply chains while ensuring patient safety throughout clinical development.

The selection process must balance multiple factors, including biological compatibility advantages of human-derived materials, regulatory acceptance pathways, and practical considerations such as supply security and cost management. Materials derived from human sources provide enhanced biological compatibility for cultivating human cells, especially in patients whose cellular function has been compromised by multiple treatment regimens.

Early-phase development should emphasize flexibility and risk assessment while ensuring basic safety requirements. Late-phase development necessitates enhanced focus on GMP compliance, supply security, and smooth transition planning. Proactive planning and supplier collaboration facilitate successful transitions between development phases without compromising product quality or timeline adherence.

FUTURE TRENDS & REGULATORY EVOLUTION

Cell therapy production continues evolving toward reduced reliance on human-derived materials, driven by regulatory preferences and supply chain considerations. However, human-derived raw materials remain widely utilized due to their demonstrated effectiveness in supporting cellular processes critical for therapeutic success.

Current regulatory frameworks continue to develop specific guidelines and standards for ancillary materials. Clear frameworks defining requirements and responsibilities for both users and suppliers will influence the pace of commercialization while ensuring patient safety and continued innovation in cell-based therapies.

When properly documented with comprehensive Certificates of Analysis, Origin, and Compliance, human-derived materials maintain acceptance for therapeutic applications. The key lies in thorough characterization, appropriate risk assessment, and robust quality systems supporting consistent material performance throughout product lifecycles.

SUMMARY

Strategic raw material selection requires balancing regulatory compliance, material performance, and supply chain reliability throughout cell therapy development. When appropriately qualified and documented, human serum albumin and male AB serum provide essential support for CAR-T cell manufacturing while maintaining regulatory acceptability.

The ARI-0001 workflow demonstrates that consistent raw material strategies can support progression from early clinical development through regulatory approval. Success depends on thorough supplier evaluation, comprehensive documentation, appropriate risk mitigation, and proactive planning for development phase transitions. As regulatory frameworks evolve, organizations must maintain flexibility while ensuring patient safety and product quality remain paramount in raw material selection decisions.

Q&A

Daniel Benítez Ribas

Q What are the main considerations when evaluating a raw material supplier, and which criteria should organizations prioritize to meet their specific needs?

DBR The primary consideration should be regulatory compliance and quality systems, as this foundation enables progression from early clinical trials through late-stage development. Material consistency and safety testing are critical for guaranteeing product safety in clinical applications. While less critical than the first two factors, supply chain reliability remains essential for ensuring adequate material availability throughout development and manufacturing phases.

Q How can proper selection, evaluation, and qualification of raw materials impact CGT products' quality, safety, and efficacy?

DBR Consistency and process performance must be considered to minimize batch-to-batch variability when considering proper raw material management, which directly impacts regulatory compliance and patient safety. Additionally, materials must demonstrate therapeutic efficacy within the specific

manufacturing system. A product that meets regulatory and safety requirements but fails to support cellular function will not be viable for the intended application.

Q What are the potential benefits and challenges of human-derived raw materials?

DBR **The primary benefit is biological compatibility.** Human-derived materials, such as serum or albumin, provide superior cell growth and function when producing human cells for therapeutic use. This is particularly important for patients with compromised cellular quality due to previous treatments, where enhanced biological compatibility can reduce mortality after processes such as lentiviral infection and support necessary expansion. Human-derived materials also benefit from regulatory acceptance and compliance pathways established with local and regional agencies.

Q What essential factors should organizations consider when choosing critical raw materials for early and late phase development, and how can this ensure a smooth transition during development?

DBR **In early phases, organizations should focus on flexibility while conducting appropriate risk assessments to guarantee safety.** The priority is determining whether the product will be effective before investing in more stringent requirements. As products advance to late-phase development, emphasis shifts to ensuring GMP compliance, material compatibility, and supply security. Smooth transitions require proactive planning, supplier collaboration, and appropriate testing of components to facilitate successful raw material transitions between development phases.

BIOGRAPHY

Daniel Benítez Ribas is the Qualified Person of the Immunotherapy Section (Immunology Department), responsible for ensuring the quality of cell therapies, including CAR-T cells such as ARI-0001 (varnimcabtagene autoleucel) and ARI-0002h (cesnicabtagene autoleucel) both approved by the AEMPS and produced under GMP regulations. He obtained his Bachelor's degree in Biology from the University of Barcelona, Barcelona, Spain in 1995 and completed his PhD at the Immunology Service of HCB in 2003. He conducted his postdoctoral research at Radboud University, Nijmegen, Netherlands, where he developed dendritic cell vaccines for metastatic melanoma. In 2008, he joined CIBERehd as a Principal Investigator at Hospital Clínic de Barcelona, supervising dendritic cell-based therapies for autoimmune diseases, including the first Phase 1b clinical trial in humans for Crohn's disease. Since 2016, he has been part of the Immunology Deptartment at Hospital Clínic de Barcelona and IDIBAPS, focusing on cell-based therapies for cancer and autoimmune diseases, and overseeing several clinical trials. He has published over 135 articles in renowned journals and holds two patents, participating in numerous antitumor immunotherapy projects.

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