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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

Expanding the range of cellular immunotherapy

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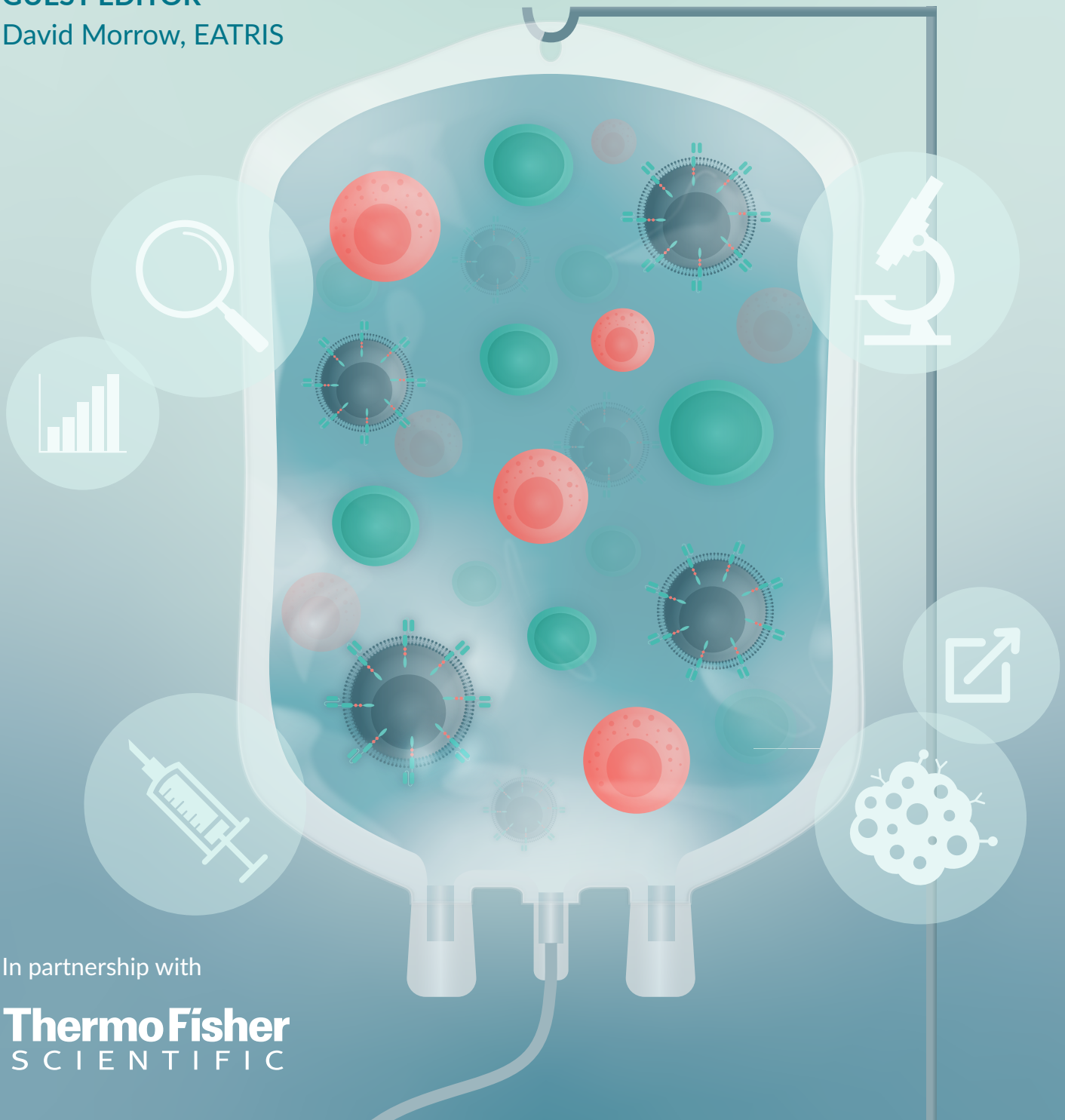

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CONTENTS VOLUME 11 · ISSUE 1

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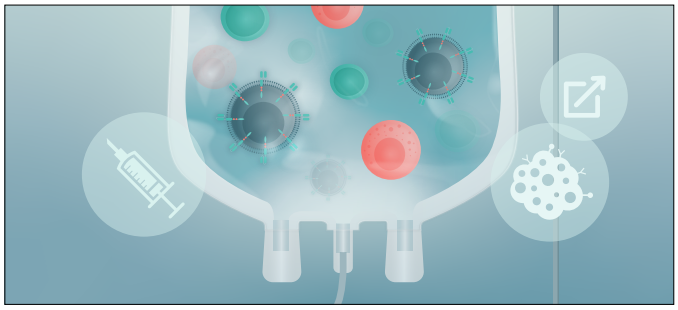

LATEST ARTICLES

CELL & GENE THERAPY INSIGHTS

EXPANDING CELLULAR IMMUNOTHERAPY

SPOTLIGHT

Breaking barriers in cell and gene therapy: advancing ATMPs through innovation and collaboration

Anton Ussi, Toni Andreu, Florence Bietrix, and David Morrow

FOREWORD

"As a community, the ATMP field needs to build a new collaborative ecosystem that leverages data to tackle complex diseases, particularly rare genetic disorders."

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In recent years, a slew of advanced therapy medicinal products (ATMPs), particularly cell and gene therapies, have demonstrated the transformative power of these therapies. CAR-T cell therapy, for example, has revolutionized the field in the treatment of patients with various types of relapsed/refractory (R/R) B-cell malignancies including diffuse large B-cell lymphoma (DLBCL), B-cell acute lymphoblastic leukemia (B-ALL), follicular lymphoma (FL), mantle cell lymphoma (MCL), and multiple myeloma (MM). CAR-T cell application in solid tumors, however, remains elusive due to a variety of challenges in the biology. This notwithstanding, results published in 2024 brought renewed optimism for the use of CAR-Ts in solid tumors, by showing that targeting two brain tumor-associated

proteins instead of one showed promise as a strategy for reducing tumor growth in patients with recurrent glioblastoma (GBM), an aggressive form of brain cancer. According to results obtained by researchers from the Perelman School of Medicine at the University of Pennsylvania and Penn Medicine's Abramson Cancer Centre in an **ongoing Phase 1 clinical trial**, this 'dual-target' approach is showing early promise that CAR-T therapies for solid tumors like GBM can potentially generate effective enduring responses. It is clear, however, that to achieve the full potential of cell and gene therapies, it is crucial to continue to strive to overcome significant barriers in the biology, clinical application and regulatory science. Examples of current obstacles include limited understanding of cell behavior in patients, complicated regulations due to the rapid pace of technological changes, lack of data standardization, poor reproducibility in early development, and inadequate communication among stakeholders.

Central to advancing ATMPs such as CAR-Ts is addressing the obvious challenge in predicting how patients will respond. There is a strong need for specific biomarker signatures to help doctors locate the best candidates for these treatments. Technologies that observe immune cell activity are vital for assessing the effectiveness and duration of ATMP responses. New methods like non-invasive imaging and computer modelling are being explored for predicting treatment success and minimizing invasive testing. Such innovations could streamline patient selection in ATMP supply chains.

Developing safe and effective cell and gene therapies needs innovative technologies and collaborative approaches to address the technical challenges in research, development, and production. Platforms that adapt to specific needs using standardized tools are becoming increasingly popular for developing treatments and diagnostics, making the process more efficient and cost-effective. In 2021, the National Institute of Health (NIH), the US FDA, and various organizations started the Bespoke Gene Therapy Consortium (BGTC) to create standards that speed up the development of personalized gene therapies. Further similar initiatives are now essential, particularly in the CAR-T space as we strive to create more effective, safer, and cost-effective development processes for streamlining their development. To achieve this goal, the right collaborations and incentives must be fostered and facilitated where industry and academia—together with patient organizations—can work together to develop such standardized platform technologies. One recently launched example of such an initiative is the **European Rare Diseases Research Alliance (ERDERA)**, which launched in September 2024. ERDERA aims to improve the health and well-being of the 30 million people living with a rare disease (RD) in Europe, by making Europe a world leader in RD research and innovation and support concrete health benefits to patients, through better prevention, diagnosis, and treatment. This partnership aims to deliver an RD ecosystem that builds on the successes of previous programs by supporting robust patient need-led research, developing new diagnostic methods and pathways, and spearheading the digital transformational change connecting the dots between care, patient data, and research, while ensuring strong alignment of strategies in RD research across countries and regions. A core component of ERDERA is to develop innovative platform technologies that facilitate viral and non-viral gene therapy development. This represents a first of its kind initiative in the EU where industry and public partners work together to develop such platform technologies with the goal to facilitate a more standardized and streamlined development process for

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gene therapies for RD in a manner similar to the successful Bespoke Gene Therapy Consortium (BGTC) in the USA.

Europe needs to effectively facilitate collaboration among research institutions, patients, healthcare professionals, industry, and regulators to fully utilize resources for developing safer and more effective ATMPs. One of the ERDERA partners, the European Research Infrastructure for Translational Medicine (EATRIS), is working to support ATMP development by providing the right innovative tools and technologies to researchers to support their ATMP development programs. This is achieved by an infrastructure of over 150 research institutions and medical centers across 14 EU countries with an ambition to support translation of innovative therapies such as ATMPs to the clinic. To achieve this, EATRIS strives in particular to connect regional ATMP infrastructures and communities to establish a strong ecosystem for ATMP creation in Europe, benefiting academia and industry alike. By creating a robust ecosystem with reproducibility and quality at its core, effective ATMPs can meet their potential and revolutionize treatments for RD, cancer, auto-immune, and other pathologies in the future.

Research continues apace on strategies to avoid or dampen adverse effects from ATMPs, including managing cytokine release syndrome (CRS), a serious complication from some treatments. This could potentially be achieved through standardized technology platforms, as mentioned above, that address for example therapy response in the individual. New and effective prognostic tools can help doctors manage CRS early for example, balancing risks and treatment efficacy. As a community, the ATMP field needs to build a new collaborative ecosystem that leverages data to tackle complex diseases, particularly rare genetic disorders. While the concept of ATMPs might seem straightforward, actual implementation is complex and needs a profound understanding of personalized treatment strategies. Addressing these complexities through targeted research and innovation should remain a priority as we seek to enhance ATMP efficacy and improve patient outcomes.

BIOGRAPHIES

Toni Andreu specialized in genetics and genomics of rare diseases. He has been working in the field of neuromuscular disorders from a clear translational perspective, from basic science to the development of cell and animal models and clinical research. During his career he has published over 180 scientific papers, numerous book chapters and supervised several PhD programs. After working at Columbia University on mitochondrial disorders from 1998–2001, he moved to Barcelona to create the Neuromuscular Lab at the Vall d'Hebron Research Institute where he became Director of the Neurosciences Research Program and later CEO of the University Hospital of Bellvitge, one of the largest health care institutions in Spain. He has also been extremely active in the field of policy-making, and has held positions as the Director of the Spanish National Institute of Health Carlos III as well as the Director General for Research and Innovation at the Catalan Ministry of Health. Toni is the current Scientific Director of The European Infrastructure for Translational Medicine (EATRIS), Amsterdam, The Netherlands a position he has held since 2018.

Anton Ussi is Operations and Finance Director at EATRIS ERIC, the ESFRI European infrastructure for translational medicine. Joining EATRIS in 2010, he was part of the team responsible for the design and statutory incorporation of the infrastructure, whose membership

counts 14 European member states. In his current role as CEO since 2015, Ussi has a background in technology transfer, with previous history in mechanical engineering and automotive design, and small business administration. He specializes in public–private and public–public collaboration and translational research in medicine. Ussi is also Principal Investigator of REMEDi4ALL, a large EU-funded initiative to develop a European medicines repurposing framework.

Florence Bietrix is the Deputy Director to the Executive Board at EATRIS. She earned her PhD in Life Sciences from Toulouse University, Toulouse, France in 2006. Following her doctorate, she worked as a study director at Avogadro, a contract research organization in Fontenilles, France, where she managed pre-clinical studies on new therapeutics for atherosclerosis and metabolic syndrome. In 2008, Bietrix relocated to the Netherlands to undertake postdoctoral research at the Academic Medical Center in Amsterdam. Her research focused on developing new therapeutic targets for atherosclerosis and nonalcoholic steatohepatitis. She joined EATRIS as a Scientific Platform Manager for Biomarkers and Advanced Therapy Medicinal Products (ATMPs) and gradually transitioned into operations. Since 2019, she has served as the Head of Operations, overseeing strategic development, quality assurance, and implementation of initiatives within the organization. Her dedication to translational research reflects a holistic approach, from preclinical study design to fostering international collaboration, ensuring scientific discoveries lead to tangible benefits for patients.

David Morrow is the ATMP and Vaccine Scientific Program Manager at EATRIS C&S. David received a BSc (Hons) in Molecular Biology from University College Dublin, Dublin, Ireland in 2001 and a PhD in Vascular Biology from Dublin City University, Dublin, Ireland in 2006. This was followed by an American Heart Association Postdoctoral Fellowship at the University of Rochester Medical Center, NY, USA from 2006–2008 which resulted in an American Heart Association Young Investigator Award in 2008. From 2009–2015 he was an NIH/American Heart funded Principal Investigator heading multiple projects in cardiovascular disease and cancer. David also holds an MBA in Health Science Management and has worked as a consultant and in technology transfer in life sciences. David has recently authored papers and being invited as guest speaker at panel discussions at international conferences discussing the major bottlenecks in ATMP development.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

EXPANDING CELLULAR IMMUNOTHERAPY

SPOTLIGHT

COMMENTARY

CAR-T cells in B-cell mediated autoimmune diseases: do all roads lead to Rome?

Dimitrios Mougiakakos

The use of CAR-T cell therapies in autoimmune diseases represents a groundbreaking shift in treatment paradigms. Building on successes in hematologic oncology, CAR-T cells targeting CD19 have shown profound efficacy in achieving durable remissions, as exemplified by the first patient treated for severe systemic lupus erythematosus. These therapies offer advantages such as active migration of a 'living' drug, deep tissue penetration, and potential immunologic reset over traditional B-cell depleting strategies. Complementary approaches, including bispecific antibodies and emerging cell-based modalities such as allogeneic CAR-T cells or CAR NK cells, are expanding the therapeutic armamentarium. While promising, challenges remain with respect to scalability, accessibility, and long-term outcomes. Ongoing research heralds a paradigm shift in personalized, durable autoimmune therapies.

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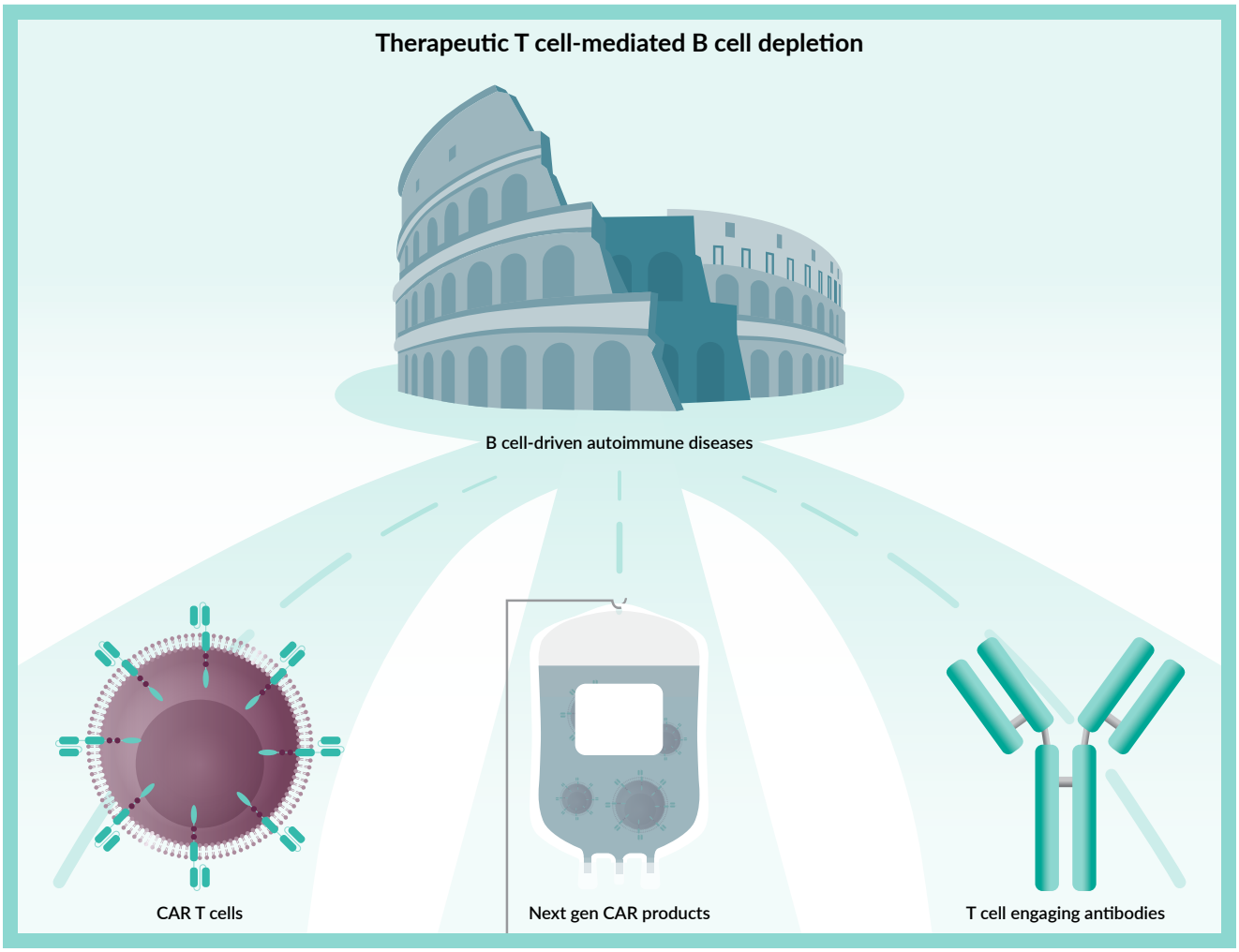
Not much longer than 3.5 years ago in 2021, our team faced significant challenges in treating a young patient with severe systemic lupus erythematosus (SLE) [1]. Multiple prior therapeutic interventions, including B-cell-depleting monoclonal antibodies, had failed to achieve sustained disease stabilization. In response, we, together with the patient, elected to pursue a novel approach: the administration of CAR-T cells targeting the CD19 antigen on B cells.

RATIONALE FOR CAR-T CELLS AND CD19 TARGETING

B cells are integral to the pathophysiology of numerous autoimmune diseases, including SLE [2]. Their pathogenic roles extend beyond autoantibody production, such as anti-double-stranded DNA (anti-dsDNA) antibodies in SLE, to include antigen presentation (of self-peptides) and the secretion of pro-inflammatory cytokines like interleukin (IL)-6. In a similar manner, multiple sclerosis



Road(s) to Rome: T cell-mediated B-cell depletion as a promising therapeutic approach for B-cell-driven autoimmune diseases. Currently, different strategies are being explored for clinical implementation: the use of established CAR-T cells, well known from real-world experience in hematology and oncology; novel CAR technologies originally developed for hematologic malignancies, now being directly evaluated in autoimmune diseases; and T cell engagers, which enable targeted immune modulation without the need for cell transfer. All these approaches share the common goal of effectively addressing the challenges in the treatment of autoimmune diseases.

(MS) is characterized by the presence of intrathecal oligoclonal bands (OCBs), which originate from clonally expanded B cells within the brain parenchyma that produce immunoglobulins. Likewise, in rheumatoid arthritis (RA), hallmark autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) play a key pathogenic role. Consequently, B-cell depletion strategies have been employed in various autoimmune conditions; for instance, the monoclonal anti-CD20 antibody rituximab is approved for RA [3] or is regularly used off-label in MS [4].

While CAR-T cell therapies have primarily been approved for hematologic malignancies, such as lymphomas and multiple myeloma, their profound and sustained B-cell depletion capabilities suggest potential applicability in B-cell-driven autoimmune diseases [5,6]. Notably, CAR-T cells exhibit active migration, longevity, and effective tissue penetration, potentially surpassing the depletion achieved by monoclonal antibodies, particularly within tissue compartments. Unlike antibodies, which rely solely on passive diffusion and have a limited half-life, CAR-T cells

actively home to target sites and persist longer *in vivo* [7]. This distinction is particularly relevant in diseases such as SLE, where we have observed that successful B-cell depletion in peripheral blood did not necessarily correlate with effective elimination in affected tissues, such as the renal parenchyma [8]. Recent studies have demonstrated deeper B-cell depletion in lymph nodes with CAR-T cells compared to monoclonal antibody treatments [9].

Targeting CD19 offers the advantage of broad expression across various B-cell differentiation stages, including plasmablasts and subsets of plasma cells [10]. This broad targeting is advantageous for addressing the diverse pathogenic B-cell functions in autoimmune diseases. On the other hand, it cannot be excluded that in certain disease entities or even on a personalized level, it may be more beneficial to also target CD19-negative, CD38- or BCMA-positive plasma cells [11,12]. To determine the optimal approach, further data are needed, ideally including the identification of predictive biomarkers. Additionally, a thorough risk–benefit assessment is required, particularly regarding the potential consequences of the resulting immunodeficiency.

CLINICAL OUTCOME AND IMMUNOLOGICAL RESET

When deciding on the treatment, one concern was the potential exacerbation of inflammation by infusing CAR-T cells into a highly inflammatory environment or the at least theoretical risk of transducing an autoreactive T cell clone. However, the patient tolerated the infusion well and showed a rapid reduction in symptoms, achieving and maintaining treatment free remission to date [13]. This finding suggests an 'immunological reset' as B cells reconstituted as predominantly naive cells without autoantibody production. In addition to the 'rejuvenation' of the B-cell population, changes in the gene expression signatures of other immune cells such as monocytes and T cells have also been observed, indicating for example reduced inflammatory activity, such as an attenuated IFN signature [14]. Overall, this process is still insufficiently understood and requires further research.

BROADER IMPLICATIONS AND ONGOING RESEARCH

This successful outcome has generated significant interest in both academic and industrial sectors, with the aim of achieving long-lasting, potentially permanent, therapy-free remissions, ideally with a single infusion of CAR-T cells [6,7]. Given the increasing incidence of autoimmune diseases, this approach holds great promise, although questions remain about future accessibility and scalability.

In the initial phase of exploration, established CAR-T cell therapies from hematology and oncology have been applied to a growing number of therapy-refractory patients and autoimmune indications with underlying B-cell pathophysiology, including SLE, systemic sclerosis (SSc), and myositis [13,15,16]. These studies have employed autologous CAR-T cells of the second generation with 4-1BB or CD28 co-stimulatory domains targeting antigens like CD19 or B-cell maturation antigen (BCMA), administered following lymphodepletion with agents such as fludarabine and cyclophosphamide [6].

Emerging data from these studies have confirmed the anticipated therapeutic potential, with some reports indicating remarkable efficacy. Notably, the safety profile in autoimmune patients appears more favorable compared to oncology settings, with lower incidences and severities of cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), hematotoxicity, and infections [5,6]. In a recently published meta-analysis of a total of 80 patients

with autoimmune disorders (i.e., SLE, SSc, idiopathic inflammatory myositis, antiphospholipid syndrome, RA, and Sjögren's syndrome) treated with B-cell-depleting CAR-T cells, it was found that all patients achieved an immunosuppression-free state at the last follow-up [17]. Regarding tolerability, grade ≥2 CRS was rare, occurring in only four cases, and ICANS was also reported in four cases, both of which were well manageable. No fatal adverse events occurred. This disparity underscores the distinct differences between patient populations and cautions against direct extrapolation from oncology to autoimmune contexts. However, it is important to note that these results are based on a limited number of studies and patients. Larger, randomized studies are needed to determine more precise efficacy rates and the incidence of adverse events such as CRS and ICANS.

The observed favorable safety profile may be attributed to factors such as differing pre-treatments (i.e., less myelotoxic), biological reserves, and notably, the lower antigen burden in non-malignant diseases. Nonetheless, isolated reports of severe adverse events in clinical studies warrant continued vigilance, especially as patient numbers increase and interindividual variability becomes more apparent.

To date, the scope of CAR-T cell therapy has expanded beyond rheumatology to include B-cell driven neuroimmunological diseases. B-cell-depleting CAR-T cells have been utilized in conditions affecting both the peripheral (e.g., myasthenia gravis [18] or stiff person syndrome [19]) and central nervous systems (CNS; e.g., MS), yielding promising results in terms of efficacy and tolerability and numerous early clinical trials are being planned or are already recruiting patients. Here, too, the paradigms from hemato-oncology need to be reconsidered, as we may have to monitor and evaluate patients with CNS involvement in a completely different way with regard to toxicities, especially ICANS (e.g., by using continuous EEG) [20].

ADVANCEMENTS IN CAR-T CELL TECHNOLOGY

Before achieving regulatory approval for CAR-T cells in autoimmune diseases, a second phase of development has commenced, leveraging nearly a decade of experience with CAR-based technologies in malignancies. This progression allows the field of autoimmune diseases to potentially bypass certain developmental stages through a 'dual track' approach.

Innovations under investigation include multispecific CAR-T cells capable of recognizing multiple antigens to mitigate the risk of immune escape. One example is BCMA-CD19 compound CAR-T cells, which are being evaluated in SLE patients [21]. In addition, mRNA-based CAR-T cells have been explored, offering advantages such as reduced risk of insertional mutagenesis and transient CAR expression, providing an additional safety mechanism in cases of CAR-T cell-induced toxicity but requiring repeated dosing. Notably, mRNA-based approaches may eliminate the need for lymphodepletion, leading to entirely 'chemotherapy-free' treatments. However, comparative efficacy between transiently and stably expressed CARs remains to be fully elucidated. Here, too, the first promising data from clinical trials with BCMA CAR-T cells in MG are already available [22].

CAR natural killer (NK) cells are emerging as a promising alternative to CAR-T cells in autoimmune disease therapy, leveraging their distinct biological properties and favorable safety profile [23]. Unlike T cells, NK cells belong to the innate immune system, offering both CAR-mediated and natural cytotoxicity, which enables them to target autoreactive immune cells effectively. CAR NK cells have shown reduced risks of severe CRS

and ICANS, as they release fewer pro-inflammatory cytokines such as IL-6. Their limited lifespan *in vivo* also mitigates long-term toxicity, providing a built-in safety mechanism. These cells are particularly well-suited for allogeneic 'off-the-shelf' therapies, as they are less prone to causing graft-versus-host disease (GvHD) and can be engineered from healthy donors or induced pluripotent stem cells (iPSCs). Challenges such as limited persistence and scalability remain, but advancements like IL-15 overexpression hold promise.

In the realm of T cell effectors, allogeneic CAR-T cells are garnering significant attention, offering the potential for readily available 'off-the-shelf' treatments [24]. Unlike autologous CAR-T cells, which require patient-specific manufacturing, allogeneic CAR-T cells are derived from healthy donors, enabling large-scale production and rapid accessibility. This approach addresses critical limitations of autologous therapies, such as long manufacturing times, complex logistics including apheresis, high costs (of approximately US$350,000–450,000), and dependence on the patient's immune cell health, which may be compromised in autoimmune diseases. Lymphodepletion may always be necessary in the allogeneic approach to ensure the persistence of the transferred cells. To overcome risks like GvHD and early rejection, allogeneic CAR-T cells are engineered with sophisticated genetic modifications. Techniques such as CRISPR/Cas9 are used to knock out the T cell receptor (TCR) to prevent GvHD and modify HLA molecules to evade immune rejection. Early clinical reports have shown that these cells can effectively target pathogenic immune populations in autoimmune diseases like SSc and myositis while maintaining safety [25,26]. The possible reduction in costs could ultimately be the decisive aspect in enabling better access to healthcare systems that are under great strain worldwide.

Further potential effector populations include γδ T cells [27] as well as professional immunoregulatory cell populations such as double-negative (DN) T cells [28], regulatory T cells (TRegs) [29], and mesenchymal stromal cells (MSCs) [30]. They are designed to recognize disease-specific antigens, dampen immune activity through cytokine release and modulation of antigen-presenting cells, and induce bystander suppression in affected tissues, which means that the target cell (i.e., B cell) is not directly destroyed and could have certain advantages in terms of preserving immune function. These cell-based therapies, currently being primarily evaluated in preclinical settings, hold significant potential, and we eagerly await the first patient data to understand their clinical impact and scalability [7].

ALTERNATIVE APPROACHES TO T CELL-MEDIATED B-CELL ELIMINATION IN AUTOIMMUNE DISEASES

In parallel with the development and clinical evaluation of CAR-T cell therapies for autoimmune diseases, the concept of T cell-mediated B-cell elimination is being explored as a complementary or competitive strategy. These efforts build on the extensive experience in hemato-oncology, where bispecific antibodies have significantly changed treatment paradigms. Bispecific antibodies, such as those targeting CD3 and CD19 (e.g., blinatumomab) or BCMA (e.g., teclistamab), establish a 'rendezvous' between the effector T cell and the target cell (e.g., CD19pos B-cells or BCMApos plasma cells). This interaction activates the T cell and triggers the destruction of the target cell in a repeatable cascade.

In oncology, the introduction of bispecific antibodies has reshaped the treatment landscape, raising recurring questions about the optimal selection and

sequencing of bispecific antibodies versus CAR-T cells [31]. While the curative potential of CAR-T cells is well established, bispecific antibodies lack sufficient long-term follow-up to confirm such results. Their advantages include off-the-shelf availability, potentially better tolerability, and lower cost for short-term use if they could replicate the efficacy of CAR-T cells in certain contexts of autoimmunity. Moreover, due to their more favorable toxicity profile, they could also be administered to patients who are not suitable for CAR-T cell therapy due to their overall condition. However, questions remain about the depth of depletion achievable with bispecific antibodies, particularly given their lack of active tissue penetration comparable to CAR-T cells.

Not surprisingly, following the early successes of CAR-T cell therapy, the first patients with refractory autoimmune diseases across the rheumatologic spectrum were treated with bispecific antibodies in CD3×CD19 and CD3×BCMA formats [32–34]. The results, although based on limited follow-up, were very promising and generated considerable enthusiasm, leading to numerous active clinical trials. Thus, the field now encompasses not only two parallel tracks involving CAR-T cell therapies, but also a third track addressing T cell-based strategies broadly in the context of B-cell-mediated autoimmune diseases.

OUTLOOK

The rapid development of CAR-T cell therapies and complementary strategies, such as bispecific antibodies and novel effector cell populations, underscores a transformative era in the treatment of autoimmune diseases. While early successes, such as durable remissions in SLE, highlight the potential of these innovative therapies, many questions remain regarding long-term outcomes, accessibility, and scalability. The introduction of 'off-the-shelf' solutions, such as allogeneic CAR-T cells and bispecific antibodies, offers promising ways to overcome logistical and cost barriers. In addition, emerging technologies, including mRNA-based CAR-T cells and CAR NK cells, herald a future in which therapies are safer, more versatile and more widely available. As preclinical research advances and more clinical trials report data, the field is approaching a paradigm shift that offers hope for durable, targeted, and personalized treatments. The bench-to-bedside journey continues to hold immense potential, and the collaborative efforts of academia and industry are poised to redefine therapeutic standards of care in autoimmune disease.

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CELL & GENE THERAPY INSIGHTS

EXPANDING CELLULAR IMMUNOTHERAPY

SPOTLIGHT

INNOVATOR INSIGHT

Worth the switch? Enhancing process performance for cell therapy manufacturing with an animal component-free raw material strategy

Phil Morton and Shanya Jiang

Historically, cell therapies have relied heavily on animal- and human-derived components—but the variability associated with these sources can lead to inconsistency and safety concerns. In recent times, focus has shifted toward utilizing chemically-defined raw materials, including animal component-free proteins. This article explores the critical role raw materials play in process performance and highlights how animal component-free raw materials can enable optimized, reliable, and efficient cell therapy manufacturing.

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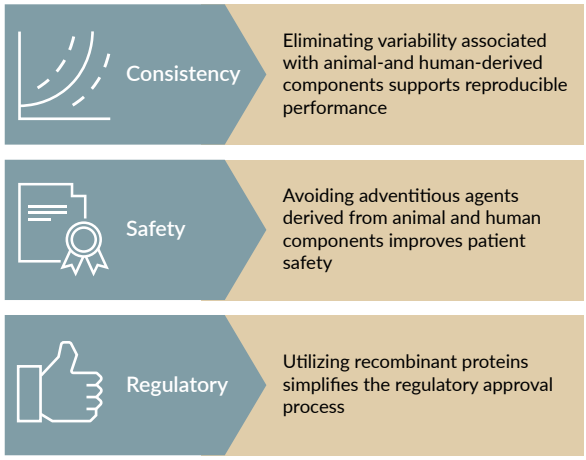
Throughout the cell therapy manufacturing workflow, various raw materials from cell culture media (including albumin) cytokines, biopreservation solutions, and stabilizing agents are utilized. While some materials, such as excipients, become part of the final formulation and others like ancillary materials do not, the quality of all materials that come into contact with cells is critically examined. This is because material quality can directly influence the stability, safety, potency, and purity of the final product. Shifting from the use of animal and human-derived raw materials to animal component-free raw materials in cell therapy manufacturing can address both safety concerns and consistency challenges (Figure 1). For example, human serum albumin often contains numerous ligands when derived from serum-based processes. These ligands can vary depending on the raw material source, geography, and supplier—and even from batch to batch, despite efforts by suppliers to minimize variability through pooled donations. By transitioning to a recombinant version, a more consistent process can be achieved. This approach also enhances safety and

FIGURE 1

Benefits of using aninal component-free raw materials in cell therapy manufacturing.



regulatory compliance, leading to faster regulatory approvals. In addition, improved performance and consistency can ultimately reduce overall cost per dose, offering a clear advantage for a manufacturing process.

Given these benefits, many raw material suppliers have expanded their portfolio to include chemically-defined components. Below we explore the advantages of an animal component-free raw material strategy, from recombinant human albumin, to cell culture media, cytokines, and cryopreservation solutions.

IMPROVED CRYOPRESERVATION AND POST-THAW RESULTS WITH RECOMBINANT HUMAN ALBUMIN

Recombinant human albumin is a critical material for many cell-based products and is widely used due to its versatility. As a carrier molecule, it can be used for cell banking, cell culture media, harvesting, formulation, and cryopreservation.

The molecule's core properties, outlined in Figure 2, provide a number of benefits. Sartorius's recombinant human albumin, Recombumin®, has supported customers in various applications from cell growth to cryopreservation. It is manufactured using a quality management system certified to ICH Q7 GMP guidelines.

In addition to to its physiochemical and biological benefits, Recombumin can be further customized to optimize its performance for different applications as it is fully recombinant, chemically defined, and ultra-pure. This allows the ligand and additive profiles to be altered for optimization to a specific process, for example, cell expansion as illustrated in Figure 3.

Building on its customizable nature, Recombumin also demonstrates significant benefits in cryopreservation, particularly for T cells. When compared to serum-derived albumin, Recombumin shows superior cryopreservation performance and improved cell recovery post-thaw due to its consistent and chemically defined composition (Figure 4).

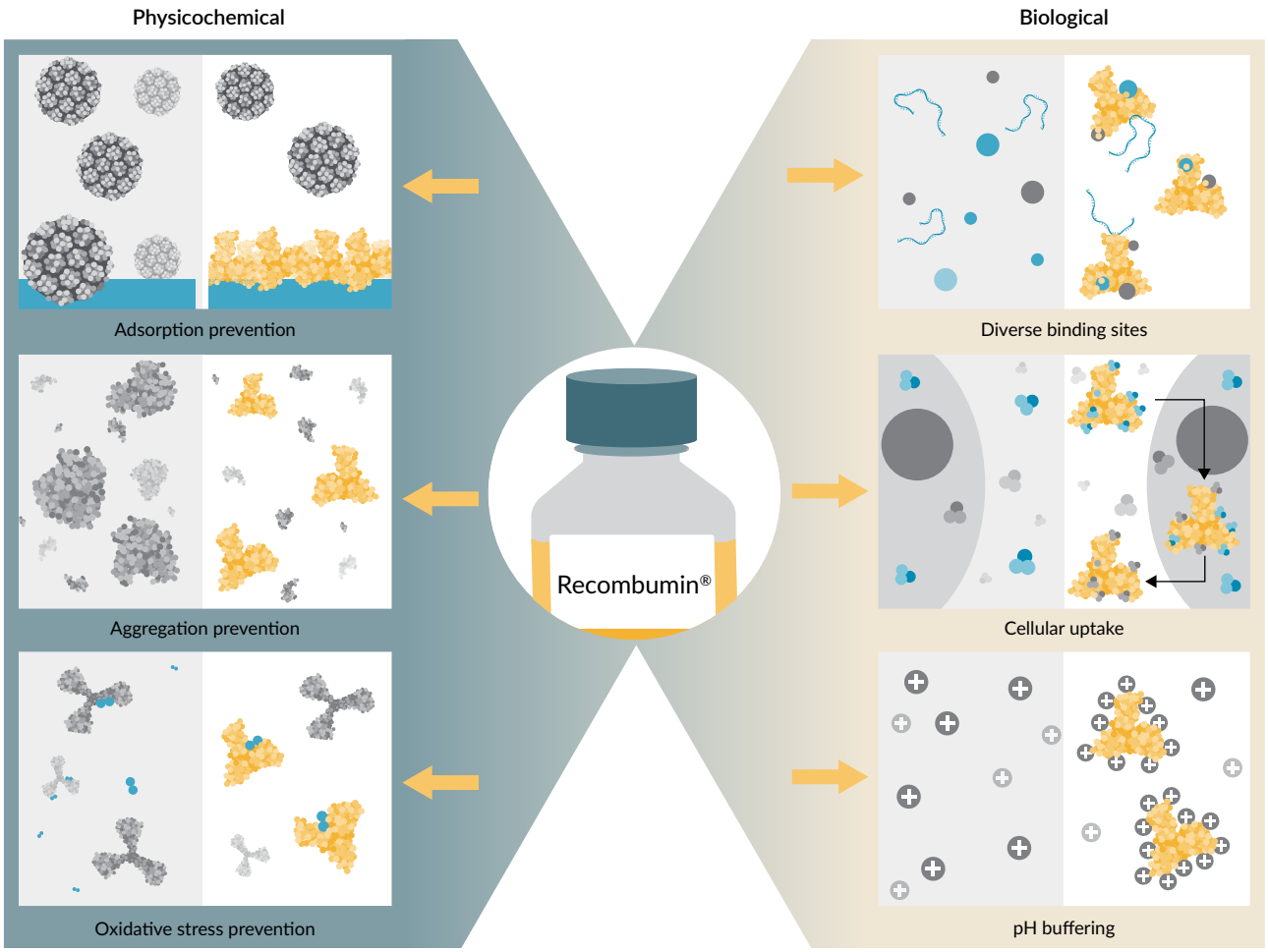
While serum-derived albumin may contain beneficial ligands, it also introduces potentially undesirable ligands that can adversely affect cells. Recombumin eliminates this variability, delivering a more reliable and effective solution.

Using Recombumin in cryopreservation also offers the possibility of reducing dimethyl sulfoxide (DMSO) content (Figure 5). There is a growing interest in reducing or eliminating DMSO from cell therapy formulations due to the negative side effects (for example, gastrointestinal, cardiovascular, respiratory, and dermatological reactions) that it can have on patients.

The advantages of recombinant human albumin extend beyond cryopreservation to the critical 2-hour window after thawing T cell therapies. As Figure 6 illustrates, Recombumin preserves a higher number of live cells while reducing the proportion of dead and apoptotic cells over the 2-hour period generally allowed for CAR-T cell therapies before administration with benefits extending up to 4 hours. This extended window provides clinicians with more time to administer these life-saving therapies without compromising their efficacy or quality.

FIGURE 2

Properties of recombinant human albumin, like Sartorius' Recombumin®.



Furthermore, Recombumin is supported by drug master files (DMFs) in the USA, Japan, Canada, New Zealand, China, and Australia. While Europe does not have such a system, there are mechanisms in place to address this. These DMFs, along with the regulatory support offered by Sartorius, provide a basis for safety in the process.

CHEMICALLY DEFINED T CELL MEDIA FOR INCREASED CELL EXPANSION AND VIABILITY

Cell culture media plays a pivotal role in providing a nutrient-rich environment for cells to grow and function properly. Traditionally, cell culture media has been supplemented with human serum as it provides the essential growth factors, hormones, and lipids for cell proliferation and growth. But concerns surrounding consistency, supply, and the risk of introducing adventitious agents have urged the field to transition towards serum-free and animal component-free alternatives. To address these concerns, Sartorius offers chemically defined T cell media, which has been developed to ensure robust performance, promote strong CAR-T cell growth, and maintain stable CAR expression without the addition of serum. This is achieved through the use of recombinant proteins, specifically Recombumin, to optimize the media formulation.

As shown in Figure 7, culturing peripheral blood mononuclear cells (PBMCs) from healthy donors with 4Cell® Nutri-T Advanced results in higher fold-expansion compared to alternative chemically defined media. It also provides comparable or better performance than media supplemented with or without serum, with viability of T cells being maintained above 90%.

Importantly, the use of 4Cell Nutri-T Advanced medium resulted in a 2.4-fold higher expansion of patient-derived CAR-T cells compared to alternative chemically defined media (Figure 8). Good viability of CAR-T cells was also maintained.

In terms of CAR expression, 4Cell Nutri-T Advanced media supports robust CAR expression over 10-day culture. The CD4/CD8 cell ratios are similar, maintaining balanced and functional cells (Figure 9).

From a functionality perspective, 4Cell Nutri-T Advanced medium produces highly cytotoxic cells, with even higher killing capacity at 1:4 effector:target ratio (Figure 10). This not only outperforms other chemically defined media but also shows slightly better results than media supplemented with human serum.

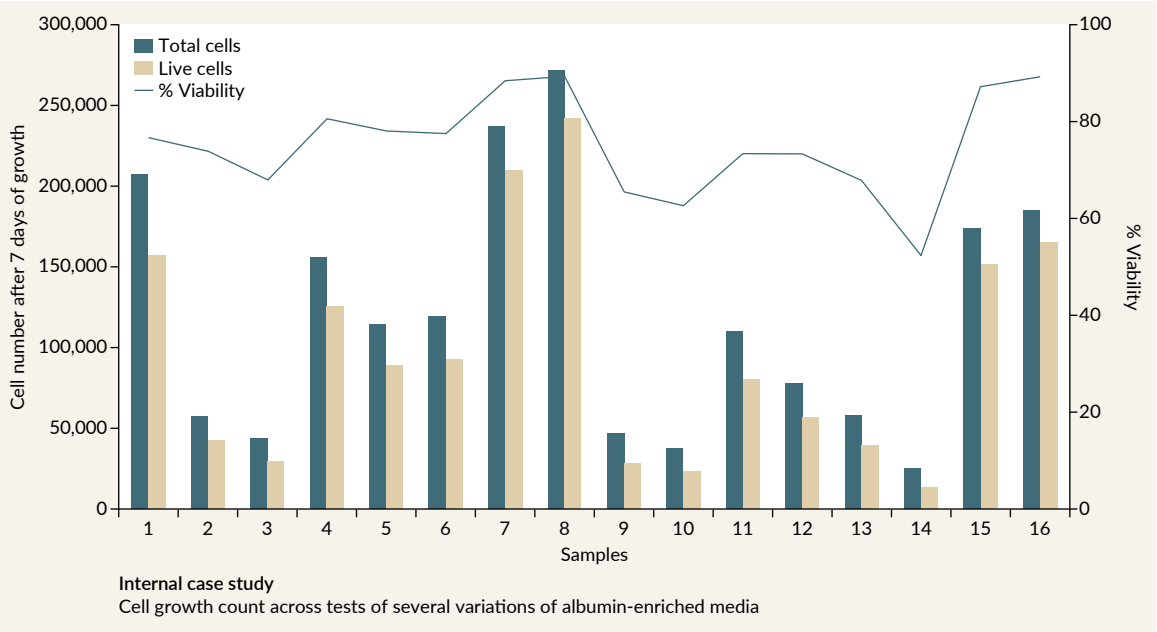
In summary, 4Cell Nutri-T Advanced medium provides superior proliferation of T cells from both healthy donors and patients, and ensures stable transduction efficiency and optimal CD4/CD8 ratios. Improved cytotoxicity and overall performance results in a more efficient process, reducing the cost per dose.

GROWTH FACTORS AND CYTOKINES TO ALLOW A SEAMLESS TRANSITION FROM R&D TO CLINICAL STAGES

Growth factors and cytokines are used throughout cell therapy manufacturing, from cell activation to expansion and differentiation. Utilizing high-quality, animal component-free cytokines is crucial for ensuring consistency, safety, and regulatory compliance in cell and gene therapy manufacturing. Sartorius produces a variety of animal component-free growth factors and cytokines available in multiple

FIGURE 3

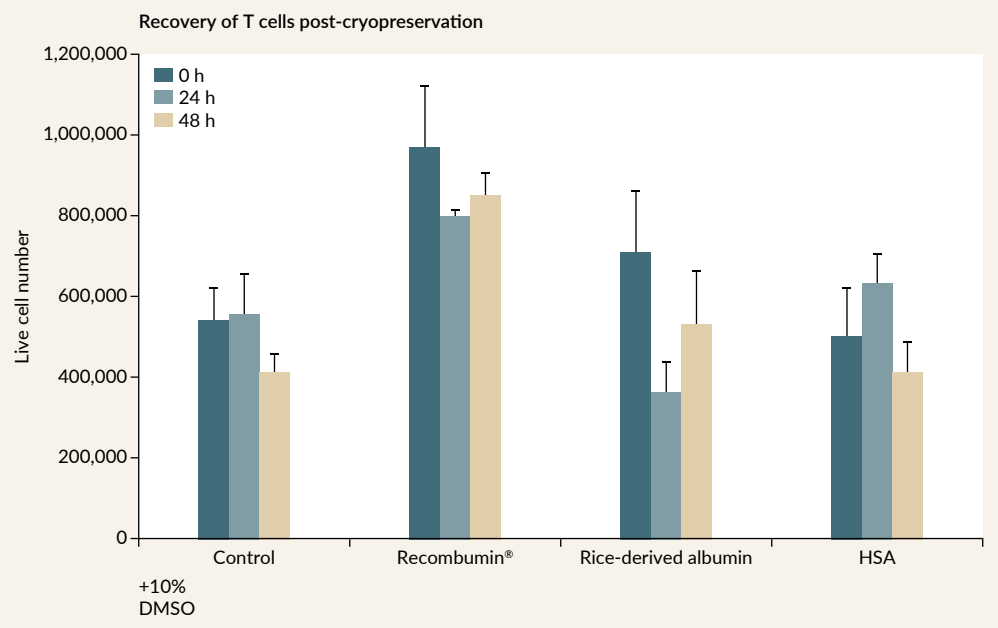
Cell growth count across tests of several variations of albumin-enriched media.



Internal case study
Cell growth count across tests of several variations of albumin-enriched media

FIGURE 4

Comparative post-thaw recovery of T cells.



grades to support a seamless transition from research to preclinical development and clinical manufacturing.

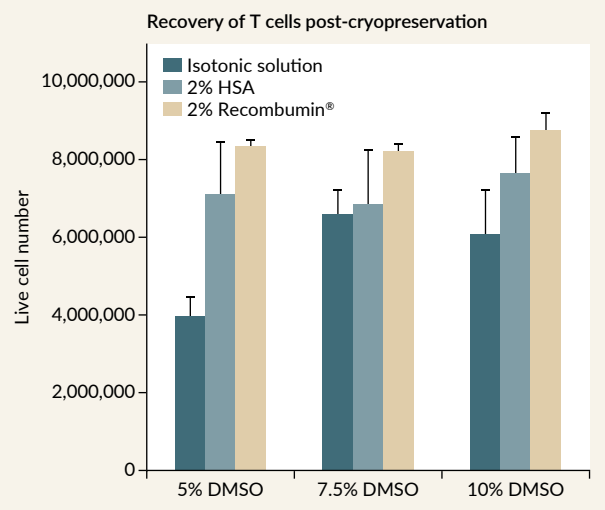
A strict animal-derived component-free (ADCF) policy is followed to ensure maximum safety of Sartorius' preclinical and GMP cytokines. As a result, no animal or human-derived components are part of any of their cytokine products. They can therefore be safely used without the need to perform time-consuming and expensive viral safety studies, thereby bringing a significant economic benefit.

Figure 11 provides a comparison of a preclinical-grade IL-2 from CellGenix® versus Proleukin®, a therapeutic IL-2, using different donors and media. The results demonstrate similar performance between the preclinical and clinical-grade IL-2, reinforcing the reliability of these products across development stages.

As these cytokines and growth factors are specifically designed with cell therapy in mind, their formulations, testing protocols, and packaging are all optimized to meet the unique requirements of this field.

FIGURE 5

Reducing dimethyl sulfoxide content for cryopreservation.



CHEMICALLY DEFINED BIOPRESERVATION SOLUTIONS

Short- and long-term storage during cell processing can pose several challenges including low post-thaw recovery.

Cryopreservation techniques are employed during cell banking, fill and finish, and long-term storage. Developers producing their own 'home-brew' cryopreservation formulations will often use human serum albumin, plasma, or serum supplemented with 5–10% dimethyl sulfoxide (DMSO). Sartorius offers chem-

FIGURE 6

Comparison of hold time and number of apoptotic cells post-thaw.

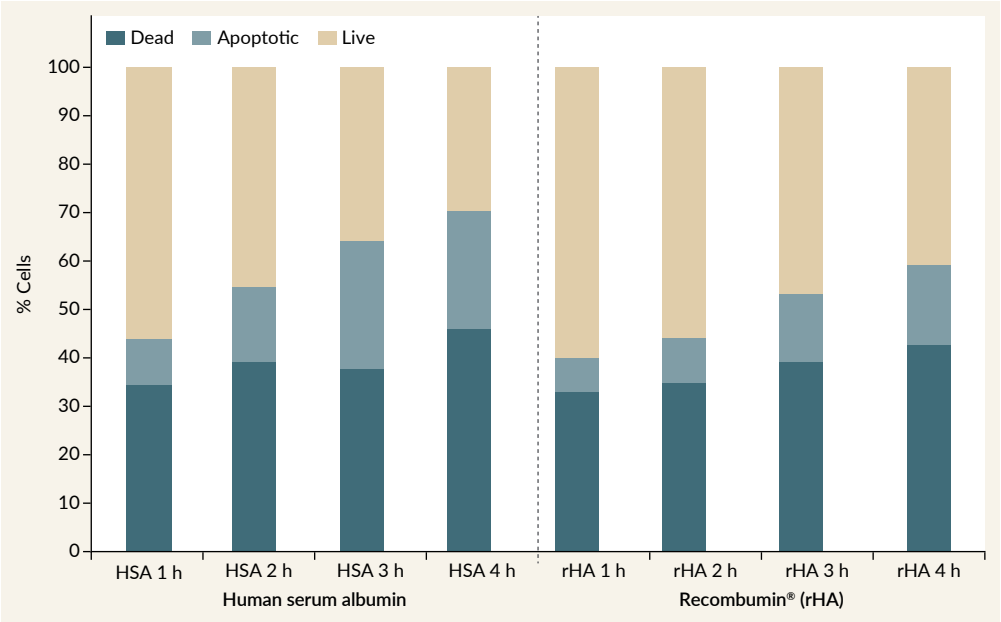
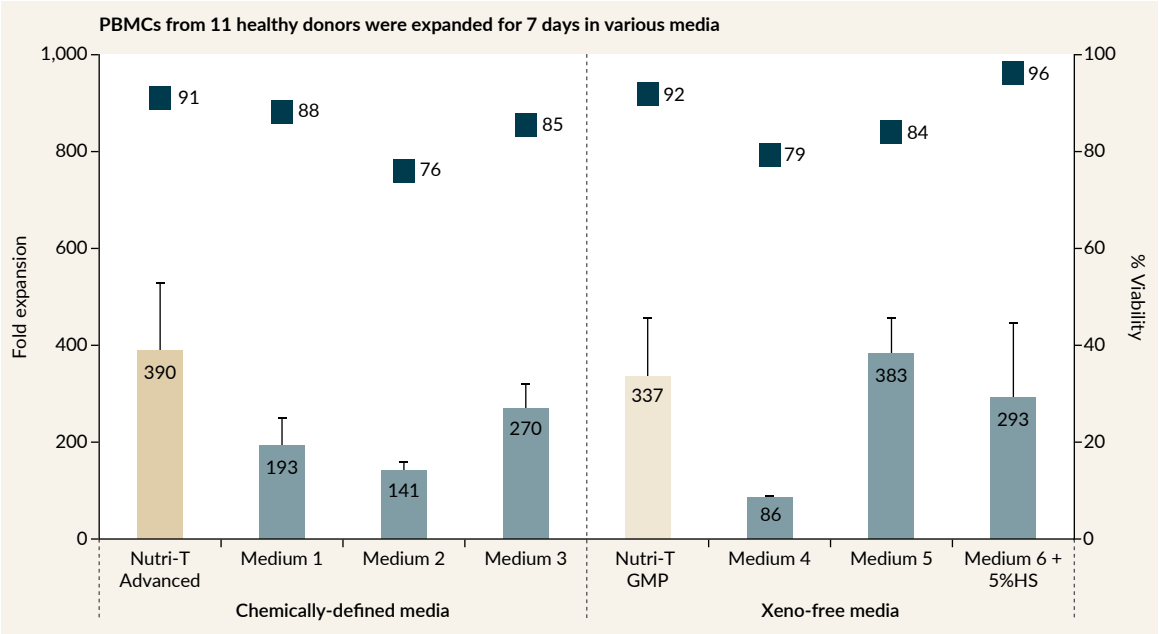


FIGURE 7

Expansion and viability with healthy donor-derived T cells.



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ically-defined, protein-free, animal component-free cryopreservation solutions NutriFreez® D5 and D10, which contain 5% and 10% DMSO, respectively. They are both manufactured in compliance with relevant cGMP guidelines and show similar results

FIGURE 8

Expansion and viability with patient-derived CAR-T cells.

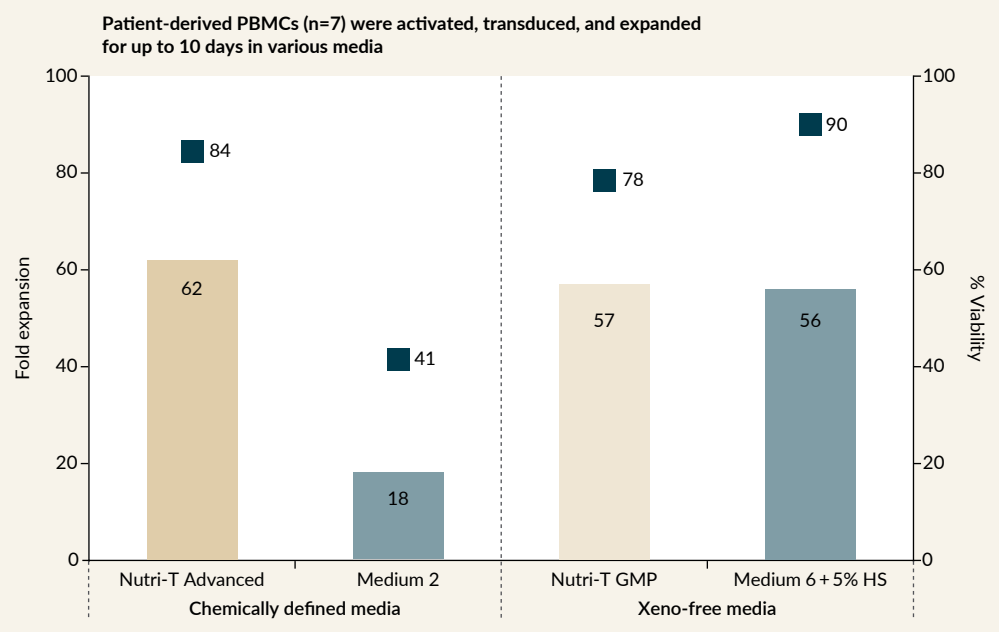

FIGURE 9

Transduction efficiency and frequency of CD8⁺ cells in patient-derived CAR-T cells cultured in G-Rex® 6M.

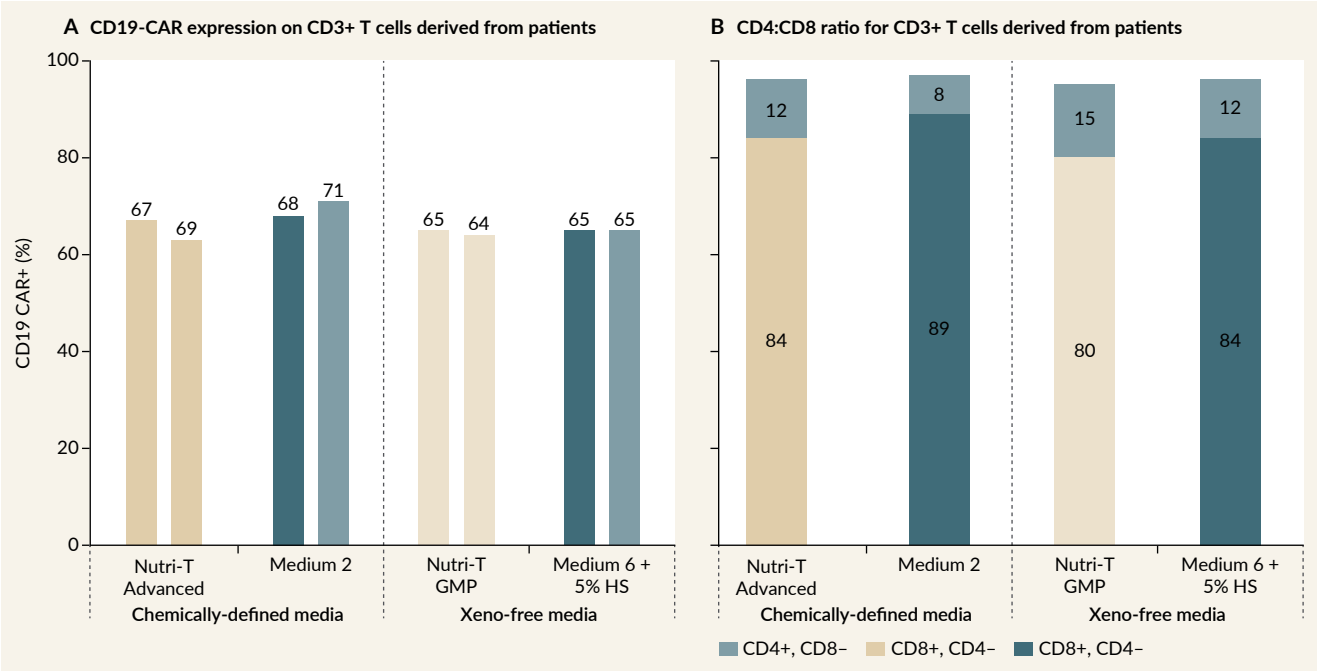

FIGURE 10

Functionality and effectiveness of CD19- CAR-T cells.

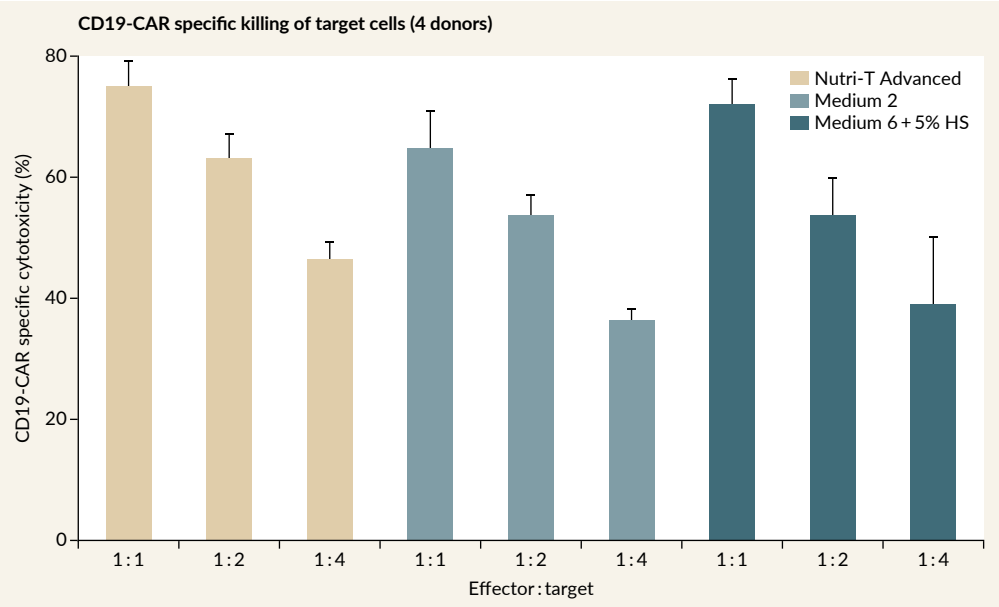

FIGURE 11

Fold expansion of peripheral blood mononuclear cells cultured using CellGenix® Preclinical IL-2 or Proleukin®.

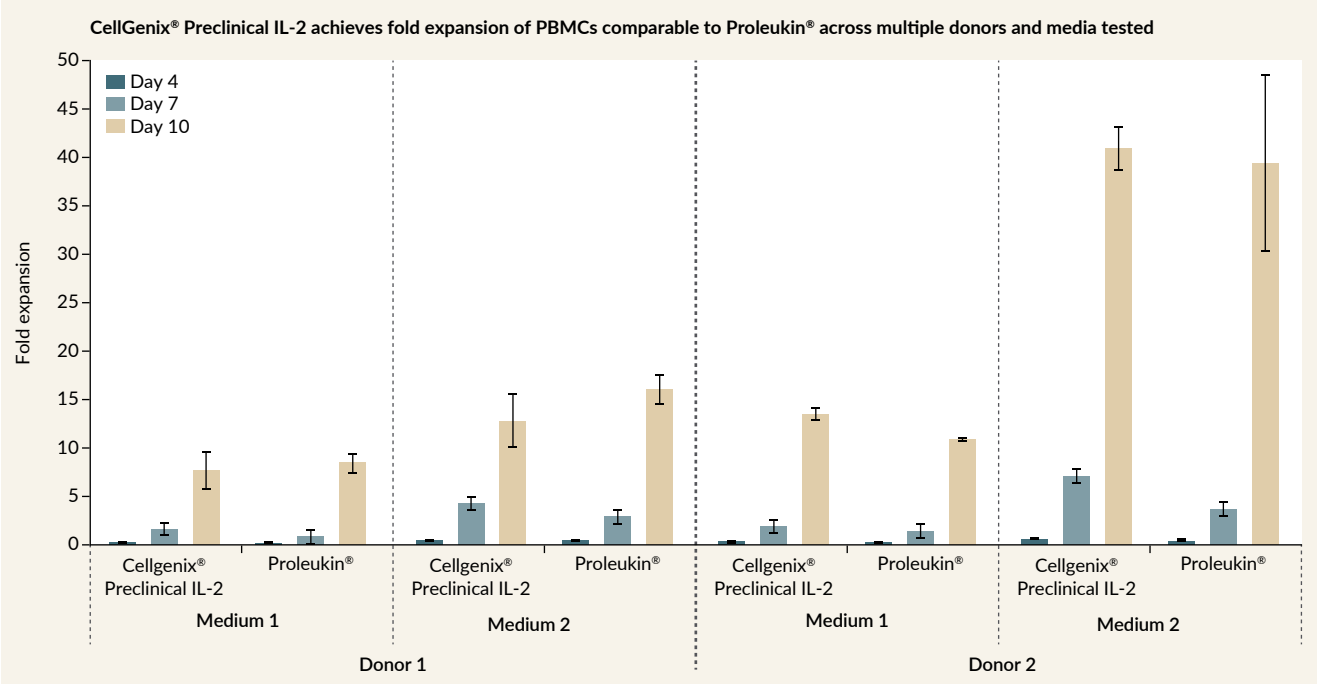

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FIGURE 12

Transduction efficiency and immunophenotype ratio in CAR-T cells 10 days post-thaw.

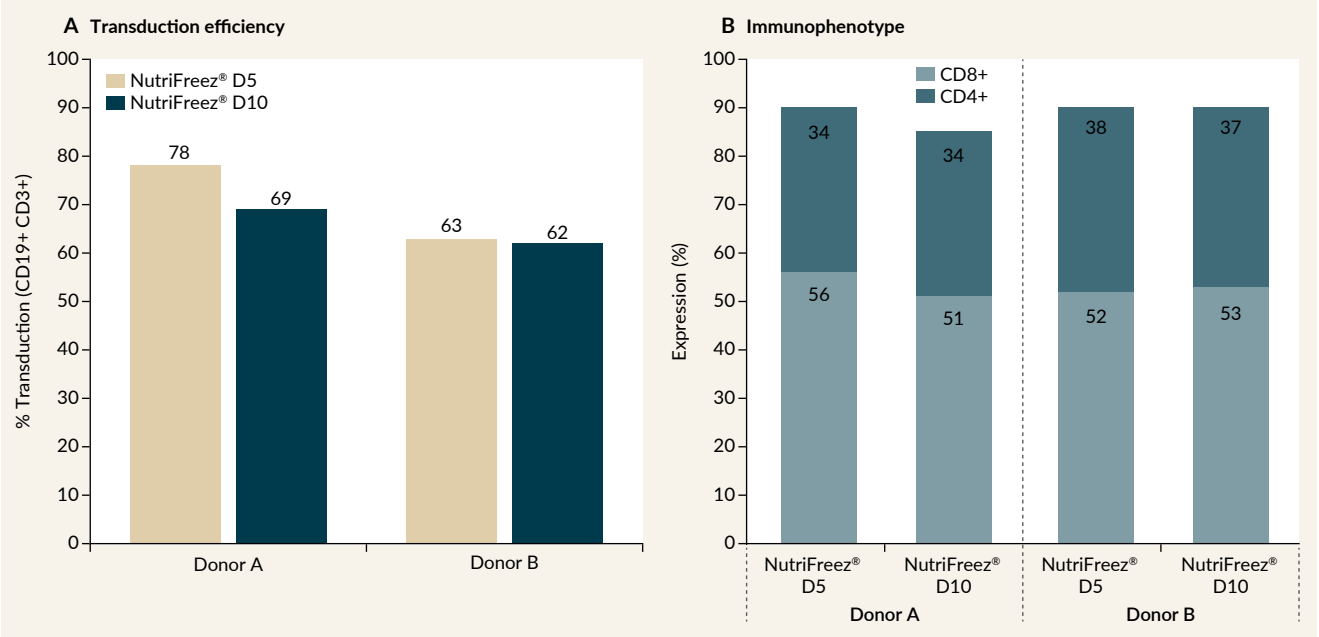


FIGURE 13

Morphology, viability, and comparative fold expansion of peripheral blood mononuclear cells with NutriStor® and competitor solutions.

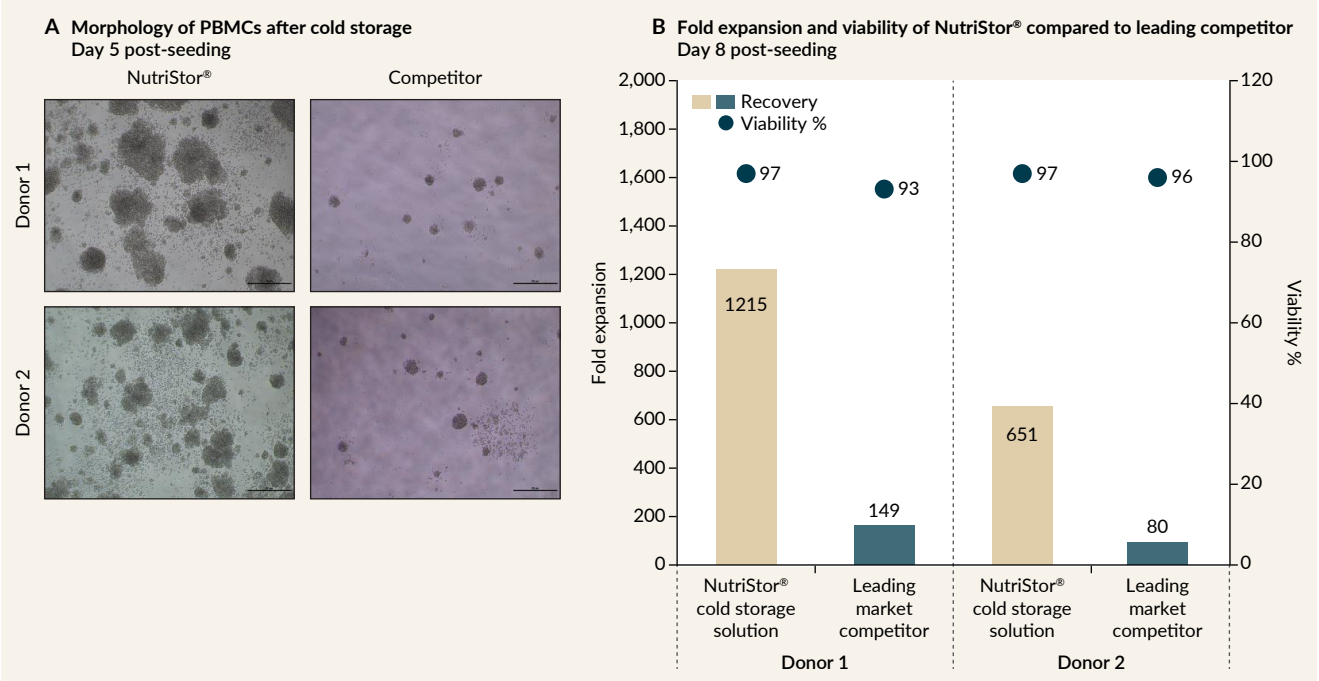
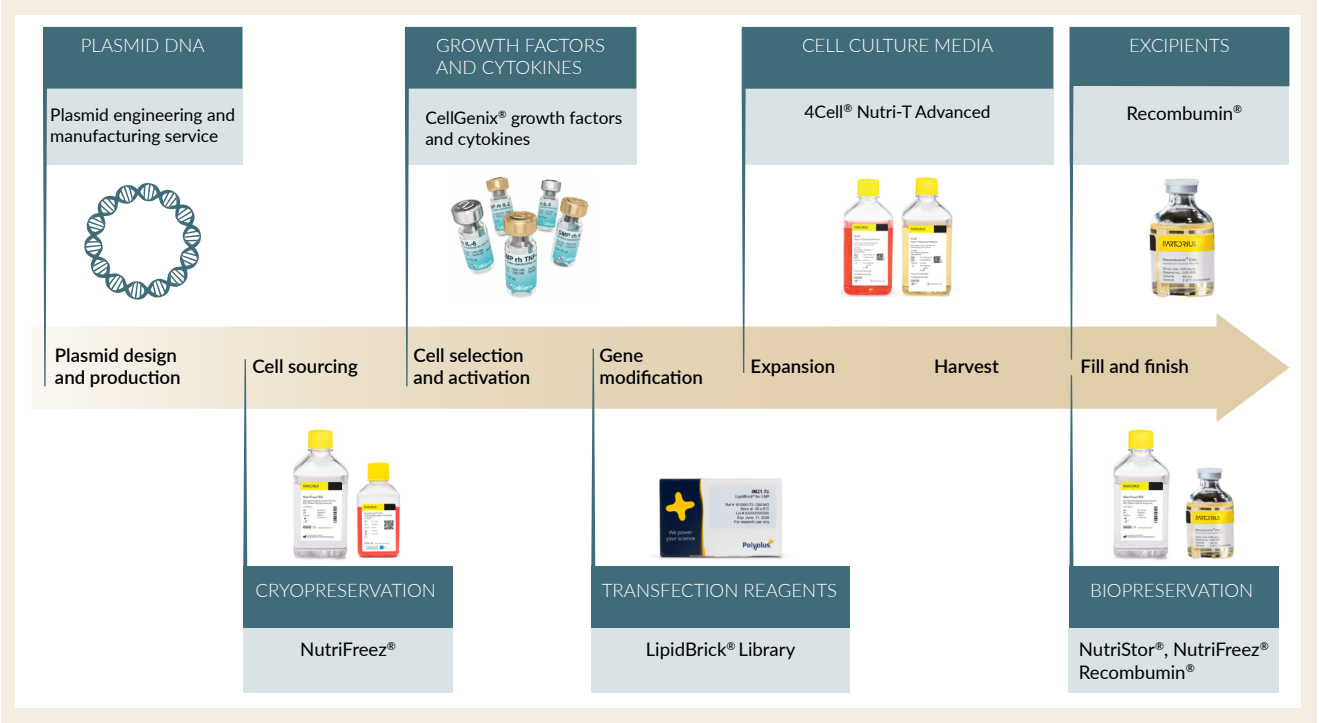


FIGURE 14

Animal-component free materials to enable safe and consistent cell therapy development and manufacturing.



in maintaining consistent transduction efficiency and immunophenotype ratio in CAR-T cells 10 days post-thaw (Figure 12). While Nutrifreez D10 is medium-based, Nutrifreez D5 is a chemically-defined, salt-based solution specially designed for freezing and thawing of cells intended for cell therapies and clinical applications.

In contrast to cryopreservation, short-term cold storage, slows down the metabolism of the cells, and therefore allows the transport of the cells without the cost-intensive and stressful effects of freeze–thaw.

NutriStor® is a chemically defined, ready-to-use cold storage solution, providing an alternative to maintain products at 2–8 °C. Used for short-term storage during expansion, differentiation, fill and finish, quality assurance, and cell transportation, this approach maintains cell viability and functionality following cold storage while mitigating freeze–thaw induced cell stress. This solution also comes with comprehensive regulatory documentation (current GMP, DMF, regulatory support file, and pre-clinical safety studies) to support patient safety.

A comparative study against a leading competitor was conducted (Figure 13) using PBMCs which were stored at 2–8 °C for 4 days and subsequently cultured. PBMCs previously stored in NutriStor demonstrated significantly better growth, higher expansion rates, and excellent viability post-storage. This highlights NutriStor's capacity to support robust cell health and functionality under non-cryopreserved conditions.

TRANSLATION INSIGHT

Transitioning to chemically defined raw materials for cell therapy manufacturing offers a number of key benefits and can offer comparable or improved results and performance compared to raw materials containing human- or animal-derived components. Working with highly defined and

consistent materials allows for optimized processes, without the variability and associated risk that comes with new batches of raw materials. Starting with the end in mind is crucial for cell therapy manufacture, as more reliable and efficient processes ultimately shorten time-to-market, help to reduce cost-per-dose, and enhance patient safety.

You can learn more about Sartorius' portfolio of animal component-free materials for cell therapy in Figure 14.

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SPOTLIGHT

Efficient non-viral engineering of immune cells for cell therapy using circular single-stranded DNA

Howard (Hao) Wu

VIEWPOINT

"The integration of precision gene editing with safe and scalable delivery systems represents a paradigm shift in how we think about and approach immunotherapy."

Cell & Gene Therapy Insights 2025; 11(1), 7–11 · DOI: 10.18609/cgti.2025.002

RESEARCH BACKGROUND

In recent years, gene-editing technologies have become central to advancements in immunotherapy, particularly in engineering immune cells for the treatment of diseases such as cancer and autoimmune disorders. However, traditional methods—especially those relying on viral vectors for gene delivery—have raised significant concerns related to safety, efficacy, scalability, and accessibility. These concerns have slowed the widespread adoption of these therapies over the globe. Recently, a Boston, MA, USA-based biotech company Full Circles Therapeutics achieved technological breakthrough to overcome many of these limitations, signaling a new era in the development of safe, scalable, efficient, and more affordable immune cell therapies.

Although CRISPR/Cas9 technology has been widely used in gene editing, enabling

efficient targeted gene knockout and single-base editing, precise and site-specific integration of large gene fragments remains a technical challenge. These current gene delivery technologies face the following issues:

- Limitations of viral vectors: including immunogenicity, which can trigger immune responses; difficulty in production, as the viral preparation process is complex and costly; payload limitations, such as the restricted gene capacity of AAV vectors; and safety concerns, as random gene integration by lentivirus and AAV can pose potential safety risks.
- Drawbacks of non-viral methods: such as low efficiency and high toxicity associated with double-stranded DNA (dsDNA), and challenges in large-scale production of linear single-stranded DNA (lssDNA).

These technological bottlenecks present significant challenges for the engineering of immune cells (such as CAR-T cells) and the clinical application of cancer immunotherapy. Therefore, there is an urgent need for a non-viral, efficient, and safe gene integration technology.

RESULTS AND CONCLUSION

The company's innovation developed a proprietary miniaturized circular single-stranded DNA (cssDNA, trademarked as C4DNA™), a non-viral technology that enables stable genomic integration without relying on viral vectors. They developed a non-viral gene writing technology (GATALYST™) platform based on the C4DNA, which has brought disruptive progress in the field of large gene fragment integration. This platform utilizes a genetically engineered phage system to produce mini circular single-stranded DNA (cssDNA) with a size of up to approximately 20 Kb. It achieves efficient and precise large gene integration through a homologous recombination mechanism.

The cssDNA non-viral vector avoids the genomic instability caused by random integration and has the potential for industrial-scale production, significantly improving manufacturing efficiency. The GATALYST™ platform can achieve efficient, precise, and safe integration of large genes in various dividing cells. It has demonstrated up to 70% knock-in efficiency in induced pluripotent stem cells (iPSCs), as well as high-efficiency site-specific integration in multiple clinically relevant primary immune cell types. The platform can edit multiple genomic loci relevant to clinical applications and is compatible with various nucleases editing systems.

Using electroporation delivery technology, cssDNA can achieve up to 50% site-specific integration of dual-targeting CAR (CD19/CD22) in CAR-T cells, exhibiting excellent anti-tumor function both *in vitro* and *in vivo*. This provides new avenues for treating relapsed or refractory multiple myeloma (RRMM).

The GATALYST™ platform, with its flexible, safe, and scalable cssDNA vector, shows great potential in the field of immune cell therapy. This platform is expected to overcome the limitations of current gene delivery methods and provide a novel non-viral solution for gene and cell therapies targeting cancer, autoimmune diseases, and genetic disorders, thus advancing the development of precision medicine.

PERSPECTIVE

The non-viral revolution: precision and safety in focus

One of the most significant challenges in the field of gene editing has been ensuring the precise and controlled integration of genetic material into the genome. Full

Circles' use of C4DNA, a miniaturized form of circular single-stranded DNA, enables targeted, accurate genomic integration. This eliminates the reliance on potentially unpredictable viral systems, providing a safer alternative for patients. The technology significantly enhances scalability, editing efficiency, and overall safety, particularly for CAR-T and CAR-NK cell therapies. These therapies, which have already demonstrated significant promise in treating cancers, could become more accessible and less expensive as the technology scales up. For autoimmune diseases where the safety bar is much higher than end stage cancers, adoption of a non-viral engineering approach is more attractive.

The company's approach directly addresses the scalability problem, which has been a major bottleneck in the widespread use of gene-edited immune cells. One of the key challenges for current therapies, such as CAR-T, is the complexity and cost involved in producing personalized therapies for each patient. Full Circles' non-viral method could lead to the development of off-the-shelf cell therapies with non-viral precise genome engineering, meaning that engineered immune cells could be produced in bulk and stored for rapid use in patients, much like other biologics, making treatments more affordable and time efficient. Full Circles is now leveraging the multiplex editing approach by C4DNA to manufacture the allogeneic CAR-NK product for autoimmune diseases.

A new path for clinical application

Full Circles' recent patent approval in Japan, a leading global market for biopharmaceuticals, is a significant milestone for the company and for the field at large. The granting of the patent underscores the technology's clinical potential, particularly in markets where gene therapies are already a focus of intense research and development. With Japan at the forefront of oncology, autoimmune disease research, and genetic metabolic disorders, the impact of this innovation could be transformative.

The granted patent, which covers the use of universal circular single-stranded DNA for targeted genomic integration, highlights the broad utility of this technology in genome editing. Importantly, this technology is designed to work in a nuclease-editor agnostic manner, a feature that improves its adaptability, programmability and target specificity.

Expanding the therapeutic landscape

The broader implications of Full Circles' breakthrough extend beyond the immediate application to CAR-T and CAR-NK therapies. The potential for using this non-viral gene-editing technology could dramatically expand the range of diseases that can be treated with engineered immune cells. By enhancing the efficacy, scalability, and cost–effectiveness of immune cell therapies, Full Circles has positioned itself to address complex and hard-to-treat conditions—such as cancers, chronic autoimmune diseases, and perhaps even genetic disorders caused by ultra-large root cause genes with scattered mutation haplotypes—that have long eluded conventional treatments.

Dr Richard Shan, the Chairman of Full Circles Therapeutics, emphasized that the company aims to make these therapies more accessible and affordable to a broader patient population. In an era where the cost of biologics and personalized medicine remains a critical barrier to widespread adoption, the scalability of Full Circles' non-viral approach offers a promising solution. By making cell therapies off-the-shelf, the company could facilitate faster and more efficient deployment of treatments, ultimately accelerating the clinical translation of these breakthrough technologies.

Looking ahead: the path to widespread adoption

The recent recognition from Novo Nordisk Bio innovation Hub, which awarded Full Circles the 'Pathbreaker' award, underscores the company's commitment to collaborating with industry partners and accelerating the accessibility of breakthrough therapies. The award is a testament to the importance of forging strategic partnerships to drive the next generation of therapeutic innovation. By focusing on patent licensing and collaborative development, Full Circles aims to expand the reach of its technology and increase its impact in the global healthcare landscape.

Ultimately, Full Circles' non-viral immune cell engineering technology could become a cornerstone in the future of cell and gene therapy, enabling a broader range of patients to benefit from cutting-edge treatments. The integration of precision gene editing with safe and scalable delivery systems represents a paradigm shift in how we think about and approach immunotherapy. With further advancements and clinical validation in the near future, we are confident that this technology could play a key role in shaping the next generation medical treatment for a wide array of diseases.

This VIEWPOINT is based on the following original publication: Xie K, Starzyk J, Majumdar I, *et al.* Efficient non-viral immune cell engineering using circular single-stranded DNA-mediated genomic integration. *Nat. Biotechnol.* 2024; published online Dec 11. https://doi.org/10.1038/s41587-024-02504-9.

BIOGRAPHY

Howard (Hao) Wu has almost 20 years of experience in gene editing technology and new drug discovery. He is specialized in overseeing R&D programs, new lab and research team set up in the biotech start-ups. Dr Wu is the co-founder and CSO of Full Circles Therapeutics, Cambridge, MA, USA where he is dedicated to developing curative gene editing based gene/cell therapy. He is responsible for generating revenue through collaboration with MNC and biotech partners. Before founding Full Circles Therapeutics, Dr Wu was leading multiple discovery biology programs and disease prioritization in the genetic disease space at Fulcrum Therapeutics Inc. (NASDAQ:FULC), a small molecule drug discovery biotech company. He had been with the company through the full development phases starting from the start-up, expansion, until post-IPO development, during which he led a cross-functional team for portfolio disease selection and prioritization of multiple disease programs including neuromuscular disease, cardiac disease, hematological and metabolic diseases. Before joining Fulcrum, Dr Wu was a Senior Research Fellow at Whitehead institute, MIT. His research focused on neurological disorders utilizing a combination of CRISPR/Cas9 mediated genomic and epigenomic editing technology and stem cell technology. He did his PhD in Biochemistry and Structural Biology at Hongkong University of Science and Technology (HKUST), Hong Kong and Bachelor's degree in Chemistry from Fudan University, Shanghai, China before he did his postdoctoral research at Johns Hopkins University School of Medicine, Baltimore, MD, USA and Howard Hughes Medical Institute, Chevy Chase, MD, USA. Dr Wu has more than 30 journal publications, patents, and research and industry grants. For his work, he has received a fellowship award from the Human Frontier Science Program (HSFP) and the NARSAD Young Investigator Award. He was also awarded the Alfred Blalock Young Investigator Award from JHMI and President's award from Whitehead Institute, MIT.

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EXPANDING CELLULAR IMMUNOTHERAPY

SPOTLIGHT

Solid tumor treatment: how can we achieve therapy efficacy and persistence in the next frontier of cell therapies?

INTERVIEW

"We aim to overcome the immune suppression that cancer can cause, so our therapeutic cells can effectively target the cancer cells."

Though there have been significant advancements in adaptive cellular therapies, researchers continue to face challenges in enhancing efficacy and targeting solid tumors. Abi Pinchbeck, Editor, BioInsights, speaks with **Sebastian Kobold**, Professor of Experimental Immuno-Oncology, LMU University Hospital, about the potential of emerging cellular immunotherapy strategies and approaches to overcoming the barriers in current treatment paradigms.

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Q What are you working on right now?

SK The primary interest of my lab is to discover and develop novel strategies to overcome limitations in adoptive cellular therapies. Historically, most of our work has focused on T cells, but we have expanded to explore other cell types.

In essence, my lab's efforts revolve around three main pillars. First, we are exploring how to target cancer cells more efficiently and selectively. We are working on both methods and receptor types that allow us to target cancer cells in a highly selective way, ideally sparing healthy cells and enabling us to reach a wide range of cancer cells.

The second area we focus on is guiding T cells to areas of interest. In our case, this is primarily the cancer or its metastases. We are developing strategies to selectively direct T cells toward these sites, with one prominent example being our work on chemokines and chemokine receptors, which is a major area of interest for my team.

Lastly, we are addressing the issue of dysfunction or immune suppression at the cancer site. We aim to overcome the immune suppression that cancer can cause, so our therapeutic cells can effectively target the cancer cells. To achieve this, we use engineering to design new receptors and modify cellular components to enhance desired functions or prevent suppressive effects at the cancer site.

Q What are the current key challenges preventing the success of CAR-T cell therapies in solid tumor indications?

SK There are, in my view, three major scientific or biological challenges to face when designing or developing a therapy for solid cancers.

The first, which is a more global drug development concept, is how to deliver the drug to the target site. Since we are dealing with therapeutic cells, which are living drugs, how can we bring these cells to the cancer site, metastases, or any critical area for treatment? This requires a selective, specific approach. If the cell therapy does not reach the necessary site, there is virtually no chance it will be effective. This concept applies to any drug: if it does not reach its action site, it will not effectively treat the disease.

The second biological challenge is enabling T cells or immune cells to recognize cancer cells properly. If these cells cannot identify their target, they cannot be effective. This issue is especially challenging in cellular therapies for solid tumors due to disease heterogeneity. Solid tumors evolve over long periods, allowing for multiple mutations and evolutionary changes before treatment is possible. We still lack a robust solution to address this heterogeneity while targeting most or all cancer cells and sparing healthy cells.

The last challenge is overcoming immune suppression at the cancer site. This is, perhaps, the most difficult challenge. Suppression can happen through direct physical interactions—physical, soluble, or otherwise—or indirectly, as immune cells may lose function due to biological limits. These cells were not necessarily designed to perform the sustained functions we expect of them in therapy. This suppression is often driven by cancer but can also result from the natural life cycle of an immune cell. We need to address both aspects when designing therapies; otherwise, even the most specific and selective therapy will not perform well if it loses functionality at the target site.

Ultimately, these biological challenges are crucial to solving the problem of cell therapies in solid tumors. But we also face technical, logistical, and financial challenges in making these therapies viable in such indication. While I am confident that solving the biological issues will eventually lead to solutions for these other hurdles, it is clear that we do not yet have all the answers.

Currently, cell therapies are costly, labor-intensive, and time-consuming to produce. Manufacturing these products requires complex logistics to transport cells to and from

"...we need solutions that make cell therapy production, availability, and process development more efficient and affordable for health systems globally."

patients, high-quality reagents (which are often in short supply), and adequate production facilities. These factors can create access barriers for patients, especially in cases where the disease progresses too quickly to allow for cell collection or expansion.

This complexity results in a high price tag, with cell therapies estimated to cost $300,000–450,000 per treatment, excluding hospitalization and other care expenses. If we hope to use cellular therapies for solid tumors, which account for 90% of cancers worldwide and affect millions each year, we need a different logistical and financial model. At the current cost and with existing logistical constraints, widespread application is not feasible. Therefore, we need solutions that make cell therapy production, availability, and process development more efficient and affordable for health systems globally.

Q How can we improve efficacy and persistence in solid tumors for CAR-T cell therapies and other cellular immunotherapies?

SK The potential of cellular therapies lies in overcoming several key challenges to achieve their promise—providing a one-time treatment that delivers lasting therapeutic effects. As I mentioned, current issues include technical, logistical, and financial challenges as well as biological ones. Re-dosing or giving additional doses of the same therapy is not typically effective if the initial treatment fails, as this likely indicates an inherent biological mismatch. However, in some specific cases, repeated dosing has shown individual success, though it is not broadly applicable.

An alternative approach may involve targeting earlier stages of cancer when the immune system is less compromised and more responsive. Evidence from immunotherapy trials, particularly with checkpoint inhibitors in diseases like melanoma, suggests that treating patients at earlier stages can enhance response rates. In hematologic cancers, moving CAR-T therapies to earlier treatment lines has shown promise in improving outcomes. Finally, a more experimental approach that many researchers are exploring is enhancing cellular efficacy and longevity through genetic and cellular engineering, potentially enabling therapies to overcome immunosuppressive barriers and extend the cells' therapeutic lifespan.

Q Can you go into more detail surrounding the add-on strategies for cellular therapies in solid tumors? What approaches are the Kobold Lab investigating, and where is promise being seen?

SK In the field, we are seeing clinical evidence that cellular therapeutics may be effective for solid tumors. For a long time, clinical successes in the

"One of the most exciting developments is that we now have two approved products for solid tumors in the USA, which, though not yet approved in Europe, indicate clear evidence of efficacy."

solid tumor space when treating patients with cells have been elusive. However, this has changed significantly over the last 2–3 years, after nearly two decades of limited breakthroughs.

One of the most exciting developments is that we now have two approved products for solid tumors in the USA, which, though not yet approved in Europe, indicate clear evidence of efficacy. One of these is the use of tumor-infiltrating lymphocytes (TILs) for melanoma patients who are refractory to immune checkpoint inhibitors. Two products have been successful here: the first is an academically developed TIL therapy from the Netherlands Cancer Institute (NKI) and National Center for Cancer Immune Therapy (CCIT) in Copenhagen. Their Phase 3 randomized trial met its primary endpoint of progression-free survival, with indications that overall survival may also be significantly improved. The second product is a commercial TIL therapy approved in the USA, which has shown similar results and underscores that cell-based immunotherapies can benefit even those patients who have failed checkpoint inhibition.

Another promising development is the approval of a T cell receptor-engineered cellular product targeting MAGE-A4 in refractory synovial sarcoma. This product, approved in the USA in the summer of 2024, is based on positive results from a Phase 2 clinical trial, representing a breakthrough for patients who previously had few or no effective options.

There are two other notable clinical achievements, though not yet approved, that are worth mentioning. One is a GD2-specific armored CAR, with added cytokine support and a safety switch, which was developed within the pediatric oncology field for neuroblastoma. This CAR-T therapy has delivered impressive response rates, including complete remissions in young patients who had no remaining treatment options. The other exciting advance is a CAR therapy combined with an mRNA vaccine, targeting claudin, which has shown promise in refractory testicular cancers. This combination suggests that cellular therapeutics could have a broad impact across different solid tumor types.

In our lab, we are working on three key areas. First, we are investigating chemokine receptors to improve T cell recruitment to tumors by restoring a match between tumor-expressed chemokines and the receptors on T cells. We have demonstrated that this approach can enhance T cell recruitment to tumor tissues and support therapeutic effects across a wide range of cancers, and we are now moving toward clinical development. Second, we are using computational techniques on large datasets to identify cancer-selective antigens, which led us to discover new targets in acute myeloid leukemia. We have already designed CARs targeting these antigens and confirmed their potential in a range of models, including patient-derived ones, and we are eager to take this into clinical trials. Finally, we are addressing immune suppression by engineering fusion receptors to convert suppressive signals into stimulatory ones. One prominent example is our PD-1/CD28 fusion receptor, which transforms PD-1's inhibitory signal into a CD28 costimulatory signal. This strategy, patented with our US partner, has already advanced to a Phase 1/2 clinical trial, showing the potential of modifying immune suppression pathways to enhance the efficacy of cellular therapeutics.

Q What technologies are used to modulate the tumor microenvironment and how can we overcome the associated physical, chemical, and biological challenges?

SK To begin, it is helpful to differentiate between technologies that can be applied to the therapeutic cells themselves and those applied directly to the tumor or patient environment. Focusing on cell engineering, especially for therapeutic applications, we have seen a remarkable expansion in techniques over recent years. This includes methods for receptor engineering—encompassing protein biochemistry, receptor design, and targeting strategies—to direct therapies to specific cell types and regions within the genome. Additionally, different delivery methods, from viral to non-viral approaches, use DNA, RNA, or nucleases to edit target cells precisely.

Beyond cell targeting, we are also exploring technologies that allow selective control over cells, such as on-off switches or adaptable modules that can manage or even remove cells if needed. This list is far from exhaustive, but it highlights some of the diverse techniques currently in use or under development for cell modification.

In contrast, the technologies available to directly modulate the patient's environment or tumor are in a much earlier stage. Traditionally, approaches like chemotherapy or radiation—applied either locally or systemically—are more generalized, though still among the best options for preparing a patient's environment to support subsequent therapeutic cell application. However, newer advancements are emerging, such as different forms and delivery depths of radiation, as well as small molecules or agents that target specific pathways locally or systemically.

It is essential to explore these options further in the context of cellular therapies, as traditional drugs (like tyrosine kinase inhibitors developed for oncogenic signaling) do not necessarily translate seamlessly to cellular therapeutic settings. While there is a promising range of strategies in development, much remains to be learned and innovated to refine these approaches effectively.

Q CAR-T cell therapies have seen much more success in liquid cancers including leukemias. Can you assess the state of affairs here?

SK In the field of hematology, CAR-T therapies have firmly established their place, with seven approved products currently available. Additionally, there are numerous other products in advanced stages of regulatory development, depending on the region of the globe. Many of these upcoming therapies target similar indications, such as B-cell malignancies, plasma cell disorders, or myeloma, and are nearing approval.

However, I am uncertain about the true innovation offered by some of these new therapies, as many still target the same antigens—namely CD19 and B-cell maturation antigen (BCMA)—that existing products already address. One significant trend we have observed is the push to use CAR-T therapies in earlier lines of treatment. Although not a large number of trials have explored this shift, those that have are showing promising results. These trials suggest that CAR-T therapies can maintain their strong efficacy even when used earlier in the treatment pathway. In some cases, these early-line CAR-T treatments may

even outperform conventional therapies, potentially reducing the need for patients to undergo the more burdensome traditional treatments before becoming eligible for CAR-T.

That said, outside the current success seen in B-cell malignancies and myeloma, the effectiveness of CAR-T therapies in other hematologic conditions—like acute myeloid leukemia—has been limited. This highlights a significant opportunity for improvement and innovation in hematology, as the success seen in B-cell-related cancers has not yet been replicated across other indications.

Q What are your key goals and priorities for the next 12–24 months?

SK One of our current initiatives is a project in Germany called the BAYCELLator, the Bavarian Cell Therapy Catalyst. This collaborative effort aims to accelerate the transition from exploratory research to clinical development by creating a plug-and-play system. The goal is to leverage established production tools and protocols, adapting them to specific research concepts. While this approach still demands significant work and funding, we believe it has the potential to speed up development timelines here in Germany.

In addition to this, a major objective is to broaden our focus to include more diseases and strategies on a larger scale. By doing so, we hope to address some of the limitations we previously identified and push the boundaries of what is possible in cell therapy.

BIOGRAPHY

Sebastian Kobold studied medicine in Homburg, Zurich and Bordeaux. He completed his doctoral training in tumor immunology with C Renner in Zurich and Homburg. Dr Kobold did his residency training in hematology, oncology and clinical pharmacology in Hamburg and Munich. Since 2014, he is a board-certified clinical pharmacologist and immunologist and serves as attending physician at the university hospital of the LMU, Munich, Germany. Dr Kobold trained as a post-doctoral fellow in Hamburg (D Atanackovic), Munich (S Endres) and Boston (K Wucherpfennig). In 2019, he was appointed Professor of Medicine and Experimental Immuno-oncology at the LMU Munich and as Deputy Director of the Division of Clinical Pharmacology at the University Hospital of the LMU. The research of Professor Kobold is focused on developing innovative immunotherapeutic strategies utilizing T cells for cancer treatment. His research has been internationally recognized with among others the Ernst-Jung Career Advancement Award, the Vincenz Czerny Award for cancer research, the Johann-Georg-Zimmermann Award for cancer research and more recently the Wilhelm Vaillant Award for Medical Research. Dr Kobold also serves as academic editor to the *Journal for the Immunotherapy of Cancer*, *ESMO IOTECH*, *Frontiers in Immunology*, and *Frontiers in Oncology* and reviews for international leading journals and grant funders including the *Nature* and *Science* publishing groups.

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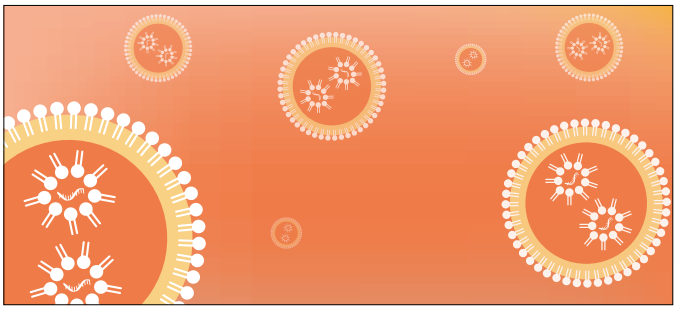
CELL & GENE THERAPY INSIGHTS

CONTENTS VOLUME 11 · ISSUE 1



CELL & GENE THERAPY INSIGHTS

REGULATORY INSIGHTS

Regulatory innovations: why is the UK an attractive hub for CGT development?

INTERVIEW

"...the UK has representation in nearly 50% of all European ATMP trials"

Jokūbas Leikauskas, Editor, BioInsights, speaks to **Rehma Chandaria**, Senior Regulatory Affairs Lead, Cell and Gene Therapy Catapult, about the UK's position as an attractive hub for cell and gene therapy (CGT) development, recent Medicines and Healthcare products Regulatory Agency (MHRA) initiatives reshaping the regulatory framework, and the global implications of post-Brexit regulatory divergence.

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Q Can you tell us a little about your background and current role, and what sparked your interest in the regulatory aspects of CGTs?

RC I started my career as a pharmacist before transitioning into the laboratory as a research scientist. I completed a PhD in tissue engineering and later worked as a scientist specializing in CAR-T cells at a biotech company. Both roles sparked my interest in CGTs and are still very relevant to my position today.

In 2018, I shifted away from the laboratory into a dedicated regulatory affairs position within a regulatory consultancy. Since then, I have focused on the regulatory aspects of CGT products, which has led to my current position as a Senior Regulatory Affairs Lead at Cell and Gene Therapy Catapult. In this role, I work as part of the broader access strategy group, which besides regulatory affairs, also includes the nonclinical, health economics and market access teams.

Together, these three teams collaborate to support CGT developers—often in early stage of development (e.g., academics or small biotech companies) but also pharma organizations that seek input around managing the unique challenges that CGTs present. We provide strategic and operational guidance to help optimize their development programs and maximize the adoption potential of their advanced therapy medicinal products (ATMPs).

In addition to supporting developers, we also engage in what we call 'environmental shaping', which involves optimizing translational processes and creating a favorable environment for the adoption of ATMPs. While our efforts have a global perspective, there is a particular emphasis on advancing these goals within the UK.

Q Why is the UK an attractive place for CGT developers, especially regarding regulatory aspects?

RC To begin with, I would like to highlight some data demonstrating the growth of the CGT sector in the UK. According to data recently published by CGT Catapult, there were 187 ATMP clinical trials ongoing in the UK in 2024, representing a 7% increase from the previous year (175 trials in 2023). The data also shows that the UK has representation in nearly 50% of all European ATMP trials [1]. This is particularly noteworthy given the regulatory changes following the UK's exit from the EU. Developers now need to submit a separate clinical trial application in the UK. The fact that so many developers are doing this indicates that there are clear advantages to including UK sites in their clinical trials.

One of these advantages is the timeline for clinical trial application (CTA) reviews. In the UK, the statutory timeline for combined regulatory and ethics review for ATMPs is 90 days, compared to 156 days in the EU. Additionally, the MHRA has been publishing performance data for CTA reviews, which shows they consistently outperform statutory timelines on average [2].

Additionally, the UK has the Advanced Therapy Treatment Centre network, coordinated by CGT Catapult. This network fosters strong relationships between the NHS and industry, increasing the capacity to deliver CGTs to patients. These efforts have improved clinical trial infrastructure, making the UK an increasingly attractive place for ATMP clinical trials.

Furthermore, the MHRA has expressed its commitment to being an 'enabling regulator'. This is evident in their pragmatic, science-driven, and risk-proportionate approach to regulatory reviews. At CGT Catapult, we have repeatedly observed this during interactions with MHRA to support developers. The agency consistently considers scientific rationales and provides risk-based guidance. A key example of this was in 2023 when the MHRA became the first regulator in the world to approve Casgevy®, the first ATMP using CRISPR-Cas9 gene editing.

"...the MHRA is piloting a clinical trial lifecycle package approach."

Q In the past couple of years, the MHRA initiated new procedures for CGT developers. How are these initiatives reshaping the field?

RC The MHRA has been launching new initiatives recently, many of which are particularly important for ATMPs. One notable example is the first-ever regulatory framework for point-of-care and decentralized manufacture, which was laid before Parliament in October 2024 and is planned for implementation in 2025. This framework aims to address logistical challenges associated with ATMPs such as autologous cell therapies, enabling manufacturing to occur closer to clinical sites rather than transporting the materials to and from a central manufacturing facility.

Another significant initiative is the MHRA's new clinical trials regulation, laid before Parliament in December 2024 and planned for implementation at the start of 2026. This new regulation will embed the single regulatory and ethics review route, which is already in use, into legislation. It will also introduce research transparency requirements, align with global ICH Good Clinical Practice guidance, and streamline safety reporting and pharmacovigilance requirements.

Additionally, the MHRA is piloting a clinical trial lifecycle package approach. This initiative is designed for developers who wish to conduct the entire lifecycle of their clinical trials in the UK, from Phase 1 through to Phases 3 and 4. Under this approach, the MHRA would provide enhanced services, including more input at key points throughout clinical development. Developers would need to submit a complete application adopting the combined phase approach and include their plan for all subsequent phases, designating the UK as the main center. As part of this approach, sponsors would receive full scientific advice meetings during Phase 1, as well as additional guidance as needed during Phases 2 and 3.

Finally, it is important to mention the Innovation Licensing and Access Pathway (ILAP). While ILAP has been active for several years, it has recently relaunched, with new, more selective criteria. The relaunch includes collaboration between the MHRA, the National Institute for Clinical Excellence, health technology assessment bodies, and the NHS. This integrated approach aims to involve all key stakeholders from the early stages of clinical development, ultimately facilitating the adoption of innovative therapies.

Q How do you see changes in regulatory alignment and divergence impacting CGT development in the UK?

RC The EU pharmaceutical reform is currently underway, which involves changes to legislation in the EU [3]. One aspect of this reform is the updated definition of a gene therapy medicinal product. Currently, both the UK and EU definitions of a gene therapy medicinal product specify that it must contain or consist of a recombinant nucleic acid sequence that is administered to regulate, repair, replace, add, or delete a genetic sequence. However, this definition is expected to be broadened under the new EU

"As the UK is not part of the EU pharmaceutical reform, this presents an opportunity to either maintain the existing definition or diverge from it."

pharmaceutical reform to include both synthetic and recombinant nucleic acids, rather than being limited to recombinant ones.

Another critical change is the distinction between gene therapies that edit the genome and those that do not. This differentiation is important because it impacts public perception of these products. Many people associate gene therapy with human genome editing, which is not always the case with the current definition that also includes therapies such as mRNA-based treatments. By explicitly differentiating between genome-editing and non-editing gene therapies, the reform could shift both the regulatory landscape and public understanding of what constitutes gene therapy.

As the UK is not part of the EU pharmaceutical reform, this presents an opportunity to either maintain the existing definition or diverge from it. The MHRA will need to evaluate how best to approach this to support the development and adoption of gene therapies.

The MHRA is addressing regulatory independence with a pragmatic, risk-proportionate approach, reducing burdens on developers through initiatives such as the recognition approach, which streamlines reviews for products already approved by trusted regulators.

Additionally, the MHRA is leveraging collaborative and work-sharing initiatives, such as the Access Consortium and Project Orbis for oncology products. These programs allow developers to submit a single application reviewed concurrently by multiple regions, streamlining the process.

Q What are your goals and priorities over the next 1–2 years in your work?

RC At CGT Catapult, one of our current initiatives is the creation and operation of the UK Regulatory Innovation Network for Advanced Therapies, which was launched with funding from Innovate UK and support from the MHRA.

The purpose of this network is to promote collaboration between regulators, industry, and researchers, to ensure a world-leading regulatory environment for advanced therapies in the UK. CGT Catapult is leading this initiative in collaboration with the University of Birmingham. We have secured funding for the next year, and we aim to sustain and expand the network beyond this initial funding period to ensure its long-term impact.

The Network aims to support the UK to build a world-leading regulatory ecosystem that welcomes and encourages healthcare innovation. By working with stakeholders across the CGT industry, we aim to identify opportunities to optimize the regulatory landscape and streamline processes to enable the timely development of, and improved access to, safe and effective advanced therapies.

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BIOGRAPHY

Rehma Chandaria joined the Cell and Gene Therapy Catapult, London, UK in November 2023. Prior to this, Rehma worked in regulatory consulting, supporting companies with the full scope of US, EU, and UK regulatory affairs. During this time, Rehma provided technical and strategic support to small and medium sized companies developing novel medicinal products included cell and gene therapies. Rehma previously worked with CAR-T cells as a scientist at Autolus and has a PhD in Tissue Engineering.

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CELL & GENE THERAPY INSIGHTS

BUSINESS INSIGHTS

CONFERENCE INSIGHT

The Nordics are dancing on

Helena Strigård

BIO-Europe celebrated its 30th anniversary in Stockholm, Sweden, on November 4–6, 2024, bringing together over 5,000 delegates from around the world.

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Stockholm in November is usually not the brightest place on earth, but this year's BIO-Europe delegates got lucky. The sun was shining over the capital of Sweden as the partnering event opened its doors to the 30th edition of the conference, filled with sharp minds from all around the world including a strong line up of cell and gene therapy (CGT) actors. In the mix of research companies, service providers, investors, and academics, the feeling of excitement and expectation was thick in the air. The ability to meet in person post-covid at these huge events is still an excitement in itself, and the ABBA-themed program contributed to the good spirit. However, another element of tension was present and tangible, as C-suite leaders were rushed off their feet in the partnering area; the sentiment of 'now or never' lingered.

WHAT'S THE NAME OF THE GAME?

In the wake of the pandemic years, the sharp decline in available funding took life science companies by surprise. As always, it has taken a while before the full spectra of ripple effects have played out in the industry. The venture capital (VC)-dependent companies did a good job in 2022 to keep up the image of business as usual, but soon enough, burn rates started to take a toll on **human capital** [1]. Still, many service providers were walking straight into the 2023 budget year with a smile on their faces, not realizing that the resource they live on—R&D companies—were already hitting the breaks.

WINNER TAKES IT ALL?

The short answer is that yes, the winner takes it all. With financial actors still holding their money in a tight grip, only the most eligible companies come out of the partnering booths with a decent shot at reaching their next milestone. It's not as easy to sell a vision as before. From the many voices that were heard to research this article, the importance of being able to show solid data came up frequently. 'Show me your ass', as

one US-based investor put it, referring to the assets that must be available up front. Grand words will no longer cut it.

MONEY, MONEY, MONEY...

The Nordic region is an often-overlooked life science hot spot where each Nordic country on its own is often considered too small of a market, yet jointly form one of the most innovative regions in the world. And with good science and accurate, somewhat humble business plans, trust is building in our offering. One promising Swedish life science company is raising 7 million SEK (a little over US$600,000) to take them to the next infliction point. The resilience and low-key aspirations for funding (combined with a genuine Nordic trait of dilution-aversion) has helped the space to stay alive. A recent publication from Swedish Innovation Agency Vinnova showed the remarkable growth of life science companies over the past two decades [2]. Even so, many companies are running on fumes and to be successful, ultimately one must go for the big money, which there is not enough of locally.

DANCING QUEEN

So, will the Nordic CGT companies be successful? Only time will tell. The local VC actors are keeping busy, networking and syndicating globally to create an influx of intelligent capital towards the hidden CGT-gems of the Nordics, using events such as BIO-Europe to showcase opportunities. Hopes are up amongst investors and R&D companies themselves. Based on the record high influx of CDMOs establishing manufacturing plants on Nordic soil [3], it seems that service providers have picked up on the tune being sent out to the world: the Nordics are dancing on.

VOICES FROM THE EXHIBITION FLOOR

Dr Eva-Karin Gidlund, CBO, NorthX Biologics
Dr Roger Lias, Global Head Commercial, Biologics, Aurigene Pharmaceutical Services Ltd

Q What's hot and what's not in ATMP? Insights from service providers, investors, and academics at the 2024 BIO-Europe conference in Stockholm.

EG The field of ATMPs has experienced a fascinating evolution in recent years, particularly as we transition out of the intense focus brought by the pandemic.

During COVID-19, mRNA became the star modality, a game-changer that accelerated timelines and delivered unparalleled results. Now, we see the landscape broadening beyond mRNA.

The current trend is not only about finding the next big modality; it is about leveraging a mix of old and new approaches. For instance, bacterial systems, long regarded as 'old-school', are regaining attention because they offer robust, cost-effective platforms that complement newer technologies. Alongside this, we see exploration into next-generation formulation methods and delivery systems. It's about pathways to success, not a singular route.

This diversification is exciting because it allows companies to find approaches tailored to their specific needs and challenges. We've observed renewed interest in recombinant proteins and exosomes, particularly for markets like the USA, demonstrating how legacy technologies can still meet critical unmet needs.

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RL Despite the current 'funding crunch' and a litany of ATMP companies reporting their struggles, the field undoubtedly remains hot as evidenced by activity at BIO-Europe and, more recently, the Biomanufacturing World Summit in San Diego. The potential for this diverse class of products remains compelling; approvals continue and clinical data continues to be compelling and even potentially 'curative'.

Putting on my 'manufacturing hat', we see that the plasmids market remains buoyant and the mRNA space has cooled and settled a little on the back of the pandemic. Gene therapies continue to push forward with encouraging results and are still largely dependent on AAV and lentivirus for their success. Manufacturing these products remains challenging but, equally, we have recently seen supply outstrip demand at specialist CDMOs and some continue to struggle. We watch the various 'non-viral' delivery technologies under development with interest.

Things get even more chaotic in the cellular therapy space, where we see a very broad range of therapeutic approaches and product types. Things can be especially challenging for autologous therapies, where the now tried and tested approaches and methods for traditional biologics manufacture simply do not apply. Allogeneic approaches have yet to play out and regenerative cellular therapies add even more complexity. While we are seeing innovative manufacturing approaches being developed, my opinion remains that, for the moment, cell therapy manufacture remains 'the wild west'.

All of this translates into a cost of goods that is currently untenable for most potential indications. This must become an area of significant focus if these innovative products are to reach patients.

Q Would you say that smaller companies are less influenced by trends now compared to the COVID years, when everything centered around RNA modalities?

EG Absolutely. During the pandemic, the urgency to respond drove smaller companies to adopt RNA modalities almost by necessity. Speed was critical, and RNA provided a pathway that fit that need perfectly. Post-COVID, however, we're seeing a shift.

I see more and more that small companies now dare to focus on what they do best, aiming for excellence within their niche areas rather than chasing trends. This is not to say they are ignoring innovation, but rather that they are more selective. Instead of pivoting to fit the modality of the moment, they are looking at what they already have intellectual property, platforms, or expertise, and asking how they can optimize these assets.

RL Yes. COVID (quite understandably) created an unusual market and many smaller companies did some significant 'lane changing' for either altruistic or opportunistic reasons. Across the biopharmaceutical industry, the COVID hangover is still having a bigger impact than I think many realize. In the current funding environment, focus becomes critical and data is king. Companies (both big and small) cannot currently afford to diversify significantly are, by necessity, focusing on core strengths. There remains a tendency to chase niches that have recently demonstrated success.

One interesting note regarding AI, which is front and center of so many discussions at the moment. Investors that I have talked to are saying that unless it is absolutely

fundamental to your model, do not even mention it in your pitch decks—simply mentioning it is not a differentiator!

Q A lot of companies here at the conference are talking about AI. But do we know how to integrate it in a truly meaningful way in ATMP?

EG AI is undoubtedly one of the hottest topics at BIO-Europe this year, and for good reason, it holds immense potential for transforming how we approach ATMP development. Not just from a technical point of view but rather from an organization and full HR perspective which will in the end affect the outcome of innovation. That said, integrating AI meaningfully requires us to address fundamental challenges in how we use and share data, work more efficiently and get help in always staying ahead with the newest trends technologies and science.

As an example, many companies now are still duplicating the same experiments, even when valuable data already exists. This inefficiency stems from a lack of frameworks for sharing and utilizing data across projects or companies. AI gives us the opportunity to break that cycle. By designing smarter experiments, using predictive models, or building on pre-existing knowledge, we can significantly reduce time and cost in development.

For AI to truly make a difference in ATMP, we need to think holistically. It is not just about solving isolated problems but creating systems that can integrate and refine the entirety of the development process, from early-stage design to manufacturing. I am a strong believer that integrating cutting-edge technologies like AI enables us, within life science and health care, to innovate and learn from the past as well as embrace the future.

RL AI is a huge topic and it can be applied in so many ways across our marketplaces, so I think the question requires significant nuance. There is clearly great excitement about the potential for AI in drug design and discovery, but while I am seeing some progress in the small molecule space, my opinion is that we are still a long way from demonstrating its utility for more complex biologics and ATMPs. That does not mean that there isn't any interesting work going on.

AI (and I think we can lump-in machine learning, digital twinning, and other broadly digital approaches) has much more immediate applicability in the manufacture of these products, although the need for consistent supporting and consistent data sets remains a challenge. We are undoubtedly, however, already reaping some 'AI benefits' in support of documentation, quality systems/analysis, and supply chain.

To answer your question more specifically: the cost per data point and the need for big data to truly push these technologies will mean that the big players will likely have to show the way with respect to integrating in a truly meaningful way. I don't think that we have yet grasped the potential and we have certainly not yet seen good implementation, but I have no doubt that it is coming!

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BIOGRAPHY

Helena Strigård is the founder of Ventures Accelerated, Stockholm, Sweden a conglomerate of angel investors and seasoned life science executives supporting life science companies on their growth journey. She has held several leadership positions in the life science industry, notably being the CEO of the Swedish life science industry organization SwedenBIO, pharmaceutical manufacturer NorthX Biologics, Executive Search Firms along with multiple board positions in the industry. Previously, Helena had assignments ranging from Head of Section at Swedish Ministry of Finance to advisor roles with the Confederation of Swedish Enterprise, Business Europe in Brussels and Innovation Agency Vinnova. Helena has a background as an economist and holds a Master's degree in Molecular Biotechnology.

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CELL & GENE THERAPY INSIGHTS

MANUFACTURING SCALE-UP

Addressing challenges in AAV manufacturing scale-up for cost-effective gene therapies

INTERVIEW

"Currently approved therapies have a very high price, and much of that is driven by the expensive raw materials and costly manufacturing processes."

Jokūbas Leikauskas, Editor, BioInsights, speaks to Andrew D Tustian, Executive Director, Preclinical Manufacturing and Process Development, Regeneron, about the advancements in AAV process development from preclinical to commercial stages, addressing challenges in scaling, automation, and cost reduction, while prioritizing the delivery of innovative gene therapies to patients.

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Q What are you working on right now? What are the key areas of focus in your group at Regeneron?

ADT The Viral Vector Process Development group at Regeneron has three major responsibilities. We produce viral vectors for preclinical studies in our pilot-plant in Tarrytown, NY, USA. We also handle all phases of AAV-based viral

vector process development. Finally, we manage the technology transfer of the processes we have developed to GMP manufacturing sites. So far, we have exclusively transferred to CMOs as Regeneron currently has no in-house AAV manufacturing capabilities.

We have just finished our first-ever formal process characterization studies, for our otoferlin program, which targets a specific gene mutation that causes congenital deafness. It is an exciting project, where a one-time treatment could potentially allow a deaf person to hear for the rest of their life. For process characterization, we have been using a quality-by-design approach, following principles outlined in Project A-Gene, a paper published by the National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) and Alliance Regenerative Medicine (ARM) in 2021 [1]. We began by defining the critical quality attributes (CQA) of our product. After this, we risk assessed which of these CQA could be impacted by each manufacturing unit operation. Finally, we risk ranked process parameters within each unit operation that might influence the CQA or other process outcomes such as yield. We designed comprehensive multivariate and univariate studies to characterize and define critical process parameters for each unit operation. This detailed process understanding is crucial for advancing the therapy towards eventual approval.

Another project we have recently completed is Regeneron's Factor IX protein (FIX) program for patients with hemophilia B. This therapy involves a combination of an AAV and a lipid nanoparticle (LNP) from our collaboration with Intellia. When we developed the initial process back in 2020, the viral titers were at a certain level, but now, with 4–5 more years of experience, we have managed to increase those titers more than tenfold by optimizing the upstream process. Since this therapy requires systemic administration, the dosage per patient is high and the previous process would have been very expensive per patient in terms of cost-of-goods.

In addition to these two late-stage programs, we have multiple early-stage programs in the preclinical phase, where we are starting process development to prepare for GMP process transfers to CMOs. In summary, we are working across the entire spectrum—from early-stage process initiation to late-stage process development, and finally process characterization.

Q Can you describe how needs differ in developing processes for the production of viral vectors from preclinical stage to late and commercial stages?

ADT For preclinical use, the material does not need to be produced in a GMP facility. However, any changes between toxicology lots and clinical lots should be assessed for their impact on product safety. In essence, even though preclinical material is made in a less regulated environment, it must follow the same process as used to produce clinical material, or at least follow a process that produces comparable material.

When you produce material for clinical or commercial use, it must be manufactured following GMP principles in a GMP-certified suite. This process is governed by regulatory guidelines. Comparability must also be established if there are any process changes or transfers in the clinical stage. For example, in our FIX program, we have developed a late-stage commercial process with modifications to maximize productivity. Because of these

"Even though many of these therapies require only small amounts of viral vector due to localized administration, the overall material requirements can still be significant."

changes, we need to show that the material produced from the late-stage process is comparable to the early-stage material.

Q What do you see as the critical key challenges in both tech transfer and the scaling up of viral vector manufacturing, specifically AAV?

ADT One of the main challenges with AAV is the high cost of raw materials, the expense of running the processing campaign in GMP suites, and the relatively low productivity one can achieve. Currently approved therapies have a very high price of millions of US dollars per dose, and much of that is driven by the expensive raw materials and costly manufacturing processes. As a result, a critical hurdle is being able to produce enough material from a manufacturing campaign to supply the doses needed to make a program feasible.

Another factor people often overlook is the demand for material needed for CMC activities and process validation prior to approval. At Regeneron, we refer to this as independent demand: material requirements independent of clinical need. Even though many of these therapies require only small amounts of viral vector due to localized administration, the overall material requirements can still be significant. For example, in our otoferlin program, the dose is administered directly into the cochlea in the ear, requiring only microliters of material. However, bioburden suitability testing, drug product filter validation studies, assay validation, and so on require large amounts of material.

Additionally, there are logistical issues with tech transfer, especially when working with CMOs. We have had situations where we transferred a process to a CMO facility, only for them to shut down that plant. We then had to transfer the process to another facility within their global organization. The issue with this is that each plant transfer triggers a comparability assessment, requiring the production of additional batches and a full comparability study. This adds considerable time and expense. Another logistical issue is CMO staff turnover, which can slow things down due to a lack of experienced manufacturing operators.

Significant complexities also arise during scale-up due to the nature of the technology itself. For instance, we use triple transient transfection in our AAV manufacturing process. The transfection step, which involves mixing the transfection reagent with the plasmid DNA and adding it into the bioreactor, requires careful consideration during scale-up. There are many scale-dependent parameters in this process that must be tightly controlled.

Finally, a significant hurdle in scaling up is the work required to qualify and validate analytical assays. In order to release lots for clinical use, you need to qualify around 30 different assays, and for commercial use, these assays must be validated. Many of these assays are time- and labor-intensive, requiring analysis of both the AAV capsid structure

"From the upstream process, the proportion of full capsids can vary significantly...The challenge lies in isolating those full capsids from the empty ones."

and the packaged genomic material as well as several residuals. It is an enormous amount of work, and it is easy to underestimate the effort involved.

Q How can these hurdles be overcome to scale up AAV manufacturing for CGT products to reach wider populations?

ADT Regarding the business challenges with CMOs, it is crucial to maintain a close, collaborative relationship with your partners. Pre-booking certain suites in advance can help ensure smoother project timelines. The same principle applies to analytics. From the very beginning, it is important to consider whether you can centralize analytics to streamline the process. In essence, starting early is key—ensuring you have enough time to qualify your analytical assays will help avoid delays in lot release.

Transfection challenges can be addressed by applying robust engineering principles. It is important to carefully assess which scalable parameters are being affected during scale-up. In particular, mixing conditions require close attention to ensure consistent performance across different facilities. It is possible to conduct dye-based studies to evaluate mixing effectiveness, and computational fluid dynamics can also be used to support scale-up.

Q What approaches are you taking/do you see being taken to address the existing challenges in viral vector production? Are there any technologies that you find particularly promising?

ADT There are two key issues that are not necessarily related to scaling up but are more general challenges within the AAV manufacturing process itself.

The first challenge is the separation of the desired product—AAV with the gene of interest encapsidated inside—from capsids that are either empty or partially filled with DNA. From the upstream process, the proportion of full capsids can vary significantly. The upstream percentage can be as low as 5%, with a typical process producing around 20% full capsids while an excellent process might yield over 60% full capsids. The challenge lies in isolating those full capsids from the empty ones.

This separation typically occurs during downstream processing, specifically through ion exchange chromatography. Traditional approaches use gradient separation, which can require large buffer volumes and be difficult to automate because of inconsistent elution profiles due to variations in load material. Isocratic methods are also employed, and can address some of these issues, although they can be very sensitive to the material loading or buffer conditions, which can be difficult to control at scale. We have explored various

technologies and ways of operating to make this step more robust, particularly with respect to loading variations.

We are about to publish a paper discussing a two-step approach to ion exchange chromatography, where the material is loaded once, followed by elution using a series of isocratic steps. We then use process analytical technology (PAT) approach to select pools enriched with full capsids. These enriched fractions are reloaded onto the column, and the process is repeated. This method has resulted in effective full capsid enrichment, even with load material containing <10% full capsids.

Another area I am excited about involves optimizing the first chromatography step in the AAV process. This step typically uses an affinity resin with specificity for AAV—most industry-leading resins feature camelid-derived antibodies attached to the resin matrix. While this step is highly effective, it can be time-consuming, as it can take quite a long time to load the material. Many new affinity formats are currently in beta-testing—not just resins, but also membranes, fiber-based technologies, and other rapid-flow systems. These technologies allow for faster material flow, significantly speeding up this manufacturing step. In addition to these, we are continuously looking at ways to increase viral titers from our bioreactors while simultaneously decreasing the raw material COGs.

Q What role do you see automation and artificial intelligence (AI) playing in the future of viral vector process development and manufacturing?

ADT Starting with automation, the primary area where I see real value is in analytics. Many analytical assays require extensive preparation, and using automated liquid handlers helps in two significant ways: firstly, by reducing variability from operators, and secondly, by decreasing the hands-on time required from technicians. This not only improves consistency but also frees up valuable time for other tasks. AAV release processes involve several labor-intensive assays that are excellent candidates for automation. Automation is also highly beneficial in process development laboratories. For example, during the process characterization for our otoferlin program, we had to run thousands of PCR assays for various studies. Automation plays a crucial role in accelerating these workflows.

When it comes to automating the actual manufacturing process, we are still in the nascent stages. These processes are currently still quite manual. Since many of the therapies target orphan indications and we are moving towards higher viral titers, the number of production runs required may not justify the effort and resources needed to automate them. However, in the future, I anticipate more and more process automation, primarily for process robustness, and particularly through the integration of PAT-based systems with automated process steps.

Regarding AI, it could be particularly useful in analyzing DNA sequences during the initial candidate selection phase. For instance, it might predict secondary structures in DNA or potential truncations during vector production. This could allow us to identify sequences likely to result in partial capsid formation that can be screened out before production. Additionally, AI could assist in analyzing next-generation sequencing data to quantify packaged host cell DNA, plasmid DNA, and other components. That said, a common challenge in applying AI to biology and biotech is the need for large training datasets which is likely to remain a limiting factor in the AAV space.

Q What are your goals and priorities over the next 1–2 years, both for yourself and for Regeneron as a whole?

ADT At Regeneron, our top priority over the next few years is to get our first program across the finish line and approved for commercial use. It is incredibly exciting to be at the point where we can bring a program to patients. Additionally, we are excited about our collaboration with Intellia Therapeutics, using LNPs to deliver CRISPR-Cas9, which cleaves DNA at a specific site to enable gene insertion. The AAV vector then delivers the gene that will be inserted. We are eager to obtain proof-of-concept in patients for this approach in the near future. Looking further ahead, we are developing a strategy to retarget AAVs using antibodies. AAVs often end up in the liver, which can lead to toxicity. To address this, we have an approach that detargets AAVs from the liver and uses antibodies to direct them to the specific part of the body required for therapeutic effect.

In the broader industry, I believe there needs to be a continued development to optimize the AAV platform, particularly in two key areas. Firstly, the ability to redose patients—allowing for an initial dose followed by subsequent doses if gene expression needs to be increased. Secondly, finding ways to dose patients who have pre-existing immunity to AAV, eliminating the need to screen out these individuals.

Personally, my current focus is on reducing the costs associated with AAV process development and manufacturing. Many of our programs target orphan indications, and to make these therapies commercially viable, we need to ensure that the production and development costs allow for patient-accessible pricing. I am particularly excited about how process development can contribute to this, whether through incorporating new technologies to increase titers and yields or through strategic approaches, such as designing 'right-first-time' early-stage processes to eliminate the need for late-stage process optimization and therefore costly comparability studies.

Ultimately, my motivation, and Regeneron's as well, is driven by the goal of bringing new medicines to patients, and seeing our programs progress toward commercial approval, which is the most rewarding aspect of this work.

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BIOGRAPHY

Andrew D Tustian leads the viral vector process development group at Regeneron Pharmaceuticals, Tarrytown, NY, USA focused on developing bioprocesses for adeno-associated virus (AAV) based viral vectors to cure diseases such as hemophilia B, congenital hearing loss, and Pompe disease. Andrew has worked at Regeneron since 2009. Prior to moving to gene therapy Andrew co-led the purification development group for protein therapeutics, working on Fc-fusion, monoclonal antibody, and bispecific processes. He has worked on the

process development for the FDA-approved drugs Dupixent®, Libtayo®, Inmazeb™, Kevzara®, Evkeeza™ and Praluent®, and helped develop the bispecific antibody platform at Regeneron. Andrew received his doctorate in Biochemical Engineering from University College London, London, UK and his undergraduate Masters in Biochemistry from the University of Oxford, Oxford, UK.

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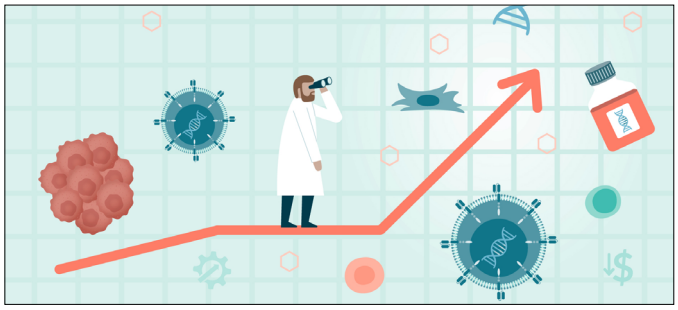
CELL & GENE THERAPY INSIGHTS

CONTENTS VOLUME 11 · ISSUE 1



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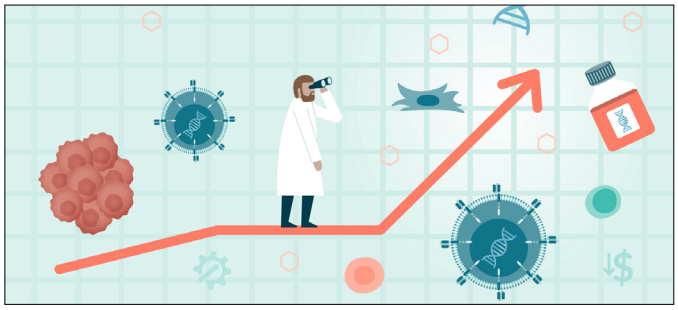
CELL & GENE THERAPY INSIGHTS

CONTENTS VOLUME 11 · ISSUE 1



LATEST ARTICLES

CELL & GENE THERAPY INSIGHTS

THE CGTI FORECAST

New frontiers for cell and gene therapy: addressing 2024 milestones and the outlook for 2025

We bring you insights from four experts across the cell and gene therapy industry, who reflect on the key events of 2024 and share their thoughts on what lies ahead for 2025. From the approval of innovative therapies, such as locally made CAR-T products and new cellular immunotherapy treatments for solid cancers, to breakthroughs in gene delivery methods, 2024 saw many milestones for the field. Looking forward to 2025, there's a shared sense of excitement about transformative clinical data and advancements that could pave the way for new, large-scale therapeutic applications.

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PANEL

Alexey Bersenev MD PhD, Director, Cell Therapy Labs, Yale New Haven Hospital, and Assistant Professor, Yale University
Florence Salmon, VP Regulatory Affairs, HOOKIPA Pharma Inc.
Jason Dowd, Global Lead Cell Therapy Platform Process, Bayer
Chris Bond PhD, Chief Scientific Officer, Notch Therapeutics, Inc.

Q What key events in the cell and gene therapy space that took place over the course of 2024 stand out to you?

Alexey Bersenev First, I want to highlight the approvals of locally made CAR-T products around the world, beyond the US and Europe. In 2024, we saw two more indigenous CAR-T products approved. In Spain, ARI0002h (Cesnicabtagene Autoleucel), an anti-BCMA CAR-T for multiple myeloma. This is an example of point-of-care manufacturing as Hospital Clínic of Barcelona, the developer of the therapy, will be central manufacturing and delivery site and provide oversight of all satellite sites including other hospitals. In China, we saw the approval of Zevorcabtagene Autoleucel, another anti-BCMA CAR-T for multiple myeloma, developed by CARsgen Therapeutics. It will be commercialized with CARsgen and Huadong Medicine in China. These indigenous products offer the advantage of independence to local governments to control the regulatory reimbursement and pricing, increasing patient access overall.

We also saw CAR-T products go to community hospitals, at least in the US. In OUTREACH trial commercial CAR-T cell product Breyanzi was administered across 18 sites including non-FACT-accredited institutions, with resulting meaningful efficacy and favorable safety in community-based medical oncology centers.

Additionally, there was the first CAR-T in vivo application in a human. In October 2024, Interius BioTherapeutics dosed the first patient with an in vivo, lentivirus-based CAR-T therapy to target CD20-expressing malignant B cells. Umoja Biopharma has also received FDA clearance of their IND application for UB-VV111, a CD19 in vivo CAR-T cell therapy for hematologic malignancies.

Finally, there was the first approval of T cell-engineered products in solid malignancies: afamitresgene autoleucel (TECELRA) by Adaptimmune, approved for unresectable metastatic synovial sarcoma.

Q What do you see as the biggest hurdle for the field as we move into 2025?

Florence Salmon Financing, especially in cell therapies. When supporting small companies with fundraising, all discussions boil down to the cost of goods and such therapies being too expensive. Not treating sick people properly is even more expensive, but nobody looks at the costs in a holistic manner like that. “The return on investment is uncertain, too slow.” The problem in cell therapies is that you have to frontload your process development before you go into the clinic and these costs are often not accepted by investors.

The financing model we have in the biotech is: “Move as quick as possible to the clinic, generate proof of concept data, and then profit.” This does not work well for cell therapies, because if you go with a bad process into the clinic, you won´t have meaningful results. Even if something is successful in the clinic, a process that is impossible to industrialize will cost a lot more money, and a lot more clinical data. Outside of big pharma, it is hard for small companies and academics to pass Phase 1 studies, which for me, is the biggest problem at the moment.

Jason Dowd Demonstrating genetic stability of engineered cells is difficult because it is impossible to prove a negative. That is, proving that a very rare event did not happen poses several challenges. The analytical technologies are certainly improving, so I am hopeful

that Regulatory-acceptable solutions can be found. For now, that question remains, especially for cell therapies that are meant to be durable and will live within patients for 10, 20, or 30+ years. We need to be sure that there isn't one cell that's going to grow into a problem. That's a challenge, but also one hurdle that we need to demonstrate.

Q What are you most excited about in the field for 2025 and why?

Chris Bond The clinical data that will emerge in the coming year on lupus, multiple sclerosis, and other impactful autoimmune disorders has the potential to be transformative for the field. We are all waiting to see how robust, durable, and meaningful these responses will be, and whether will we see the profound activity we observe in lymphoma and hematological malignancies translate into B-cell autoimmune disorders.

Another critical question is how we approach treating these patients, as they represent a very different patient population. In the lymphoma setting, these patients are typically older, have undergone multiple rounds of chemotherapy, and are in an oncology setting, which is a very acute, life-threatening situation. The safety window and tolerance for cytokine release syndrome, immune effector cell-associated neurotoxicity syndrome, and other side effects in these patients are very different from, for example, a 20-year-old patient with lupus. Therefore, we must understand how safe the therapies will be, and how we can select the patients to give them the best chance of therapeutic success.

Several companies are moving in the same direction, so it will be interesting to see how broad these responses are. Early signs suggest that autologous therapies are showing promising responses, but it is still uncertain how allogeneic CD19 CAR and pluripotent stem cell (PSC)-based platforms will perform, considering only a small number of patients have been treated with these therapies so far.

Florence Salmon Seeing CAR-T cell therapies moving outside of liquid cancers and into solid tumors and autoimmune disease is very exciting. I am curious to see the development of in vivo CAR-Ts or the emergence of other genetically-modified cells in new disease areas.

BIOGRAPHIES

Alexey Bersenev received his medical education and certification as a general surgeon in Russia. He holds a PhD in Transplantation/Pathology. He had a post-doctoral training in the US in Philadelphia at the Thomas Jefferson University and the Children's Hospital of Philadelphia, where he studied human immunology and stem cell biology. He worked as a cell manufacturing specialist at the University of Pennsylvania and trained in clinical cell processing in a GMP cell manufacturing facility and was involved in the manufacture of CAR T-cell products for clinical trials and technology transfer to industry. He has expertise in manufacturing of cellular products for clinical trials and regulatory submisisons. In addition to his position as Director of the Advanced Cell Therapy Lab at Yale-New Haven Hospital, he is an Assistant Professor of Clinical Laboratory Medicine at the Department of Laboratory Medicine at Yale University, New Haven, CT, USA.

Florence Salmon has a PhD in Molecular and Cellular Biology from the Université Louis Pasteur, Strasbourg, France. She gained considerable experience in cell and gene therapies development and regulatory affairs, bringing many of the early cell and gene therapies from the bench to the clinic and to marketing authorisation. She worked, among other companies, at uniQure on the approval of Glybera (first approved gene therapy) in 2012 and supported the development of a number of AAV-based gene therapy vectors for various disease areas. At Novartis she worked on the worldwide submission and approval of Kymriah (first CAR-T), on the worldwide approvals of Luxturna and Zolgensma, and advised on the development of the cell and gene therapies pipeline. She has participated in building-up several biotech start-ups and in the development of a number of new products in the cell and gene therapy field.

Jason Dowd received his PhD from the University of British Columbia Michael Smith Biotechnology Laboratories, Vancouver, Canada in Biological and Chemical Engineering and has recently completed his MBA, specializing in technology management. Jason has directly mentored and grown progressively larger development & manufacturing teams with over 20 years of experience in diverse companies, launching new services, R&D, and commercialized therapeutic products with multi-hundred million dollars per year revenues. Jason is enabling development, platform and GMP teams to reach their potential and goals. Dedicated to innovation and 'let's make it happen', Jason joined Bayer in August 2023.

Chris Bond is currently the CSO of Notch Therapeutics, Seattle, WA, USA. Dr Bond has worked in the biotech and pharmaceutical industry for over 18 years including work at Genentech, Oncomed, Juno Therapeutics, Celgene, and Kite. Chris has extensive experience in the discovery and development of adoptive cell therapies, monoclonal antibodies, as well as cellular engineering and genome editing. At both Juno and Kite he led the discovery and pre-clinical development of CART and TCR programs targeting solid and hematological tumors. While at Kite Chris oversaw the engineering and discovery of allogeneic cell therapy platforms utilizing T-Cells from both donor derived 3rd party sources as well as induced pluripotent stem cells. Chris has led collaborations with industry leading genome editing companies including Editas Medicine and Sangamo Therapeutics. Prior to his work in adoptive cell therapy Chris led monoclonal and bi-specific antibody discovery and engineering programs targeting cancer stem cell antigens at Oncomed Pharmaceuticals.

Chris received his PhD in Biochemistry from the University of Washington, Seattle, WA, USA in 2000. He is an inventor on numerous patents and has published papers in the fields of protein structure and engineering, immunology, and oncology.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Enabling allogeneic cell therapy development: scaling up generation of PSC-derived NK cells

Mark Kennedy

Induced pluripotent stem cells (iPSCs) provide a potentially unlimited source of cells capable of differentiating into any cell type, making them highly valuable for the development of cell therapy. However, manufacturing of iPSCs is complex and presents many challenges, particularly when scaling up cell cultures to meet the growing demand. This article explores strategies to overcome these challenges and introduces a novel feeder-free protocol for differentiating iPSCs into specialized cells, including induced natural killer (iNK) cells, which have potential applications in allogeneic cell therapies. The article includes a case study demonstrating the functionality of iNK cells developed using the novel differentiation protocol in cancer treatment applications with patient-derived human colorectal cancer tumoroids.

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ADDRESSING CHALLENGES IN PSC CULTURING AND MANUFACTURING

Pluripotent stem cells (PSCs) have the potential to differentiate into nearly any cell type and exist in two forms: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). iPSCs are generated by forced expression of transcription factors that revert somatic cells such as fibroblasts or peripheral blood mononuclear cells (PBMCs) to an ESC-like state, restoring their pluripotency. In essence, iPSCs provide a potentially unlimited cell source that can differentiate into any cell type, making them highly useful for numerous applications, such as studying basic stem cell biology, modeling diseases *in vitro*, drug screening, regenerative medicine, and cell therapy development.

However, translation of monolayer PSC cultures to manufacturing settings may be impractical for some workflows. Hurdles include: the limited availability of fit-for-purpose reagents that meet clinical manufacturing requirements; protocols that can support the scalability needed for manufacturing; the currently limited ability to differentiate PSCs at scale; and the difficulties in maintaining stable PSC characteristics in stressful workflows. To

address these challenges, it is important to utilize regulatory requirement-compliant reagents and media systems, implement protocols that are compatible with bioreactors and suitable for cell differentiation at larger scales, and adopt PSC characterization assays to monitor cell quality and stability.

CELL THERAPY SYSTEMS FOR CLINICAL AND COMMERCIAL MANUFACTURING

Cell Therapy Systems (CTS™) are GMP-manufactured products that have undergone all necessary safety testing, including extensive qualification and testing of critical raw materials, and final product testing for mycoplasmas, endotoxins, and sterility. CTS products are supported by regulatory documentation suitable for reference in regulatory filings, such as Drug Master Files, Regulatory Support Files, validation summaries, and regulatory agency letters. These systems are specifically engineered for clinical and commercial cell and gene therapy (CGT) manufacturing.

A typical PSC workflow is built around the following stages: generation of iPSCs from initial cell type (e.g., fibroblasts) by reprogramming followed by cell expansion, passaging, banking and recovery, and finally, cell differentiation. Each step requires robust characterization methods, and CTS products can be utilized for this purpose. This includes media systems and reagents such as Gibco™ CTS™ StemScale Medium, Gibco™ CTS™ NK Xpander™, and Gibco™ PeproGMP™ cytokines.

SCALING UP PSC MANUFACTURING WITH SUSPENSION CULTURE SYSTEMS

PSCs can be cultured in two different ways: the conventional adherent format and suspension culture. Depending on the cell yield required, adherent culture may be suitable, although this method typically requires more time, effort, and reagents, compared to suspension culture. For example, a suspension culture can achieve $4.5–5 \times 10^9$ PSCs over five passages, whereas adherent culture would require seven passages to reach a similar yield, and thus more time- and reagent-consuming. Overall, suspension culture can be an efficient method to achieve target cell yields while reducing both time and labor.

Gibco CTS StemScale Medium was developed to support PSC growth in suspension culture and enable rapid scale-up. This approach has some key differences from traditional PSC culturing in adherent culture. Firstly, once PSCs are removed from plate surfaces, they need to form aggregates, which can then propagate as cellular aggregates or spheroids. However, the free-floating spheroids require careful handling during media exchange to avoid aspirating stem cells. At smaller scales (e.g., 1–3 L) a gravity settling method with a manual aspiration of the spent medium can be utilized. For cultures exceeding this volume, the gravity settling method becomes impractical, requiring instead perfusion capabilities with specific bioreactor setups.

Another important consideration for suspension culture is understanding when and how to passage the cells, as monitoring confluency is no longer feasible. Instead, the focus shifts to maintaining spheroids at an average maximum size of approximately 400 µm in diameter as an indicator for passaging. Spheroids that are >400 µm in diameter may develop gradients of nutrients, oxygen, and metabolic waste, which can lead to poor culture health, making dissociation of PSC spheroids more challenging.

DEVELOPMENT AND OPTIMIZATION OF PSC SUSPENSION MEDIUM

To support suspension culture and manufacturing workflows, we engineered Gibco

CTS StemScale Suspension Medium to meet the requirements for translational and clinical applications. This culture system is xeno-free and designed specifically to maintain PSC aggregates in suspension without microcarriers. Furthermore, the StemScale Suspension Medium has high expansion capability within a simplified workflow that does not require as many ancillary reagents and tools as some other systems. Lastly, the medium is compatible with various culture formats and vessels, including bioreactors up to at least 3 L. Compatibility with larger formats is currently being assessed.

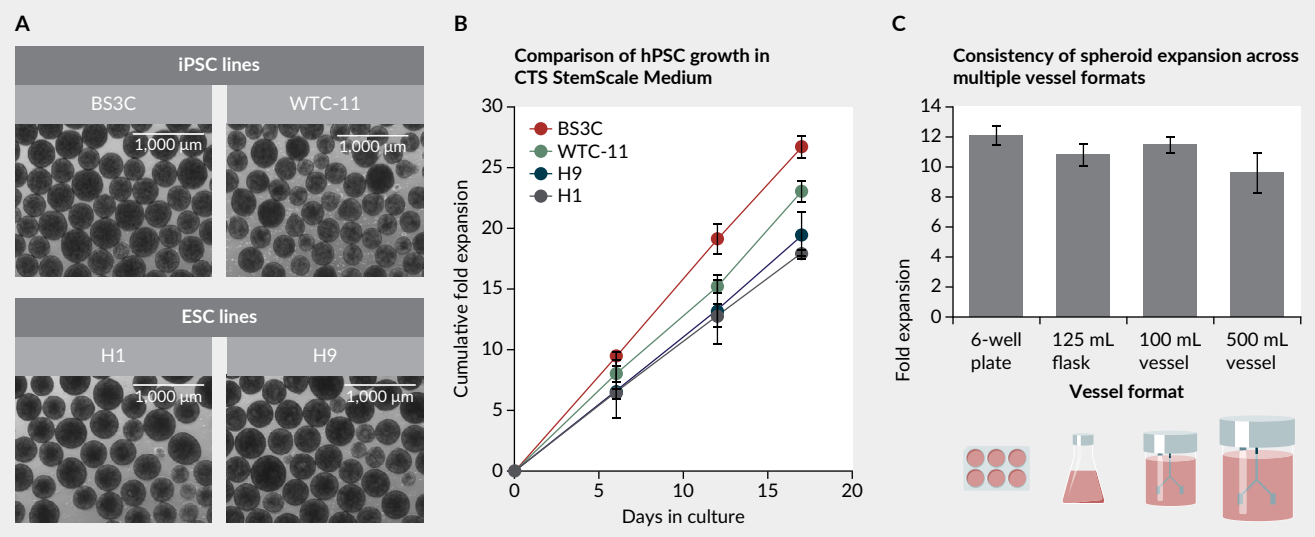
During the development of CTS StemScale Medium, the Research Use Only (RUO) version was re-engineered, and new user protocols were developed. This included redesigning the media formulation while considering changes to recommended seeding density, shake speed, and dissociation methods. Design of Experiments (DoE) was used to explore various medium components and their impact on cell growth in two different PSC lines during media optimization. The components that were found to be beneficial for growth were included in the formulation, while the components that were neutral or had minimal impact were either selected for further analysis or omitted to simplify the final formulation. Similarly, a range of seeding densities was also tested to maximize cell output while minimizing input.

Based on studies analyzing the cumulative fold expansion of PSCs, it was discovered that seeding at 100,000 cells/mL produced the best fold expansion, although it also generated the smallest yield. In contrast, seeding at 300,000 cells/mL resulted in a low cell expansion but a high cell yield. As a result, a starting point of 200,000 cells/mL is recommended for new users, although optimization of a specific cell line is advised. This example highlights the importance of not over-interpreting the fold expansion metric alone, but rather balancing it with cell yield to determine optimal seeding density.

In a further study, shake speed optimization was evaluated. PSCs, when seeded into suspension culture, are typically placed on an orbital shaker that provides agitation to the media, helping cells to

FIGURE 1

iPSC spheroid expansion across multiple cell lines using CTS StemScale Medium.



A and B: the growth of four different PSC cell lines; C: fold expansion of PSCs in different vessels.

aggregate and grow. The shake speed must be optimized, as it can vary across different orbital shaker platforms. Variations in lateral displacement between instrument models can drive differences in the fluid dynamics, affecting spheroid size and growth as well as PSC aggregation. Since passaging is recommended when spheroids reach 400 µm in diameter, this will likely affect the time between passages. Based on internal studies with multiple PSC cell lines it was discovered that the typical time between passages is 5–6 days.

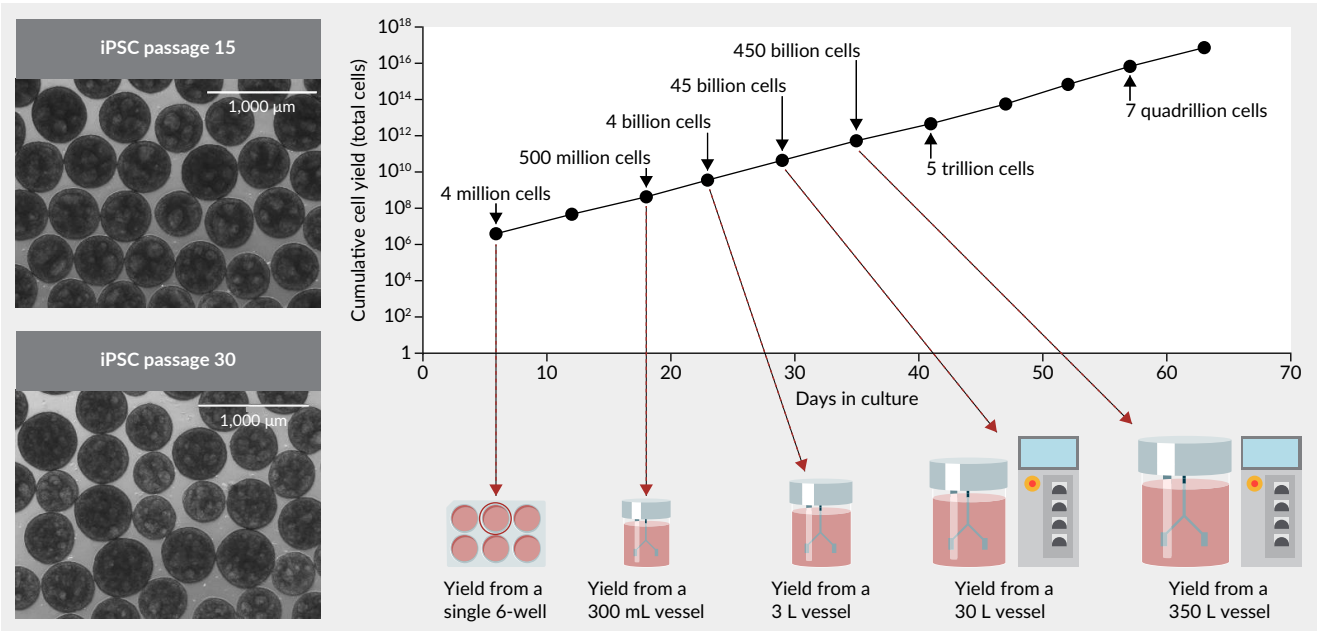
ENSURING CONSISTENT GROWTH AND SCALABILITY OF PSCS ACROSS MULTIPLE CELL LINES

CTS StemScale Medium promotes consistent serial expansion across multiple cell lines, although there are some cell-line-specific growth differences. In this study, four different cell lines were tested using the CTS StemScale Medium (Figure 1A and B). The analysis demonstrated that spheroid morphology and cell growth were both consistent across all cell lines. Additionally, consistent growth was observed regardless of the culture vessel utilized. For example, similar fold expansion was observed in cultures such as a 6-well plate, 125 mL flask, 100 mL vessel, and 500 mL vessel, and in cultures up to 3 L (Figure 1C). This linear trend enables easier scale-up, as it requires fewer steps to re-optimize at each increase in culture volume and allows researchers to project the culture volume necessary to produce a desired cell yield.

The next study found that long-term growth in CTS StemScale Medium was consistent over 30 consecutive passages. Additionally, spheroids maintained round morphology over the culture duration, and large cell yields were possible when seeding new vessels with the entire harvest from the previous passage. A theoretical 10-passage study was also outlined to predict cell yields based on the assumption that all cells are carried forward in the seed

FIGURE 2

Examples of long-term spheroid morphology (left) and the estimated number (right) and of obtainable PSC cells over 10 passages in different vessels using CTS StemScale Medium.



train (Figure 2). This graph underscores the massive cell yield that should be achievable when scaling from 3 L up to volumes that are critical for large-scale manufacturing and commercial workflows. Scaling beyond 2–3 L cultures requires perfusion to maintain healthy spheroid cultures. Preliminary data (not shown here) suggest that perfusion will almost certainly enable significant increases in cell densities, and the likelihood is that the yield for 30 L shown in this projection is an underestimation.

Beyond media system scalability, it was equally important to address whether the CTS StemScale Medium maintains key PSC characteristics. As shown in Figure 3, three different assessments of stem cell pluripotency were carried out. The immunocytochemistry for pluripotency marker Oct4 was assessed and was shown to be expressed in virtually every cell in culture, which was further supported by looking at the co-expression of Oct4 with another pluripotency marker, NANOG (Figure 3A and B). These markers were analyzed by flow cytometry, which revealed that >90% of the cells were double-positive.

Furthermore, Applied Biosystems™ PluriTest™ Assay™ was utilized to analyze the gene expression of a larger cohort of pluripotency-associated genes compared to other gene sets. As shown in Figure 3C, the iPSCs gene expression profiles were highly similar to other PSCs while being highly dissimilar to non-PSCs.

FIGURE 3

Immunocytochemistry assay for pluripotency marker Oct4 (A); flow cytometry assay to determine co-expression of Oct4 marker with NANOG (B); pluripotency assay of pluripotency-associated genes (C); and genomic stability assay of PSCs (D).

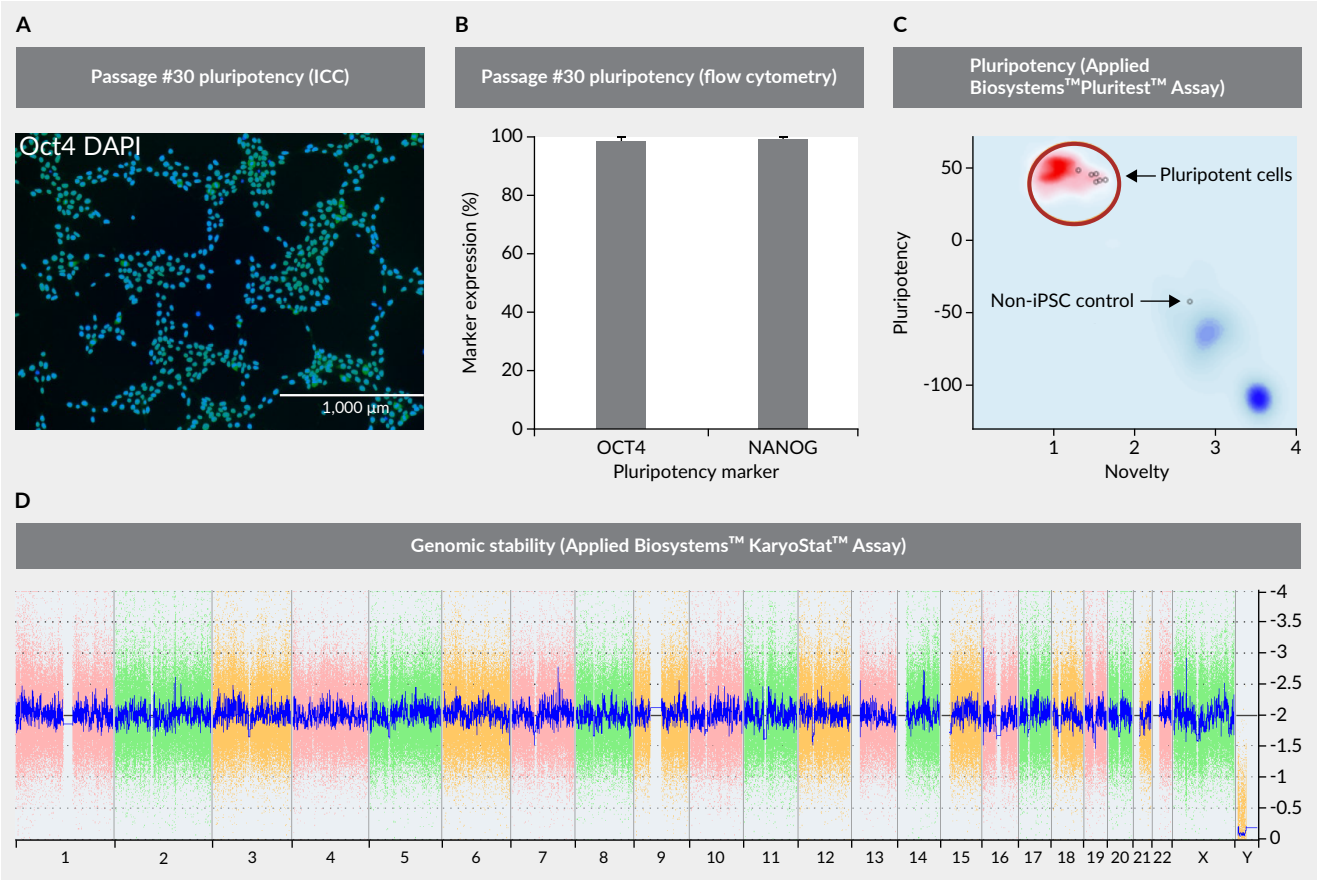
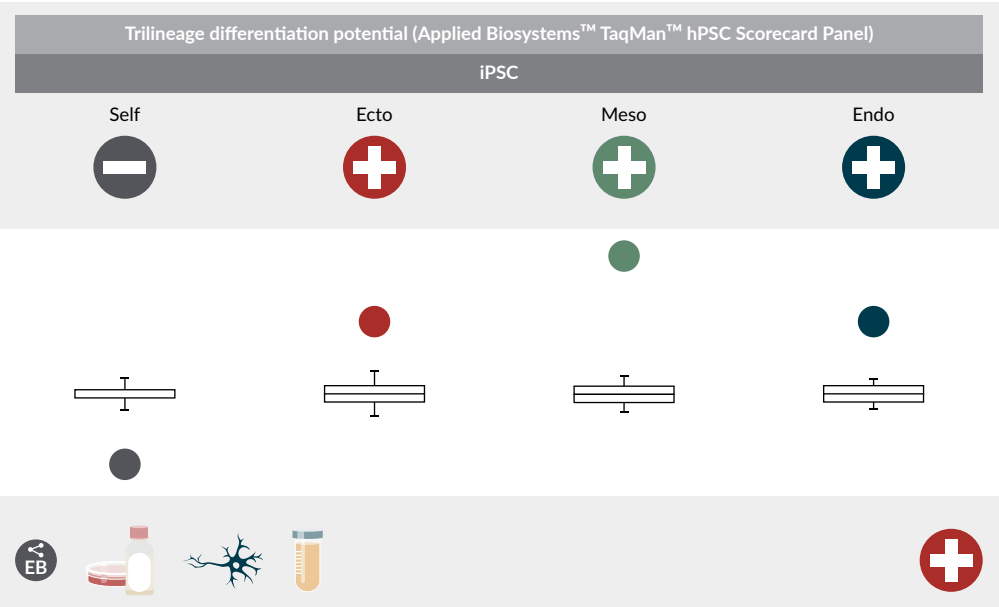


FIGURE 4

Trilineage differentiation potential of PSCs using Applied Biosystems™ TaqMan™ hPSC Scorecard Panel.



If the colored dot is above the box plot, it indicates the cells have upregulated the expression of genes for a given germ layer. If the colored dot is below the box plot, it indicates that the cells either lost expression or have only slightly upregulated gene expression markers (not shown) for each cell type.

Finally, the Applied Biosystems™ KaryoStat™ Assay™ (Figure 3D) was carried out to detect any chromosomal abnormalities. This assay has a resolution of approximately 1 Mb, providing better resolution than traditional G-banded chromosome analysis, and offers whole-genome coverage, allowing for the detection of most genomic or chromosomal abnormalities. The data demonstrated that spheroids remained pluripotent after 30 consecutive passages in suspension culture, indicating that the cell line did not develop culture-induced abnormalities. This is an important QC measure that should be regularly performed on PSC banks and long-term cultures, especially if they are intended for use in cell therapy workflows.

ASSESSING PSC DIFFERENTIATION POTENTIAL

After generating high-quality PSCs, it was crucial to confirm that the cells retained their tri-lineage potential to minimize issues when differentiating them later. PSCs can differentiate into three germ layers that give rise to all cell types. In order to determine the tri-lineage potential of these long-term cultured cells, CTS stem cell cultures were spontaneously differentiated, and gene expression patterns were assessed using the Applied Biosystems™ TaqMan™ hPSC Scorecard Panel, which is a quantitative RT-PCR assay that examines gene expression for a set of reference genes for each of the different germ layers, as well as for markers of self-renewal.

This method provides a readout on whether the cells are differentiating and, if so, into which germ layers. As shown in Figure 4, after the cells were subjected to a spontaneous differentiation assay, upregulation of markers from all three germ layers was observed while markers of self-renewal were downregulated, demonstrating that cells grown in CTS StemScale

Medium continue to exhibit tri-lineage differentiation potential.

DEVELOPMENT OF FEEDER-FREE DIFFERENTIATION PROTOCOL FOR INK CELLS

After confirming the trilineage differentiation potential in CTS StemScale cultures, another experiment was carried out to evaluate whether these cells could be further differentiated in suspension culture into a more clinically relevant cell type. Therefore, iPSC differentiation into induced natural killer cells (iNK) was assessed, as iNK cells are potential candidates for the development of allogeneic cell therapies due to their ability to target cancer cells in an antigen-independent manner.

A feeder-free differentiation protocol was developed to maintain the PSCs in a scalable suspension format (Figure 5). The differentiation of PSC spheroids to mature iNK cells takes approximately 38 days and requires the application of various growth factors and cytokines.

Firstly, PSCs were differentiated into induced hematopoietic progenitor cells (iHPCs) for 8–9 days. Afterwards, cultures were harvested and resuspended in iNK cell induction media, to initiate downstream differentiation. After approximately 20 days in culture, the iNK cell phenotype was monitored to track how well the differentiation is progressing. More specifically, CD56 was utilized as a key marker for iNK cells, along with the absence of CD3 expression. The cytotoxic marker CD16 was also monitored. Internal studies demonstrated that this technique works efficiently for adherent and suspension culture formats.

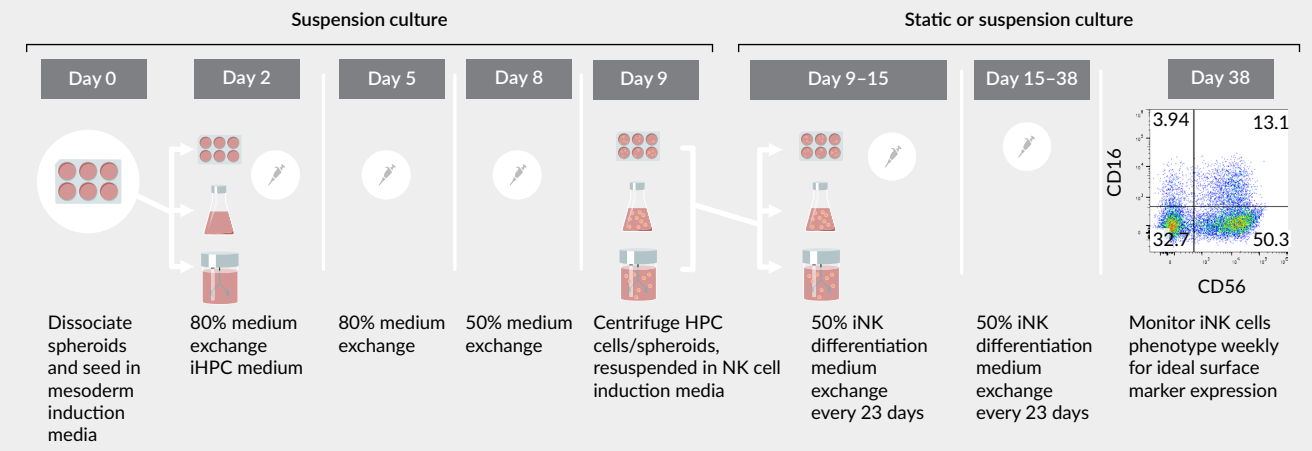
Essentially every reagent used was a CTS or equivalent GMP product, including the PeproGMP™ growth factors and cytokines, except for the StemPro™-34 Medium system, which is currently an RUO product but will be available in a CTS version in 2025.

PHENOTYPIC CHARACTERIZATION DURING INK CELL DIFFERENTIATION

Throughout the differentiation process, the cells were phenotyped to monitor the dynamic changes in iHPC and iNK marker expression, as shown in Figure 6. The emergence of a population of CD34-positive cells was observed around day 9, marking the iHPCs. As differentiation continued, the acquisition of CD56 expression in the absence of CD3 expression was observed,

FIGURE 5

iNK cell differentiation protocol using CTS products.



as well as a slight increase in CD16 expression around day 21. Both CD56 and CD16 markers continued to rise over the final weeks in culture, with >60% of the cells being CD56-positive.

ENRICHMENT OF INK CELLS USING CTS NK-XPANDER MEDIUM

Regarding iNK cell enrichment, Gibco CTS NK-Xpander Medium supports the rapid expansion of primary NK cells as it enables cell expansion without feeder cells while preserving robust cytotoxic activity, making it suitable for translational and manufacturing workflows. Additionally, the NK-Xpander Medium maintains CD56 and CD16 expression, and is designed to meet regulatory compliance requirements for ancillary materials in CGT manufacturing.

In order to demonstrate the capabilities of CTS NK-Xpander Medium, differentiated cells were transferred to CTS NK-Xpander Medium and cultured for another 10–14 days, aiming to achieve peak expansion. As shown in Figure 7A and B, both CD56 and CD16 populations significantly increased over the expansion period, which enabled further purification and enrichment of the cell population. Ultimately, the CD56-positive/CD3-negative cell population was 85–95%, and there was a marked increase in the number of cells expressing CD16, ranging from 40–60% of the CD56-positive population.

Another important point to emphasize is the significance of choosing the optimal PSC line for the differentiation process. While in this experiment multiple iPSC lines were successfully differentiated into iNK cells, each line is slightly different and should be closely characterized. For

FIGURE 6

Phenotypic flow cytometry-based characterization of iHPC and iNK cell culture markers over 38 days.

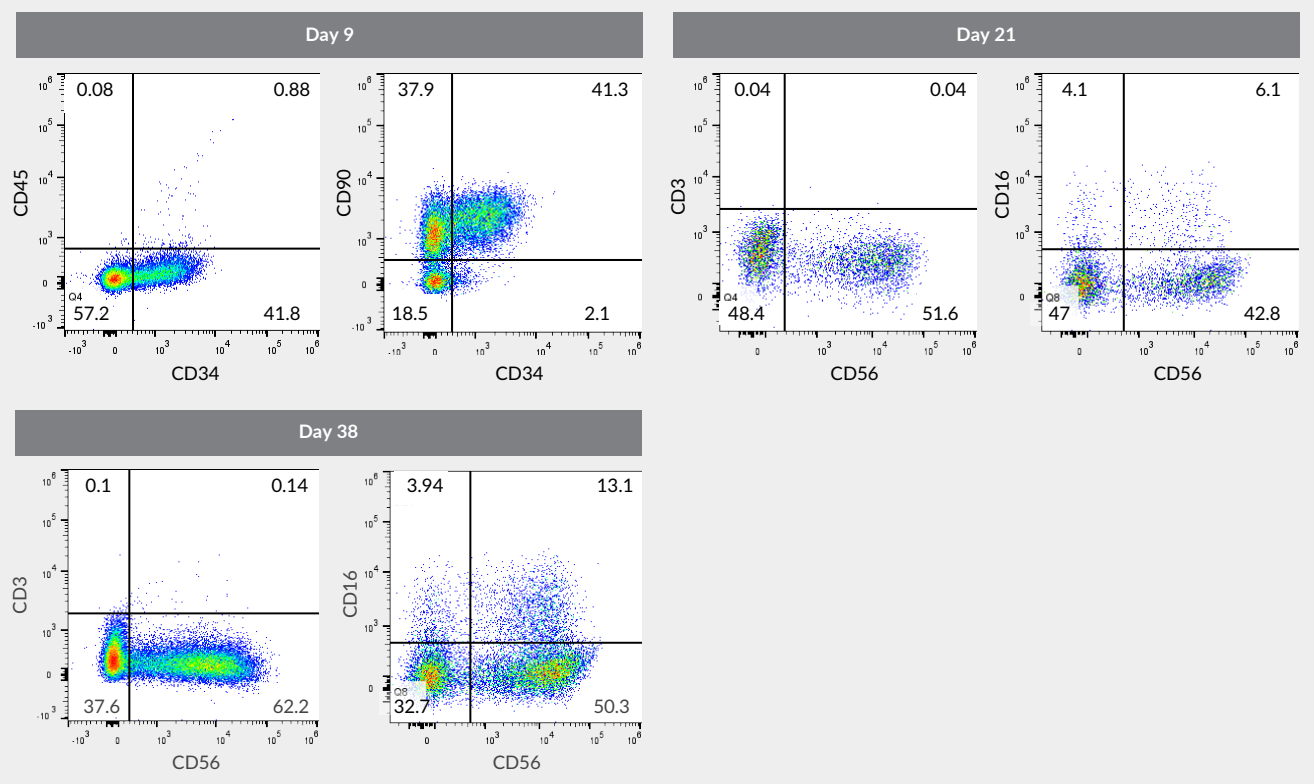
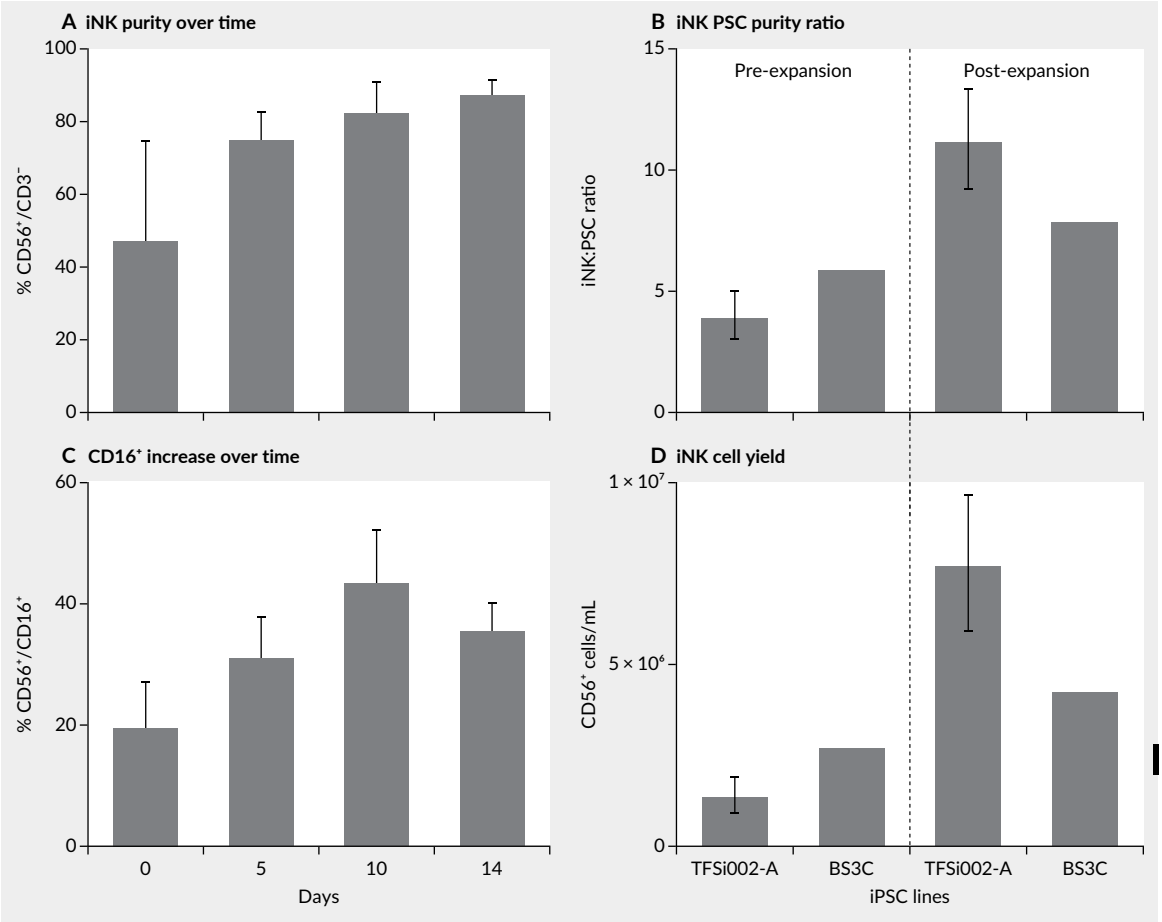


FIGURE 7

Enrichment of iNK cells using CTS NK-Xpander Medium.



A: iNK purity over 14 days; B: CD56 and CD16 cell populations over 14 days; C: iNK PSC ratio pre- and post-expansion using two iPSC cell lines; D: iNK cell yield pre- and post-expansion using two iPSC cell lines.

example, the TFSi002-A and BS3C cell lines (Figure 7C and D) both successfully differentiated, but their expansion potential was notably different. By comparing pre- and post-expansion iNK numbers, it was found that the TFSi002 cell line generated much higher yields than the BS3C cell line.

In another experiment, the activation status of the pre-expansion iNK cells was studied more broadly (Figure 8). In addition to the expression of the CD16 subset of the differentiated cells, the expression of activation markers DNAM-1, NKG2D, and NKp46 was detected. Low expression of the inhibition markers CD158 and PD-1 was also observed. Although some level of changes was noted in the culture, these markers indicate the activation and inhibitory profiles of the iNK cells.

CRYOPRESERVATION AND RECOVERY OF INK CELLS FOR DOWNSTREAM APPLICATIONS

PSC differentiation protocols are often lengthy and do not always provide convenient stopping points where cells can be paused, cryopreserved, and recovered for continued differentiation or downstream applications. However, utilizing Gibco™ CTS™ PSC Cryomedia to cryopreserve cells following differentiation allows for the recovery at later time points and expansion

prior to use. Based on an internal study examining the iNK cells after recovery and expansion, it was observed that the CD56, CD3-negative population recovered and reached >90% purity within 10–14 days. Furthermore, these cells retained high viability and similar expansion potential to iNK cells that were not cryopreserved. The CD16 marker expression also increased, suggesting that these iNKs had cytotoxic potential.

CASE STUDY: TARGETING PATIENT-DERIVED CANCER MODELS WITH PSC-DERIVED NK CELLS

Beyond iNK cell growth and phenotyping, the remaining question is: how to demonstrate their functionality, since a significant population of iNK cells expressing various activation markers, including CD16, was detected?

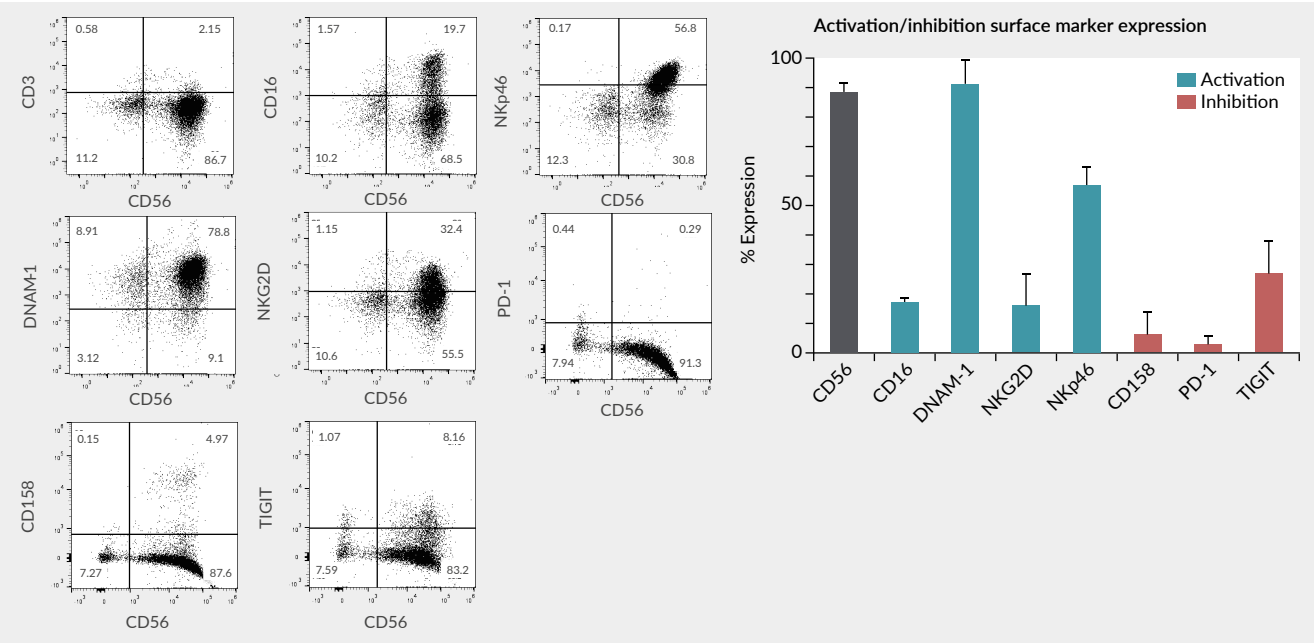
To address this question, an *in vitro* immuno-oncology application assay was developed using patient-derived cancer cells. These cancer cells were isolated from primary tumors and cultured as 3D tumoroid microtissues in the Gibco™ OncoPro™ Tumoroid Culture Medium. This media system allows the culture of tumoroids, either in suspension culture or embedded in an extracellular matrix, and was developed as a simplified system compatible with multiple cancer indications.

One of the strengths of this system is that tumoroids preserve many key characteristics of the primary tumor, making them more representative of the original tumor than traditional cancer cell lines. Through detailed DNA and RNA sequencing-based characterization methods, it was confirmed that these tumoroids retain the heterogeneity and genetic makeup of the original tumor. As a culture model, tumoroids can be used for a variety of applications, including the assessment of the killing potential of iNK cells.

Therefore, an assay was devised using a human colorectal cancer tumoroid line,

FIGURE 8

Data on the activation and inhibition of iNK surface markers.



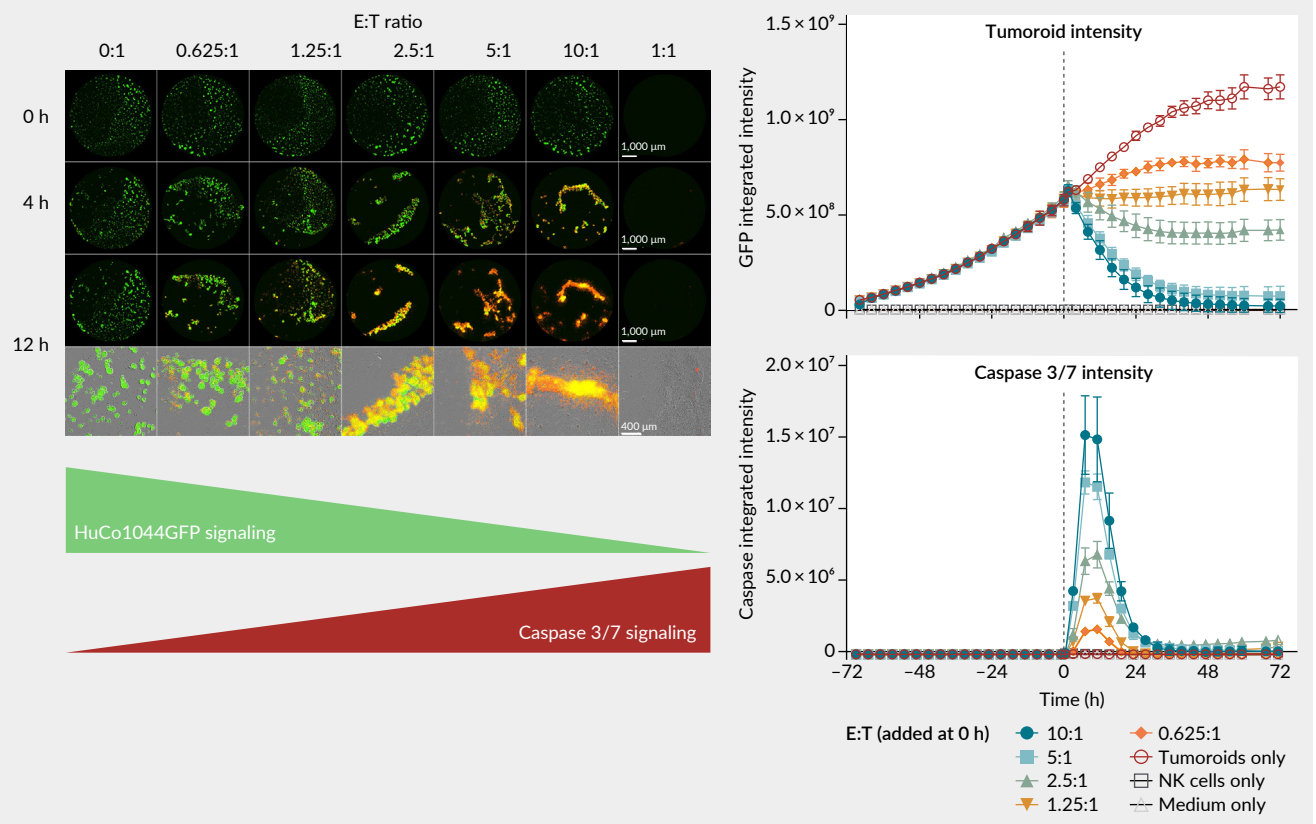
which was genetically engineered to express green fluorescent protein (GFP) to track live cells under normal culture conditions. The process involved forming tumoroids, allowing them to grow for 2–3 days, and then mixing them with varying amounts of iNK cells for co-culture over a few more days.

During the co-culture, GFP was monitored as a readout of live cells, and red fluorescence was measured using the CellEvent™ Caspase-3/7 dye, which helped to assess dead and dying cells. Typically, if the tumoroid cells are healthy and proliferating, an increase in green fluorescence is observed over time. However, if the cells start to die, a decrease in green fluorescence, accompanied by an increase in red fluorescence, is apparent, indicating cell death. As shown in Figure 9, HuCo1044–GFP tumoroids were grown in OncoPro Tumoroid Culture Medium, and the ratios of effector iNK cells (E) and target tumoroid cells (T) were measured at different time points. The results of this assay showed that, as predicted, the iNK cells were highly functional.

As illustrated in Figure 9A, the cells in green fluorescence survived and proliferated, although as the effector cells were mixed in, the loss of green fluorescence was observed along with an increase

FIGURE 9

Assessment of iNK cell functionality against HuCo1044–GFP tumoroids grown in OncoPro Tumoroid Culture Medium.



Top left: The fixed numbers of tumoroid cells with different ratios of iNK cells are shown. The E:T ratio is increased from left to right, where the effector cell (E) is the iNK cell, and the target cells (T) are the tumoroid cells. The top row shows time 0, while the second row was taken approximately 4 hours after culture. The bottom two rows represent 12 hours of culture, with the lower row showing a higher magnification overlaid with a phase image. Top right: quantification of fluorescence over time plotted based on tumoroid intensity. Bottom right: quantification of fluorescence over time plotted based on Caspase 3/7 intensity.

in red fluorescence, which continued to rise over time. As shown in Figure 9B, the tumoroid-only cells, represented by the line with red circles, increased over time. Additionally, a clear E:T ratio correlation was observed, with a loss of green fluorescence over time. The two highest E:T ratios (5:1 and 10:1) led to the highest numbers of killed tumoroid cells within 30–40 hours. Similarly, results illustrated in Figure 9C support the previously demonstrated phenotyping, confirming that iNK cells generated through the CTS-based differentiation protocol are highly functional.

SUMMARY

In summary, the CTS StemScale PSC Suspension Media and the associated CTS-based protocol can facilitate the transition from discovery to clinical and commercial iNK cell manufacturing by enabling the rapid, feeder-free, scalable expansion of PSCs. Based on results from a case study, it was demonstrated that the CTS-based differentiation protocol can be utilized to produce highly functional iNK cells capable of killing human colorectal cancer tumoroids, showing promise for potential applications in cell therapy manufacturing.

Q&A

Marcus Bunn

Q Have you carried out the tumoroid-killing assay on previously cryopreserved iNK cells? Would you expect any differences compared to iNK cells that were not cryopreserved?

MB We have tested the tumoroid-killing assay in both cases: testing iNK cells that have been cryopreserved, expanded, and then co-cultured with the tumoroids, as well as the opposite approach, where differentiation and expansion are completed all at once, followed by co-culture. No differences were observed in the killing efficiency of iNK cells, proving to be a very beneficial stopping point.

Q What are some of the challenges encountered while developing the CTS-based protocol, and could you share any related advice for anyone who wants to utilize it?

MB The first thing to consider when following the protocol is intellectual property (IP). Additionally, when choosing the cell line, it is important to ensure they efficiently grow and differentiate in StemScale Medium. If one starts developing iPSCs in a 2D or adherent platform such as StemFlex™ Medium and wants to transition to suspension culture, I would not recommend moving directly into the differentiation protocol. Instead, I suggest carrying out at least three adaptation passages in CTS StemScale first, then moving into the differentiation.

Furthermore, it is critical to consider the size of spheroids before starting the differentiation. We recommend keeping the spheroids in StemScale for 1–2 days, or until the general population of spheroids reaches about 200 µm in diameter. Anything <200 µm will result in very low yields of iNKs, and anything >200 µm will prevent differentiation, as the cytokines will not permeate through the larger-diameter spheroids.

Q How robust, repeatable, and consistent is this protocol, and what is the impact of cell line variability?

MB The CTS-based protocol is very robust. It all starts with consistency in the starting PSC cultures, making sure they are all relatively uniform. Consistency can be proven by passage numbers. Regarding cell lines, any differentiation will exhibit some variability. It is known that donor-to-donor variability is a significant factor even

with primary cells. However, while there will always be some variability between cell lines, it is crucial to ensure that the starting point for the PSCs is consistent.

Q What are some of the limitations of the current protocol, and how can it be improved?

MB The current limitation of the CTS-based protocol is the static step going from the HPCs to the iNKs. This step is not conducive to scaling up and then plating down, which can seem counterintuitive. We are currently in the process of optimizing this step to make the entire workflow suitable for suspension culture. The data is preliminary thus far, but we are seeing good results already.

Q What are the main challenges while scaling the differentiation method?

MB Firstly, the difficulty in scaling arises from the media changes, as these can become tedious and costly, especially when working with larger volumes. We are exploring ways to scale up while keeping costs reasonable, which is currently our biggest concern. Once we optimize the differentiation for <3 L volumes, scaling beyond that will present additional challenges.

Q What is the viability of iPSCs in 3D culture at different shake speeds?

MB The 70–90 rpm shake settings can affect the size of the spheroids as well as the total cell yields, which ultimately impacts viability. As you go lower than 70 rpm, the results tend to worsen, but viability remains >60%. Going above 90 rpm can lead to shear force that is too high for the PSCs, which lowers viability. In essence, we found that 70 rpm works well for our shaking platform.

Q Is the Sendai virus used for iPSC reprogramming GMP-compliant?

MB Yes, the CTS™ CytoTune™-iPS 2.1 Sendai Reprogramming Kit includes the necessary traceability documentation, as well as the Certificate of Analysis and Certificate of Origin to support regulatory filings.

Q How many different iPSC lines have been tested in the development of CTS StemScale?

MB We tested several ESC and iPSC lines in the development of CTS StemScale. Additionally, whenever we conduct long-term or short-term packaging studies, we always send those cell lines for genomic stability studies.

Q Is the 2-week iNK expansion culture feeder cell-free?

MB Everything in the CTS-based protocol is feeder cell-free. We aim to avoid using any feeder cells in our systems to maintain GMP compliance and to help simplify the cultures.

Q Have you also monitored CD57 in the iNKs?

MB We plan to monitor CD57 in the iNKs shortly, as this marker is commonly examined and regularly reported in the literature. Since we are still focused on scaling up our protocol, we aim to limit the number of markers we evaluate, although CD57 is certainly one that we are interested in.

Q How do you cryopreserve the final NK cells? What is the percentage of remaining NK cells 24 hours post-thaw?

MB We cryopreserve our cells in CTS PSC Cryomedium at a density of 2.5×10^6 cells/ml. When we thaw them, we observe good recovery of the cells. Typically, >80% of the cells that were frozen come out of cryopreservation. This recovery rate remains stable for approximately 3-4 days post-thaw, after which we start to see some expansion. 24 hours after cryopreservation, we typically observe approximately 80% of the cells that were thawed.

Q Are the media and reagents used for cell differentiation regulatory-friendly and suitable for cell therapy?

MB Everything in the CTS-based protocol is cell therapy manufacturing-grade, except for the MEM α, which we are working to replace. However, all other media components, as well as the cytokines from PeproGMP, are cell therapy manufacturing-grade.Atque autero iussatum en vidi pere nost acciptem pro Catiliciam viteruncemor quodier untrorudet viusquam se conirio ndeniquam nostiquo eterorum simihilla senium iam conferi patque adestere menata miu ide publis.

BIOGRAPHIES

Mark Kennedy joined Thermo Fisher Scientific, Frederick, MD, USA as an R&D scientist in 2016 focused on 2D and 3D cell culture models development, including pluripotent stem cells. He received his PhD from Memorial University, St John's, NL, Canada and subsequently trained at the National Cancer Institute, NIH. Dr Kennedy has a long-standing interest in understanding gene regulatory networks controlled by growth factor signaling in models of mammalian development and stem cell biology. Currently, Dr Kennedy leads a R&D team dedicated to the development of new tools and reagents to support the advancement of pluripotent stem cells and their applications.

Mark Kennedy, Staff Scientist, Thermo Fisher Scientific, Frederick, MD, USA

Marcus Bunn joined Thermo Fisher Scientific, Washington DC, Baltimore area, USA in 2022 as a Research and Development Scientist, focused on pluripotent stem cell applications. He earned his PhD in Comparative Medicine and Integrative Biology, with a focus on immunology, from Michigan State University, MI, USA. During his doctoral studies, Bunn concentrated on the energetics of immune cells in various autoimmune diseases.

Marcus Bunn, R&D Scientist III, Thermo Fisher Scientific

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Maximizing cell therapy process flexibility and efficiency with next-generation technologies

Over the last two decades, the field of cancer treatment has undergone a revolution. Innovative cell therapies are offering new treatment options for many previously incurable diseases, including several types of blood cancers. Many patients who are ideal candidates for cell therapies may have exhausted every other treatment option or are facing disease recurrence. As such, they require rapid access to safe and efficacious treatments. One such innovative treatment is CAR-T cell therapy.

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In CAR-T cell therapy, T cells from a patient or donor are genetically engineered to recognize and bind to cancer cell antigens. When infused into the patient, the T cells trigger an immune response to find and destroy the cancerous cells [2]. These therapies have been shown to reduce, and even completely eradicate, tumors in people with advanced cancers [3]. In some cases, the treatment responses have lasted for years, with high levels of CAR-T cell persistence [1,4].

In line with these successes, the industry has made great progress. Since 2017, seven CAR-T cell therapies have been approved by the US FDA for treating aggressive blood cancers, some forms of leukemia, and multiple myeloma [3]. In fact, to keep pace with the number of promising cell-based therapeutics, the FDA's Center for Biologics Evaluation and Research opened its new Office of Therapeutic Products in 2023 [5]. Other global regulatory bodies are also following suit, with additional CAR-T cell therapies being approved by the Chinese National Medical Products Administration (NMPA) and the Indian Central Drugs Standard Control Organisation (CDSCO) [6].

Despite significant advancements, challenges remain in CAR-T cell therapy manufacturing, particularly in terms of speed and cost. For some patients, a delay in access to treatment can be life-threatening, making accelerated manufacturing processes critical. Additionally, the high cost of cell therapy production limits patient access to these transformative treatments. To address these issues, there is a vital need for innovations that can enhance production efficiency and help reduce costs, broadening access to these life-saving therapies.

DRIVING PERFORMANCE WITH NEXT-GENERATION MAGNETIC CELL SEPARATION

Within the cell therapy manufacturing process, isolation and activation of target cells are vital steps, ensuring that only the desired cell population is used for treatment in order to maximize the therapeutic's efficacy. Magnetic beads that passively dissociate over time have been commonly used for carrying out isolation and activation in clinical and commercial therapies. Thermo Fisher Scientific's Gibco™ CTS™ Dynabeads™ CD3/CD28 passive-release beads have been driving progress in the industry for nearly 20 years, being used in over 200 clinical trials and several commercialized cell therapy manufacturing workflows [7,8].

In 2023, Thermo Fisher innovated further with the launch of its Gibco™ CTS™ Detachable Dynabeads™ platform. This technology utilizes a unique active release mechanism to allow manufacturers to control the detachment of beads at any point in their workflow. Designed to increase flexibility and scalability in both allogeneic and autologous workflows, CTS Detachable Dynabeads can help reduce manufacturing times by removing the need to wait for passive dissociation. The active release mechanism employed by the Detachable Dynabeads platform is the first of its kind for clinical trial and commercial manufacturing use. This innovation has helped give manufacturers greater control over their processes, accelerate their workflows, and drive cost savings while helping to deliver high cell purity, yield, and viability.

The Detachable Dynabeads are conjugated to highly specific, single-domain VHH antibodies. These antibodies are targeted to specific cluster of differentiation (CD) markers, enabling them to selectively isolate the T cell phenotypes displaying these markers. The specialized Gibco™ CTS™ Detachable Dynabeads™ Release Buffer is then used to actively detach the Dynabeads via a competitive binding mechanism, allowing them to be magnetically removed with the Gibco™ CTS™ DynaCellect™ Magnetic Separation System.

The first product released to use this technology was the Gibco™ CTS™ Detachable Dynabeads™ CD3/CD28 magnetic beads, which was created for the one-step isolation and activation of CD3+ and CD28+ T cells. Studies have shown that achieving a high-quality pool of early T cell phenotypes is crucial for enhancing the consistency and durability of therapeutic results [9]. The active release mechanism of the CTS Detachable Dynabeads CD3/CD28 facilitates the preservation of T cells with optimal early phenotypes, helping to maximize the efficacy of the final CAR-T product.

THE EVOLVING ROLE OF T CELL SUBSETS IN CAR-T DEVELOPMENT

There is ever increasing knowledge about the role of—and interplay between—different T cell subsets, and how they can impact therapeutic efficacy, safety, and persistence [10]. Of particular focus are two groups of cells: CD8+ cytotoxic T cells and CD4+ helper T cells. CD8+ cells are responsible for directly attacking and killing cancer cells, which is crucial for a CAR-T treatment to be effective. CD4+ cells, on the other hand, play a supporting role, driving the activation and function of other immune cells and producing cytokines that help sustain the immune response.

Although many therapeutic strategies have initially focused on isolating CD8+ T cells, trends are evolving to include CD4+ T cells—either separately or in combination with CD8+ cells—to fine-tune the resulting therapies [11]. Some workflows aim to achieve predefined CD4:CD8 ratios that require isolation of single T cell subsets in parallel. Others are looking to isolate single subsets of CD4+ or CD8+ T cells, while some therapies require isolation of all T cells.

In response to the growing need for T cell subset isolation, Thermo Fisher has released its Gibco™ CTS™ Detachable Dynabeads CD4 and Gibco™ CTS™ Detachable Dynabeads CD8 for use in clinical and commercial manufacturing. This allows developers to isolate CD4+ and CD8+ T cells, either separately or together, facilitating more precise control of CD4:CD8 ratios. These new products allow cell therapy manufacturers to fine-tune their therapies for maximum efficacy, safety, and persistence.

DRIVING SCALABILITY THROUGH AUTOMATION

By helping overcome the key challenges of speed and cost, the CTS Detachable Dynabeads platform is driving increased production efficiency and shortening critical timelines in the cell therapy process. However, with several approved therapies on the market and increasingly promising data on efficacy and persistence, there is a desire for CAR-T cell therapies to be prescribed earlier and to more patients [12]. With this predicted increase in demand for CAR-T cell therapies, manufacturers are looking for further ways to drive improved process efficiency.

Within this area, there are numerous issues that need to be addressed. One challenge is scaling up production to meet clinical and commercial demands without compromising on therapeutic efficacy and quality. The cell therapy manufacturing process is also intricate, involving numerous specialized instruments, reagents, and consumables. As such, these workflows can often be time-consuming, labor intensive, and costly, resulting in long manufacturing times and high per-patient costs [13].

Automated systems are playing a key role in helping to tackle these challenges by helping developers streamline and expedite their workflows, resulting in more efficient processes. The CTS DynaCellect Magnetic Separation System is a modular and closed automated manufacturing system that has been designed to work optimally with the CTS Detachable Dynabeads portfolio. As a stand-alone instrument or as part of an integrated workflow, the operator-independent system can help manufacturers isolate the right cells and minimize production failures while providing increased robustness and precision.

Through the integration of automated systems, manufacturers can enhance flexibility across workflows, generating high-quality outputs with reduced variability and error. These systems will also help manufacturers more efficiently produce efficacious cell therapies, reducing production costs and supporting long-term commercial success.

THE PATH FORWARD FOR CAR-T CELL THERAPY MANUFACTURING

CAR-T cell therapies are revolutionizing cancer treatment, offering hope for patients around the world. However, despite the improvements in therapeutic efficacy, there are still several manufacturing challenges that need to be addressed, including high production costs and lengthy manufacturing processes, which can limit treatment speed and availability. As such, further innovation in CAR-T manufacturing will be necessary to support continued progress.

With its portfolio of cell therapy solutions, Thermo Fisher is committed to advancing cell therapies by addressing the most pressing challenges in CAR-T manufacturing. For example, CTS Detachable Dynabeads are helping developers shorten manufacturing times and increase process flexibility, resulting in more efficient and cost-effective workflows. Similarly, the integration of automated modular systems, such as the CTS DynaCellect Magnetic Separation System, is facilitating greater scalability and consistency, essential for expanding the accessibility of cell therapies.

Ultimately, these next-generation solutions are set to play an instrumental role in shaping the future of the cell therapy industry. By supporting workflow optimization, they can help developers and manufacturers meet the increasing demand for safe and efficacious CAR-T cell therapies and help ensure that more patients can access these critical treatments.

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INNOVATE YOUR CELL THERAPY WORKFLOW

DRIVE YOUR PERFORMANCE FORWARD

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Make more lentivirus and make it right the first time

Katherine Schewe

Lentiviral vectors (LVs) are increasingly used in gene-modified cell therapy manufacturing due to their capacity for large genetic payloads, stable gene delivery, and low immunogenicity. However, their manufacturing presents several challenges, including safety, performance, and purification stability. This article examines key hurdles such as achieving accurate vector measurements, maximizing viral titers, and optimizing purification processes. It highlights advances in LV production and explores the significance of design of experiments (DOE) in optimizing transfection processes and the importance of scalability in developing robust manufacturing strategies.

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CHALLENGES AND CONSIDERATIONS IN LV MANUFACTURING

Lentiviral vectors (LVs) are typically the preferred delivery platform for gene-modified cell therapies due to their ability to carry large genetic loads, offer reliable and stable gene delivery, and exhibit low immunogenicity. All these properties are ideal for targeted oncolytic immunological therapies, and the treatment of genetic disorders.

However, LV manufacturing poses several challenges due to the physiological and physicochemical characteristics of the virus. The most common hurdles can be grouped into these three categories: safety, performance, and purification stability.

The key safety challenges include achieving total and functional vector measurements, choosing assays that provide virus safety assurance, and ensuring product and process impurity removal.

Regarding performance, the most common challenges include maximizing viral titers, finding alternatives to using a multi-plasmid packaging system, overcoming physicochemical conditions to optimize lentivirus recovery and functionality, and reducing the high costs of key steps such as affinity chromatography.

Finally, in order to overcome the purification stability challenges of LV manufacturing, it is crucial to find the most optimal final formulation buffer that maintains a stable monodisperse population, and address hurdles such as thermo-instability and sensitivity to freeze-thaw cycles.

EXPLORING ADVANCES IN LV MANUFACTURING

Overcoming the aforementioned challenges is crucial, especially as the cell and gene therapy (CGT) market is experiencing significant growth and activity. For lentivirus specifically, oncology remains a major clinical focus, and there has been an extraordinary 600% increase in the number of lentivirus-based clinical trials over the past 5 years [1]. The preclinical pipeline is also strong, with lentivirus being widely explored for gene-modified cell therapies targeting cancer indications, as well as a growing number of non-oncological applications.

The CGT industry is actively seeking solutions to address the challenges in LV manufacturing and support drug product developers in bringing their therapeutics to the market. For example, there has been a recent shift from adherent to suspension cell culture-based manufacturing, with a focus on optimizing the transient transfection process to improve lentivirus production outcomes. The primary goal is to de-risk the manufacturing process by providing a scalable, defined workflow that delivers high yield and recovery, reduces manufacturing time, lowers cost of goods, and integrates analytical tools to support the process. Over the next few years, the adoption of technologies that further optimize processes to reduce cost of goods, boost productivity, and shorten the time needed to deliver therapies to patients is expected. These emerging technologies include stable production systems, process intensification platforms, and rapid quality evaluation methods.

OPTIMIZED CLINICAL AND COMMERCIAL LV PRODUCTION

The VirusExpress® Lentiviral Platform from MilliporeSigma was developed with two primary goals: to manufacture higher quantities of the virus and to ensure accurate and consistent production from the start. To achieve this, the platform utilizes their optimized packaging plasmids that are readily available off-the-shelf. MilliporeSigma has also established a partnership with an external CDMO to support the manufacturing of the gene-of-interest (GOI) plasmids on behalf of the therapeutic developers.

With the optimized plasmids, GMP-banked HEK293T cell line, and chemically-defined cell culture medium, the LV platform achieves consistent and reproducible titers in the 4×10^8 transducing units (TU)/mL range. Additionally, it is possible to achieve a 20% recovery after the final filtration stage.

Together, these elements create a platform that delivers consistent, high-quality results at any scale: 50 L, 200 L, and beyond. MilliporeSigma also offers fill-finish capabilities at their viral vector development and manufacturing facility in Carlsbad, CA, USA.

Furthermore, as part of the platform, a comprehensive analytical toolbox provides consistent characterization of the product, as well as monitoring productivity, yield, and purity. Lastly, regulatory support services offer customized assistance at each critical milestone, as the therapy progresses through the regulatory pathway toward commercialization.

Designed for rapid IND submission, the optimized, end-to-end lentivirus platform increases productivity and titers while lowering overall operational costs, and compressing project timelines, which in turn can significantly reduce the cost per dose, making therapies more accessible to patients.

HIGH-TITER LV PRODUCTION

As a critical component of the VirusExpress Lentiviral platform, commercially available packaging and internally developed plasmids were extensively tested in various combinations. One combination was

found to enhance the platform's performance nearly 20-fold compared to the original setup (Figure 1).

Utilizing MilliporeSigma's internally developed packaging plasmids and a commercially available transfer plasmid along with the HEK293T cell line, chemically defined media, and optimized upstream workflows viral titers in the 4×10^8 TU/mL range were achieved at harvest. These optimized transfection parameters and titers have not only been validated at small scales but also reproduced at larger, clinically relevant production scales.

MAXIMIZED LV YIELDS

Beyond upstream workflows, VirusExpress Lentiviral platform has optimized downstream processing workflows to maximize lentivirus recovery. Three key areas of downstream processing that have the greatest impact on step recoveries were targeted: ion exchange chromatography, tangential flow filtration (TFF), and sterile filtration unit operations.

For ion exchange chromatography, the sodium chloride concentration and the elution step were optimized in order to determine the conditions that would yield the highest infectious particle recovery. Regarding TFF, both concentration and diafiltration processes were optimized. For sterile filtration, the optimization of the formulation buffer was the main focus to understand the impact of pH, salt concentration, stabilizers, hold times, and temperatures that may occur during the final processing and storage of the LV.

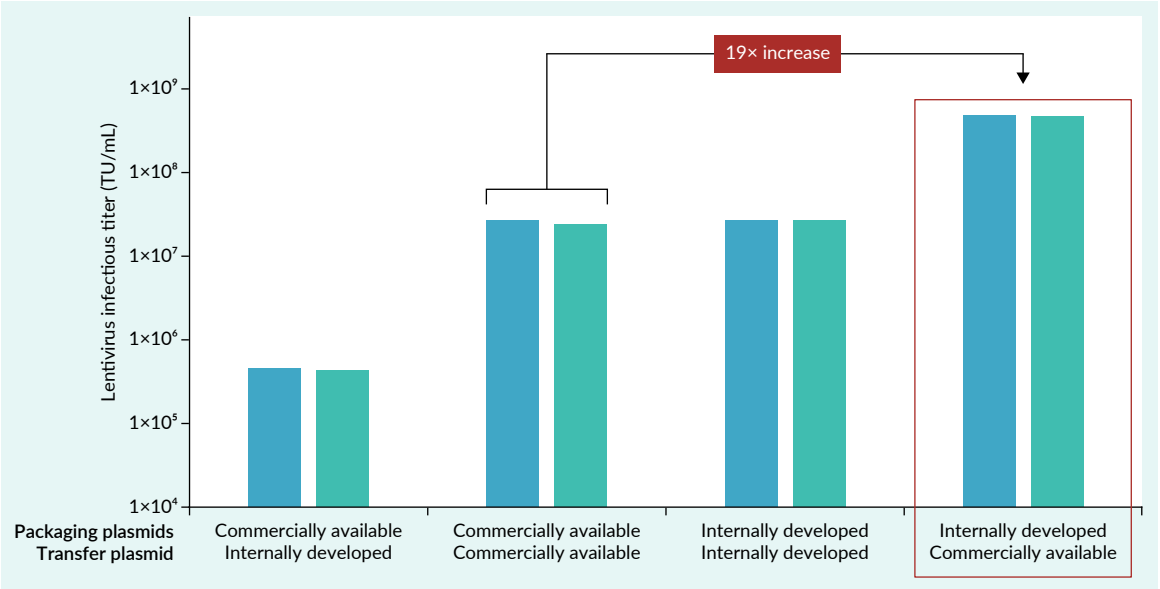
High repeatability and consistent step yields were achieved, leading to an overall 20% lentiviral vector recovery after the final filtration stage. These optimized conditions were also validated at larger production scales, confirming scalability and consistency.

CLINICAL RELEVANCE AND EFFICIENCY

In order to examine the clinical relevance and impact for therapeutic developers,

FIGURE 1

Comparison of different lentivirus infectious titers when utilizing different combinations of commercially available and internally developed packaging and transfer plasmids.



performance outcomes at 50 L and 200 L scales were calculated. Given the productivity of 4×10^8 TU/mL for the harvest titer, the number of doses that could be achieved using the platform was estimated, assuming a multiplicity of infection (MOI) of three. For a 50 L scale, approximately 1,300 doses could be generated, while at the 200 L scale, the number of doses is approximately 5,000.

In a real-world scenario, the VirusExpress Lentiviral platform was utilized to manufacture a gene-modified cell therapy for an autoimmune indication for a therapeutic developer. Streamlining the path to clinic, process development and GMP manufacturing were completed in under 12 months, delivering the product ahead of schedule.

Accelerating to GMP is always a key consideration in CGT manufacturing. When planning the path from concept to commercialization, a traditional timeline can take up to 3 years, while a streamlined, comprehensive approach such as the VirusExpress Lentiviral platform can be utilized to optimize and de-risk the workflow, saving time and improving efficiency.

ADVANCED ANALYTICS FOR LV MANUFACTURING

Comprehensive analytics for the VirusExpress Lentiviral platform include off-the-shelf assays to support LV characterization. Overall, more than 18 methods were developed to ensure the concentration, potency, purity, safety, quality, and integrity of the lentiviral vector in-process and final product.

Furthermore, MilliporeSigma's viral vector analytical development team provides comprehensive support for assay development, method validation, and comparability studies to provide accurate and reliable methods for each stage of development. These methods are designed to transfer seamlessly to manufacturing. Furthermore, the team also supports process development through design of experiments (DOEs) and conducts in-depth virus testing using advanced characterization technologies.

THE IMPORTANCE OF SCALABLE PROCESS DEVELOPMENT AND A PURPOSE-BUILT MANUFACTURING FACILITY

MilliporeSigma's process development teams incorporate a customizable, virus-specific holistic approach to develop a robust and scalable process. Automation and small-scale studies are performed to reduce the risk, accelerate timelines, and optimize the upstream and downstream workflows for the specific GOI. Additionally, the onsite pilot scale lab ensures the high performance of the processes, which are well-characterized at multiple single-use bioreactor scales. Yields and product quality profiles achieved at the representative laboratory and pilot scale are directly translatable to large-scale production runs due to the same equipment being used. Overall, the pilot scale lab ensures the scalability of the process and mitigates the risk of unforeseen scale-up challenges, preventing timeline delays, and enabling efficient, streamlined, and de-risked tech transfer from process development to manufacturing.

The custom-designed manufacturing facility was specifically built to mitigate risks in viral vector production, offering suspension cell culture manufacturing through flexible operations and suite models. These processes are fully scalable from vial thaw to downstream operations and fill-finish. The current scale supports volumes from 50–2,000 L, covering the entire lifecycle of a molecule, from clinical development through commercial production.

The fill/finish suites are equipped with a state-of-the-art dual chamber isolator and a fully automated fill line capable of

processing 3,600 vials per hour. A typical 1.2 mL fill volume runs at about 40 vials/mL, with 100% inline weight checks. The flexible fill line accommodates a range of vial sizes starting from 2 mL, and the isolator uses grade A vaporized hydrogen peroxide sanitization, meeting both FDA and EMA regulatory requirements. The fill/finish area is directly connected to the visual inspection and labeling suite, ensuring a streamlined workflow that supports the aseptic fill process.

MANUFACTURABILITY ASSESSMENTS AND FEASIBILITY STUDIES FOR LV DEVELOPMENT

In order to identify and evaluate potential improvements in LV development, the manufacturability assessment is a key initial step. The 6–8-week assessment involves a thorough review of the process, during which various areas for enhancement are examined. This includes gathering information about the gene of interest and plasmids, performing upstream studies with different transfection parameters, building process development and manufacturing strategies, and testing different analytical method approaches.

Additionally, feasibility studies can be performed to test and develop a strategy that ensures the VirusExpress Lentiviral platform delivers the most optimal therapeutic results. The goals of the feasibility studies are to evaluate multiple conditions including different transfection reagents, enhancing reagents, or boosting reagents, and select the most optimal transfection reagent.

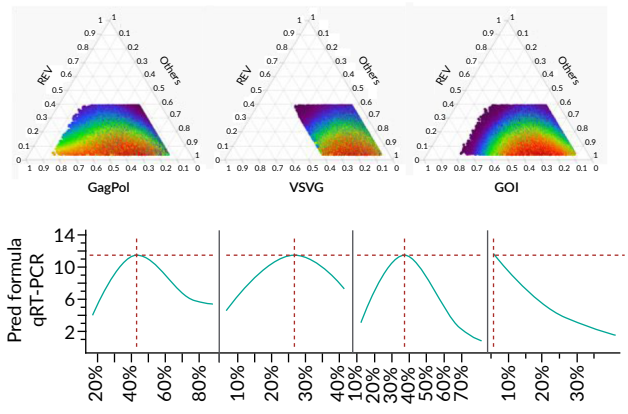
CASE STUDY: DOE APPROACH FOR OPTIMIZING LV TRANSFECTION

There are numerous parameters that may be crucial for optimizing the transfection process. A major advantage of using a DOE approach through statistical software for experimental design or data modeling is the ability to study multiple parameters simultaneously, along with their potential interactions. This approach minimizes the number of experiments compared to the traditional one-parameter-at-a-time approach. The key parameters of the DOE approach include screening transfection reagent, viable cell density, DNA concentration, transfection reagent to DNA ratio, transfection complex formation time, and volume.

In an internal case study, the Ambr® 15

FIGURE 2

An example of the prediction profiler using JMP software, which illustrates how the responses shift as the levels of different factors are adjusted for lentivirus production.



	Plasmid qty %				Calculated molar ratio (normalized to pREV)				Sample titer	p24
Condition	VSVG	pGOI	GagPol	REV	VSVG	pGOI	GagPol	REV	GC/mL	ng/mL
1										
2										
3										
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system was utilized for plasmid ratio DOEs in order to optimize transfection for lentivirus production. As part of the transfection process and platform improvement efforts, different variables were explored, such as transfection viable cell density, the ratio of transfection reagent to DNA, and total DNA concentration. For these experiments, a standard PEI-based transfection reagent was utilized, and a response surface design was applied, which enabled the development of a model that identifies optimal transfection conditions. As illustrated in Figure 2, optimal conditions were identified, leading to an approximately 60% increase in genome titers compared to the baseline conditions.

SUMMARY

In conclusion, the challenges associated with LV manufacturing underscore the need for innovative solutions that enhance safety, performance, and purification stability. As the demand for gene-modified therapies continues to rise, platforms such as VirusExpress Lentiviral platform offer promising advances in manufacturing processes, ensuring high yield and quality, while reducing costs and timelines. By embracing these technologies, the cell and gene therapy industry can facilitate faster and more efficient pathways to bring transformative treatments to patients.

Q&A

Katherine Schewe

Q What is the origin of the MilliporeSigma packaging plasmids?

KS The GMP-grade packaging plasmids come from a bank that was developed internally and in partnership with a leading high-quality pDNA CDMO.

Q Which nuclease do you use, and what are the optimal salt conditions for its operation?

KS The nuclease used is Benzonase. Our findings indicate that performing a single-step evolution in 1–1.5 M sodium chloride concentrations improves recovery rates. Additionally, this approach results in high removal of host cell proteins.

Q What recovery rate can be achieved with the VirusExpress Lentiviral platform?

KS The VirusExpress Lentiviral platform consistently achieves a 20% recovery rate after the final filtration. This allows developers to produce high-quality batches with greater yields, thanks to the advanced downstream capabilities.

Q Can the VirusExpress Lentiviral platform be used to test for residuals?

KS We have successfully developed and established several testing methods that allow us to assess not only raw materials but also cellular debris. We can test for residual Benzonase®, as well as residual host cell proteins and host cell DNA, which enables us to gain a thorough understanding of the impurity profile.

Q What is the current status of stable cell lines in the market?

KS There are several stable-producing cell lines available in the market, with some designed specifically for lentivirus production and others tailored for AAV production. Currently, we are assessing various options to determine the best fit for our capabilities. However, these specific cell lines are not part of our current offerings.

Q What is the ideal phase to start a manufacturing assessment with our platform?

KS For the manufacturing assessment, any phase can be a suitable time to start. However, to optimize the time to market and establish the best process early, it is most beneficial to begin at the very beginning when developing the construct. This approach can save both time and money as the therapeutic progresses toward commercialization. The manufacturability assessment can determine the most effective processes to support the program and achieve key milestones, ensuring that the product reaches patients after commercialization.

Q What is the maximum fill size of the isolator?

KS Our equipment can accommodate multiple fill configurations and sizes without any limitations. We can handle various types of fill containers, whether they are vials or bags, and we can adapt the correct configuration for your specific needs. The main limiting factors are the stability of the virus, and the time required for visual inspection of the material. As long as the product is stable and the inspection is completed on time, we can meet nearly any requirement the developers may have.

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INNOVATOR INSIGHT

Optimizing for the future: streamlining the production of T cell-based immunotherapies

Soong Poh Loong and Paula Lam

Despite its high potential to treat rare conditions, T cell-based immunotherapy manufacturing faces challenges in terms of cost, quality/safety, standardization, scalability, and speed of innovation. This article explores the development of a 7-day, closed, viral-based CAR-T cell production process, and introduces a novel soluble transduction enhancer for optimizing lentiviral-based immunotherapy manufacturing. By improving scalability, reducing cytotoxicity, and enhancing CAR-T cell efficacy, this approach offers the potential to enable better outcomes for patients with cancers such as chronic lymphocytic leukemia, helping optimize both safety and cost-effectiveness in immunotherapy.

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CHALLENGES AND OPPORTUNITIES IN CELL THERAPY MANUFACTURING

In recent years, significant advancements have been made in clinical trials for cell and gene therapies (CGTs), particularly within the field of immuno-oncology. However, numerous manufacturing challenges, such as therapeutic costs, patient safety, standardization, automation, scalability, and speed of innovation must yet be addressed to enable widespread clinical adoption and application.

Delivering affordable, safe, efficacious, and sustainable cell therapies requires substantial investments in system and product innovation, the standardization of manufacturing processes, and the implementation of automation. These efforts aim to enhance process efficiencies and support the successful translation and scaling of innovative therapeutics into GMP settings.

Gibco™ CTS™ PORTFOLIO FOR CGT MANUFACTURING

The Gibco™ Cell Therapy Systems (CTS™) portfolio of CGT products, which are tested for safety and backed by regulatory documentation such as Drug Master Files, Regulatory Support Files, validation documents, and regulatory agency letters, are designed to support the transition from

discovery through clinical and commercial manufacturing. Additionally, CTS™ products are manufactured under global quality standards such as Quality Management System ISO 9001 and 13485, and they are already utilized extensively in over 200 clinical trials and several commercialized therapies.

BioCell INNOVATIONS CASE STUDY

BioCell Innovations Pte Ltd is a Singapore-based clinical-stage biotech focused on delivering affordable cell and gene therapy products to patients in need. Alongside a number of industry collaborators, BioCell develops both CAR constructs and innovative manufacturing processes for both autologous and allogeneic cellular immunotherapies.

Developing CAR-T cell immunotherapies for lymphoid neoplasms

Between 2017 and 2021, lymphoid neoplasms ranked as the fourth or fifth most common cancer among the top ten cancers affecting both men and women in Singapore [1]. Each year, approximately 300 new cases of diffuse large B-cell lymphoma (DLBCL) are diagnosed in Singapore, with 30–40% of these cases being relapsed or refractory to traditional R-CHOP combination therapies. Globally, DLBCL accounts for a third of all non-Hodgkin lymphomas, with a range of 20–50% between countries. United States cancer registry data reports an age-standardized incidence rate for DLBCL of 7.2/100,000 [2].

Seven CAR-T cell immunotherapies have been approved by the US FDA since 2017. Of these therapeutics, five target CD19 and two target B-cell maturation antigen (BCMA). The overall response rates for these therapies range from 72–98%. For example, the first patient to receive CAR-T cell therapy for acute lymphoblastic leukemia in 2012 has remained cancer-free to date. This patient received a CD19-targeted CAR-T therapy derived from the FMC63 monoclonal antibody, which is specific to CD19. However, the use of a non-human single-chain variable fragment (scFv) in this treatment may induce a human anti-mouse antibody response. This immune reaction could limit the persistence and effectiveness of CAR-T cells in patients. Another challenge of CAR-T therapy is cost, which can start at a minimum of US$375,000 per patient.

The potential of CAR-T cell therapies in treating autoimmune diseases

Recent studies have also highlighted the potential benefits of CAR-T therapies in treating autoimmune diseases. Autoimmune diseases affect approximately 600,000 individuals in Singapore, which is 11% of the entire population [3]. Globally, systemic lupus erythematosus (SLE) incidence has been estimated at 43.17/100,000, with the disease affecting 3.41 million people [4]. Of the various types, SLE, immune-related myopathies, and systemic sclerosis could all potentially benefit from CAR-T cell therapies. By targeting and eliminating autoreactive B-cells, CD19 CAR-T cell therapy may reduce immune-mediated damage, potentially benefiting patients with severe forms of SLE, systemic sclerosis, and myositis

The development of a second-generation anti-CD19 CAR-T vector

At BioCell, a second-generation CAR vector was constructed, consisting of a humanized anti-CD19 scFv with a CD8 transmembrane domain followed by a 4-1BB CD3ζ costimulatory activation domain. This construct is denoted as BC-hCD19

FIGURE 1

The cell-killing capabilities of BC-001, FMC63, and C2146 constructs.

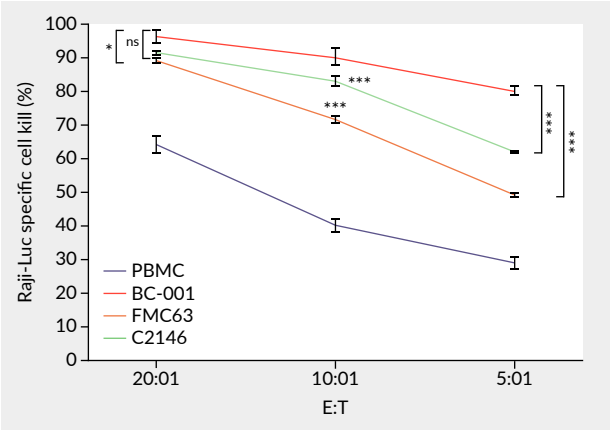
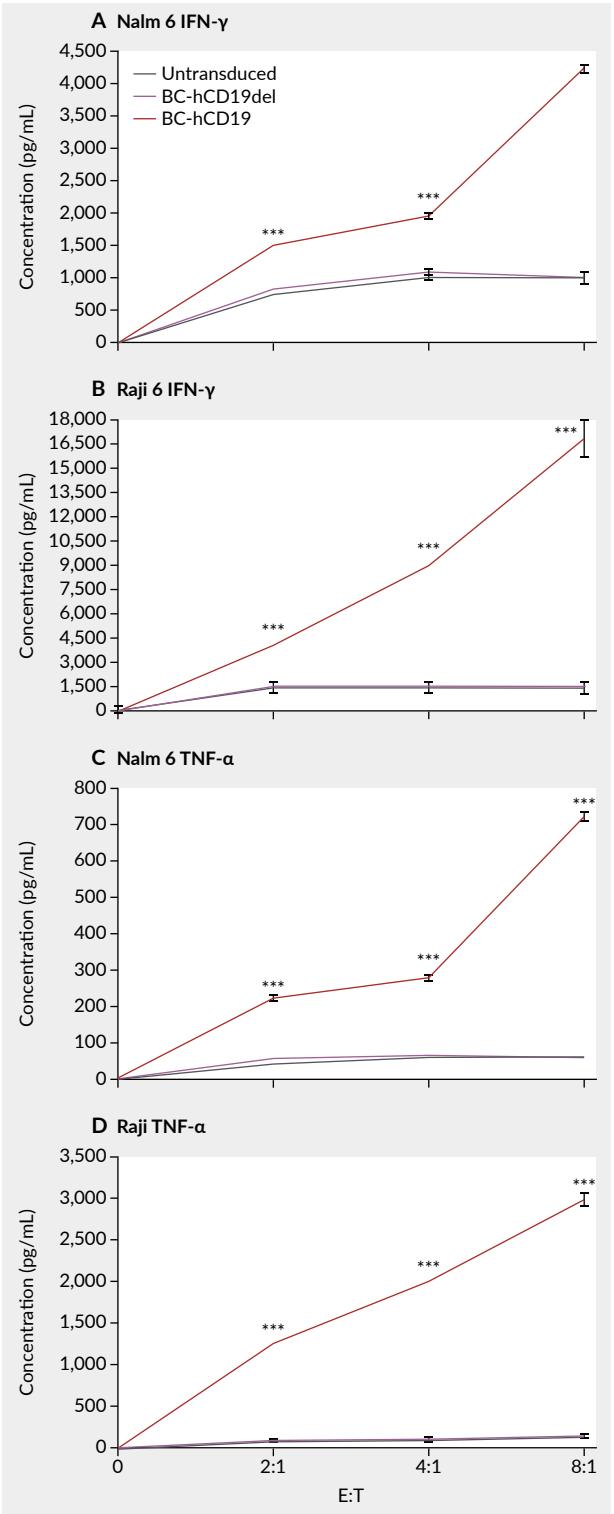


FIGURE 2

The levels of IFN-γ and TNF-α in BC-hCD19-CAR-T cells co-incubated with target cells.



IFN-γ: interferon-gamma, TNF-α: tumor necrosis factor-alpha.

or BC-001. The costimulatory activation domains were deleted to generate a mutant construct, which serves as a matched control. Additionally, a soluble transduction enhancer, referred to as BC-TE, was found to increase CAR expression and enhance the populations of naïve T cells and T memory stem cells (T_{scm}), both of which are key for persistence. More importantly, CAR-T cells prepared in the presence of BC-TE have shown a suppressive effect on cytokines related to cytokine release syndrome (CRS), such as interleukin-6 (IL-6).

As shown in Figure 1, the cell-killing activities of BC-001 were compared against FMC63 and C2146, both of which have the same vector backbone as BC-001. In a co-culture study with Raji tumor cells expressing luciferase, the results showed that BC-001 exhibited enhanced cell-killing ability compared to FMC63 and another humanized CD19 CAR-T construct, C2146.

Assessing cytokine responses in CD19 CAR-T cells co-cultured with cancer cell lines

In another study, levels of pro-inflammatory cytokines, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) were subsequently measured in

CD19 CAR-T cells co-cultured with Nalm6 and Raji cells. Nalm6 cells were originally derived from a patient with acute lymphoblastic leukemia, while Raji cells were derived from a patient with Burkitt lymphoma. The supernatant was analyzed by multiplex ELISA. The results showed that both IFN-γ and TNF-α were significantly elevated in both cell line models. In contrast, the cytokine levels secreted by cells transduced with the CD19 deletion control vector were not significantly different from those of the untransduced cells (Figure 2).

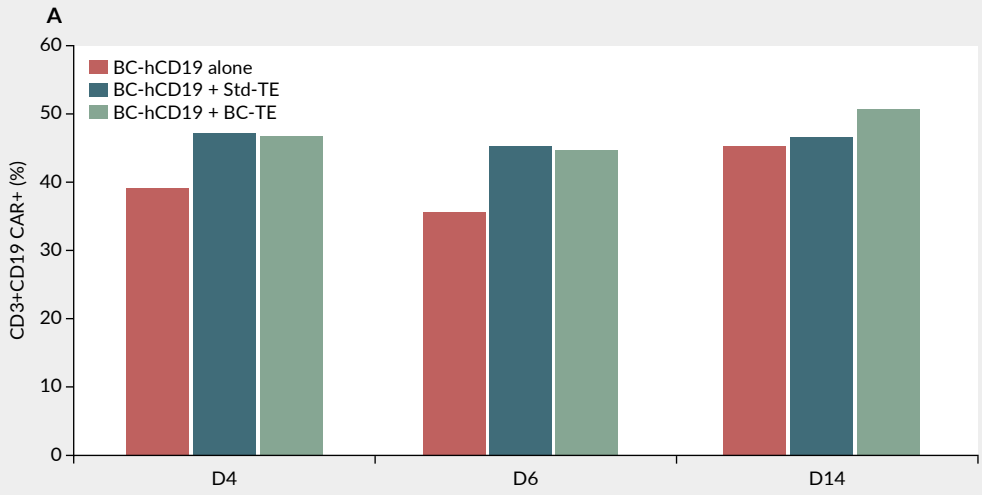
Enhancing lentiviral transduction efficiency with a novel reagent

One of the current strategies to help improve viral gene transfer involves the inoculation of cells with the Retronectin reagent. However, using Retronectin as a standard transduction enhancer (STD-TE) is often challenging for clinical applications because of the required pre-coating. Additionally, the method is difficult to standardize, cumbersome, and time-consuming. Quiescent and resting T cells are recalcitrant to the diffusion of lentiviral (LV) vectors, meaning that a high multiplicity of infection with LV vectors is required to overcome the limitations. Therefore, the identification of a safe, soluble, and easy-to-manipulate additive with a potent capacity to enhance the transduction efficiency of target cells using VSV-G pseudotyped antiviral vectors is essential.

In a proprietary study, which involved screening and domain mapping transduction enhancers, a soluble reagent (BC-TE) demonstrated similar transduction efficiency to Retronectin while enhancing CD19 CAR expression in a dose-dependent manner. Additionally, it was found that BC-TE reduces the quantity of CAR

FIGURE 3

Options and possible issues for RSV prevention.



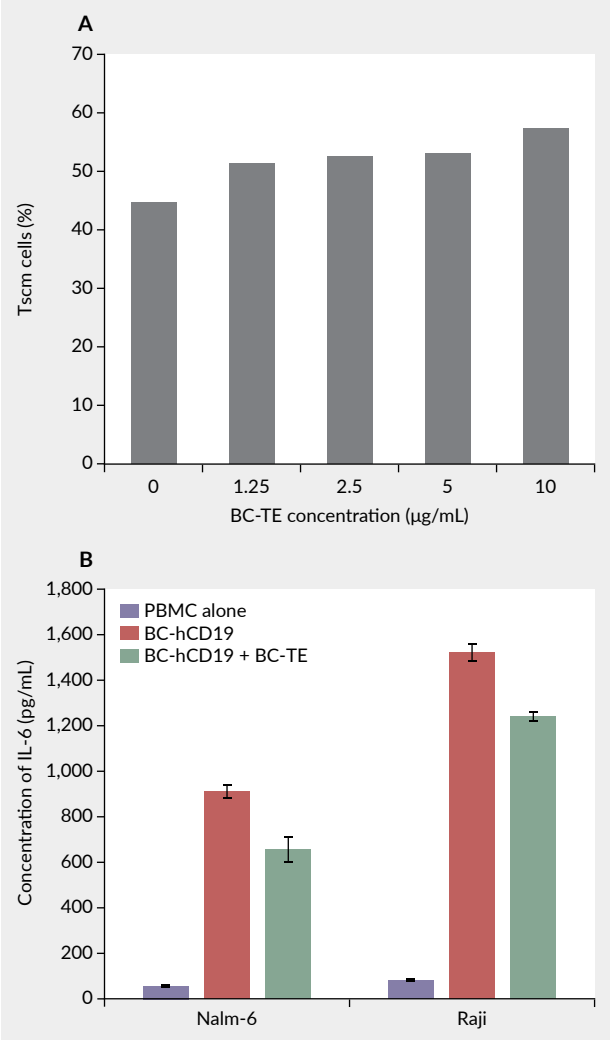
Cumulative fold change	D4	D6	D14
PBMC Ctrl	5.2	21.5	181.8
BC-hD19	3.9	11.4	167.3
BC-hD19 + Std TE	3.8	11.1	145
BC-hD19 + CV-TE	3.5	11.7	185.7

lentiviral vectors required to achieve good transduction efficiency, thus meeting the recommendation from the US FDA to maintain vector copy number/cells at less than five copies/genome. Reducing the use of clinical-grade lentiviral vector also resulted in significant cost savings.

As shown in Figure 3, CAR expression was enhanced and maintained over 2 weeks in the presence of BC-TE, without compromising the ability of T cells to proliferate and expand.

FIGURE 4

A: fraction of Tscm cells with increasing concentration of BC-TE; B: the suppressive effect of BC-TE on IL-6.



IL-6: interleukin-6.

Furthermore, it was found that BC-TE not only increases CAR expression but also promotes a slight increase in the percentage of T_{scm} cells (Figure 4A). Long-lived, cell-renewing T_{scm} and T central memory cells are likely key players in maintaining superior tumor regression. However, their low frequency in the blood of cancer patients presents a major hurdle for clinical CAR-T production. Therefore, this increase in the percentage of T_{scm} cells is a desirable feature for CAR-T therapies. When the supernatant from the cell culture study was examined, a reduced level of IL-6 was detectable in CD19-transduced cells prepared in the presence of BC-TE, but not in those prepared with the standard transduction enhancer, as shown in Figure 4B.

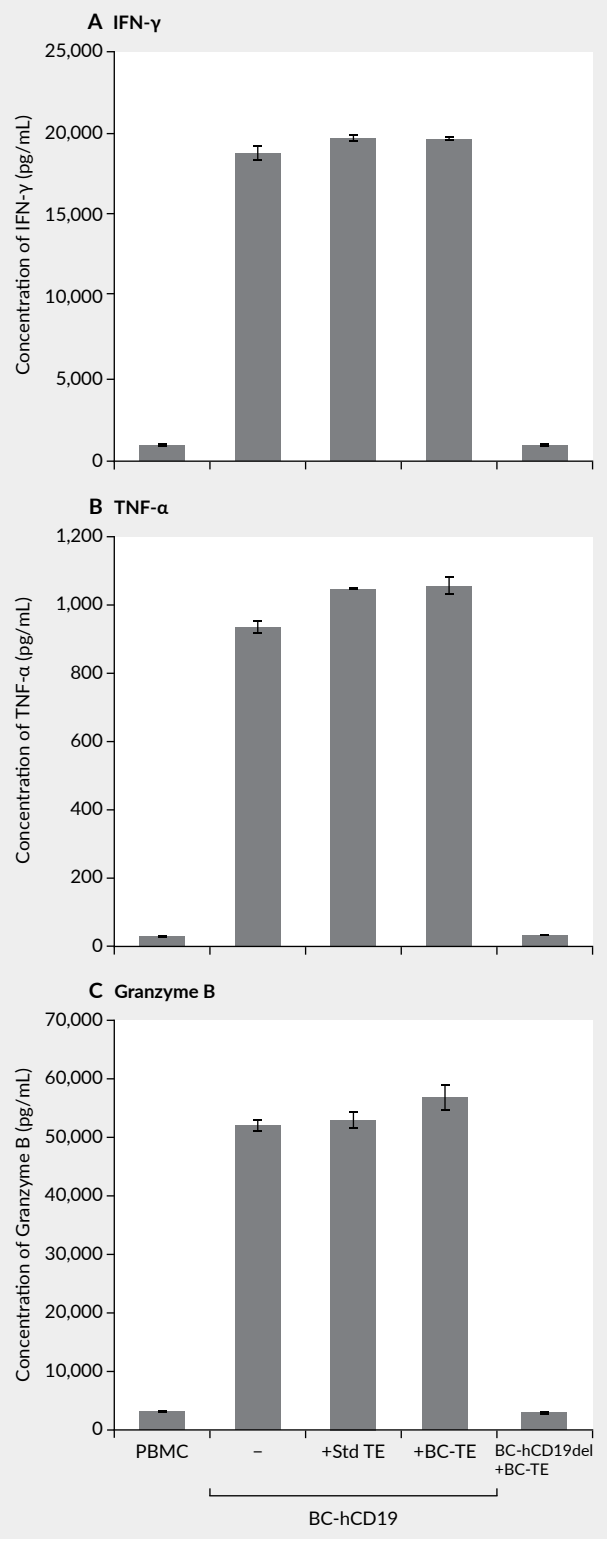
Beyond IL-6, the expression of TNF-α and IFN-γ was also profiled. No significant difference was observed between CAR-T cells prepared in the presence of BC-TE and those prepared with the standard Retronectin (Figure 5A and Figure 5B). The results also indicated that an increased level of granzyme was secreted by CAR-T cells prepared in the presence of BC-TE compared to the control groups, which included viral vectors alone or the standard transduction enhancer (Figure 5C).

Addressing cytotoxicity in CAR-T therapies with BC-TE

Tocilizumab is a monoclonal antibody that targets the IL-6 receptor. The inhibition of IL-6 binding to its receptor helps control inflammation, which is commonly used to treat CRS. However, it can also lead to secondary neurotoxicity when used alone, or increase the risk of opportunistic infections in patients. Currently, 17.4% of patients receiving CAR-T cell therapy succumb to CRS or cardiovascular and pulmonary adverse events [5]. In a recent case report, two patients with severe CRS were treated with tocilizumab with only one surviving. This particular example shows

FIGURE 5

Selected cytokine profiles in the presence and absence of BC-TE.



IFN- γ: interferon-gamma, TNF- α: tumor necrosis factor-alpha.

that cytotoxicity remains a significant challenge in CAR-T therapies. To address this hurdle, genome editing strategies to silence IL-6 and CD19 in CAR-T treatment are currently undergoing early-phase clinical trials.

In an internal co-culture cytotoxicity to evaluate potency, PBMCs were transduced with humanized CD19 vectors using either a standard transduction enhancer or BC-TE, or left un-transduced and later incubated with the tumor cells. As illustrated in Figure 6A, the tumor cell-killing mediated ability of BC-TE-enhanced humanized CD19 CAR-T cells was significantly increased compared to cells transduced with the standard transduction enhancer or without any enhancer. Notably, the level of IL-6, measured by ELISA, in cells prepared with BC-TE was only half that of cells prepared using the standard transduction enhancer or no enhancer at all (Figure 6B). In summary, this study presents a novel approach that significantly diminishes IL-6 cytokine levels without affecting tumor cell-killing capabilities.

Pre-clinical evaluation of BC-TE-enhanced CD19 CAR-T therapy

Next, a pre-clinical animal study was performed using NOD scid γ (NSG) mice. The mice were irradiated and injected with one million Nalm-6 luciferase-expressing tumor cells. The mice were subsequently randomized into groups and systemically infused with either phosphate buffered saline (PBS), PBMCs, or PBMCs transduced with humanized CD19, prepared either in the presence of BC-TE or without it. Tumor cells were effectively eradicated in the PBMCs transduced with humanized CD19 CAR-T, regardless of the presence of BC-TE, as anticipated. Tumor cells in the PBS-Control groups continued to proliferate. However, reduced IL-6 was observed only in the serum samples from the mice treated

with CD19 CAR in the presence of BC-TE and not in the PBS-control group, suggesting that CD19 CAR-T cells could potentially translate to a safer immunotherapy product without compromising efficacy.

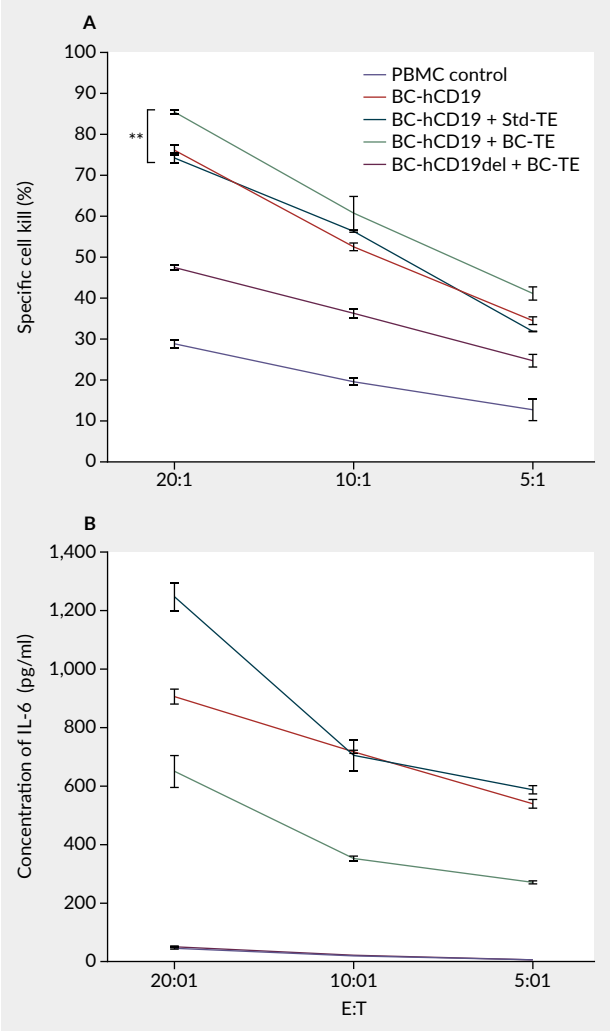
Suppressing IL-6 in CAR-T cells with BC-TE

In another proprietary study, the desirable properties of preparing CAR-T cells in the presence of BC-TE were tested with another type of CAR, denoted as NKG2D. These cells were genetically modified to express the NKG2D receptor, enabling them to kill cancer cells that express corresponding ligands, which are often present on cancerous or stressed cells but not typically found on normal human cells. The results were consistent with previous findings, showing that CAR expression increased in the presence of BC-TE, as shown in Figure 7A and Figure 7B. Additionally, IL-6 was significantly suppressed (Figures 7C and Figure 7D) while efficient cell kill capabilities were retained (Figure 7E and Figure 7F), suggesting that the biological effects of BC-TE are independent of the type of CAR construct used.

A subsequent study sought to determine whether the diminished IL-6 modulation observed with CD19 CAR T cells containing a 4-1BB costimulatory domain could also be seen with CAR constructs incorporating other commonly used TNF-α superfamily costimulatory domains, such as CD28 or ICOS. The results showed that cells prepared in the presence of BC-TE exhibited a statistically significant reduction in IL-6 across all tested costimulatory domains compared to the control group without BC-TE, suggesting that the inhibitory effect on IL-6 was not specific to a single type of costimulatory domain.

FIGURE 6

Tumor cell-killing capability and levels of IL-6 in CD19 CAR-T cells produced in the presence and absence of BC-TE.



CASE STUDY: A 7–DAY, SCALABLE CD19 CAR-T CELL MANUFACTURING PROCESS USING CTS WORKFLOW

A closed, automated system was utilized to manufacture CAR-T cells in seven days utilizing the CTS™ DynaCellect™ Magnetic Separation System and CTS™ Rotea™ instruments. Firstly, a quarter of a frozen Leukopak from a healthy donor was thawed and loaded onto a transfer bag for processing. T cells were then isolated and activated using CTS™ Dynabeads™ CD3/28 magnetic beads at a bead-to-cell ratio of 3:1, facilitated by the CTS

DynaCellect Magnetic Separation System. Subsequently, the isolated cells were collected in complete CTS™ OpTmizer™ One media supplemented with cytokines, and then transferred to either a 1 L G-Rex100M-CS vessel or a small-scale G-Rex6M for expansion. After 24 h, humanized CD19 lentiviral vectors were added to the G-Rex100M-CS vessel for transduction. On day 7, the process involved volume reduction by GatheRex, de-beading by CTS DynaCellect, and harvesting of the CAR T cells using counterflow centrifugation with the CTS Rotea system. These steps demonstrate the practicality of upscaling the production process while maintaining the critical parameters required for effective CAR-T manufacturing.

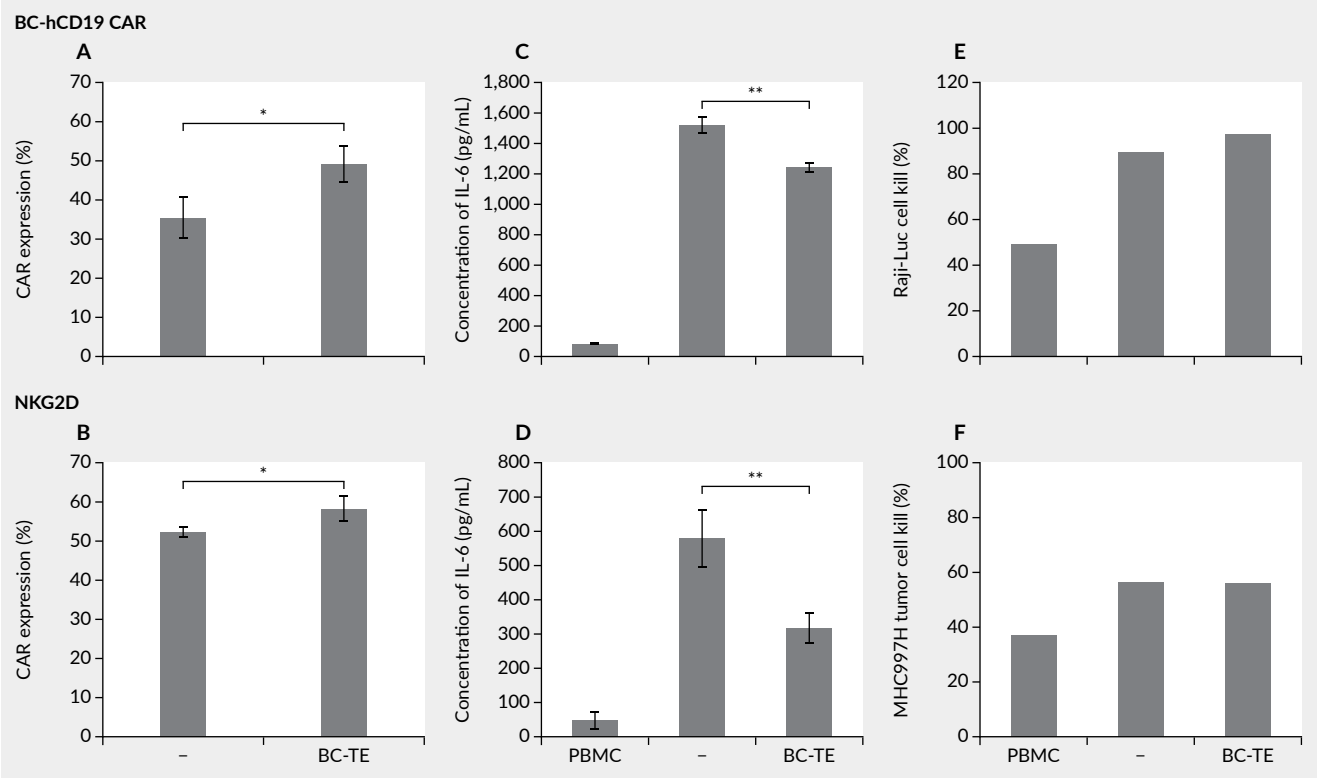
Flow cytometry was employed to assess immune cell composition throughout the entire process. The data shown in Figure 8 are representative of a scaled-up CAR-T cell expansion step. At day 7 post-harvest, over 96% of the T cell population was pure, with a distinct increase in T cells compared to day 0 (Figure 8A). Cell viability also improved from 74 to 85% (Figure 8C). T cell recovery after de-beading and harvest was 78, 74, and 88%, respectively (Figure 8B). By day 7, the fraction of CD4+ cells exceeded that of CD8+ cells (Figure 8D). In summary, these data support the feasibility of implementing the CTS-based manufacturing process in clinical settings.

Small-scale CD19 CAR-T cell expansion

In a small-scale experiment, G-Rex6M deep-well plates were used for CAR-T cell expansion. Activated PBMCs from healthy donors were transduced with humanized CD19 CAR LV vectors, either in the presence or absence of BC-TE, or left non-transduced. It

FIGURE 7

The effects of BC-TE on various CAR constructs.



was discovered that in the presence of the soluble transduction enhancer, CD19 CAR expression increased from 62–90%, with a corresponding 10% increase in T_{scm}.

Subsequently, a lactate dehydrogenase cytotoxicity assay was performed to evaluate the potency of humanized CD19 CAR-T cells. It was discovered that CAR-T cells prepared in the presence of BC-TE exhibited higher cytotoxicity at target-to-effector ratios of 10:1 and 20:1, compared to those prepared without BC-TE. No difference was observed at the lower effector-to-target ratio of 5:1. Furthermore, IL-6 levels were consistently reduced in the presence of BC-TE.

Scaling up CD19 CAR-T cell expansion and QC testing

In order to scale up the 7-day CAR-T manufacturing process, a 1 L G-Rex100M-CS vessel was used for expansion instead of G-Rex6M deep-well plates. It was discovered that 64% of the cells were CD19 CAR-positive, with a corresponding 12% increase in T_{scm}. Similar tumor cell-killing activity mediated by humanized CD19 CAR-T cells was observed, and a decrease in IL-6 levels was also noted when BC-TE was added to the scaled-up CAR-T expansion study.

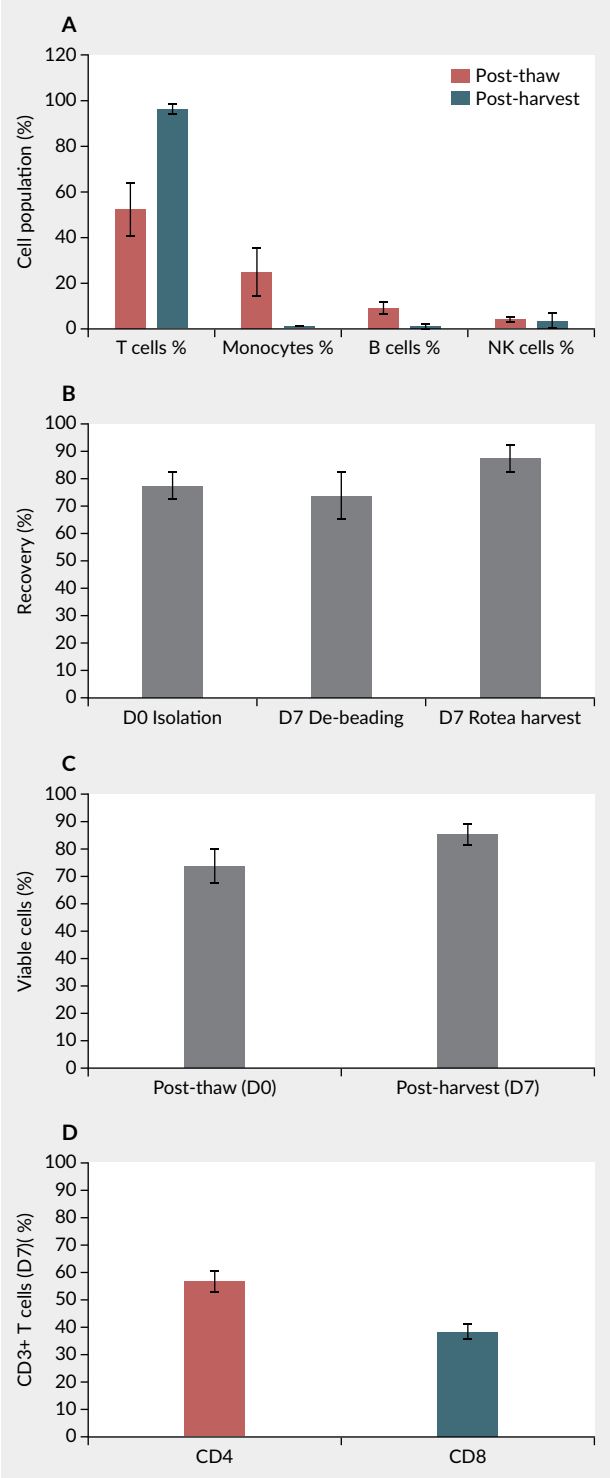
Furthermore, a 7-day sterility culture was performed on the final product harvested on day 7. The results were negative for microbial growth, replication-competent lentiviral vectors, and mycoplasma. Both endotoxin levels and CD19-positive cells remained low, indicating that the final CAR-T cell product is relatively free of contaminants and replication-competent lentiviral vectors.

CLOSED, END-TO-END CAR-T CELL MANUFACTURING WORKFLOW USING CTS INSTRUMENTS

Closed and modular CTS instruments such as the CTS DynaCellect, CTS Rotea, and CTS™ Xenon™ systems can support robust CGT manufacturing processes, enabling

FIGURE 8

T cell population, recovery, viability, and comparison between CD4 and CD8 cells.



D: day.

flexibility and ensuring sterility throughout fully closed manufacturing workflows. The instruments are designed for all cell therapy manufacturing steps, including cell isolation, activation, bead removal, cell processing, viral or non-viral engineering, expansion, wash, formulation for characterization, and lot release. Additionally, segregating the incubation step enables the instruments to be repurposed for subsequent or alternative tasks.

Automated cell isolation and activation with the CTS DynaCellect System

The Gibco CTS DynaCellect Magnetic Separation System is a closed, automated solution designed for cell isolation and bead removal processes in CGT development and manufacturing. Together with its fit-for-purpose consumables, the system ensures high levels of cell purity, recovery, and viability. Whether utilized as a standalone instrument or integrated into a workflow, the operator-independent system enhances manufacturing robustness and precision by isolating target cells effectively and minimizing potential failures. The system is designed specifically for use with magnetic beads. The results from an internal study, illustrated in Figure 9, show that CD3+/CD28+ T cells that were directly isolated and activated in a Leukopak bag using CTS Dynabeads magnetic beads without any washing steps were over 97% pure.

The CTS DynaCellect system consists of an intuitive programmable interface, an integrated rocker, and fluidics panels, enabling high cell recovery across a wide range of reaction volumes. The system delivers optimal efficiency, achieving cell purity rates over 95% without compromising cell viability. Additionally, the process flexibility is supported by sterile, single-use consumables and an available software upgrade that ensures compliance with 21 CFR Part 11, allowing for seamless scaling from development to clinical and commercial manufacturing.

Flexible cell processing with CTS Rotea counterflow centrifugation system

The CTS Rotea is a compact, closed, automated cell processing system specifically designed for cell therapy manufacturing. Utilizing proprietary counterflow centrifuge technology, the instrument enables flexible operations, including size-based cell separation, washing, concentration, buffer exchange, and formulation. The system's unique chamber design allows for a very low output volume of 5 mL, enabling over 95% cell recovery with no loss in viability. Lastly, the interface of the system supports compliance with 21 CFR Part 11.

The counterflow centrifugation process usually begins by introducing the cells into the chamber through a narrow tube with a high fluid entry velocity ensuring that the cells exit the tip and enter the chamber (Figure 10A). Simultaneously, the chamber spins at an opposing G-force, preventing the cells from exiting the chamber. When the flow rate and G-force are balanced, or in equilibrium, a fluidized bed of cells forms and is maintained in the chamber. At this stage, media exchange, cell washing, separation by size, and concentration can be performed.

The conical shape of the chamber, along with the narrowing at the top, increases the fluid velocity, which forces smaller cells to exit the chamber first. The G-force and flow rate settings can be adjusted to facilitate the separation of smaller cells, a process known as elutriation. As shown in Figure 10B, the G-force is lowered, allowing smaller cells to exit through the top of the chamber into the back. Instead of elutriating smaller cells, unwanted small cells can be depleted. For example, when a Leukopak is loaded, small platelets immediately exit the chamber, while the PBMCs are retained. This enables

platelet depletion to be achieved with a single-step sample loading process.

EXAMPLES OF CTS ROTEA APPLICATIONS

Monocyte depletion and lymphocyte enrichment

The CTS Rotea can be utilized to help reduce monocytes from patient apheresis samples, serving as a debulking step before CD3+ T cell isolation. In proprietary studies, this approach has allowed lymphocyte enrichment to increase from 40 to 72% while maintaining extremely high cell viability.

CAR-T cell wash and formulation

One of the key features of the CTS Rotea is its ability to perform thorough cell washes in an automated and enclosed manner, ensuring that residual components are efficiently removed before the final formulation of the product. In internal studies, it has been demonstrated that over 90% of common cell culture components, such as cytokines like IL-2, glucose, residual human serum albumin, and dimethyl sulfoxide, are removed during this process.

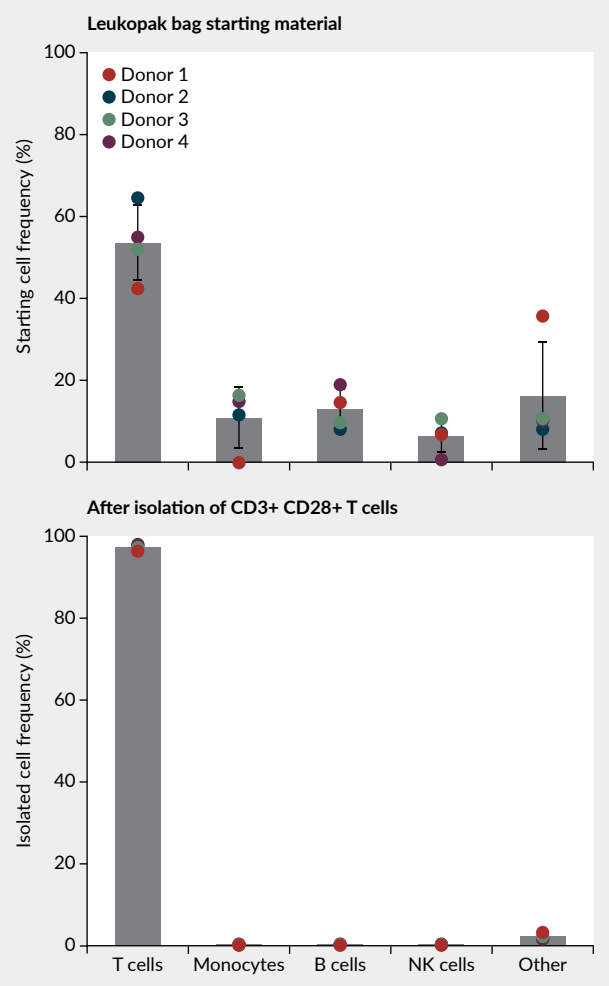
ROBUST T CELL EXPANSION WITH CTS OPTMIZER ONE MEDIA

One of the key recent trends in terms of efforts to enhance CAR-T cell therapy has focused on improving both the persistence and safety of these therapeutic products by identifying and developing specific reagents and components that support the formulation of fit-for-purpose media. This enables the cultivation of a higher population of naïve and T_{scm}.

In a proprietary study, T cell activation and expansion were evaluated using the serum-free, animal origin-free (AOF) CTS OpTmizer One media formulation, and compared to two other supplier formulations (CM1 and CM2) as well as a chemically defined AOF media (CM3). As shown in Figure 11, T cells were activated on day 0 using CTS Dynabeads CD3/CD28 magnetic beads at a 3:1 ratio with IL-2, and cultured until day 10 with IL-2 added on day 3 and a 50 to 60% media exchange on days 5 and 7. By day 10, cell viability remained comparably high at 84% with CTS OpTmizer One media, and ranged from 76 to 88% with other types of media. In summary, the CTS OpTmizer One media consistently supported higher cell counts, with an increase of up to 214% compared to CM1, CM2, and CM3 formulations.

FIGURE 9

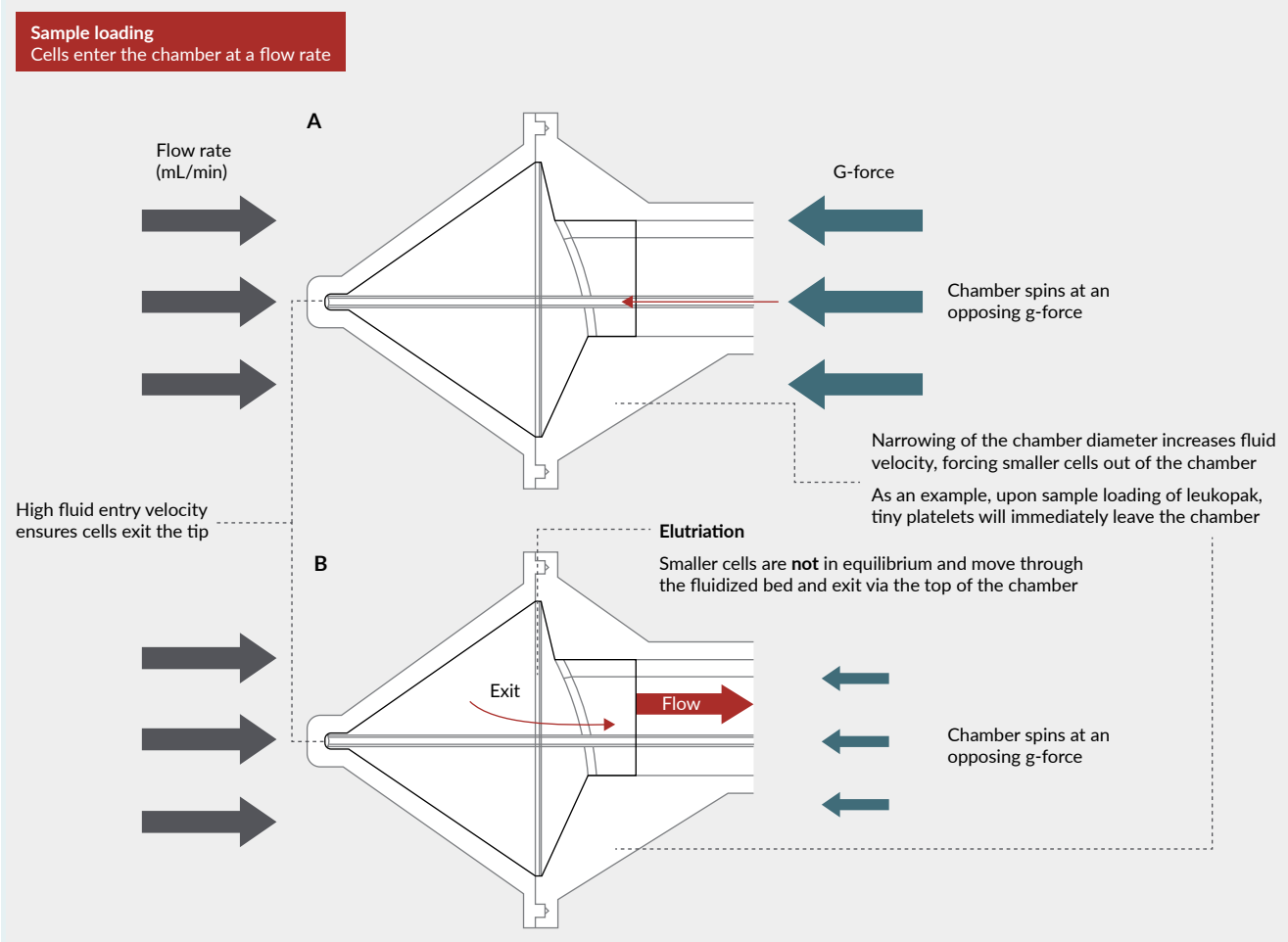
Starting and isolated cell frequency (%).



n=4 donors.

FIGURE 10

Counterflow centrifugation process.



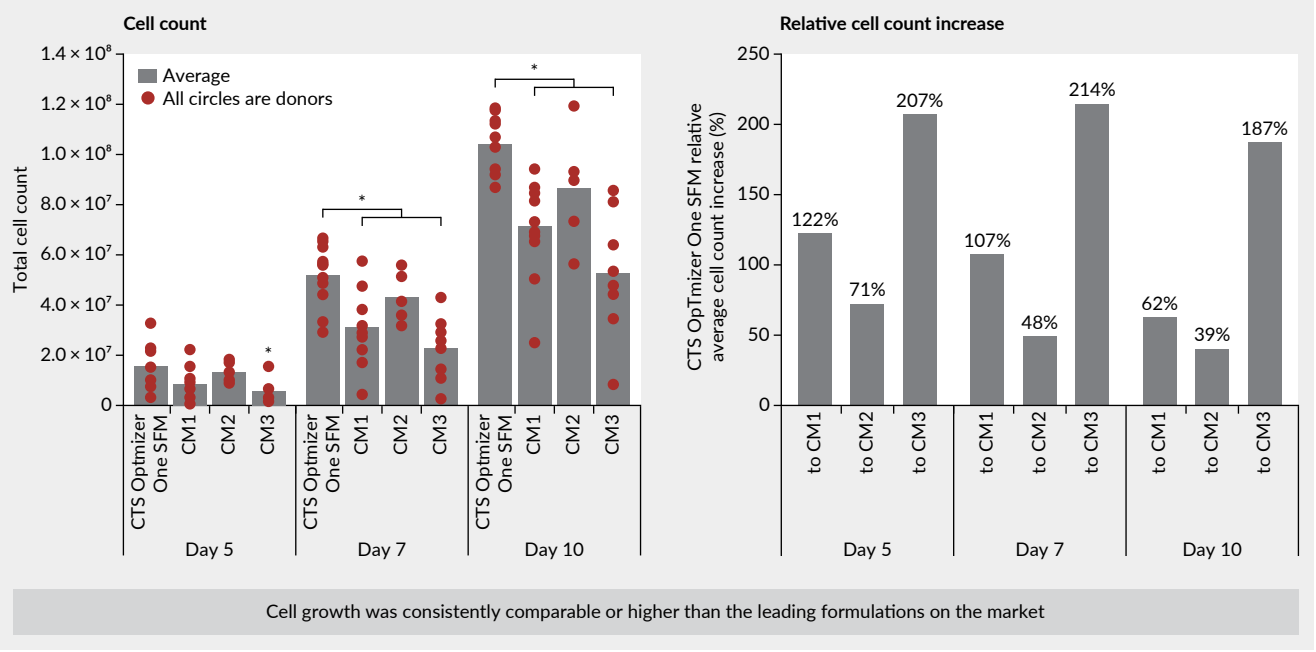
Furthermore, by day 10, T cells cultured with our CTS OpTmizer One media demonstrated a comparable or statistically higher average expression of early cell phenotype stem and central memory markers. Notably, despite the highest growth observed with CTS OpTmizer One, there was no loss in the desired early memory phenotype of the cells.

CELL EXPANSION AND VIABILITY WITH CTS OPTMIZER ONE MEDIA IN LEUKEMIA PATIENTS

In another experiment, it was assessed whether CTS OpTmizer One was compatible with patient material. More specifically, lymphocytes were isolated from two disease donors with acute myeloid leukemia. The results demonstrated that CTS OpTmizer One media supported more consistent and higher cell expansion by day 10 compared to other supplier media (CM1 and CM3). Additionally, CTS OpTmizer One maintained 85% cell viability and ensured more consistent preservation of the early cell phenotype compared to the other media. Similarly, for two chronic lymphocytic leukemia disease donors, cells cultured in CTS OpTmizer One media exhibited a higher average expansion (84%) by day 10 compared to those in CM1 media, with viability remaining high across all conditions (For Research Use or Manufacturing of Cell, Gene, or Tissue- Based Products. CAUTION: Not intended for direct administration into humans or animals.).

FIGURE 11

Comparison of cell growth using CTS OpTmizer One SFM and other media on the market.



CTS OpTmizer One SFM: n = 11 donors, CM1: n = 11 donors, CM2: n = 5 donors, CM3: n = 8 donors, * = $p < 0.05$.

THE IMPACT OF CTS OPTMIZER ONE MEDIA ON LENTIVIRAL TRANSDUCTION EFFICIENCY

Another proprietary study evaluated whether CTS OpTmizer One impacted LV transduction efficiencies. Donor cells were transduced with a GFP-based LV construct and observed from day 7 to day 14. The results showed that GFP+ cells demonstrated comparable or even higher LV transduction efficiency with CTS OpTimizer One media compared to the other supplier's CM1 media. Lastly, it was observed that transduction efficiency did not diminish over time, and the culture was not overrun by non-transduced cells.

SUMMARY

In summary, Thermo Fisher Scientific has developed a 7-day viral-based CAR-T production process using CTS instruments, which is designed to help reduce both overall COG and time to patients while enhancing process efficiency, robustness, and simplicity for developers.

The BioCell Innovations pilot study demonstrated the feasibility of scaling up a 7-day process for humanized CD19 CAR-T cell production using a closed system that included the CTS DynaCellect Magnetic Separation System, CTS Rotea System, and G-Rex100M-CS vessels. Additionally, preliminary data on a second-generation humanized CD19 CAR construct demonstrated its functionality and safety both *in vitro* and *in vivo*. Notably, the addition of the soluble transduction enhancer BC-TE increased CAR expression, promoted Tscm cells, and reduced IL-6 expression, potentially leading to a safer, more cost-efficient, and more effective final drug product.

Q&A

Soong Poh Loong (left), **Paula Lam** (right)

Q What multiplicity of infection (MOI) was used for transducing the cells cultured in CTS OpTmizer One media?

SPL We used an MOI of 5. In our in-house R&D tests, we also evaluated the LV vectors at an MOI of 2 and obtained similar efficiencies.

Q Can the CTS-based process be used for non-viral-based T cell and natural killer (NK) cell production?

SPL For non-viral approaches, including knock-ins, knockouts, or gene editing techniques such as CRISPR/Cas9, CTS systems can be seamlessly connected in a sterile manner to our CTS Xenon electroporator in an automated, closed process. This enables the efficient non-viral delivery of constructs to the cells. Additionally, modular closed systems can also be used to isolate and modify NK cells.

Q Does the humanization of the CD19 CAR improve the overall response rates in patients undergoing CAR-T cell therapy?

PL A clinical trial conducted by Dr Shannon Maude at the Children's Hospital of Philadelphia investigated the use of humanized CD19 CAR-T cell therapy in children and young adults with relapsed or refractory B-cell malignancies. The results demonstrated that the humanized CD19 CAR-T cell products achieved durable remission and long-term persistence, even in patients who had previously failed multiple CAR-T cell therapies [6].

Q Are there any safety and toxicity concerns associated with using the soluble transduction enhancers during the manufacturing of CAR-T cells?

PL Regarding safety and toxicity, no weight loss was observed in mice that were administered humanized CD19 CAR-T cells, either prepared with BC-TE or

Cell & Gene Therapy Insights 2025; 11(1), 41–58 · DOI: 10.18609/cgti.2025.007

without it, compared to untreated controls. In both the small-scale and large-scale runs, cell viability remained high when using transduction enhancers, in contrast to the controls without enhancers. While the risk of toxicity appears to be low, further investigation is needed to confirm these findings.

Q How do strategic industry partnerships drive innovation and commercialization in cell therapy manufacturing?

PL Strategic industry partnerships involve extensive brainstorming and a great exchange of experiences and ideas while working towards a common goal. For example, the shared goal of developing a safe and effective CAR-T product to help patients was the main driving force behind this partnership, which has proven to be highly successful.

SPL There is a significant gap between basic research and clinical product application, and this is where stronger collaboration with manufacturers, product developers, and clinicians is crucial to understanding real needs.

When we combine the expertise of the clinicians and target patients with these strengths, a more defined and efficient route is formed—one that overburdens neither manufacturers nor patients. We are focused on driving the benefits of a modular approach, which helps streamline processes while allowing product developers to focus on the safety of their therapeutics.

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BIOGRAPHIES

Soong Poh Loong is a Field Application Scientist at Thermo Fisher Scientific, Singapore, supporting the Cell and Gene Therapy portfolio across Asia-Pacific and Japan. He manages a team of Application Scientists with a focus on providing technical consultations and process optimization support to early clinical-stage developers. Poh Loong also works in collaboration with Thermo Fisher's scientists to lead and implement collaboration projects.

Prior to joining Thermo Fisher, Poh Loong had over 20 years of experience and expertise in human stem cell research, GMP cell lines generation, and cardiac tissue engineering, Poh Loong's translational research interests center around stem cell-based disease modeling, heart regeneration, and repair. He is a member of the ISSCR, European Society for Cardiology, and the German Center for Cardiovascular Research (DZHK) and obtained his PhD from the Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Germany, with a summa cum laude thesis and patents on Engineered Heart Muscles for heart failure treatment. This multi-centered work has since progressed to a first-in-human clinical trial of the BioVAT-HF therapeutic device for terminal heart failure. Poh Loong also co-founded a Singapore biotech startup that develops high-speed platforms for cellular characterization, disease modeling, and drug safety screening.

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Paula Lam graduated from the University of Leeds, Leeds, UK majoring in Molecular Biology and Biochemistry. Following this, she undertook postdoctoral training at St Jude Children's Research Hospital, Memphis, TN, USA and Massachusetts General Hospital, Boston, MA, USA before returning to Singapore as a group leader at the National Cancer Center where her research focus lies in developing vectors with enhanced specificity and potencies against human cancers. She is currently the Chief Scientific Officer of BioCell Innovations, Singapore, a clinical-stage biotech, with a primary objective to bring cell and gene therapies to patients-in-need. BioCell responsible for the development of in-house CAR lentiviral vectors and cell-based immunotherapies by leveraging a novel manufacturing platform.

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AUTHORSHIP & CONFLICT OF INTEREST

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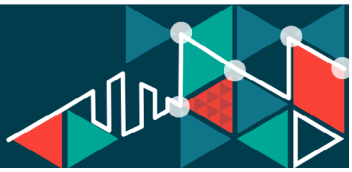


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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Toward clinical-scale nonviral gene-edited CAR-NK cells for cell therapy

Namritha Ravinder and Krishanu Saha

Improving the accessibility and effectiveness of cell therapies is essential to meet the growing demand for cancer treatments. Engineered natural killer (NK) cells represent a promising modality, offering safety, effectiveness, and the possibility of allogeneic use. This article highlights gene editing techniques such as CRISPR-Cas9 for enhancement of NK cell functionality, and explores nonviral transfection methods and scalable manufacturing processes. Additionally, a case study on anti-Meso3 CAR-NK cells targeting ovarian cancer cell line is explored, showcasing the enhanced efficacy of engineered CAR-NK cells for killing cancer cells and their potential for clinical scale applications.

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ADDRESSING LOW ACCESSIBILITY OF CELL THERAPIES WITH ALLOGENEIC APPROACHES

Cell therapies have demonstrated remarkable responses in patients and are established as an important new modality for treating various diseases, particularly blood cancers. However, one of the key obstacles to achieving a broader impact for cell therapies is their cost. Currently, the cost of an autologous cell therapy in the USA is approximately $400,000 per dose, limiting access to many patients. Certain regions in the USA with varying socioeconomic statuses, as well as racial and ethnic differences, experience unequal access to cell therapies. Additionally, projections indicate that the US population is aging, which will likely further increase the demand for such therapies.

Currently, most cell therapy products utilize autologous strategies, where blood sample is collected from a patient, and immune cells, such as natural killer (NK) and T cells, are extracted, engineered *ex vivo* to recognize and kill specific cancer cells, and reinfused into the same patient. However, autologous cell therapies are costly and complex to manufacture and administer.

In contrast, an allogeneic strategy involves taking a blood sample from a healthy donor, and engineering and expanding the cells *ex vivo* to achieve doses for hundreds or thousands of patients. The vision behind this approach is to create a set of cells stored in an infusion bag, allowing clinicians

to access them 'off the shelf' upon their decision to treat a patient. At the time of diagnosis, these types of modalities are readily available for infusion, potentially expanding access to patients.

Another key advantage of allogeneic cell manufacturing is the ability to genetically engineer the cells with multiple functionalities. This ability is largely due to the extended timeframe available in allogeneic workflows for quality control (QC), unlike the limited QC window in autologous manufacturing.

EXPLORING NK CELLS AS POTENT CELL THERAPIES

NK cells are part of the innate immune system, playing a vital role in the body's first line of defense against infections and tumors. They can be obtained from multiple sources, including donor or patient leukapheresis, cord blood, induced pluripotent stem cells (iPSCs), and established NK-92 cell banks [1]. Additionally, NK cells are human leukocyte antigen (HLA)-agnostic and pose little to no risk of graft-versus-host disease, meaning they are generally regarded as safe.

Studies have shown that engineering NK cells to express a synthetic chimeric antigen receptor (CAR) can increase their potency [1]. The insertion of a CAR into NK cells has already generated promising outcomes for patients with blood cancer. For example, in a MD Anderson Cancer Center Phase 1/2 trial with anti-CD19 CAR-NK cells, a 64% complete response was observed [2]. Similarly, in Nkarta's Phase 1 trial with CD19 CAR-NK cells, a 70% complete response was achieved [3]. Additionally, no severe adverse events were observed.

CHALLENGES AND ADVANCES IN GENE EDITING FOR CAR-NK CELL THERAPY

Research in CAR-NK cell therapy has primarily focused on blood cancers, although the field is now shifting towards solid tumors. Solid cancers present a more complex and challenging tumor microenvironment (TME) for cell therapy due to the presence of several inhibitory molecules, such as TGF-β, vascular endothelial growth factor, fibroblast growth factor, and certain checkpoint molecules that are typically activated in the TME. Altogether, these molecules can lead to decreased function and persistence of NK cells. As a result, even the cells that reach and act on the tumor may not remain there, or they may be eliminated or become dysfunctional. One of the inhibitory checkpoints of interest has been NKG2A, which binds HLA-E [4]. In order to deliver transgenes into primary NK cells, the Wisconsin Institute for Discovery aims to genetically manipulate this pathway through utilization of CRISPR-Cas9 genome editing.

While the CRISPR-Cas9 strategy has been the key focus, other approaches for delivering transgenes to CAR-NK cells have been employed. One alternative method for NK cell manipulation includes utilizing viral vectors, although there are concerns that the viral transgene can disrupt endogenous genes, raising issues of insertional mutagenesis. Additionally, electroporation may be utilized to deliver transgenes via mRNA, transposons, and combining CRISPR-Cas9 with AAV or DNA templates. However, it was discovered that the mRNA-based technique tends to result in transient expression rather than the durable expression associated with genomic integration. Similarly, transposons offer less control compared to CRISPR-Cas9 regarding the insertion sites within the genome. Finally, strategies involving AAV vectors, such as the SLEEK technique, have shown efficacy but require viral production and selection, which may pose additional challenges [5].

Numerous studies have explored the combination of CRISPR-Cas9 genome editing and nucleic acid-mediated delivery. Double-stranded DNA (dsDNA) is favored due to more straightforward QC and the

ability to manufacture high concentrations of the template. Some studies have reported approximately 8% knock-in efficiency with a green fluorescent protein (GFP) transgene [6]. Furthermore, more recent data indicated that a larger construct, such as a CAR, achieved knock-in rates of approximately 5–10% [7]. These rates are lower compared to some viral vector results, such as 25% knock-in efficiency with lentiviral vectors, demonstrating that there is still room for improvement in enhancing the genetic manipulation of NK cells with CRISPR-Cas9 genome editing.

NONVIRAL ENGINEERING OF NK CELLS TO TARGET SOLID TUMORS

A recently released preprint, based on a study led by Keerthana Shankar, focused on engineering primary NK cells obtained from healthy donors using guide RNA (gRNA) targeting the gene KLRC1, which inhibits the tumor checkpoint [8]. A template encoding CAR was also utilized. This approach was designed not only to knockout the receptor, thereby enhancing the potency of NK cells, but also to express a CAR that targets the disialoganglioside (GD2) antigen. GD2 is a molecule that is expressed on several solid tumors, including glioma, neuroblastoma, and osteosarcoma, which exhibit some of the highest expression levels (approximately 50%, as reported in the *Cancer Cell Line Encyclopedia* [9]). One of the advantages in this field of research is that a glycolipid, GD2 structure is common to both human and rodent tumors, allowing for robust comparisons of CAR functionality in preclinical studies.

The transgene utilized in this study was a second-generation CAR [8]. The results from electroporation of Cas9, gRNA, and the template encoding the CAR across five donors indicated a knock-in efficiency of 15–25%. In the control experiment, which utilized an mCherry fluorescent reporter without the CAR, the knock-in efficiency was similar. Non-transfected cells showed no expression of either transgene or CAR. The mean fluorescence intensity was relatively high, which can be attributed to the KLRC1 promoter, considering the transgene did not contain a promoter. Instead, the transgene was knocked into the locus, regulated by native KLRC1 elements.

Furthermore, in order to examine whether KLRC1-CAR-NK cells exhibit off-target genomic outcomes, a comprehensive analysis was conducted using the CHANGE-Seq method developed by Shengdar Tsai's Laboratory at St Jude Children's Research Hospital [10]. This technique assisted in identifying several sites in the genome to monitor mutations as well as transgene integration. The analysis showed minimal disruption and almost no CAR transgene integration, as assessed through PCR and whole-genome long-read sequencing.

Finally, an *in vitro* potency assay was carried out to compare the potency of KLRC1-CAR-NK and unmodified NK cells. The engineered cells demonstrated significant cytotoxicity against M21-HLA-E melanoma cell lines that express both GD2 and HLA-E *in vitro*, which allowed for testing the effects of both knocking out the receptor and expressing the CAR. As shown in **Figure 1**, the highest cytotoxicity was observed in the cells expressing the CAR (KLRC1-CAR) compared to KLRC1-No CAR, KLRC1-CAR-knockout, and unmodified cells.

SCALING CAR-NK CELL MANUFACTURING FOR CLINICAL APPLICATIONS

All of the research conducted in the preprint was performed at a research scale, involving approximately 1 million cells/electroporation. However, efforts have been made to scale this type of CAR-NK engineering towards clinical manufacturing. One of the main goals is to increase the aforementioned research-scale capacity by 1–2 orders of magnitude through utilization

of reagents appropriate for both IND and Phase 1 studies. The Gibco™ CTS™ HiFi Cas9 Protein was tested and yielded promising results, including both knock-in and knockout efficiencies at favorable levels, as well as the expansion of cells over 2 weeks. This technology is being adapted to scale up the manufacturing of nonviral multifunctional NK cells.

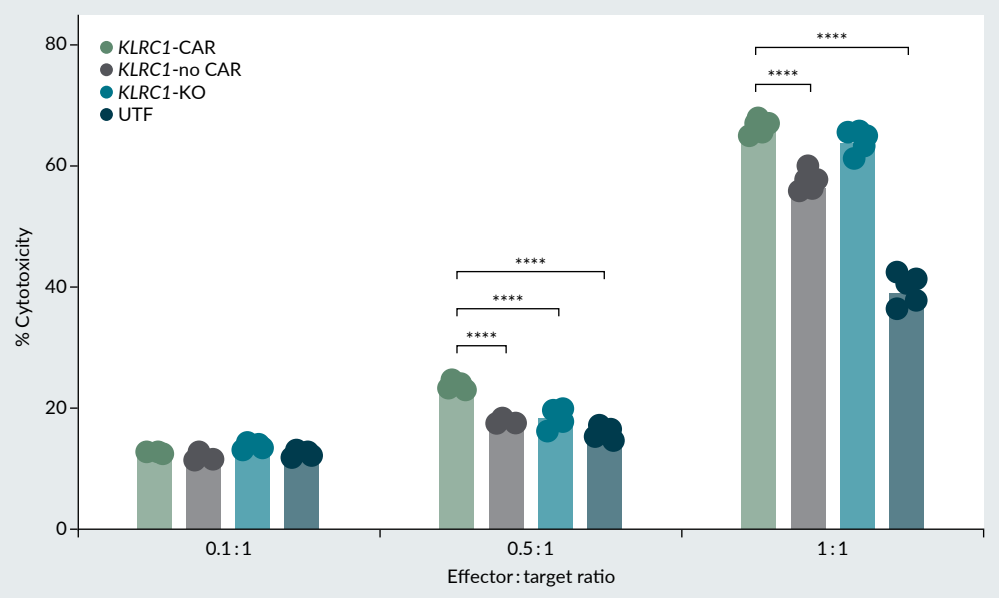
STREAMLINING CLINICAL-SCALE CAR-NK CELL MANUFACTURING WITH CLOSED SYSTEMS

Utilizing closed and modular instruments alongside GMP-grade reagents designed to meet clinical-scale cell therapy manufacturing can support a seamless transition from research to process development through commercial manufacturing phases.

The large-scale engineered NK cell manufacturing workflow involves cell isolation, activation, and bead removal utilizing magnetic bead-based technology. Subsequently, cell processing involves transduction with viral vectors or transfection with large-scale electroporation, and engineering with CRISPR-Cas9 or TALEN™ gene editing tools. Next, the cells are expanded using GMP-compliant reagents and bioprocessing equipment. The wash and buffer exchange steps pre- and post-gene editing steps can be performed using closed automated platforms like CTS™ Rotea™ Counterflow Centrifugation systems. Finally, the analytics and characterization are performed using genomic, proteomic, and cell analysis tools, as well as identity, purity, and sterility assays. The Gibco™ CTS™ Cellmation™ software powered by DeltaV™ platform can be utilized to facilitate digital integration, enabling workflow automation during the NK cell engineering process.

FIGURE 1

Potency assay of engineered NK cells and unmodified NK cells against M21-HLA-E melanoma cell line.



The Y-axis in the graph represents cytotoxicity, with the green bars indicating the engineered cells featuring the knockout of NKG2A through the KLRC1 insertion. The 'no CAR' condition includes only the knockout of NKG2A together with the insertion of an mCherry fluorescent reporter, while the knockout condition lacks any insertion.

FACTORS IMPACTING DELIVERY AND CELL ENGINEERING

Various factors and variables can impact gene editing efficiency, the expansion of NK cells, and the quality of the final cell therapy product. Therefore, it is crucial to firstly ensure appropriate cell source and culture conditions. Typically, different donor samples need to be screened to identify one capable of providing viable NK cells.

For the gene editing step, selecting the appropriate gene of interest (GOI) and, gene editing payload and gRNA with minimal off-target effects and maximum editing efficiency is vital. Based on an internal study, the Cas9-RNP system with high-fidelity (HiFi) Cas9 and synthetic gRNAs yields the best results. The Gibco CTS HiFi Cas9 employed in this context provides exceptionally high efficiencies while minimizing off-target effects.

Another key attribute that must be optimized before proceeding with cell engineering workflows is the quality of the donor DNA. Ensuring that the donor DNA is designed with the correct sequences and homology arms, as well as maintaining high-quality standards, is essential for achieving the highest gene editing efficiencies while preserving cell viability and functionality.

CAR-NK CELL ENGINEERING AND EXPANSION WORKFLOW

The first step of the NK cell engineering process involves isolating peripheral blood mononuclear cells (PBMCs) from healthy donor Leukopaks. In this step, the Gibco CTS Rotea Counterflow Centrifugation System is utilized for PMBC enrichment. Afterwards, negative selection methods are used to isolate NK cells, which are then expanded over 6 days using the Gibco™ CTS™ NK-Xpander Medium supplemented with human AB serum and interleukin-2 (IL-2).

Following cell expansion, the cells are prepared for the cell engineering step by washing and concentrating them using the same CTS Rotea Counterflow Centrifugation System into the genome editing buffer for electroporation. The cells are then resuspended in this buffer and payloads are delivered using either the Invitrogen™ Neon™ NxT or the Gibco™ CTS™ Xenon™ Electroporation System. (Neon NxT is used for research purposes to optimize various conditions for gene editing prior to advancing to clinical-scale gene electroporation with the CTS Xenon system).

For gene editing, the CTS HiFi Cas9 protein and synthetic gRNA along with donor DNA payload of choice (ssDNA, dsDNA, or nanoplasmids) are co-delivered using CTS Xenon electroporation. After delivery and gene editing using the Cas9 RNP and Xenon Electroporation System, the cells are expanded again in the Gibco CTS NK-Xpander Medium, supplemented with human AB serum and IL-2. The cells are then monitored throughout this expansion process for up to 20 days. During the expansion, the cells are analyzed at different time points to assess NK cell markers, viability, morphology, and potency. These analyses are conducted using either the Attune™ NxT Flow Cytometer or the EVOS™ Cell Imaging System.

ISOLATION AND PROCESSING OF NK CELLS WITH THE CTS ROTEA COUNTERFLOW CENTRIFUGATION SYSTEM

The CTS Rotea Counterflow Centrifugation System is a closed, automated cell processing system providing multiple advantages for cell therapy manufacturing. Firstly, the system offers process flexibility, allowing for the concentration and washing of cells in a desired buffer, enabling concentration into as little as 5 mL of the selected buffer or media. Cells processed through this system demonstrate high cell recovery (>95%) and

viability. Additionally, the system comes equipped with all necessary packages to support clinical manufacturing needs, including regulatory documentation.

IMMUNOPHENOTYPING PBMCs AND NK CELLS

In order to ensure that the screened donors possess the appropriate level and quality of NK cell population, it is essential to immunophenotype the PBMCs and isolated NK cells. For instance, markers such as CD56 and CD16 can be evaluated using flow cytometry to confirm that the donor derived NK cells exhibit the required viability for progression to the next step.

According to the study results, the NK cells derived from PBMC via negative selection demonstrated excellent purity and a high percentage of viable cells (Figure 2).

CELL CULTURE AND EXPANSION WITH GIBCO CTS NK-XPANDER MEDIUM

Once the quality of NK cells has been confirmed, the next step is to expand $CD56^+$ and $CD16^+$ cells in Gibco NK-Xpander media for cell culture. The cells expand in the CTS NK-Xpander Medium without feeder cells. Furthermore, the medium is provided in bags compatible with closed automated workflow designed to comply with industry standards, and backed with required regulatory documentation package including Drug Master Files, Certificate of Analysis, and Certificate of Origin.

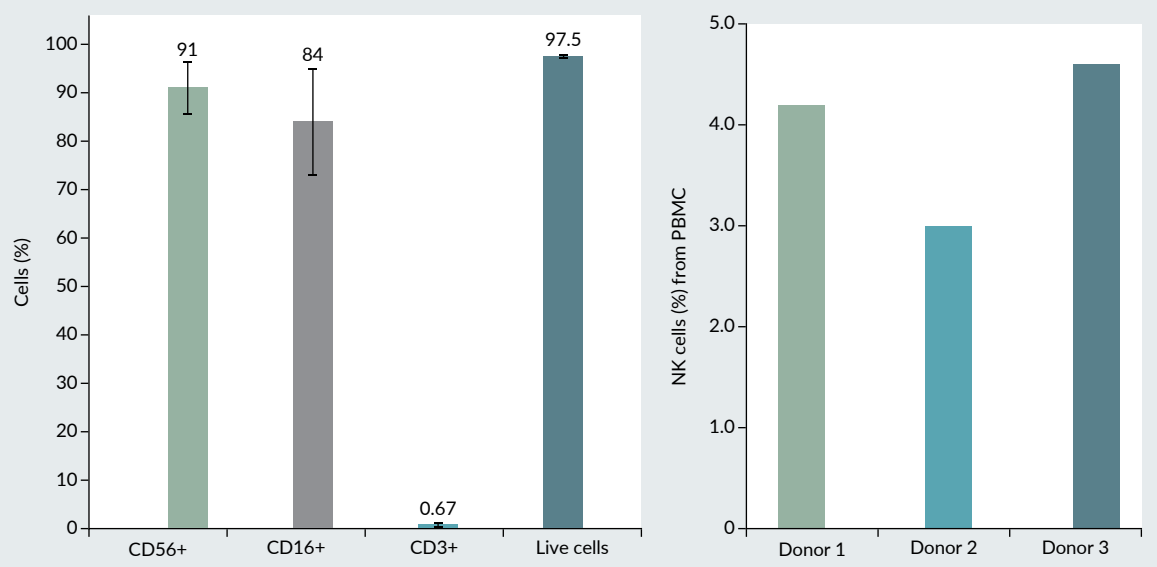
Three different donors were screened to obtain good quality NK cells with reasonable expansion rate. In each case, isolated NK cells were characterized for relevant surface markers—specifically, CD56 and CD16. Additionally, the $CD3^+$ populations were assessed to confirm the appropriate balance between NK and non-NK cells. As shown in Figure 3, robust cell expansion was observed from day 1–20 in the feeder-free NK-Xpander media and expansion rates could differ from donor to donor.

NONVIRAL GENE DELIVERY IN NK CELL ENGINEERING

Gene delivery methods for NK cell engineering include both nonviral and viral

FIGURE 2

Purity and viability assay based on immunophenotyping of NK cells using flow cytometry.



Cell & Gene Therapy Insights 2025; 11(1), 69–84 · DOI: 10.18609/cgti.2024.009

approaches. Nonviral approaches are gaining lot of traction in the cell engineering field due to the limitations posed by viral systems, such as safety concerns, increased testing burden, payload limitations, and production cost. The research-use electroporation platform, Neon NxT, and the cell therapy manufacturing-compatible CTS Xenon System are nonviral-based gene delivery methods, which enable efficient and scalable protocols for research-use to GMP applications. The clinical scale Xenon system comes with all necessary documentation and is manufactured under GMP ISO 13485 conditions. Another advantage of the electroporation method for gene delivery is its capability to deliver a broad range of payloads. For example, in a proprietary study, the HiFi Cas9 RNP system with the respective GOI-targeting gRNA was utilized to demonstrate delivery efficiency with various payload formats such as dsDNA, linear ssDNA, and circular dsDNA. This approach not only showcased the versatility of electroporation systems but also enabled the assessment of which payloads are the most effective for the specific GOI under investigation.

SELECTING OPTIMAL ELECTROPORATION CONDITIONS

The Neon NxT system offers 24 different pre-set electroporation programs as well as additional customizable programs that can be added for further optimization. During the workflow, the gRNA of interest is selected and tested together with the CTS HiFi Cas9 and CTS Xenon Genome Editing Buffer across 24 conditions to identify the optimal balance between editing efficiency and cell viability.

It is also important to identify the optimal buffer for gene editing and precise insertion of payloads at specific loci within the NK cells. In an internal study, two buffers were compared—the Xenon Gene Editing (GE) Buffer and the Xenon Electroporation Buffer (R). The GE Buffer demonstrated superior performance, achieving knockout efficiencies of nearly 80% as well as the desired expression of cell surface markers.

SCALING UP NK CELL MANUFACTURING WITH THE CTS XENON SYSTEM

After selecting optimal electroporation conditions and buffer, the process was scaled up to the CTS Xenon System. Four specific conditions from the Neon NxT System were selected—5, 9, 16, and 24. Additionally, a higher voltage condition was manually configured in Neon NxT and added to the test parameters to yield better results for challenging cell types. These five conditions were scaled up from Neon NxT to CTS Xenon using the pre-set conditions available on CTS Xenon.

The protocols between Neon NxT and CTS Xenon systems were compared using the HiFi Cas9 RNP system. The results indicated that both systems exhibited comparable editing efficiencies, with the CTS Xenon System performing slightly better overall. Specifically, protocols 6 and 1 yielded the best results, and were chosen for further downstream applications.

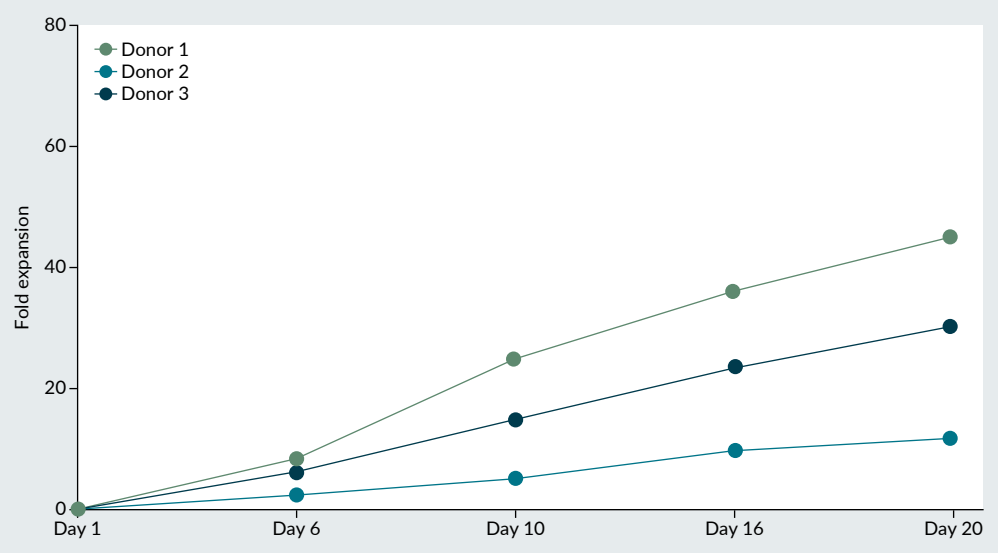
ASSESSMENT OF ELECTROPORATION AND EDITING EFFICIENCY IN CAR-NK CELLS

Electroporation and editing efficiency at B2M locus were assessed at varying NK cell densities (50×10^6 and 100×10^6 cells/mL NK-Xpander media) using the same experimental conditions. Following this, the single-shot 1 mL consumable on the Xenon System was utilized.

Post-electroporation cells were analyzed for relevant NK cell surface markers (CD56, CD16) and B2M receptor knockout efficiency using Attune NxT™ Flow Cytometry. The results demonstrated high knockout efficiency, with the cells maintaining the

FIGURE 3

Expansion of NK cells over 20 days in the CTS NK-Xpander Medium with 5% hAB serum and 500 U/mL IL-2.



essential NK cell surface markers and remaining viable.

Following optimization of electroporation conditions for knock-out experiments, protocols were evaluated for nonviral-based transgene knock-in using linear dsDNA encoding GFP, designed to target AAVS1 locus of NK cells. The first step was to select the most efficient gRNA. Of the five gRNA options tested, one performed poorly while the others achieved between 40–50% knockout efficiency at this locus. The most efficient gRNA was selected to proceed with the GFP knock-in at the AAVS1 locus. Over a 31-day monitoring period, a knock-in efficiency of 5–10% was achieved among the engineered viable cells.

Afterwards, a different locus, Rab11a, was investigated using a minicircle dsDNA encoding GFP from GenScript. Similarly, the electroporation systems with HiFi Cas9 protein and synthetic gRNA were utilized for this locus. The gating strategy included selecting live cells and analyzing the $CD56^+$ population for the GFP+ expression. After confirming the desired knock-in efficiencies, cell expansion was monitored over 15 days. Notably, the CTS Xenon System exhibited significantly higher knock-in efficiency compared to the Neon NxT, and the cells were expanded efficiently.

In a further experiment, HiFi Cas9 along with a synthetic Rab11a-specific gRNA was co-transfected along with ssDNA encoding GFP, using both CTS Xenon and Neon NxT systems to evaluate alternate DNA payload formats. It was discovered that ssDNA showed higher knock-in efficiency compared to linear dsDNA and minicircle DNA. The ssDNA contained CRISPR-specific sequences that allowed Cas9 ribonucleoprotein (RNP) to recognize and facilitate the integration at the Rab11a locus.

Similarly to previous experiments, NK cell surface markers within the live-gated cell populations were the key focus—specifically, the $CD56^+$ cells with GFP knock-in at Rab11a locus. By day 3, the editing efficiency was 4–7%. As the cells expanded from day 3 to day 20, both significant cell proliferation and an increase in GFP knock-in efficiency, reaching approximately 22–25% by day 20, were observed with the CTS Xenon System. This

experiment was conducted in two different healthy donor samples, and consistent results were observed across both systems.

Finally, the viability of the engineered NK cells using both Neon NxT and CTS Xenon systems was analyzed. Based on the results, robust populations of edited NK cells with high viability were observed (Figure 4). Furthermore, the NK cell surface marker CD56 was well maintained in all cases.

CASE STUDY: ENGINEERING ANTI-MESO3 CAR-NK CELLS FOR ENHANCED CYTOTOXICITY AGAINST OVARIAN CANCER

In order to investigate a practical application of the CAR-NK manufacturing workflow, the method was tested to produce anti-Meso3 CAR-NK cells. Mesothelin (MSLN) has emerged as a promising antigen because of its low expression on normal tissues and high expression on various solid tumors. Therefore, constructs expressing the Anti-meso3 CAR as ssDNA were generated and delivered along with the Cas9 RNP during CAR-NK cell engineering.

To generate CAR-NK cells using the nonviral genome engineering methods described here, 50×10^6 NK cells/ml were utilized, together with anti-Meso3 encoding ssDNA, HiFi Cas9, and relevant gRNA targeting the AAVS1 locus. The anti-Meso3 ssDNA, was also tagged with a V5 epitope, enabling the analysis of knock-in efficiencies using flow cytometry. For the Neon NxT System, a 100 µL pipette tip was employed, while for the CTS Xenon System, a 1 mL SingleShot electroporation consumable was used.

The live cell population for $CD56^+$ and V5+ cells was subsequently analyzed by immunophenotyping edited NK cells using Attune NxT Flow Cytometer. More specifically, two different donor derived NK cell samples were tested: one was a lower-performing sample, while the other was a high-performing sample from the initial screening of three donors. This comparison highlighted the variability in editing efficiency between different donor samples. In this particular case, donor 1 demonstrated significantly better performance than donor 2. Following the electroporation and gene editing process, the cell expansion was monitored over 20 days. The knock-in efficiencies improved from 2–6% to 12% in both donors. Notably, all these experiments utilized ssDNA.

Furthermore, the viability of the edited NK cells was assessed by conducting the immunophenotyping assay to evaluate both CD56 and CD16 markers. Once the presence of viable cell populations with a sufficient number of edited NK cells was confirmed, functional analyses on these samples were carried out.

The cytotoxicity of the anti-Meso3-CAR NK cells (effector cells) was tested against the ovarian cancer cell line, SKOV3 (target cells), which express Mesothelin 3. Different ratios of effector cells and target cells were assessed to evaluate the potency of the engineered NK cells compared to non-engineered NK cells. As shown in Figure 5A, the anti-Meso3-CAR-NK engineered cells exhibited higher potency, likely due to their enhanced recognition of the Meso3 receptors on the SKOV3 cells.

As seen in the immunophenotyping analysis in Figure 5B, both the degranulation activity of NK cells and the efficiencies of the CAR-NK cells were examined. Specifically, CD107a expression was assessed for degranulation, as well as CD56+ cells. Within this population, the presence of $CD56^+$ and $CD107^+$ cells correlated with the robust target cell killing potency of the CAR-NK cells.

Notably, the CAR-NK cells generated using these protocols were able to efficiently kill 80% of the target SKOV3 cells expressing Meso3 within 6 hours of incubation.

FIGURE 4

Viability and phenotype of engineered NK cells with Neon NxT and CTS Xenon systems.

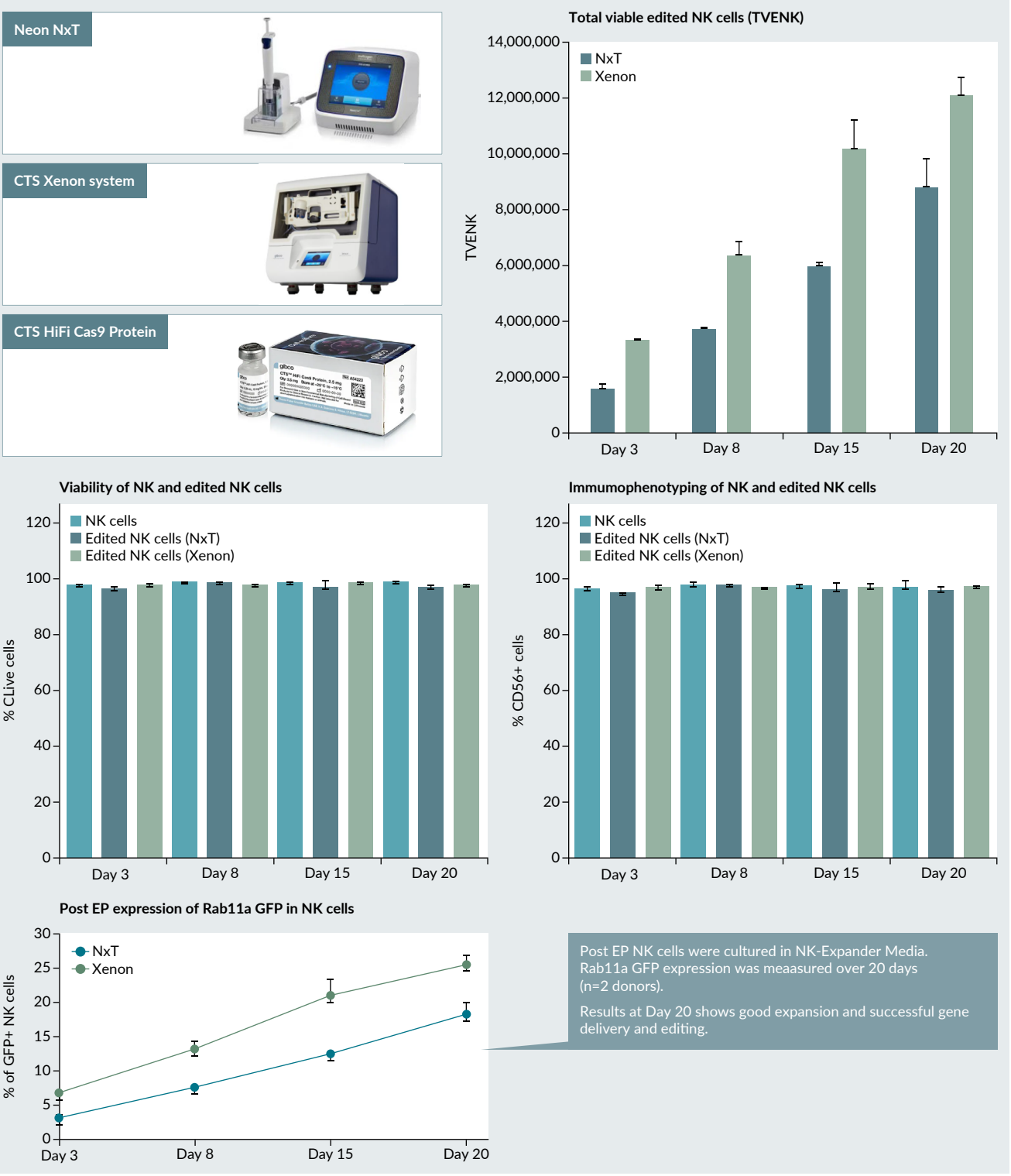
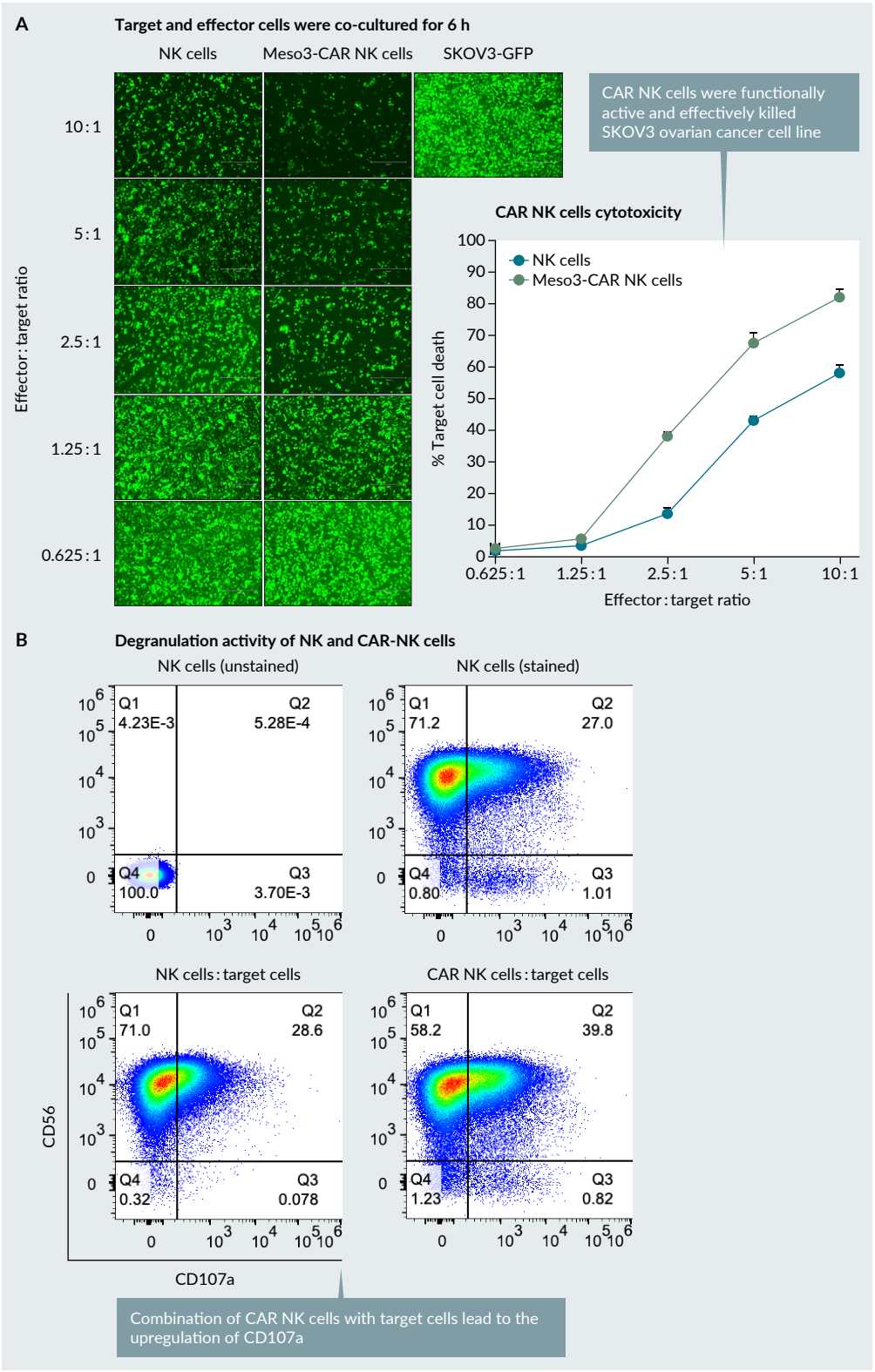


FIGURE 5

Enhanced killing efficiency of anti-Meso3-CAR-NK cells against ovarian cancer cell line SKOV3.



CONCLUSION

In summary, the closed, automated, modular instrument platforms together with the GMP-grade media systems and HiFi Cas9 reagent can effectively facilitate robust gene delivery and editing using a broad set of payloads. Furthermore, PBMCs can be successfully isolated using the CTS Rotea System, achieving an excellent profile of desired cell population with high cell recovery after counterflow centrifugation processing. Both Neon NxT and CTS Xenon electroporation systems efficiently deliver a wide range of payload formats, with knock-in efficiency of up to 20% for Neon NxT and 26% for CTS Xenon system.

Additionally, the data showcased in this article demonstrated the importance of screening healthy donor samples to ensure optimal cell populations. Once the appropriate donor samples were selected, consistent editing efficiencies and expansion of the NK cells throughout the 20-day monitoring period were observed. All cells in the experiments were cultured and expanded in the CTS NK-Xpander Medium, and the cells maintained the desired phenotype, viability, and functionality.

Collectively, the protocols and instruments discussed in this study offer robust nonviral-based NK cell engineering solutions for clinical manufacturing applications.

Q&A

Namritha Ravinder (left), **Krishanu Saha** (right)

Q Which key factors contribute to efficient nonviral editing of NK cells?

KS One of the key factors is ensuring that NK cells are dividing, which facilitates homology-directed repair (HDR). We also found that using certain small molecules helped promote the HDR mediated integration of CAR template. Additionally, it was important to ensure the cells were properly prepared, both before and after electroporation, as well as post-small molecule treatment. Finally, the quality and format of the DNA template is also important. In this case, we utilized the linear dsDNA template.

Q How do you check the copy number of inserted genes and edited NK cells after electroporation?

NR Typically, based on the GOI and its size and the intended application, either targeted sequencing, qPCR, or digital PCR is performed.

Cell & Gene Therapy Insights 2025; 11(1), 69–84 · DOI: 10.18609/cgti.2024.009

KS Apart from the methods mentioned by Namritha, long-read sequencing has been employed to ensure that the junctions between the cut site and the templates are well-preserved, enabling precise insertion through homologous recombination. Whole-genome sequencing can also be utilized to help us track any potential off-target integration. Thus far, we have not seen any off-target integrations. Additionally, droplet digital (dd)PCR is also considered for deep profiling of the copy number.

Q Can a knockout and knock-in be performed simultaneously in a single electroporation?

KS The electroporation system performs both the knockout of KLRC1 and the knock-in of CAR. A similar type of gene editing for other checkpoints would involve finding a suitable gRNA to knock out the gene likely in the early exons. Designing a template with homology arms around the cut site and ensuring that the transcript is stopped with a PolyA transcriptional terminator is also important.

Q Why was HiFi Cas9 selected for this application and what are its advantages?

NR The CTS HiFi Cas9 was specifically designed for increased fidelity, ensuring that when a particular gRNA is used, the efficiency is maximized while minimizing off-target effects. Additionally, the CTS HiFi Cas9 comes with all the required regulatory packages and is manufactured in a GMP facility, making it easy to integrate into clinical manufacturing applications.

Q What is the difference between the GE and R buffers?

NR The formulations for the GE and R buffers are similar. However, the GE buffer was specifically optimized to improve knock-in efficiency.

Q Switching to a different media system can be time-consuming and expensive. Given these challenges, what compelling benefits have led several key developers in the field to choose NK-Xpander Medium?

NR The NK-Xpander Medium is designed to produce high yields of NK cells without the feeder-based system. The system supports robust growth, expansion, and scalability from small to large volumes based on workflow needs. The medium was designed and manufactured without cytokines and growth factors, providing flexibility to users who can add their own preferred cytokines or growth factors to supplement

the medium. For example, in the workflows presented in this article, NK-Xpander Medium was supplemented with human AB serum and IL2 to support NK expansion during gene editing protocols.

Additionally, the necessary regulatory requirements, including USP <1043> Ancillary Material Requirements for Cell, Gene, and Tissue-Engineered Products, have been fulfilled. This ensures a seamless transition from process development to clinical manufacturing.

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BIOGRAPHIES

Namritha Ravinder graduated from the University of Alabama in Huntsville, Huntsville, AL, USA with a doctorate degree in Biotechnology followed by a post doctorate in HIV Virology from Children's Hospital in Los Angeles, LA, CA, USA. She joined Thermo Fisher Scientific, Carlsbad, CA, USA in 2008. During her early years at Thermo Fisher Scientific, Namritha led several custom product service offerings including cDNA library synthesis, RNAi screening, lenti virus production, and cell engineering. Following that, she led a product development team focused on gene editing and modulation tools, specifically TAL mRNA, CRISPR Cas9 plasmids and mRNA systems, CRISPR gRNA libraries, and lncRNAi libraries. In her current role, Namritha manages an R&D team dedicated to developing closed automated instrument platforms to address cell processing, gene delivery, and cell engineering needs within cell and gene therapy manufacturing workflow. Her team also supports viral vector engineering and residual assay development efforts for lentivirus and AAV virus production systems.

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Cell & Gene Therapy Insights 2025; 11(1), 125–129 · DOI: 10.18609/cgti.2025.014

THE RACE TO COMMERCIALIZATION

For biotech companies working in the cell therapy sector, optimizing speed-to-market is critical. This is essential to minimize costs and meet commercial goals, as well as to increase the number of potentially life-changing cell therapies available to patients. To achieve an optimized pace, developers must maximize efficiency at every stage—from R&D and process development to scale-up, scale-out, and regulatory filing.

Alongside reduced development timelines, accelerated manufacturing workflows are also vital. Many patients who are candidates for cell therapies are critically ill and have often exhausted other treatment options. This makes timely access to therapeutics imperative to improve the likelihood of successful treatment. Moreover, by shortening *ex vivo* cell isolation, activation, and expansion phases, manufacturers can reduce T cell differentiation [1]. This can help maximize the population of T cells with early memory phenotypes, which have been shown to be associated with increased therapeutic efficacy [2].

STREAMLINING EACH STAGE

Ex vivo cell isolation and activation are typically carried out using paramagnetic beads that selectively isolate and/or activate target T cells. These cells are then separated, and the beads are removed using an instrument with magnetic separation capabilities.

The activated cells can then be expanded using an optimized cell culture medium. The overall aim of this process is to generate a highly pure population of viable T cells with the optimal phenotype, for further

manufacturing into a safe and efficacious therapeutic product.

While previous isolation, activation, and expansion solutions have driven considerable advances, they have had limitations. Most notably, many are outdated, as they have not incorporated the most recent industry knowledge, technologies, and priorities into their design. This is most apparent in areas such as automation, scalability, and flexibility. There are also increasingly stringent regulatory requirements that must be met to enable an easy transition to commercial production. As such, to truly unlock accelerated workflows and drive long-term success there is a need for next-generation alternatives.

INCREASING EFFICIENCY THROUGH ADVANCED SOLUTIONS

In response to the need for improved efficiency, there has been a widespread drive across the industry to advance solutions that are designed to optimize development and manufacturing workflows. Suppliers are also focusing on enhancing their support services and manufacturing capabilities to increase confidence in their ability to support streamlined operations.

Given the range of options available, it can be challenging for developers and manufacturers to find the right solution to meet their process requirements. To accelerate the development of an efficient workflow and support long-term success, there are several key capabilities that developers should look for when evaluating potential solutions and suppliers.

Automated processes

When considering instrumentation for cell isolation, activation, and expansion, automation is a feature that can offer a wide range of efficiency benefits. Next-generation instruments are currently being designed with automation capabilities that can help significantly reduce processing time compared to manual approaches.

For example, the Gibco™ CTS™ DynaCellect™ Magnetic Separation System, a closed, automated platform for isolation, activation, and bead removal, can complete cell isolation typically in under 100 min when used with Gibco™ CTS™ Dynabeads™ CD3/CD28. Similarly, its automated protocol can shorten the time required for magnetic bead removal from around 5 h to under 1 h, without affecting cell viability.

Automation can also remove the need for manual interventions and enable a closed-system environment to be maintained throughout the process. Consequently, automation can help lower contamination risks, along with reducing the potential for human error or operator-to-operator inconsistency. Together, these can help reduce the risk of manufacturing failures and treatment delays.

Implementation of automation within workflows can also help biotech companies optimize resource management and overcome talent shortages. By removing the need for scientists to conduct time-consuming manual work, their time can be utilized in other areas, resulting in overall efficiency gains throughout development and manufacturing.

Modular instrumentation

Modularity is another key capability to look for when assessing instrumentation. This is crucial to efficiently manage the diverse requirements of specific workflows, such as the different scalability targets of allogeneic and autologous therapies. Additionally, for developers with existing workflows, modular equipment that can be easily integrated into current processes can help accelerate optimization. Within this area, modular instruments designed for both R&D and clinical manufacturing

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can offer further benefits. By enabling the same process to be used from development to production, developers can avoid delays associated with changing systems when scaling up or out.

To streamline processes when using modular solutions, developers should look for a software solution that can provide digital connectivity across the workflow. Off-the-shelf, open platform solutions, such as Gibco™ CTS™ Cellmation™ Software, can offer considerable advantages by reducing the need for time-consuming development and validation, while enabling integrated control and automation of multiple instruments.

Following the transition to clinical and commercial production, these solutions can also help enable regulatory compliance by facilitating traceable, reproducible, and secure data storage.

Flexible solutions

In addition to modular instrumentation, to improve process efficiency and enable specific workflow requirements to be met, flexible media and reagents are equally important. For instance, choosing an agile cell expansion medium, such as Gibco™ CTS™ OpTmizer™ One SFM, with a formulation optimized for compatibility with multiple platforms, culture vessels, and workflows, can help simplify development. To support increased production volumes, this flexibility should also extend to the range of packaging options available. In particular, media should be available in large bioprocess containers to avoid the need for pooling of media during production, helping to increase efficiency and reduce contamination risks.

Another area where flexible, next-generation solutions can increase manufacturing efficiency is cell separation reagents. The Gibco™ CTS™ Detachable Dynabeads™ platform can offer considerable time savings by enabling users to actively detach the magnetic beads rather than wait for passive dissociation that can often take 5 days. Additionally, the platform's flexibility can offer control over the duration of activation, helping manufacturers reduce differentiation and preserve the early memory T cell phenotypes that are correlated with higher treatment efficacy.

Regulatory support

During cell therapy development, applying for regulatory approval is a critical milestone and avoiding delays at this stage is necessary to maintain development timelines. Suppliers can play a key role in supporting this by providing easy access to required traceability documentation, including certificates of analysis (COAs), certificates of origin (COOs), safety data sheets (SDSs), and certificates of compliance (COCs). Some products may be supplied with additional documentation such as regulatory support files (RSFs) and drug master files (DMFs), which can streamline the filing process by enabling suppliers to liaise directly with regulatory bodies on their customers' behalf. As such, choosing a vendor with an experienced, proactive technical support team can prove highly advantageous.

Supply assurance

To further support a streamlined progression to clinical and commercial manufacturing, the vendor's supply assurance should also be assessed. To minimize the risk of supply interruptions and potentially life-threatening production delays, developers and manufacturers should look for a supplier with a multisite network offering manufacturing redundancy. The network should be supported by a robust site-to-site equivalency program—harmonizing the raw materials, manufacturing equipment and processes, and quality control measures used. This is useful to provide assurance that batch-to-batch product

consistency can be maintained between sites.

ACCELERATING PROGRESS WITH A TRUSTED SUPPLIER

Successfully establishing a cell therapy workflow requires developers and manufacturers to be able to implement solutions that can support accelerated processes—from shorter processing times to efficient scale-up or scale-out.

Working with a knowledgeable global supplier that can offer a diverse portfolio of advanced solutions with the modularity, flexibility, and scalability to meet their specific workflow needs can help dramatically simplify this process. Using their experience and extensive capabilities, the supplier can also offer trusted guidance and dependable supply assurance from R&D to commercial production. This collaboration can enable developers and manufacturers to overcome previous limitations, enhance therapeutic safety and efficacy, and ultimately increase the number of life-changing therapeutics that are available to patients.

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Maximizing CAR-T cell yields: scalable solutions with perfusion and stirred-tank bioreactors

Julia Hengst, Qasim Rafiq, Rukmini Ladi, and Pierre Springuel

The primary drivers impacting cell and gene therapy (CGT) time-to-market and COGs include productivity, enabling process control, and ensuring the quality of the final cell product. Achieving these goals requires a functional supply chain and the ability to seamlessly scale-up. This article will discuss the collaborative study between Satorius and University College London, exploring process intensification strategies in stirred-tank bioreactors for CAR-T manufacturing.

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CHALLENGES IN THE CGT SPACE: AUTOLOGOUS CAR-T MANUFACTURING

It is widely recognized within the CGT space that manufacturing presents one of the greatest obstacles to the sustainability, adoption, and development of advanced therapies, especially CAR-T and gene therapies. More broadly, there is a consensus that the manufacturing process as a whole requires further investigation. This research is crucial for advancing the field and producing therapies at costs that the market is willing to accept.

One key aspect of the CGT space is that all currently approved products are autologous. This presents several challenges such as many processes being highly manual, not easily scalable, and lacking adequate control and monitoring capabilities. These issues often result in high costs.

There are also significant challenges associated with patient-specific therapies, particularly regarding patient variability, disease burden, and the fact that many patients undergo intensive chemotherapy regimens before being eligible for CAR-T therapy.

When examining the current market, a variety of platforms are being utilized. This ranges from basic options such as culture bags and flasks to more automated systems such as the CliniMACS Prodigy, Lonza Cocoon, and wave bioreactors. Looking to the future, considerations need to be made for both autologous and allogeneic therapy approaches, with a focus on optimizing scalability, accessibility, and therapeutic efficacy.

Generating a universal donor that can be administered to multiple patients offers clear benefits. This model could be off-the-shelf, requiring minimal manufacturing time since batches can be prepared in advance. Additionally, it enables scalable production, enhancing efficiency while reducing costs. As a result, the expansion and production of cells have become fundamental drivers of research and innovation.

THE FUTURE OF CGT MANUFACTURING: STIRRED-TANK BIOREACTORS AND CAR-T

There are three key areas to consider when considering the future of the CGT manufacturing space. First, automation and decentralized manufacturing, which is gaining popularity in the form of point-of-care production. Second, bioprocess optimization and control. Third, the development of digital tools, such as digital twins, AI models, and machine learning. Notably, this study mainly focused on the bioprocess optimization and control areas of manufacturing, particularly in stirred-tank bioreactors.

Stirred-tank bioreactors are well-established in the biopharma industry, producing thousands of biological products, including recombinant proteins, vaccines, and a wide range of other biotech products. These platforms are geometrically similar and scalable. A bioreactor at the 15 mL or 150 mL scale can be taken to establish a comparability process that allows for the scale-up of potentially over 10,000 L.

Effective mixing and mass transfer are also critical components. Focusing on these aspects is essential for producing a homogeneous product, while effective parameter control can be achieved, especially when process analytical technologies are integrated into the platform. This integration allows for the monitoring and control of pH, temperature, metabolite analysis, and cell viability in real-time.

This study focused on three main phases. The first involved a QbD approach to optimize critical process parameters for CAR-T production in static culture, utilizing a DOE. The second phase focused on optimizing critical process parameters (CPPs) for stirred-tank bioreactor perfusion, also employing a DOE approach. Finally, the third phase explored multi-liter scale-up and the implementation of automated cell harvesting in a fully closed workflow.

PHASE 1: IMPACT OF CRITICAL PROCESS PARAMETERS AND EXPERIMENTAL PLAN

In Phase 1, the primary goal was optimizing the CPPs for CAR-T production. The study aimed to identify parameters based on experiences and existing literature to understand how these CPPs affect key cell quality attributes. The parameters investigated included the number of activations, seed train time, seeding density, and the concentration of IL-2, as seen in Table 1.

Table 2 shows the responses linked to critical quality attributes that were analyzed, including cell growth, viability, activation markers, exhaustion markers, and CAR expression.

G-Rex® system were utilized to examine one or two activations, seed train length, and other relevant parameters. The approach involved performing the first activation in the upstream process, transducing the cells, and then conducting a seed train for 3, 5, or 7 days, depending on the specific CPP being investigated. This would then proceed to a second activation if appropriate for that condition.

PHASE 1 KEY FINDINGS: IMPACT ON CELL YIELD AND QUALITY

The data collected showed that a repeated activation process, higher seeding density, and longer seed train times had a significantly negative impact on CAR-T cell yield.

TABLE 1

Study parameters to assess the effect of CPPs on cell quality attributes.

Factors	Levels			Factor rationale
Number of activations	1		2	Maintaining growth versus over-activation
Seed train time (days)	3	5	7	Initial growth lag versus primary cell exponential growth limits
Seeding density (cells/cm^2)	0.25×10^6	0.5×10^6	0.75×10^6	Positive cell signaling from cells being too sparse versus negative cell signaling from cells being too dense
IL-2 (IU/mL)	30		100	Activation versus potential over activation

CPP: critical process parameter.

Specifically, affecting cumulative population doublings. When the effects of each model coefficient were plotted, it became clear that the number of activations was the key factor resulting in the largest adverse impact on cell yield, as seen in Figure 1.

Importantly, this concern extended beyond yield, and similar effects on cell quality were observed, as shown in Figure 2. Quality attributes, including exhaustion, activation profiles, and phenotypic markers. Consistent with our findings on cell yield, we found that the number of activations and longer seed train times significantly impacted activation and exhaustion responses. This highlighted factors that could positively or negatively affect CAR-T yield and quality.

PHASE 1 KEY FINDINGS: TRANSLATING FINDINGS TO STIRRED-TANK BIOREACTORS

While small-scale studies in the G-Rex® system were informative, further studies were needed to determine whether these findings would translate to stirred-tank bioreactors. In the same Phase 1 study, the optimized parameters were applied to a stirred-tank bioreactor. Using a single activation and

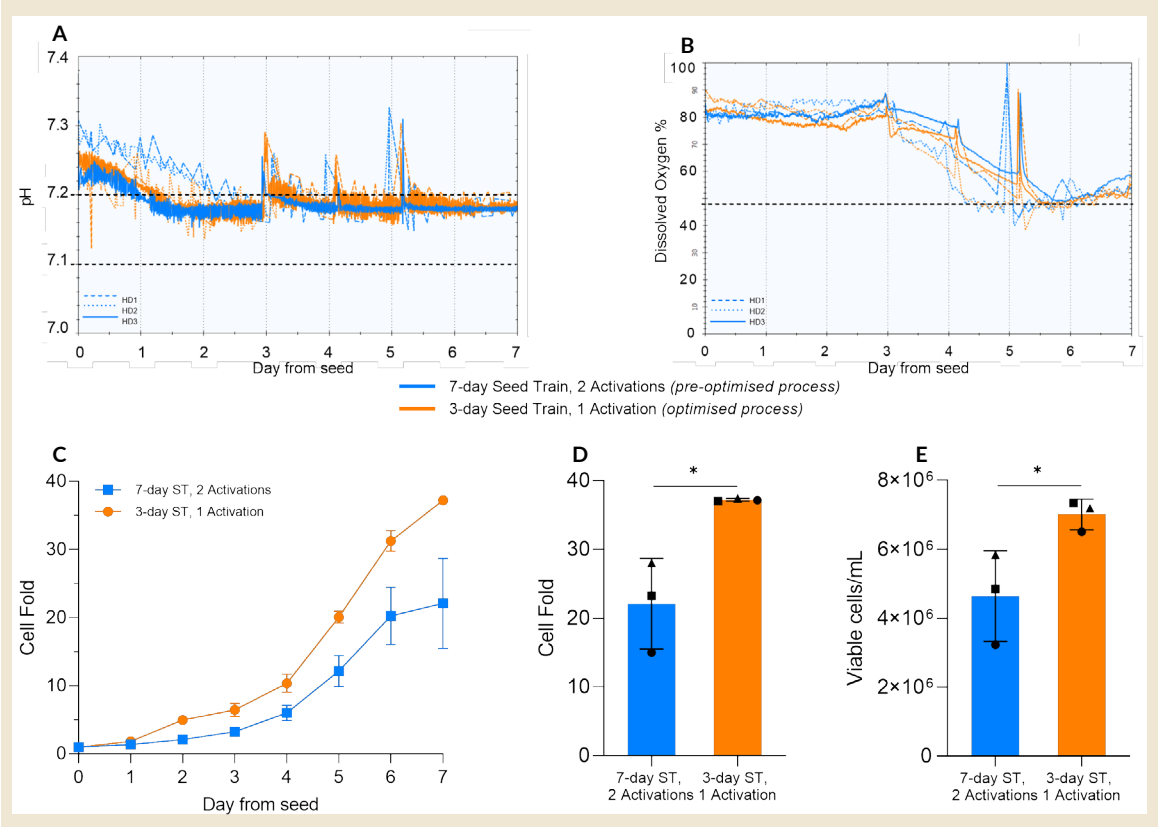
TABLE 2

Analyzed critical quality attribute linked responses.

Response	Measurement	Response rationale
Cell growth	Cells/mL	Represents cell yield—essential to achieve CAR-T dose
Viability	Percentage viable cells	Cell yield often based on viable cells; minimum viability often CAR-T product release criteria
Metabolites	Glucose, lactate, glutamine, ammonia	Shows nutrient availability in medium; consumption and productions indicative of cell state and metabolism
Phenotype	CD3, CD4, CD8	Identification and classification of T-cell subset
Memory differentiation	CCR7, CD45RO	Less differentiated cells associated with CAR-T clinical efficacy
Activation	CD69	Less differentiated cells associated with CAR-T clinical efficacy
Exhaustion	LAG3, PD1	Less exhausted cells associated with improved CAR-T function and clinical efficacy
CAR expression	CAR marker	CAR+ cells influencing therapeutic potency

FIGURE 1

Activation process, seeding density, and seed train time effects on CAR-T cell yield.



shorter seed train time resulted in significantly better outcomes in both cell yield and quality. For example, the cell fold, shown in orange in Figure 3, was much higher with a 3-day seed train and a single activation compared to a 7-day seed train with two activations. This trend was consistent across cell quality metrics as well.

In this first phase it was demonstrated that by employing a DOE approach, key culture parameters in small-scale G-Rex® system and static cultures could be pinpointed. This led to successfully translating these findings to agitated stirred-tank bioreactor conditions.

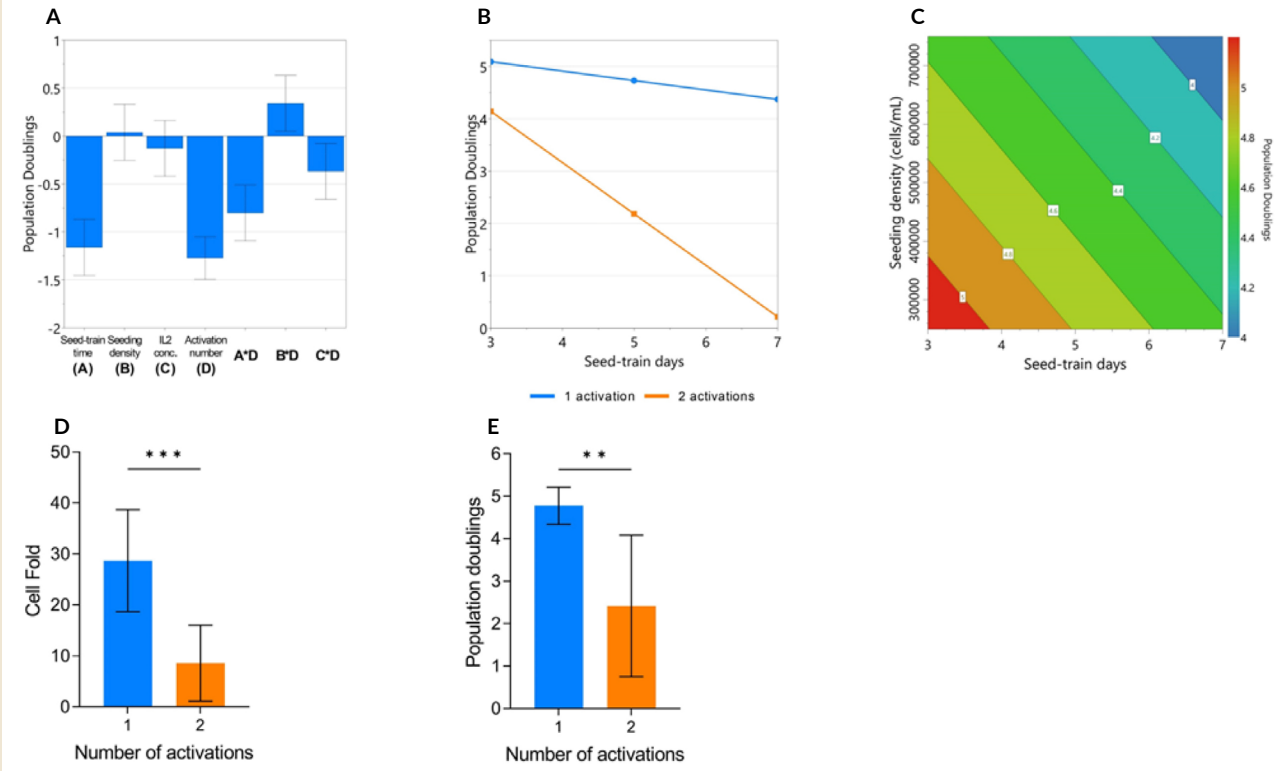
PHASE 2: ATF PERFUSION AND KEY METHODS

In Phase 2, a solid fed-batch process for CAR-T production in stirred-tank bioreactors was established. Additionally, the advantages of perfusion were recognized beforehand. Figure 4 shows how perfusion was implemented using the Ambr® 250 High Throupghput (HT). An alternate tangential flow (ATF) perfusion system was employed and following initial perfusion, a continuous exchange of culture medium was implemented. The cells were then retained while the spent medium—devoid of cells—was washed away, and fresh medium was then added back into the bioreactor. This proven perfusion process has been successfully used for various biological applications, including CAR-T cells.

In this phase of the study, the focus was to investigate the impact of key perfusion parameters on maximizing CAR-T expansion and quality in the Ambr® 250 HT stirred-tank bioreactor. A DOE approach was utilized to examine different perfusion

FIGURE 2

Activation process, seeding density, and seed train time effects on CAR-T cell quality.



parameters and assess the influence of various donors. Two distinct perfusion parameters were evaluated and included multiple donors, categorizing each parameter into low, medium, or high values.

The overall process included a thaw, activation, and transduction step over several days. This was followed by a seed train to generate sufficient cells for inoculation into the Ambr® 250 HT stirred-tank bioreactor. The study culminated in a 7-day perfusion culture. For CAR-T production, activation was applied with Dynabeads®, followed by the administration of 30 IU/mL of interleukin-2. Various responses were measured daily, including cell number and viability, gene expression, and memory and exhaustion phenotypes. The bioreactor operated at 50% dissolved oxygen and a pH of 7.3, with a maximum working volume of 210 mL.

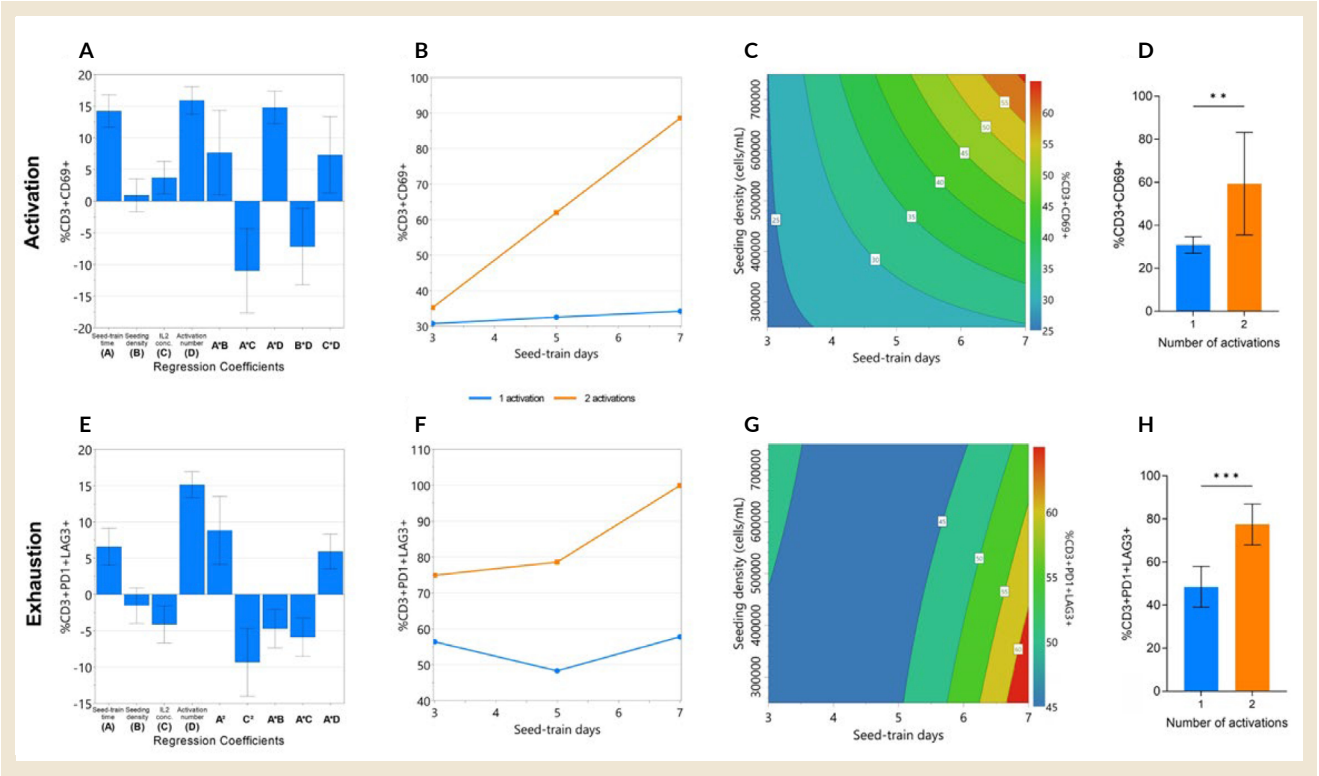
PHASE 2: OPTIMAL PERFUSION PARAMETERS AND CPPS

More than 32 bioreactor runs of perfusion were conducted. Each run represented a unique condition based on the perfusion parameters of three healthy donors under investigation. A range of outcomes depending on the perfusion parameters were observed, however, there was a clear trend indicating that certain parameters consistently yielded better results. The findings showed that in the majority of cases, perfusion led to significantly improved cell yield and viability compared to the fed-batch process. By leveraging DOE modeling, the impact of various perfusion parameters could be more precisely pinpointed.

As shown in Figure 5, for all three donors, it was observed that lower values for perfusion parameter A and higher values for

FIGURE 3

Optimized parameters of CAR-T cell production when applied in a stirred-tank bioreactor.



perfusion parameter B correlated with greater fold expansion. While some donor variability was anticipated, the critical takeaway was the consistent trend, which this contour plot effectively demonstrates.

Conducting DOE studies enhances understanding of the process by highlighting the individual and combined effects of the tested parameters on the measured responses. The perfusion parameters and donor viability were successfully explored, and the findings will be showcased in an upcoming paper.

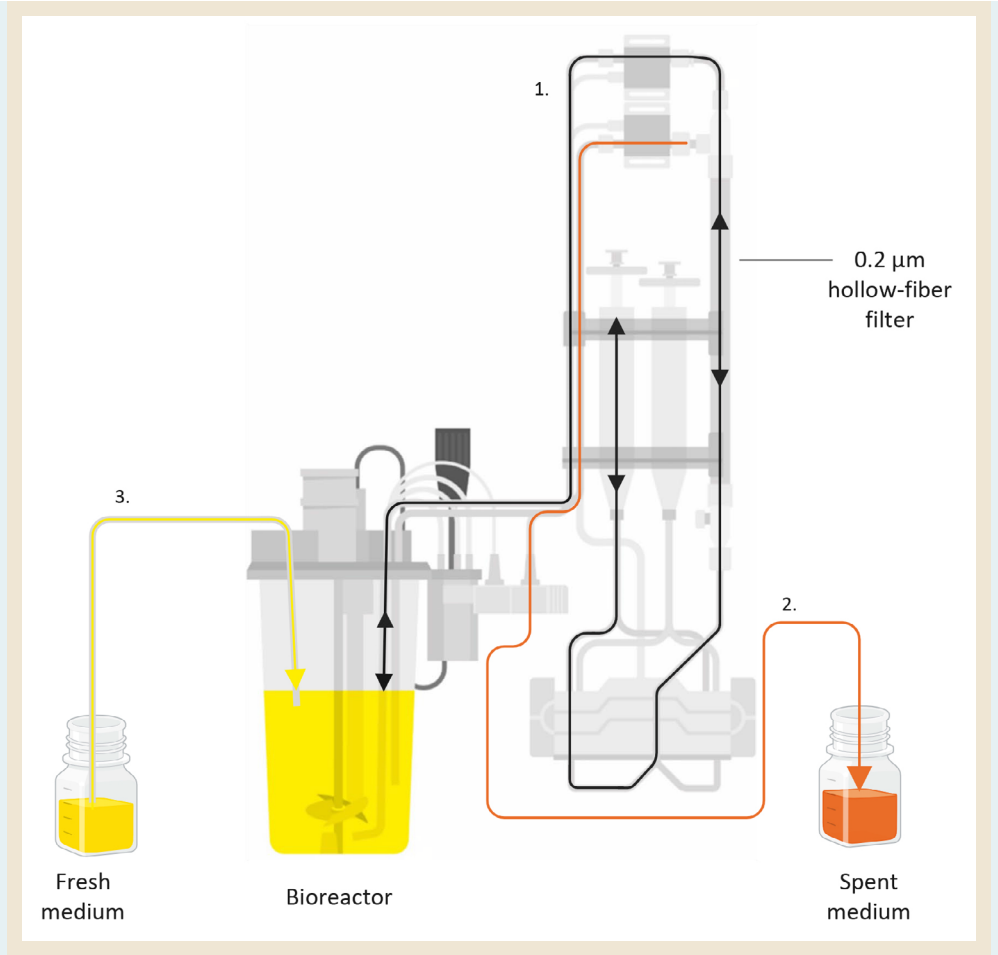
When comparing the fed-batch and optimal perfusion outcomes, the fed-batch process achieved more than a billion cells after 7 days, while the perfusion process yielded over 4.5 billion cells in the same timeframe. This translatates to approximately the amount of cells needed for one dose generated with the fed-batch process compared to four doses with the perfusion method, which can be seen in Table 3. Additionally, the perfusion process enabled the number of cells needed for a single dose in 3 days to be reached, compared to approximately 7 days for the fed-batch. Importantly, this did not require significantly more media, addressing common concerns about media usage in perfusion. In terms of quality, the cells maintained CAR expression, with no significant impact from the perfusion parameters after harvest.

PHASE 2: KEY FINDINGS AND ONGOING WORK

From a memory phenotype perspective, it was demonstrated that over 90% of harvested cells retained either naïve or central memory phenotypes, as seen in Figure 6. These were the more desirable and less differentiated state of cells. A minimal transition to effector memory or

FIGURE 4

Application of an ATF perfusion system using Ambr 250 for CAR-T cell production.



effector phenotypes was observed which was a crucial outcome given the growing recognition of the importance of memory phenotype for engraftment and *in vivo* efficacy. In terms of cell exhaustion, fewer than 3% of the harvested cells exhibited triple expression of exhaustion markers PD-1, LAG-3, and TIM-3 at the end of the bioreactor run.

Phase 2 of the study successfully investigated and optimized various perfusion parameters, achieving an improvement of at least 4.5 times in cell yield, with some cases exceeding this figure. Importantly, the harvested cells maintained a desirable phenotype, which opens avenues for further optimization. The T cells demonstrated retained viability and functionality in a stable environment using ATF perfusion.

Looking ahead, the next goal is to further optimize perfusion parameters. Previously, a 4.5 times improvement was shown with the most effective DOE run in perfusion; in current work, over nine times improvement is being achieved. This could have a significant influence when approaching CAR-T manufacturing moving forward.

PHASE 3: SCALE-UP STUDY OUTLINE

In the third and final phase, the study focused on multi-liter scale-up and the implementation of automated cell

FIGURE 5

Perfusion parameters across three health donors on fold expansion.

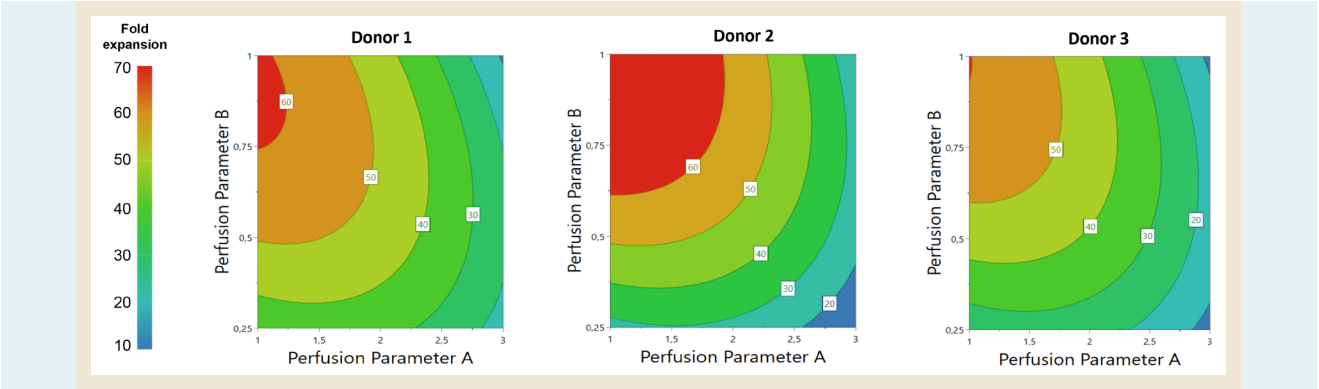


TABLE 3

Cell yield and dose of fed-batch versus perfusion of CAR-T production in stirred-tank bioreactors.

	Fed-batch	Perfusion
Final T-cell yield (7 days)	1.1×10^9	4.5×10^9
Total doses produced (7 days)	1	4
Time to reach first dose	Approximately 7 days	Approximately 3 days
Media consumption to reach first dose	Approximately 250 mL	Approximately 400 mL

Assumes one CAR-T dose = 200×10^6 CAR-T cells, and 20% of harvested cells are CAR+.

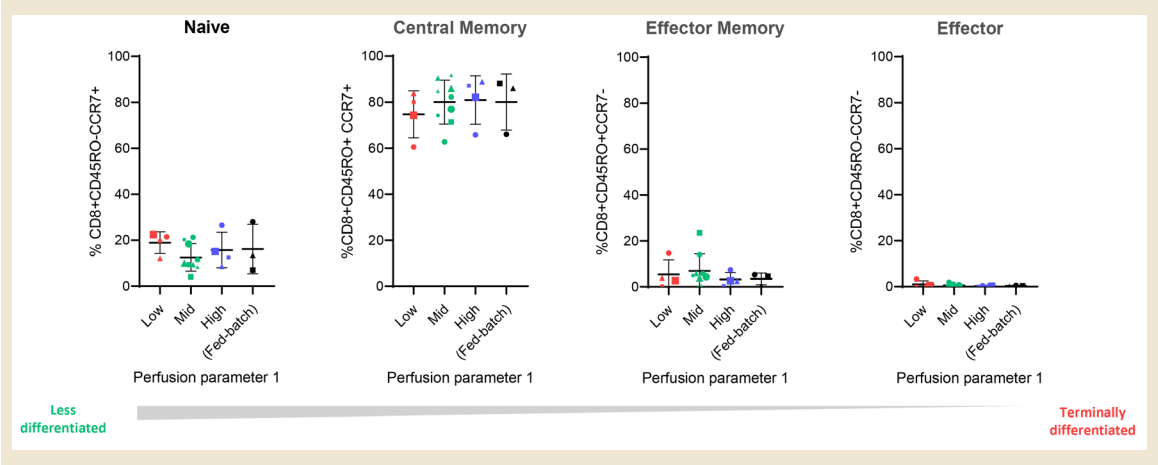
harvesting. The initial studies in Phases 1 and 2 concentrated on small-scale culture, particularly for autologous processes. Additionally, it investigated allogeneic CAR-T cell production. After gathering substantial data, these studies demonstrated the ability to scale this process and create a closed workflow for automated cell harvesting following the expansion process. This provided the basis for Phase 3 of the study.

This phase aimed to assess the scalability of the processes from the 250 mL scale using the Ambr® 250 HT bioreactor cultures to 2 L single-use platforms. It also evaluated the impact of scalable automated cell harvesting in a completely closed workflow. A similar protocol to Phases 1 and 2 was conducted over 5 days. This included thawing, activating, and transducing cells, followed by a seed train to generate enough cells for either the Ambr® 250 HT or the Univessel® single-use (SU) 2 L platform. This was followed by a 7-day fed-batch culture. However, it should be noted that this run did not utilize perfusion. In this case, it was run under fed-batch and harvested on day 12 by the Ksep® 400.

The Univessel SU 2 L is a benchtop stirred-tank bioreactor that operates with a working volume between 0.6 and 2 L. It features on-line pH, dissolved oxygen, and temperature control. The Ksep® 400 is a scalable centrifugation and buffer exchange platform designed to accommodate a range of cell density cultures, utilizing single-use consumables in a cGMP-compliant closed platform.

FIGURE 6

T cell phenotype of harvested cells under perfusion parameters, from less differentiated to terminally differentiated.



PHASE 3: KEY FINDINGS OF T CELL GROWTH AND CELL HARVESTING

The data generated from the experiments revealed that regardless of whether the Ambr® 250 HT or the Univessel SU 2 L was used, running identical processes on these platforms demonstrated almost identical results, as shown in Figure 7A. This allowed for the development and optimization at the small scale (250 mL) with confidence that the findings would translate effectively to the 2 L scale. These results were further evident in the fold expansion results, shown in Figure 7B. A nearly identical fold expansion in viable cell numbers was observed, with the Univessel showing slightly better, though not statistically significant.

Importantly, almost identical phenotypic profiles across both platforms were maintained, with over 90% of harvested cells falling within the naïve or central memory phenotypic ranges, and very few exhibiting effector memory characteristics.

The harvesting process also showed significant results. Prior to the processing with the Ksep® 400, there was a working volume of 1,950 mL, which was reduced to 141 mL after centrifugation. This achieved a 12-fold concentration, yielding a total of 110 million cells/mL. Importantly, there was minimal loss in viability, decreasing from 94 to 92.5%, with over 90% cell recovery.

There was a particular interest in assessing whether the quality of cells changed post-harvesting. It was found that the Ksep® 400 harvesting process did not adversely affect the quality of the cells. Both manually harvested and Ksep-harvested cell populations maintained their preferred phenotypes and similar exhaustion profiles, as shown in Figure 8. Additionally, the ability of the cells to grow post-harvest was examined.

Both manually harvested and Ksep®-harvested cells exhibited comparable growth trajectories, indicating that the automated harvesting process did not compromise cell quality.

INDUSTRY-ACADEMIA COLLABORATION TO ADVANCE CAR-T PROCESS DEVELOPMENT AND OPTIMIZATION

The key outcomes of the study not only involve driving innovative technologies

to improve process and product development but, most importantly, fostering an exchange that enhances understanding of cell therapy process development and manufacturing needs from the end-user perspective.

One of the major strengths of this work, and a primary reason such extensive and transformative data sets have been generated is due to the industry-academic partnership between Sartorius and University College London (UCL). It has provided a unique opportunity to influence and shape future technology development in the CGT space while integrating multiple platforms into a cohesive workflow.

As the collaborative study demonstrated, the Univessel SU 2 L system can be successfully combined with the automated Ksep® 400 system. Additionally, it can be

FIGURE 7

(A) Cell density and (B) fold expansion of harvested cells following identical processes on the Ambr 250 and Univessle platforms.

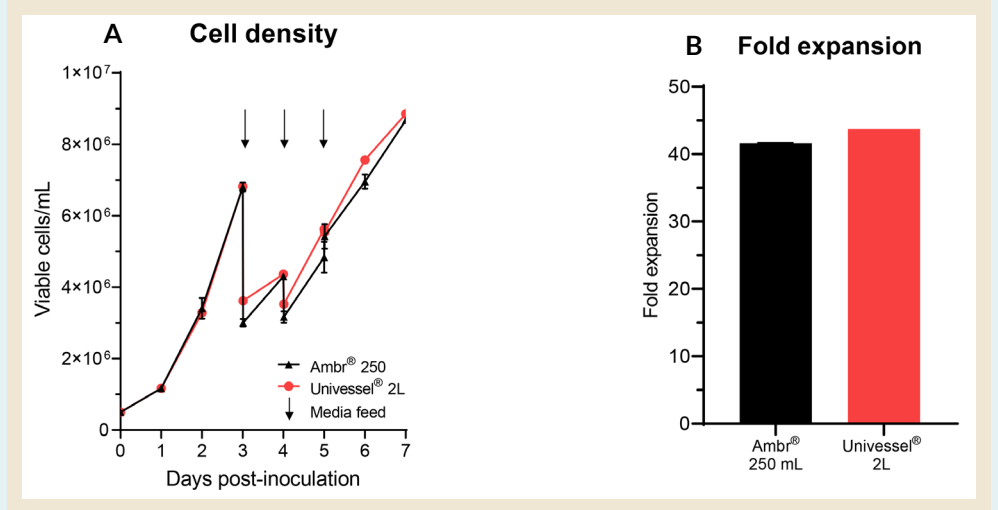
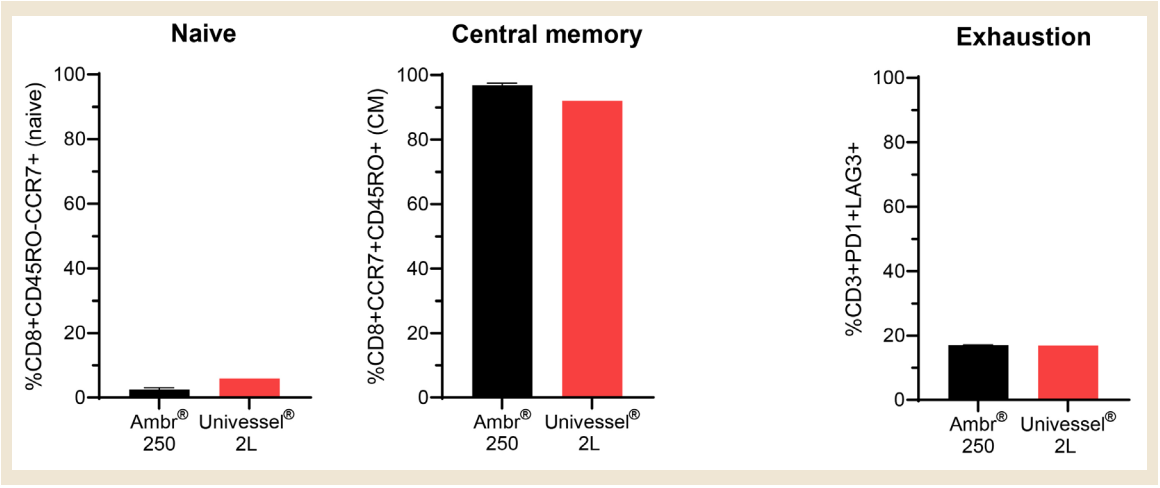


FIGURE 8

Post-harvest quality of cells (naïve, central memory, exhaustion) in manual versus Ksep harvesting.



combined with several other key technologies and platforms. This includes the use of animal component-free or serum-free media, as well as analytic platforms focused on modeling and software.

Finally, UCL has been able to draw upon Sartorius's expertise in technology utilization and operation, which has helped to address larger, more commercially and industrially relevant research challenges that are emerging across the sector. Addressing these bioprocessing and manufacturing challenges requires a collaborative, sector-wide approach that can benefit the entire industry.

CHEMICALLY DEFINED ADVANCED MEDIUM FOR PRIMARY T CELLS AND PATIENT-DERIVED CAR-T CELLS

The data presented from this study used 4Cell® Nutri-T GMP media, which is xeno-free, within the Ambr® 250 HT. However, a novel chemically defined version, 4Cell® Nutri-T Advanced, is now available.

The Nutri-T Advanced was developed in collaboration with university hospitals to ensure strong expansion performance with healthy peripheral blood mononuclear cells (PMBCs), isolated T cells, patient-derived T cells, and CAR-T cells—all without any serum supplementation. This ready-to-use formulation leads to consistent expansion of T cells, CAR-T cells, and tumor-infiltrating lymphocytes.

The medium includes recombinant human albumin which helps address regulatory concerns. Figure 9A presents the fold expansion data of $CD3^+$ T cells isolated from four healthy donors. The T cells were cultured for 10 days in 4Cell® Nutri T Advanced, animal component-free competitor media (Media A and B) or in CTL supplemented with 5% human serum. The results demonstrate superior T cell expansion in Nutri-T Advanced and high viability (Figure 9B) in all tested media.

PBMCs that were activated, transduced, and expanded for up to 10 days in various media were also assessed. It can be seen from Figure 10 that the Nutri-T Advanced

FIGURE 9

(A) The fold expansion data of $CD3^+$ T cells isolated from four healthy donors. The T cells were cultured for 10 days in 4Cell® Nutri T Advanced, animal component-free competitor media (Media A and B) or in CTL supplemented with 5% human serum. The results demonstrate superior T cell expansion in Nutri-T Advanced and high viability (B) in all tested media.

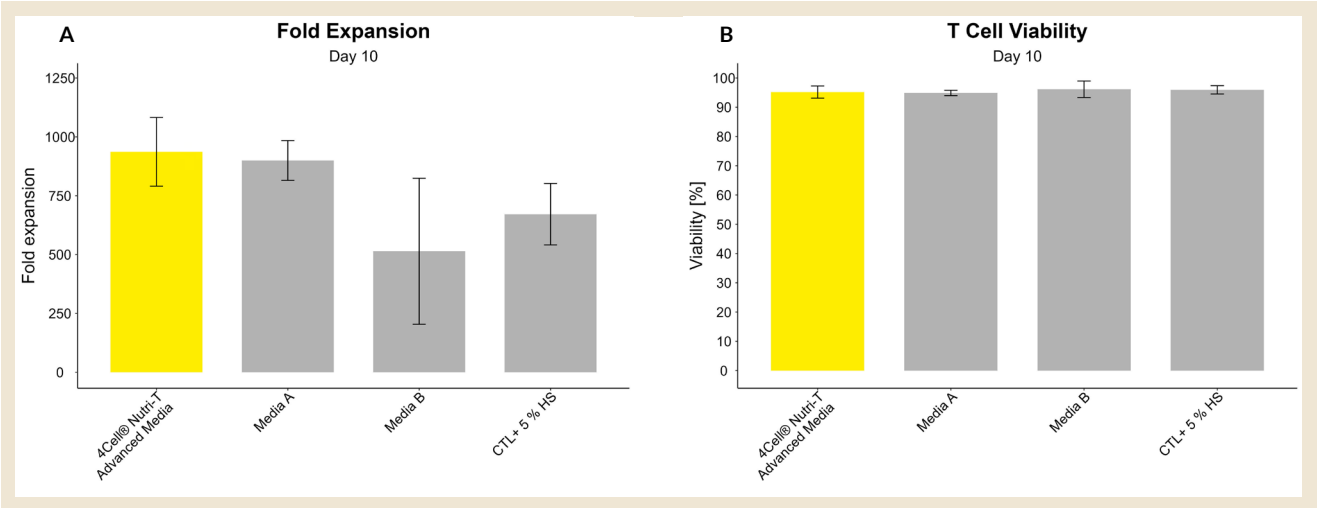
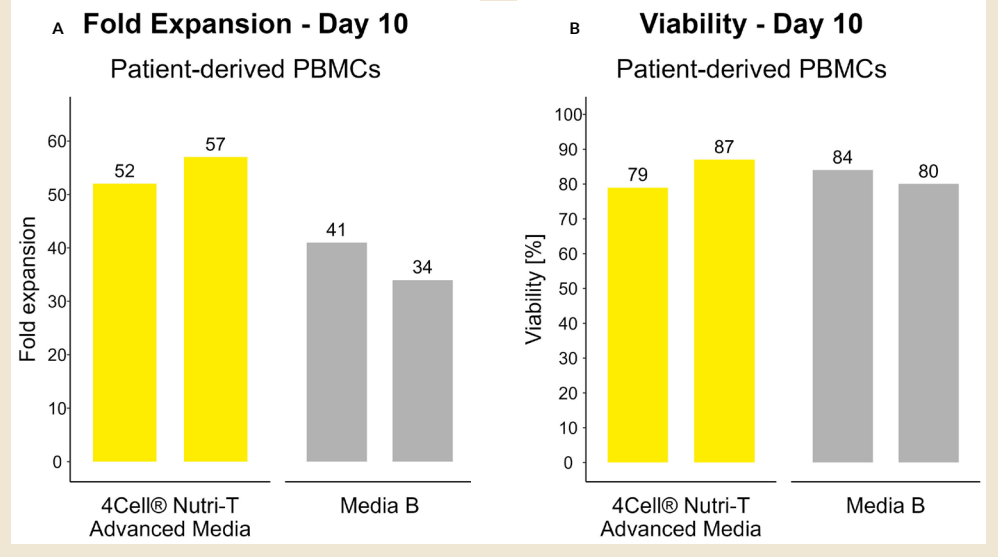


FIGURE 10

(A) The fold expansion and (B) the viability of PMBCs isolated from 2 patients. PBMCs were activated with OKT3 and IL-2, lentivirally transduced, and expanded for 10 days in either 4Cell(R) Nutri-T Advanced Medium and an animal component-free competitor T cell medium



showed superior growth of CAR-T cells, with the cells maintaining high viability. Additionally, for CAR-T cells derived from two donors, the phenotypic data showed excellent transduction efficiency across all media tested.

Moreover, the CD4 ratio was comparable across all media, demonstrating that this serum-free, animal component-free medium supports effective CAR expression, maintains a stable CD4 ratio, and promotes robust CAR-T cell expansion.

TRANSLATIONAL INSIGHTS

Moving forward, there are three main focus areas for this collaboration to maximize CAR-T cell yields in the CGT space. The first is to maintain a focus on the scalability of the optimized perfusion process at the 2 L scale. All the necessary data required for this has been generated and is now in the final stages of manuscript preparation.

Second is the development and assessment of an adaptive perfusion process that accommodates patient variability. This approach is based on specific metrics or process analytical technology measurements and is aimed at reducing media costs while enhancing production capacity and efficiency for CAR-T therapies tailored to individual patients.

The last future goal is to streamline the overall CAR-T manufacturing process in stirred-tank bioreactors. This includes integrating additional steps, such as activation and transduction, directly within the stirred-tank bioreactor.

The study demonstrated that utilizing a DOE approach provides significant insights into the CAR-T expansion process when using stirred-tank bioreactors. The results from the study provide a promising scalable process for CAR-T expansion and harvest in a completely closed workflow using single-use consumables.

Q&A


Julia Hengst (left), **Qasim Rafiq** (right)

Q Can you explain why stirred-tank reactors (STRs) would be preferred over rocking motion bioreactors or other closed systems?

QR First, it should be acknowledged that there are several platforms currently used for CAR-T production, especially at the autologous scale. These include systems like Prodigy, Cocoon, and the newer IRO platform by Ori Biotech, as well as the wave bioreactor, which is used in some approved processes.

While each system has its merits, STRs offer a combination of unique advantages that make them particularly valuable. One of the most important factors is that STRs are well-established for producing a variety of biopharmaceuticals at different scales. This means they are proven, reliable technologies available from multiple suppliers, reducing supplier risk. For instance, Sartorius is one of several companies offering these systems, ensuring more options and flexibility.

Another critical advantage of STRs is scalability. As mentioned, the study demonstrated scalability from 250 mL up to 2 L with consistent performance. This is now advancing to multi-liter scales as allogeneic applications are considered.

From a process engineering perspective, STRs also offer superior monitoring and control capabilities, which are invaluable for maintaining process consistency and quality. In STRs, CCPs such as temperature, pH, dissolved oxygen, and dissolved carbon dioxide can be easily measured in real-time. Even more advanced analytical capabilities are emerging, such as measuring cell viability and density via cell impedance or capacitance, as well as monitoring metabolites with Raman probes. This level of control ultimately results in higher product quality and yields.

Flexibility is another significant benefit of STRs. For example, strong yields were initially achieved with a fed-batch process and the addition of perfusion–an adjustment that is straightforward in an STR setup–further enhanced the outcomes of the study. Few other platforms offer this combination of process control, flexibility, and proven efficacy in the biotech sector.

Moreover, STRs have an extensive regulatory track record, with thousands of processes approved by the FDA. It has been shown that T cells, once thought to be too shear-sensitive, can grow well in STRs, confirming their suitability for these applications. That said, a current limitation is the lack of GMP-certified STR platforms at small scales. For example, the Ambr® 250 HT has been excellent for development but is not available in a GMP-certified format at 250 mL. At larger scales, however, multiple GMP-certified STR platforms are available, which is a big advantage for clinical and commercial manufacturing.

Q What is the impact of STR culture on the quality of CAR-T cells, and how does it help advance the processing of patient-derived material?

QR Initially, many of our colleagues in clinical and immunology fields were concerned about using STRs for T cells, even though they are suspension cells. There was a belief that T cells would not tolerate the agitation, as the shear stress generated in STRs might damage them. However, through research, it has been shown that this concern is unfounded.

T cells can be grown at high quality in STRs without issues related to shear stress. Importantly, the clinical-grade phenotype is maintained, and cell exhaustion is avoided, which is a significant achievement. Ultimately, the goal is not just to produce billions of cells but to produce billions of cells with the right quality attributes.

JH Additionally, we will be presenting further data in January 2025, showing that functionality was assessed alongside phenotype. Specifically, CAR-T cell expansion in the Ambr® 250 HT and the Univessel SU 2 L bioreactor was measured, along with their effectiveness in killing target cells, and their cytokine secretion. This functionality is key for demonstrating that the cells are not only abundant but clinically relevant.

Q With the current shift towards shorter manufacturing times and *in vivo* CAR-T, how do you view the role of STRs moving forward?

QR This is an area where there continues to be significant changes in how CAR-T therapies may look in the future. There is a strong trend toward reducing processing times, with some approaches aiming for minimal cell expansion or, in certain cases, no expansion at all.

Our work with STRs has demonstrated a significant reduction in expansion time to reach target cell counts. This advancement is largely due to our use of perfusion, which has allowed for a significant speed-up of the manufacturing process. We were recently awarded a £2.5 million grant which will aid in further accelerating CAR-T manufacturing by minimizing both expansion and quality assurance/quality control times.

Regarding *in vivo* CAR-T, the future goal is that the cells will not need to be extracted from the patient. Instead, therapeutic mRNA could be directly delivered *in vivo* using lipid nanoparticles. This approach would redefine what is considered a CAR-T product and fundamentally change how it is manufactured. In that scenario, the focus shifts to optimizing lipid nanoparticles, improving targeting efficiency, and ensuring effective mRNA production.

However, if *in vivo* CAR-T becomes widespread, there will still be a need for *ex vivo* manufacturing for other cell-based therapies, such as CAR-NK cells, macrophages, and hematopoietic stem cell therapies. Many of these therapies require gene modifications that are best-achieved *ex vivo*. While *in vivo* CAR-T could reshape the field, STRs and other *ex vivo* systems will remain essential platforms for manufacturing a wide range of cell therapies in the future.

Q What are the benefits of optimizing a perfusion process for CAR-T cell expansion?

JH As Qasim mentioned, the data from the study shows that shifting from fed-batch to perfusion can nearly quintuple the final cell yield. We are currently working on further optimizing this process to either increase yield or reduce media consumption—an especially important factor when considering scale-up. Large volumes of media are required for these processes, so any reduction in media use directly benefits customers by lowering costs and resource needs.

Additionally, the Ambr® system is excellent for small-scale optimization and process development. Various perfusion settings in Ambr® can be tested which allows for a seamless transition to larger bioreactors, ultimately speeding up CAR-T manufacturing. This approach not only accelerates the production process but also reduces development and clinical translation times.

Q What are the benefits of using DOE approach in CAR-T process development?

JH Several colleagues have provided valuable support to set up the DOE for this perfusion process. DOE is crucial as it provides a structured and efficient way to plan and analyze experiments, helping to interpret relationships between different variables and their effects. This approach is beneficial as it generates new data, allowing for the creation of models that map the relationships between factors. DOE allows maximum data to be gathered while reducing the number of experiments required, minimizing time, cost, and resource use, including media, consumables, and reagents, during process development and optimization. Especially for CAR-T where quantity of cells might be limited a mindful use is key for a successful development.

For example, we are currently analyzing key perfusion parameters, A and B, which will be disclosed in an upcoming publication, alongside the final CAR-T cell yield as the response variable. By using DOE, deeper insights into the process were gained, which helps define optimal operating conditions and identify a design space for the process. Through DOE, CPPs have been successfully identified and understood and their impact on key quality attributes of our product.

Using software like MODDE has allowed for direct observation of the individual and combined effects of CPPs on these quality attributes.

QR The scientific and commercial communities would benefit from fully adopting DOE in all studies. The majority of research in my group is DOE-based. DOE maximizes the ability to explore various parameters and levels, ultimately reducing development time and cost compared to the traditional one-factor-at-a-time approach. It is a transformative approach and is increasingly encouraged by regulatory bodies, as seen in the International Council for Harmonization quality guidelines, which leads to high-quality, efficient science.

Q What is the next phase of your research, and how do you plan to further improve your process?

QR: One of the primary focuses is on adaptive manufacturing and adaptive perfusion, specifically, how this can be adjusted in the process in real-time based on certain measurements to account for patient-specific material variability. This would enable a more consistent and optimized outcome across different patient samples. There are also further studies assessing ways to adapt perfusion to reduce media consumption and costs, which is critical as the space moves forward.

Another important area is applying these methods to patient material. All the data that has been shared so far is based on healthy donor material, but it must be recognized that patient material will likely present new challenges with reduced consistency or yields. However, there remains hope that STRs, combined with perfusion and enhanced control, will still yield more consistent results compared to other platforms. We are fortunate to have access to patient material now through multiple partnerships.

Additionally, the process is now being scaled-up. As successful scale-up from 250 mL to 2 L in fed-batch has been demonstrated, and similar work with perfusion is being carried out, the aim is to scale up further to 10 L.

Finally, as the field demands GMP-compliant solutions, the focus remains on transitioning to animal component-free and serum-free media. Much of the work is already serum-free, and attention is now on eliminating all animal-derived components, including recombinant proteins and cell media.

BIOGRAPHIES

Julia Hengst is an External Collaborations Manager at Sartorius, Göttingen, Lower Saxony, Germany where she builds and leads partnerships through scientific collaborations around immune and stem cell therapies. She holds a PhD in Immunology from Hannover Medical School, Hannover, Germany and has several years of experience in product management for biomedical research and cell and gene therapy clinical applications.

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Qasim Rafiq is a Professor in Bioprocess Engineering at University College London, London, UK with a focus on novel therapeutic modalities including cell and gene therapies. He is a multi-disciplinary engineer working at the life science, engineering and commercial interfaces with a research focus on the bioprocessing, automation and biomanufacture of cell and gene-based therapies. He currently leads a research portfolio of over £7.5 million as Principal Investigator and leads a dynamic interdisciplinary research group that collaborate internationally with high-caliber academic institutions, industry partners, and leading clinicians. Qasim is both a chartered engineer (CEng) and chartered scientist (CSci) and sits on multiple scientific and engineering committees including the BIA's Cell and Gene Therapy Advisory Committee.

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AUTHORSHIP & CONFLICT OF INTEREST

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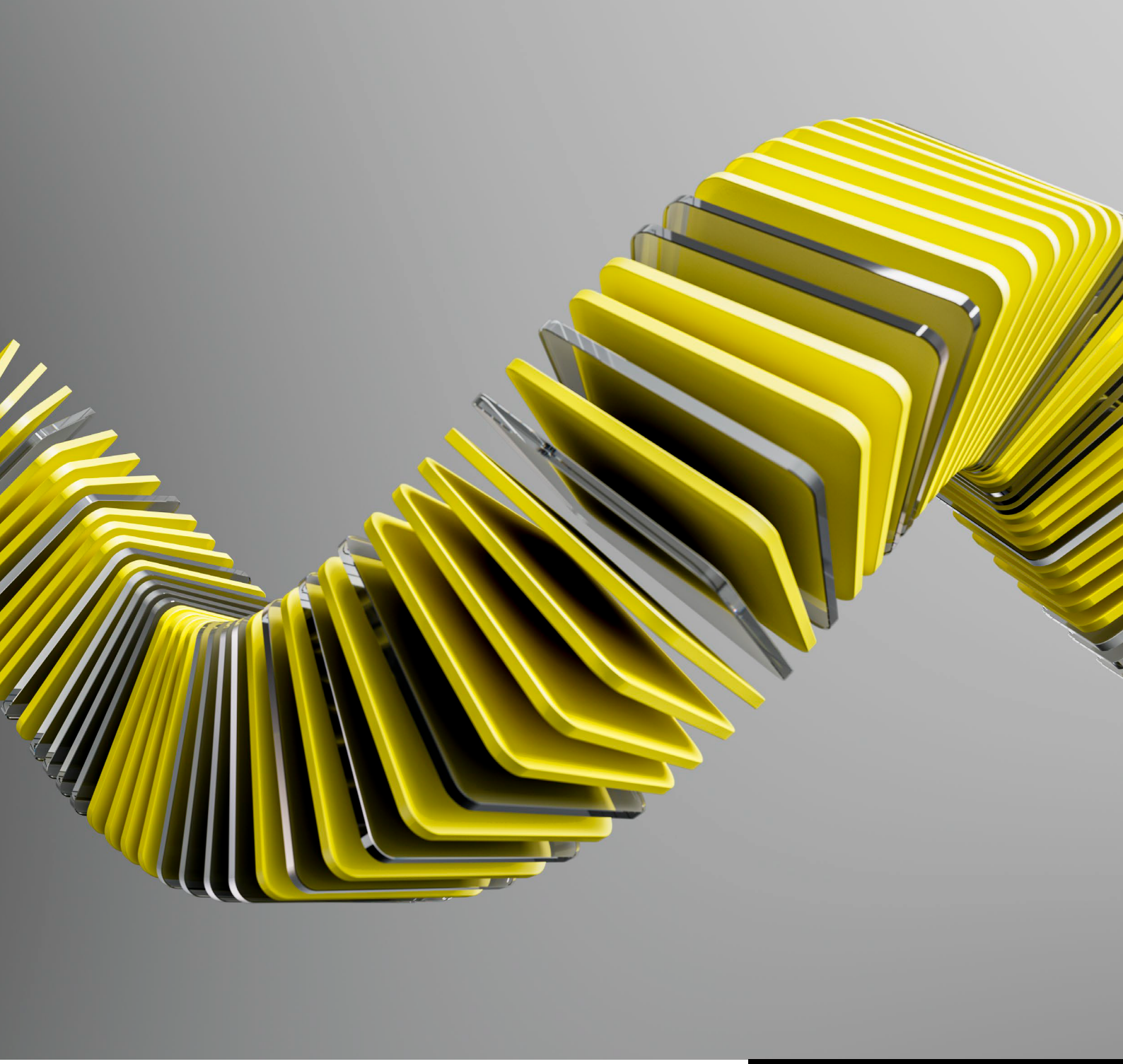

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INNOVATOR INSIGHT

Enhancing CAR-T therapy development: harnessing cell selection flexibility

Tamara Laskowski and Nuala Trainor

Clinical advancements in personalized cell therapy, particularly CAR-T cell therapy, have created a demand for innovative, closed, automated, and space-efficient manufacturing methods. Given that each therapeutic product requires a unique manufacturing process, flexibility in automation platforms is essential to accommodate these varying needs. This article will explore recent developments in the Cocoon® Platform, an automated system for CAR-T manufacturing, highlighting the ability to adapt to different processes including positively or negatively selecting T cells directly from apheresis. Enabling direct cell isolation from the input material eliminates the need for additional upstream processing equipment, thus reducing costs, time, and space requirements. Automatic transfer to an expansion chamber for extended culture enables further reduction in manual processing.

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T cell therapies are the leading cell therapy modality and the most widely pursued in the context of therapeutic assets for oncology and autoimmune indications. Within T cell programs, human peripheral blood mononuclear cells (PBMCs) and peripheral blood are the primary sourced starting material from which T cells are extracted. In the context of manufacturing CAR-T cell therapies, the input material plays an important role in process development. PBMCs and peripheral blood are known to be very heterogeneous materials, comprising a number of cellular impurities (i.e., non-T cells) that must be controlled or removed to ensure robust manufacturing. This can be done within the process itself during cell culture and expansion, or through additional upstream steps such as cell selection.

Cell selection strategies can be categorized into two main groups. First, positive selection, which describes how T cell subsets can be selected directly through binding reagents that target molecules on the T cells themselves, including CD4/CD8 and CD3/CD28. Positive selection is advantageous in that it enables direct targeting of the cell type of interest. Moreover, it allows for the ability to modulate the T cell subsets selected and enables simultaneous selection-activation through engagement

of CD3 and CD28 receptors on the surface of T cells. Challenges with this modality of selection include the potential to influence T cell biology, given the interaction with surface receptors, and the fact that steps to remove the selection reagent from the culture must be integrated into the manufacturing process.

The second group of selection strategies is negative selection. In negative selection, the strategy is to target all non-T cell cellular subsets for removal, thereby enriching T cells in the 'untouched' negative fraction. Because in a negative selection approach there is no direct T cell engagement, this reduces the impact on T cell biology. Potential challenges with this strategy include the complexity of reagent formats (often comprising multiple antibodies formulated at different concentrations) possibly leading to increased costs, and lower process flexibility to modulate T cell subsets selected or to leverage single-step selection and activation.

A FUNCTIONALLY CLOSED SYSTEM FOR AUTOMATING CELL THERAPY MANUFACTURING

A standard cell manufacturing process comprises a number of steps that are critical to transforming a T cell into a T cell therapy. Cell selection is an optional step that has become valuable to enable robust and reproducible manufacture of T cell products. Manufacturing processes can be streamlined by automating multiple unit operations into a single platform, thereby decreasing operator touchpoints and reducing the risk of errors.

The Cocoon® Platform helps to consolidate several steps into a single operating functional unit. The platform comprises a single-use cassette wherein manufacturing takes place. The cassette is compatible with adherent and suspension cells and is applicable to viral and non-viral processes. The second component is the bench-top environmental unit with integrated pH/dissolved oxygen (DO) sensors for in-process monitoring. Moreover, the Cocoon® Platform dual-zone temperature control enables storage of media and reagents at 4 °C while maintaining cell culture at 37 °C. The final component is the software that controls these functionalities and records all manufacturing process data into electronic batch records, which can be extracted at the end of each manufacture.

POSITIVE T CELL SELECTION WITHIN THE COCOON® PLATFORM

In this study, we demonstrated how to implement positive selection capabilities within the Cocoon® Platform in a streamlined and automated way to enhance CAR-T cell manufacturing. The key aims of this study were to understand the impact of impurities and address unique requirements of the input material; to maximize bead-binding efficiency whilst maintaining high cell viability and robust recovery; and to enable high T cell selection efficiency whilst reducing hands-on time.

The initial iteration of this application as a baseline protocol, leveraging a PBMC input and standard CTS™ Dynabeads™ CD3/CD28, is highlighted in Figure 1.

As shown in Figure 2, irrespective of the percentage of $CD3^+$ T cells in the input material, robust enrichment of T cells directly in the Cocoon® Platform is possible through our positive selection strategy.

Monocytes are a cellular impurity that has been shown to interfere with CAR-T manufacturing, and this selection strategy also enables a decrease in the percentage of monocytes. Moreover, as observed in the final product, the approach implemented in the Cocoon® Platform is also consistent at delivering high T cell purity levels.

During the full-scale protocol run, we observed the expansion of the selected product in the Cocoon® Platform. For the

FIGURE 1

T cell therapy manufacturing process in the Cocoon® Platform comprising integrated T cell selection.

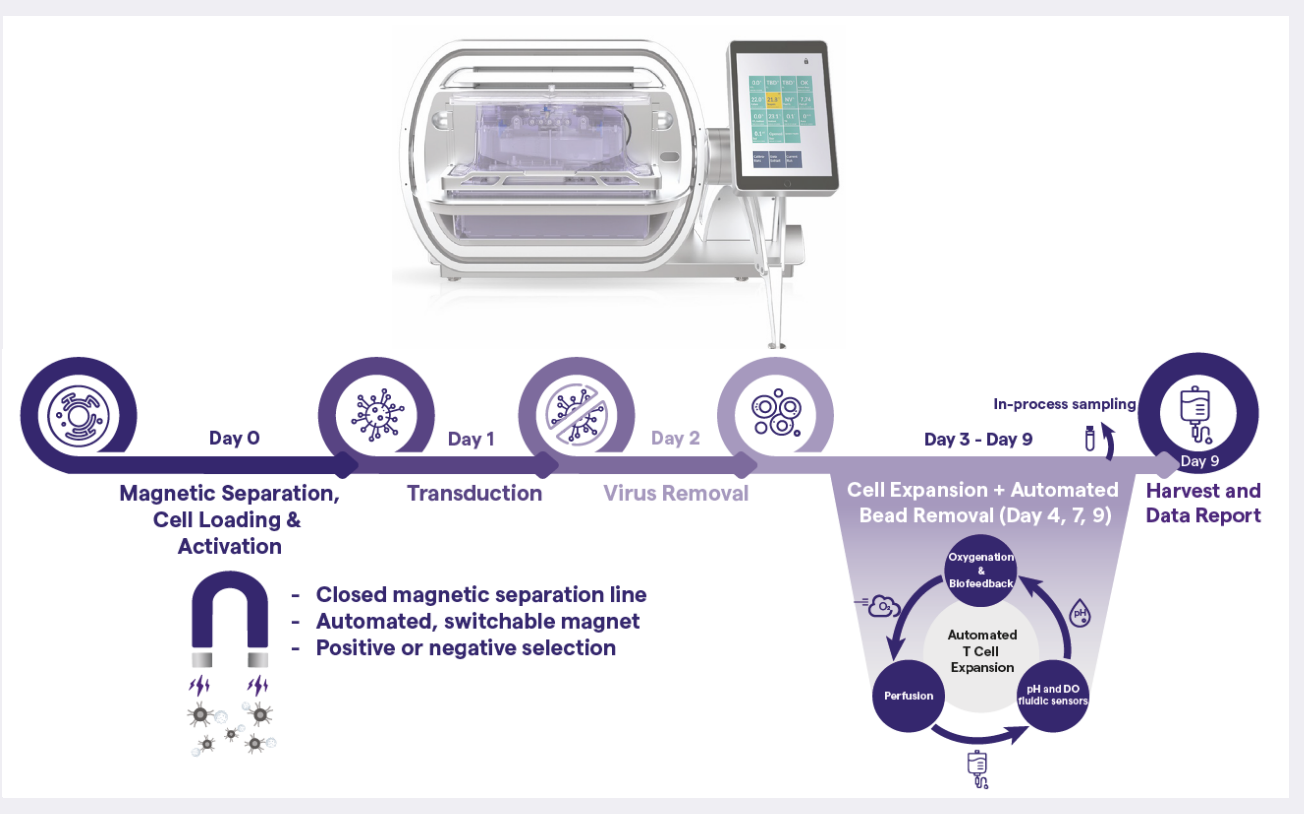
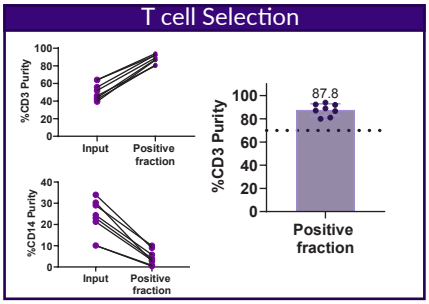
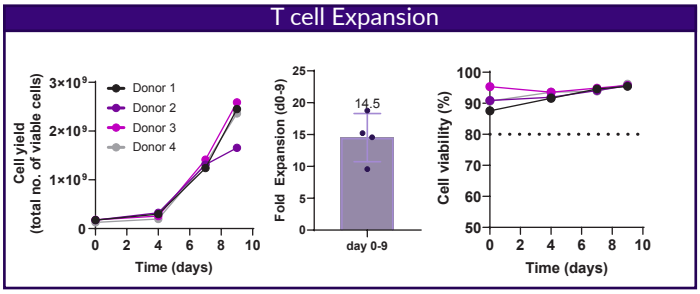


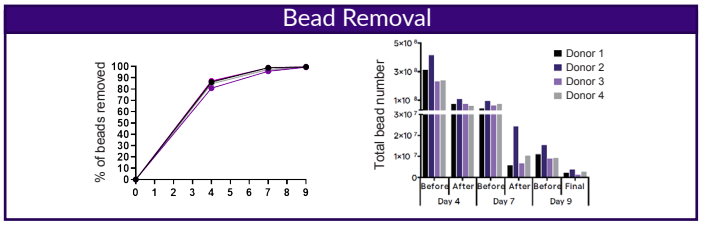
FIGURE 2

Expansion of standard CD3/CD28 Dynabead-selected T cells in the Cocoon Platform.



Robust T cell selection from PBMC using CD3/CD28 CTS™ Dynabeads™





four donors, similar trends of growth and proliferation were seen over the 9 days of manufacture. Over 14-fold expansion was achieved, maintaining high product viability throughout the entire manufacturing time.

Automated bead removal steps were integrated on days 4, 7, and 9 to remove residual beads from the product. Almost all of the beads that we added during the selection process were successfully removed at similar efficiency levels across all four donors, independent of donor-to-donor variability.

The new Gibco™ CTS Detachable Dynabeads™ reagent offered an opportunity to further enhance this application, enabling full control of when to release the beads from the cells post-selection. In the next study, the aim was to validate this reagent in the context of the same application already developed for the Cocoon® Platform. The results are outlined in Figure 3.

The starting material used was a processed PBMC that contained 59% CD3 T cells, which was enriched to approximately 95%. With the Detachable Dynabeads™, the same or better performance was achieved compared to the standard beads.

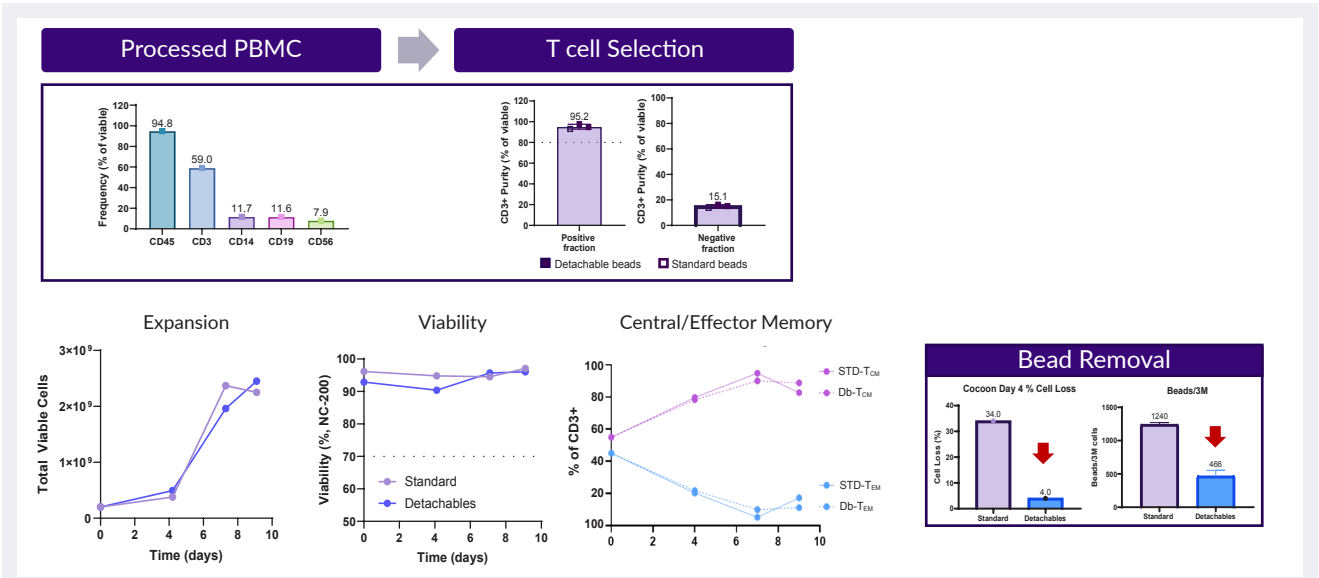
When we further compared the standard Dynabeads to the Detachable Dynabeads™ protocol adaptation, similar process performance was observed. The expansion plot shows that the cells proliferated quite similarly and achieved essentially the same overall total viable cell yield. High viabilities were maintained throughout the process for both groups.

Additionally, for both Detachable and Standard Dynabead-selected input materials, the cells maintained high central memory and low effector memory phenotypes, a profile that suggests a more robust product. Importantly, through the use of Detachable selection reagents, we were able to remove beads early in the process and to reduce overall bead residuals.

With the standard Dynabeads, cell loss of 34% was observed while performing the automated bead-residual removal steps. This is likely due to a subset of cells that are still bead-bound on day 4 (first bead-removal step) and are, therefore, captured

FIGURE 3

T cell selection and activation with CD3/CD28 CTS Detachable Dynabeads™ to enable streamlined manufacturing in the Cocoon Platform.



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along with the trapped beads. Cell loss during bead residual removal was greatly reduced to 4% by using the Detachable Dynabeads™ reagent (Figure 3).

PROOF-OF-CONCEPT: MANUFACTURING CAR-T CELLS USING INTEGRATED POSITIVE T CELL SELECTION WITH SIMULTANEOUS ACTIVATION

The process with the Detachable Dynabeads™ reagent was transferred into a CAR-T manufacturing protocol. A Lonza research-grade lentivirus vector carrying a third-generation CD19 CAR was used for this proof-of-concept work. The results are outlined in Figure 4.

For the three donors tested, good total yield was observed at final harvest on day 9. Donor 2 is a PBMC while donors 1 and 3 are diluted apheresis. Fold expansion for all donors was consistent with previous observations, with very high harvest product viability and percentage of T cell in the final drug product. Despite using a low multiplicity of infection (MOI) of 0.5, CAR-T cell yields were also consistent with the efficiency of transduction previously observed for this vector. Moreover, CAR expression was similar in all three donors.

To measure product potency, manufactured CAR-T cells were co-cultured with tumor cells, in this case, a NALM6 tumor cell line that expresses the CD19 marker that the CAR-T cell is engineered to target. As shown in Figure 4, both products can equally detect and eliminate the tumor cells, following similar response kinetics. Moreover, untransduced T cells from the same donor material were unable to eliminate NALM6 tumor cells, indicating that the anti-tumor response was CAR-mediated, and thus demonstrating the manufacture of functional and robust CAR-T cell products.

These data suggest there is an opportunity to bypass processed PBMCs, and move directly from apheresis to T cell selection, leveraging these reagents in a fully automated Cocoon® Platform protocol. When the starting material is unprocessed

FIGURE 4

Standard 9-day manufacturing of CAR-T cells in the Cocoon Platform using integrated positive T cell selection with simultaneous activation.

T cell selection using CD3/CD28 CTS™ Detachable Dynabeads™

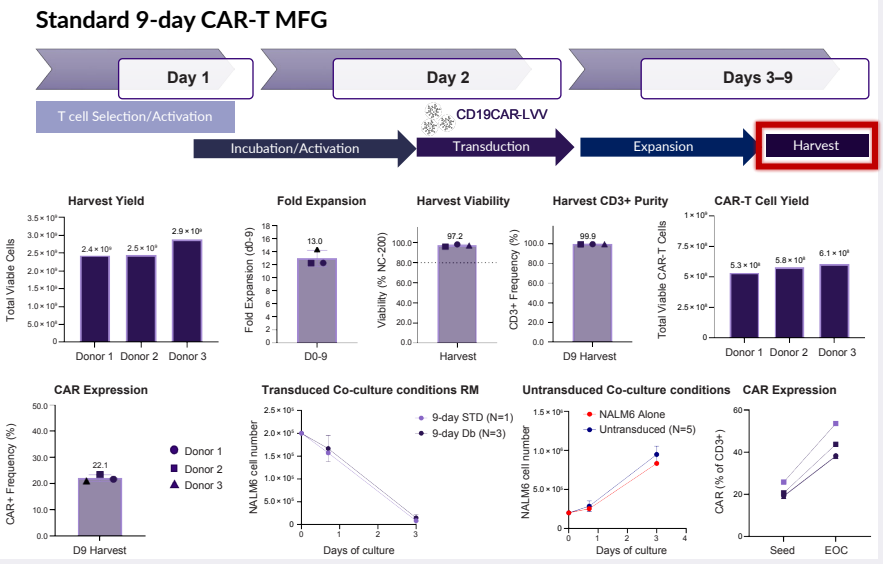
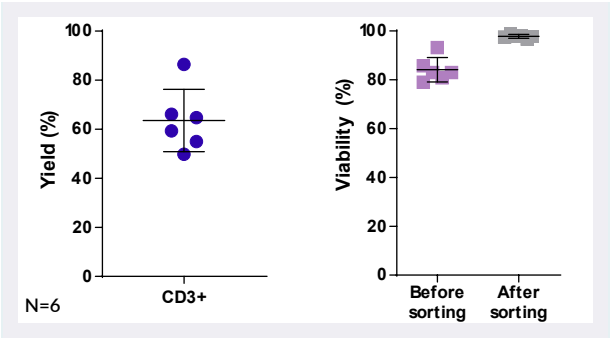


FIGURE 5

Day 0: T cell yield and T cell viability post-BACS on Cocoon Platform.



apheresis, there is the possibility of greater variability between manufactures. It is, therefore, important to ensure that the process is sufficiently robust to accommodate this variability. To test our process, we performed various manufacture runs comparing cryopreserved PBMCs to cryopreserved unprocessed apheresis product, and observed no statistical difference in the ability to enrich T cells to nearly 100% in the positive fraction in both settings.

These developments have been integrated into protocols that are now available for CD3/CD28 selection using the Detachable Dynabeads™ in the Cocoon® Platform, as well as protocols that apply CD4/CD8 selection using Detachable Dynabeads™. These same selection steps have also been added to rapid manufacturing solutions providing the opportunity to shorten the CAR-T cell manufacturing process. Our Rapid Cocoon® CAR-T™ protocols enable T cell selection directly from apheresis followed by activation, transduction, and product harvest in just 3 days, yielding a robust product that maintains high percentage of memory T cells (data not shown).

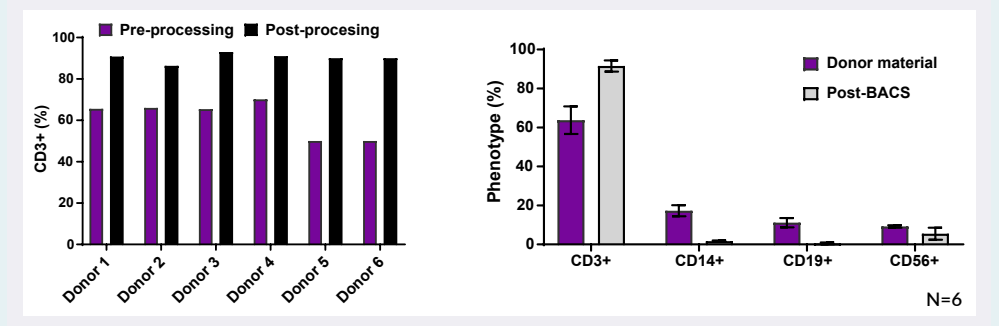
NEGATIVE T CELL SELECTION WITH THE COCOON® PLATFORM: CASE STUDY DATA

The Cocoon® Platform is designed to enable a variety of processes, including negative selection protocols. In this case, negative selection was performed through the use of Akadeum's Buoyancy Activated Cell Sorting (BACS™). BACS works through a process of mixing the sample, antibodies, and microbubbles. The microbubbles capture the cells and the bubble bound cells float, allowing enriched T cells to remain in the subnatant.

One goal of Cocoon® Platform integration with this method was to avoid the need for any pre-processing before going into the Cocoon® Platform. There was no washing of the products before they were attached to the Cocoon® Cassette. The antibodies used in this specific process were CD14, CD16, CD19, CD20, CD36, CD56, CD123, and CD235a.

FIGURE 6

Day 0: T cell yield and T cell viability post-BACS on Cocoon Platform.



For this experimental set-up, a frozen 1/4 Leukopak was thawed, cell counted, and antibodies were added. Bags (Leukopak and microbubbles) were attached to the Cocoon® Cassette before T cell isolation. The Cocoon® Platform mixed cells and antibodies before the cells were transferred to the microbubble bag for further mixing. Cell separation and transfer of the negative fraction was performed prior to cell seeding. This whole process is automated and takes around 40 minutes.

Figure 5 shows the day 0 performance; the yield and viability achieved on the Cocoon® Platform using this BACS approach. 50–86% of the CD3 cells were recovered from the starting material and the viability was higher than 97% in all conditions after negative selection. It was sufficient in separating out the dead cells from the live cells.

The purity of the population before and after separation is represented in Figure 6. The starting material input ranged from 50–70% $CD3^+$ cells and increased to 88–97% $CD3^+$ cells after negative selection. The non-target T cells remaining in negative fraction were reduced to 1.7% $CD14^+$, 0.5% $CD19^+$, and 5.5% $CD56^+$ cells.

To ensure there was no negative impact on downstream steps, day 0–9 performance of the T cell subsets was further analyzed. Figure 7 shows that a high $CD3^+$ population was maintained throughout culture (95.8 ± 3.5% on harvest), and $CD3^+/CD4^+$ and $CD3^+/CD8^+$ populations were within the expected range.

Figure 8 shows the T cell expansion and viability post-BACS. After enrichment, 1×10^8 $CD3^+$ cells were seeded and expanded to ~2.6×10^9 viable $CD3^+$ cells. High viability was maintained throughout the process.

The memory phenotype and exhaustion of these cells were also assessed. Figure 9 shows that the BACS process was effective in maintaining high stem memory T cells and low effector cell populations throughout the expansion process, in addition to low exhaustion maintained throughout expansion process.

FIGURE 7

Day 0–9 performance: T cell subsets post-BACS on Cocoon Platform.

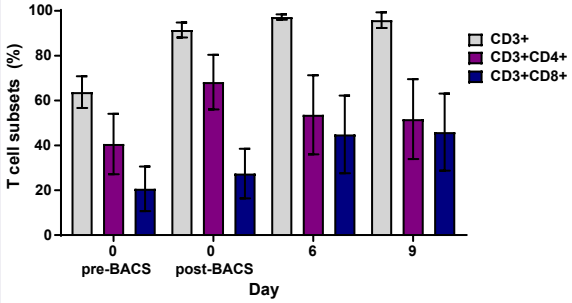


FIGURE 8

Day 0–9 performance: T cell expansion and viability on Cocoon Platform.

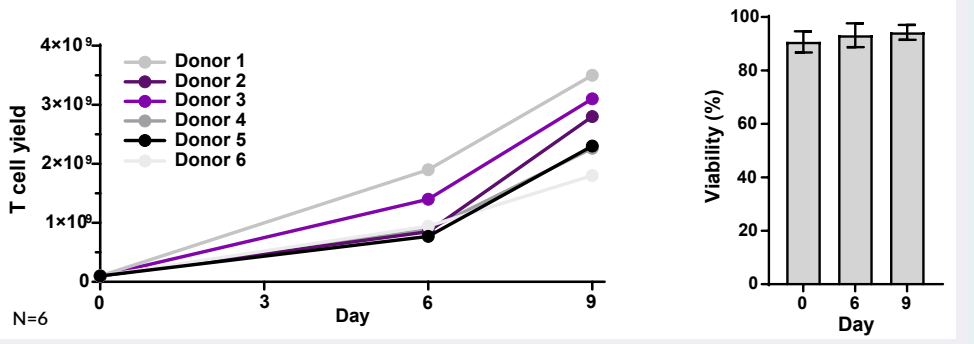
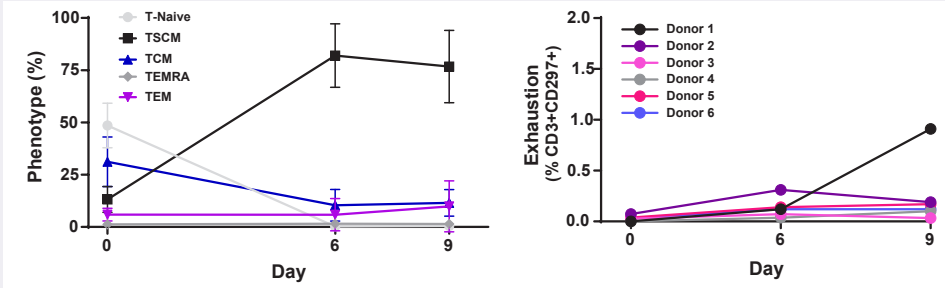


FIGURE 9

Day 0–9 performance: memory and exhaustion during cell expansion on Cocoon Platform.



N=6 TSCM=Stem memory T cells TCM=Central memory T cells TEM=Effector memory T cells TEMRA=Effector memory T cells re-expressing CD45RA

In summary, the Cocoon® Platform enables isolation of T cells directly from cryopreserved Leukopaks without any additional upstream equipment and reduced operator interactions. The negative selection approach also enables flexibility over the type of activator used. Post-separation purity of the T cells was 92% with a recovery of 64%, and the expansion was an average of 2.6 billion T cells in 9 days. There are opportunities for further process refinements and additional use cases to be optimized.

Q&A

Tamara Laskowski (left), **Nuala Trainor** (right)

Q Have you performed a comparison between products generated using different selection reagents to assess whether there is an impact on T cell phenotype or function?

TL Yes, we have. My group is carefully looking at CD3/CD28 bead selection-based reagents, which also activate the T cells, and comparing that to other methods of activation that are not related to a bead in the context of this selection. We are also comparing that to other agents that activate cells in different ways, such as with soluble antibodies, or with other activators downstream from selection.

Cell & Gene Therapy Insights 2025; 11(1), 111–123 · DOI: 10.18609/cgti.2024.013

Q You mentioned the ability to perform positive T cell selection based on CD3/CD28 or CD4/CD8 in the Cocoon® Platform. What are the advantages and disadvantages of each option?

TL The two reagents both work well at selecting and enriching T cells robustly. The best strategy for T cell selection depends on what you want to accomplish with that selection. With the CD3/CD28, we see a number of users who prefer to have a single step that both selects and activates the cells.

A second set of users prefers to modulate their subtypes. They might need an even distribution of CD4 and CD8, and that can be accomplished with the CD4/CD8 reagents, which can be tailored to the unique needs of each therapy. There is a little more flexibility with the CD4/CD8 reagents.

Q The data presented focuses on the use of 1/4-sized Leukopaks. Could the Cocoon® Platform use this method of separation for larger or smaller starting materials?

NT The 1/4-sized Leukopaks were selected as a use-case study, but there is no reason that we could not either scale up or scale down. We are currently looking at separating with full Leukopaks. Scaling down will also be possible. The software is designed to enable flexibility to make modifications relatively easily.

Q What method of activation should be used following negative selection?

NT We allow our customers to use their preferred kind of activation. In the specific case presented here, we used TransAct, but this is flexible.

Q As input material, especially patient material, can be variable, have you evaluated strategies to assess the impact on selection performance?

TL We have evaluated strategies to assess the impact on selection performance. We are looking closely at understanding what impurities are more likely to be present in an apheresis, as compared to a processed PBMC. We want to understand both the level of impurities and the impact of these cell subsets on selection. We can point to some culprits that make selection more difficult, and we target any upstream steps to specifically remove those cell types. We understand the thresholds the process can tolerate and apply the right solutions to address those needs.

Q With the 3-day rapid production system, how do you guarantee that there are no lentiviruses at the end of the process?

TL Lentiviral vectors will be cleared, but there might be a window that is necessary to completely clear the episomal expression of these vectors. We do monitor the vector copy number closely, and we monitor our ability to detect when it is stable. There are a number of initiatives in place to solve that question. We are working closely with leaders in the field to position what those analytical tests need to be to ensure product safety, for example when presenting data to the US FDA or other regulatory agencies.

Q How does formulation of input starting material influence the cell recovery and/or function?

NT In the negative context, specifically, depending on the makeup of the starting material, you may need to make some modifications, such as changing the antibody concentration or volumes. In the testing we have performed, using the off-the-shelf products was successful. Potentially, we would have to make changes, if for example, there was a very high frequency of unwanted cells.

TL From the perspective of positive selection, there are formulations for specific chemicals and reagents that are added into the apheresis or other starting material that may be incompatible with some of these reagents. We know what actions to take to mitigate these potential incompatibilities to make it work with the strategy that you have selected for your process. The formulation can have an impact, but we have solutions for this, and we are happy to work with you on that.

Q You indicate these runs were performed with frozen Leukopaks. Could this method of separation be used for fresh Leukopaks?

NT We expect that this could be used for fresh, and we are currently working on doing so. We initially selected frozen Leukopaks as we assumed that these would represent a worst-case scenario. Theoretically, it should work with fresh as well, and as we collect more data, we will be able to provide updates on the results from those tests.

Q How do you categorize T cell stemness? Which specific markers are you looking at, positive and negative?

TL We use a number of panels. We have a combined flow cytometry panel with 13–15 markers. In the combined panel, we look at the activation together with exhaustion and memory to understand the impact of the expression of these multiple markers on the same cell.

Cell & Gene Therapy Insights 2025; 11(1), 111–123 · DOI: 10.18609/cgti.2024.013

We also have more extensive panels, which are comprehensive 13/15-marker panels that are specifically focused on memory. In this context, we take a dive into the subsets of cells and the differentiated transitional states. We report not only on the expression patterns, but also transitional patterns of expression that indicate that these cells, albeit not yet an effector, are trending towards an effector. We have a comprehensive marker list that helps to delineate specific changes in phenotype over time.

Q With the negative selection, can we still use the standard cassettes, or are specialty cassettes required?

NT For these studies, we used the standard cassettes. One of our criteria was to not to require any cassette changes, as we did not want to have to redesign new cassette to enable this functionality. People will be able to use this with the standard cassettes, and connect the tubing to the various port locations.

Q Have you tried these selection methods during any electroporation-based production?

TL The selection methods have not yet been tried during electroporation-based production. We are planning to take the selection products into non-viral gene transfer approaches, not just limited to electroporation, but also to non-electroporation, non-viral systems. We have a comprehensive group within our R&D organization that are also looking at nucleofection as an option. Enriched T cells is one item on that investigation list.

BIOGRAPHIES

Tamara Laskowski is the Senior Director and Head of Clinical and Process Development at Lonza, Houston, TX, USA. In her role, she supports the development of novel T cell and NK cell therapies targeting solid and haematological malignancies and the transition of processes from the pre-clinical stage into clinical manufacturing. Moreover, Laskowski oversees the implementation of enhanced analytics for monitoring critical attributes associated with product fitness and function. Laskowski received a doctorate degree in the fields of human molecular genetics and immunology from the University of Texas Health Science Center at Houston, TX, USA, where her work focused on targeted genome editing of patient stem cells to correct genetic mutations linked to immune disorders. Laskowski was awarded a fellowship to the National Science Foundation Innovation Corps program dedicated to training scientists to develop strategies for expanding the economic and societal benefits of innovative ideas that have commercialization potential. She was the sole recipient of an award for outstanding performance upon completion of the program.

Tamara Laskowski PhD, Senior Director, Clinical Development, Lonza, Houston, TX, USA

Nuala Trainor is the Director of Innovation at Lonza—Personalized Medicine, Kingston, Ontario, Canada, where she leads a team focused on creating cutting-edge solutions for next-generation cell-based personalized medicine. Her primary emphasis is on the Cocoon® Platform, for which she oversees the system architecture. Trainor is responsible for evaluating both internal innovations and external technologies that complement and enhance the platform. Prior to her current role, Trainor led Lonza's Personalized Medicine Biology team and later the R&D team. She has been instrumental in the design of the Cocoon® Platform since 2009, which was subsequently acquired by Lonza. Before joining Lonza, Trainor worked as a Scientist at the UK Science and Technology Facilities Council (Rutherford Appleton Laboratory), where she specialized in designing cell culture automation systems. Trainor holds a PhD in Biomedical Engineering from the University of Oxford, Oxford, UK and a Bachelor of Applied Science in Chemical Engineering and Applied Chemistry from the University of Toronto, Toronto, Canada.

Nuala Trainor PhD, Director of Innovation, Personalized Medicine, Lonza, Kingston, Ontario, Canada

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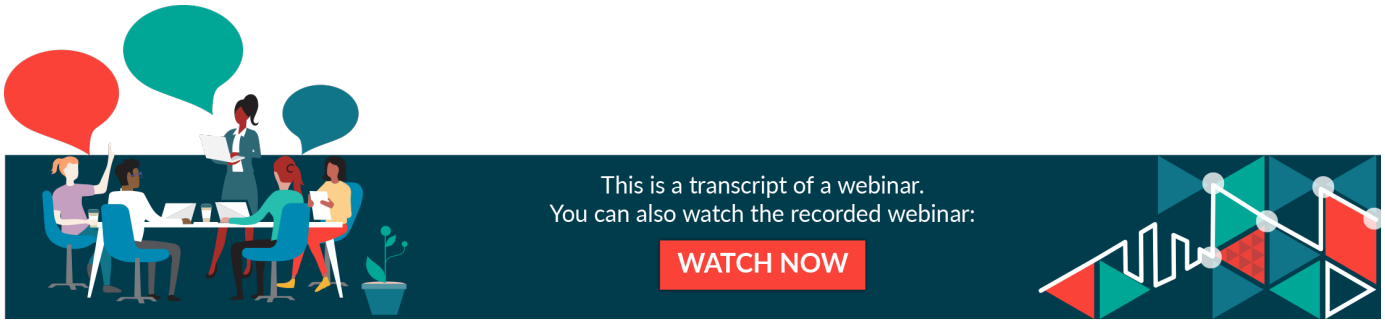

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INTERVIEW

Driving innovation in cell and gene therapy: the role of AI and robotics

INTERVIEW

"Over the past 36 months, with the rapid development and advancement of AI, the pharmaceutical industry has been one of the first to adopt this technology."

Jokūbas Leikauskas, Editor, *Cell & Gene Therapy Insights*, speaks with OmniaBio Inc.'s Kenneth Harris, Chief Strategy Officer and Head of AI, Mitchel Sivilotti, President and CEO, and Anthony Rotunno, Senior Vice President and General Manager/Site Head, about the innovative advancements in cell and gene therapy, focusing on how AI, automation, and robotics are revolutionizing manufacturing processes by reducing costs, increasing production capacity, and ultimately, improving patient outcomes in the rapidly evolving cell and gene therapy field.

Cell & Gene Therapy Insights 2025; 11(1), 59–67 · DOI: 10.18609/cgti.2025.008

Q What makes cell and gene therapy (CGT) a pivotal advancement in the evolution of modern medicine, and how do you envision this field evolving in the coming years?

KH A lot of us have all grown up taking pills and receiving injections for diseases. Unfortunately, traditional, one-size-fits-all medicines are not always effective for many rare diseases. There are over 7,000 rare diseases affecting humans, and it is often not appealing to pharmaceutical companies to target patient populations that might only consist of 10,000 or fewer people worldwide. CGTs can address this gap. It is often referred to as precision medicine–highly specialized treatments with individual doses tailored to the specific needs of each patient. While this approach can lead to higher costs, it is also far more efficacious and, in some cases, can even offer curative therapies, compared to the more generalized small-molecule pills and injections.

Q What is the main mission of OmniaBio?

MS OmniaBio is a service organization specializing in the development and manufacturing CGT products. In essence, OmniaBio assists CGT developers with their processes from pre-clinical work and early-stage manufacturing to large-scale production, and helps establish their processes and analytical testing systems. Ultimately, the main mission is to manufacture CGT products for therapeutic developers from early-stage clinical trials up to commercial stages.

Our main mission is to be an exceptional partner to advanced therapy innovators, and we have multiple key areas of focus. One of them is being a technology leader, which involves acquiring the know-how and capabilities needed to provide services for manufacturing cutting-edge biologics. Another area of focus is collaboration between CGT developers and our technical teams to create new solutions that can be implemented into their manufacturing programs. Lastly, excellence in execution is crucial, and timely operations are part of OmniaBio's DNA.

KH OmniaBio's mission is to manufacture life-saving therapies, particularly CGTs, focusing on rare diseases. The main goal is to reduce costs so that patients can access these therapies at an affordable price. As patient populations grow and the drugs become more effective, more people will become eligible for treatment. Consequently, the demand will increase, and we must maximize throughput in a single facility to meet that demand. OmniaBio's current mission is to reduce the cost of therapies for rare diseases by 50% and quadruple the number of doses produced at the facility we have already built.

Q What sets OmniaBio apart in the field of CGT manufacturing?

MS What differentiates OmniaBio is a strong emphasis on technology. We have been working in the CGT field for over 10 years. From the very early days to today, we have developed extensive experience, expertise, and know-how, which we share with manufacturers to help them bring their products to market. We achieve this by

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> "A lot of us have all grown up taking pills and receiving injections for diseases. Unfortunately, traditional, one-size-fits-all medicines are not always effective for many rare diseases."
>
> **Kenneth Harris**

heavily investing in technology–both in scientific areas, such as biologics in the laboratory, and in technologies related to robotics and automation. These technologies are leveraged to make the products high-throughput, low-cost, and high-quality, and to ensure rapid turnaround to meet developers' needs.

AR OmniaBio stands out in North America primarily because of two key aspects. Firstly, all our employees have very strong backgrounds, and our operators undergo an extensive 4–6-week training course on aseptic technique handling, which is the key to our operations. The second differentiator is the focus on innovations such as AI, automation, and robotics—we are very quickly headed that way. The newly built facility is set up with all the knowledge that we have gained previously in our experience to easily integrate these technologies into the workflows.

KH The core values of OmniaBio have always centered around innovation and rapid development, and AI aligns perfectly with these core values. Mitchel Sivilotti, our CEO, has been an entrepreneur throughout his career, and one of his goals for OmniaBio has always been to maintain that innovative edge. Additionally, as a Canadian organization, there is a strong focus on helping mankind. Therefore, OmniaBio's values are not only about innovation but also about contributing to society and the well-being of people. Combining these elements creates a rare formula in this space.

Q How are you implementing AI and robotics to improve CGT manufacturing?

KH Over the past 36 months, with the rapid development and advancement of AI, the pharmaceutical industry has been one of the first to adopt this technology. Finding drug targets and deciding whether they will be effective is not only a time-consuming process but also a very expensive one. For example, before testing a drug in humans, pharmaceutical companies can spend a decade in pre-clinical testing, investing billions of dollars before the drug is ever evaluated in human trials. AI can compress this decade-long process into months, providing a high probability of predicting whether a drug is a good candidate.

As AI and robotics continue to transform the CGT industry, one of the key challenges is that as these therapies get taken on by clinicians, the demand for doses will increase. However, building the infrastructure to produce even a small number of doses for the global market is a very long process. Therefore, OmniaBio plans to leverage the combination of AI and robotics to automate the production of advanced therapies.

Consequently, it can reduce manufacturing costs by 50–55% and quadruple our output in the same facility compared to what we had anticipated when the facility's construction

"The second [OmniaBio] differentiator is the focus on innovations such as AI, automation, and robotics—we are very quickly headed that way."
Anthony Rotunno

began three years ago. By implementing robotics, we are essentially removing humans from the risk profile. For example, one of the main benefits is a reduction in human error, less human movement, and fewer associated contaminants. Humans naturally shed microbes from our skin, hair, and breath, introducing potential contaminants into the environment, which can be significantly reduced with robots handling more tasks. Additionally, robots typically do not make mistakes and are consistent, which is much harder for humans to achieve.

Q What are the challenges and concerns regarding AI and how are you planning to address them?

KH We often get asked about the security of AI, and I think it is important to separate fear from reality regarding how we apply this technology. There are a lot of concerns in the market about the safety of AI and the security of the data it uses. At OmniaBio, we have designed our system with robust security measures from day one. The software driving our AI-based platform is highly secured and stored in what could be described as a 'vault,' accessible only by a select number of OmniaBio employees. The customer data used by the AI is also protected and anonymized, ensuring it cannot be traced back to an individual.

For example, one would not know that a specific dataset belonged to 'Ken.' It is simply a set of anonymized data that is completely de-identified based on source and time. At OmniaBio, we use straightforward, simple mathematical models, and separate the AI used for manufacturing from generative AI models, such as ChatGPT, which we only use for informational purposes. These models help inform scientists and operators, but they are never involved in the day-to-day production of the drug. In our manufacturing processes, we rely on simpler AI models with mathematical algorithms to manage robots. These algorithms ensure the robots execute commands correctly, and if something goes wrong, the system will stop the process and notify a human operator to inspect the issue.

Q What excites you the most about the future of OmniaBio in the context of the rapidly evolving CGT industry?

AR What excites me most about OmniaBio's future is that we already have a purpose-built facility and a high-performing team. The patients who will receive our therapies are often extremely sick, and being able to deliver tens of thousands of doses to those in need of life-changing and life-saving therapeutics gives us a purpose. OmniaBio provides hope to patients who desperately need these treatments, and our strategic goals are focused on growing both locally and internationally.

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"What excites me most about OmniaBio's future is our position in the biotech industry. We are providing cutting-edge solutions that directly benefit patients, and we get to witness the positive impact."
Mitchel Silvilotti

KH I am excited about the market OmniaBio is in. I think precision medicine is the future of healthcare, and the adoption of AI in this field is already a key focus in academic medicine today. OmniaBio is at the forefront of this shift, and I believe we will continue to lead in this area. We are a first-mover organization in this space, which not only gives us advantages but also opportunities to learn and adjust as we progress.

MS What excites me most about OmniaBio's future is our position in the biotech industry. We are providing cutting-edge solutions that directly benefit patients, and we get to witness the positive impact.

When we combine that with innovation and the new technologies our collaborators are developing, we are not just advancing biotechnology–we are also improving healthcare outcomes. Seeing these innovations make a real difference in patients' lives is incredibly fulfilling.

BIOGRAPHIES

Kenneth Harris has expertise spanning 35 years in innovation and leadership in technology, medical devices, and therapeutics. Harris currently serves as the Chief Strategy Officer and Head of AI at OmniaBio Inc., Hamilton, Ontario, Canada. In this role, he leads the company's corporate strategy and AI team, ensuring sustainable growth and cutting-edge implementation, safety, and scalability in healthcare robotics. Previously, Harris spearheaded the Academic Medicine and Public Health vertical within Amazon Web Services, developing the business from its inception leading a dynamic team comprised of principal trusted healthcare advisors, physician advisors, health informaticists, and healthcare IT architects. Prior to his tenure at Amazon, Harris notably founded and successfully took public a pioneering cell and gene therapy precision medicine company, marking a milestone in the advancement of cell therapy in cardiovascular medicine. Additionally, he played C-level and board roles with a Stanford University cell and gene therapy start-up, and led Pall Corporations global biomedical business for 13 years (now a Danaher company).

Kenneth Harris, Chief Strategy Officer and Head of AI, OmniaBio Inc., Hamilton, Ontario, Canada

Mitchel Sivilotti is a respected leader in the life sciences industry and serves as the President and Chief Executive Officer of OmniaBio Inc., Hamilton, Ontario, Canada. Most recently, Sivilotti was the Chief Operating Officer at CCRM, a Toronto-based cell and gene therapy accelerator focused on cell therapy and viral vector manufacturing innovation, company creation and early-stage venture investments. Over his 6 years at CCRM, Sivilotti was responsible for creating a CDMO with 200 employees, which grew to become OmniaBio. Before joining CCRM, Sivilotti was the President of TotipotentRX Corporation, which designed and commercialized sterile disposables for the cell and gene therapy market, and developed a therapeutic pipeline of candidates for cardiovascular diseases. Sivilotti's professional career

began at Pall Corporation, a Danaher company, where he held several business management and technical positions focused on process equipment and sterile consumables for the advanced therapies market. He also sits on the board of the Standards Coordinating Body for Regenerative Medicine and is a regular contributor to the Forum on Regenerative Medicine at the National Academy of Sciences.

Mitchel Sivilotti, President and CEO, OmniaBio Inc., Hamilton, Ontario, Canada

Anthony Rotunno is an executive leader with over 46 years of pharmaceutical manufacturing expertise that covers building facilities, optimizing operations, and supporting therapeutics developers from clinical to commercial scale. Rotunno currently serves as the Senior Vice President and General Manager/Site Head at OmniaBio Inc., Hamilton, Ontario, Canada. With deep experience in all facets of operations, revenue growth, and quality assurance, Rotunno has developed a strong track record in ensuring company and client success. While serving as Vice President of US manufacturing operations at Dendreon, MA, USA , Rotunno oversaw the construction and commissioning of two large-scale dedicated cell therapy manufacturing facilities. He guided the production of over 30,000 commercial lots, including Provenge, the first approved personalized immunotherapy for men with prostate cancer. With his knowledge in manufacturing and specific cell therapy experience firmly established, Rotunno founded Lykan Bioscience, where, as CEO, he built and led a therapeutic manufacturing facility and launched a CDMO focused on cell therapy manufacturing.

Anthony Rotunno, Senior Vice President and General Manager/Site Head, OmniaBio Inc., Hamilton, Ontario, Canada

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We hope you enjoyed reading this interview.
You can also listen to the recorded podcast here:

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Flexible, scalable technology solutions

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OmniaBio goes beyond being just a CDMO – we are your innovation partner, dedicated to bringing your groundbreaking therapies to life for patients in need.

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Reliable Partner	Over a decade of experience supporting 60+ clients across 150+ projects, including three pivotal programs, through our partnership with CCRM.
Comprehensive North American Capacity	Cell-based and gene-delivery capabilities spanning from preclinical development to large-scale commercial manufacturing with supply chain ease of access to U.S. and global markets.
Personalized Client Services	Collaborative and proactive client-centric planning with end-to-end program management.
Experienced CGT Talent	Industry-leading team with significant breadth and depth of specialized expertise, including 120+ combined years of leadership experience in GMP.
Operational Excellence	Seamless operational execution and continuous improvement in compliance with quality and regulatory standards.
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WEBINAR DIGEST

Control your AAV titers with in-line UV-Vis analysis and PAT-driven UF/DF systems

Brandon Goldberg, Senior Bioanalytical Application Specialist, Repligen Corporation

Biomanufacturers face numerous challenges in tangential flow filtration (TFF), including process fragmentation and measurement variability. This poster explores a novel TFF system that leverages real-time, in-line titer measurement through in-line variable pathlength spectroscopy to control the ultrafiltration/diafiltration (UF/DF) process. Case studies outlined in this poster demonstrate improved efficiency and accuracy in AAV titer determination, enhancing process control and reproducibility.

OVERCOMING CHALLENGES IN TFF WITH IN-LINE UV-VIS ANALYSIS AND PAT-DRIVEN UF/DF SYSTEMS

The most common challenges posed by mass-dependent TFF systems include fragmented processes, variability in measurements, and a high risk of human error. Solving these challenges requires novel methods with features such as real-time in-line product samples and testing, automation instrumentation, analytical assays, continuous bioprocessing, and validation services. Two existing Repligen products—the KrosFlo® KR2i system and the CTech™ FlowVPX® System—can successfully be used in combination to achieve these goals and address the challenges of TFF systems.

The KrosFlo KR2i TFF system is an automated, lab-scale TFF system used in downstream applications, while the CTech FlowVPX system is an in-line UV-Vis spectrometer with the unique ability to vary pathlength. Together, they make up the 'real-time process management' (RPM™) System, which provides process management to the UF/DF process through concentration measurement control.

Figure 1. AAV viral titer determination using in-line VPT technology.

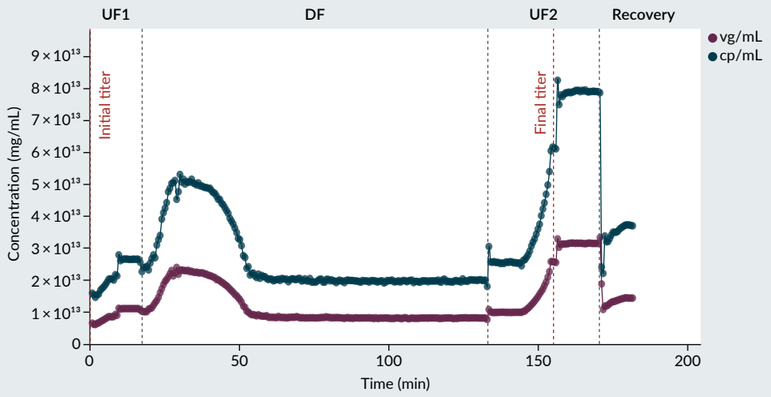
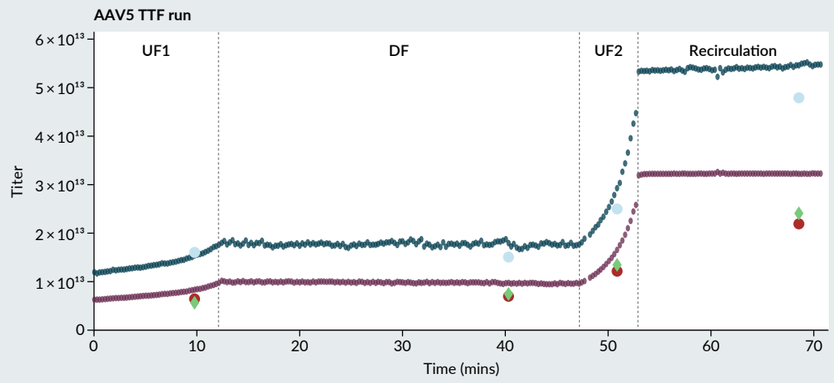


Figure 2. AAV5 viral titer determination using in-line VPT technology, and comparison with ddPCR and ELISA results.



Step	KR2i RPM: DNA (vg/mL)	KR2i RPM: capsid (vg/mL)	ELISA: capsid (vp/mL)	ddPCR: DNA (vg/mL)	ddPCR: GOI (vg/mL)	% Diff. capsid	% Diff. ddPCR	% Diff. ddPCR GOI
UF1	8.24×10^{12}	1.54×10^{13}	1.60×10^{13}	6.35×10^{12}	5.86×10^{12}	−4.03	−22.92	−28.86
DF	9.66×10^{12}	1.79×10^{13}	1.50×10^{13}	6.88×10^{12}	7.68×10^{12}	19.19	−28.78	−20.50
mid-UF2	1.65×10^{13}	2.93×10^{13}	2.50×10^{13}	1.21×10^{13}	1.37×10^{13}	17.09	−26.56	−16.84
end-UF2	3.23×10^{13}	5.48×10^{13}	4.80×10^{13}	2.19×10^{13}	2.44×10^{13}	14.08	−32.15	−24.40

CASE STUDY 1: AAV VIRAL TITER DETERMINATION USING IN-LINE VPT TECHNOLOGY

In order to demonstrate how the RPM System can provide key insights to ultimately make the UF/DF process more efficient, several case studies were performed. In the first, the FlowVPX was used to monitor AAV titer results through a UF/DF process (**Figure 1**). The viral titer (vg/mL) was compared to qPCR during the initial and final concentration steps. As shown in **Figure 1**, there is good comparability between the two systems. More importantly, this case study revealed that FlowVPX can give more insights to the user throughout this process.

CASE STUDY 2: USING IN-LINE VPT FOR DIFFERENT AAV SEROTYPES

In the second case study, a standard CDC TFF run was performed for the AAV5 serotype, and the data was compared to ELISA and ddPCR data. As demonstrated in **Figure 2**, the FlowVPX recorded data independent of serotype, successfully tracking and ending the run at the final UF2 target. Though there was a percent difference in results between methods (RPM, ELISA, and ddPCR), this is likely attributable to the fact that two users conducted off-line testing over 2 weeks versus the VPX's real-time data. The multiple users and turnaround time may have caused variations in the results. However, FlowVPX titer readings were still comparable to ddPCR and ELISA, and the difference (%) operated within a predictable output.

SUMMARY

The KrosFlo KR2i RPM System can strengthen process controls, generating high-quality and highly reproducible results, and greatly reduces the risk of error by using automated controls. Additionally, the RPM system increases process efficiency and reduces cycle time through in-line analytics with the KrosFlo RPM software platform.

Watch the webinar here | Read the full transcript here

CELL & GENE THERAPY INSIGHTS

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IN PARTNERSHIP WITH REPLIGEN INSPIRING ADVANCES IN BIOPROCESSING

EXPERT ROUNDTABLE

Why peer review matters: insights from the scientific community

Despite their invaluable expertise and thoughtful feedback, peer reviewers often go unrecognized and are not formally acknowledged in publications. This raises important questions: what makes participating in peer review worthwhile, and can this process be improved? Jokūbas Leikauskas (Editor, BioInsights) speaks to a panel of experts about their experiences participating in the BioInsights peer review process, the value to authors of constructive feedback, and the importance of independent peer review in upholding the integrity of scientific literature.

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PANEL

Atul Goyal PhD, Senior Scientist, Pfizer
Alexis Cockroft PhD, Director and Regulatory Consultant, Lex Regulatory Ltd
Panteli Theocharous PhD FRCPath, Chief Therapeutics Officer and Head of Medical and Scientific Affairs, Garuda Therapeutics
Vijesh Kumar PhD, Lead Scientist, Spark Therapeutics
Hemant Dhamne PhD, Associate Director, Process Development, Autolus Ltd

Q Do you participate in the peer review process often? If so, what motivates you to keep doing it despite the time commitment?

Atul Goyal I actively participate in peer reviews due to the opportunity to contribute to the advancement of scientific knowledge by ensuring the accuracy and quality of research.

Additionally, it allows me to stay updated on the latest developments in my field while also learning from diverse perspectives. By gaining insights from others' research, I can apply the same logic or scientific advancements to my own work. In essence, peer review is incredibly beneficial both for my own professional growth and that of the broader scientific community.

Alexis Cockroft I peer review a few times a year. My motivation comes from believing in the value peer-reviewed papers add to the scientific community. Additionally, contributing to this process keeps me up to date with the new developments, as I get to review interesting articles.

Panteli Theocharous I have had the privilege and the honor of being part of editorial boards and participating in the peer review process for journals in the haematology, oncology, and cell and gene therapy (CGT) fields. I have reviewed publications and manuscripts, providing both scientific and clinical perspectives as part of a broader process.

Peer review adds significant value and credibility to the manuscript, and it has been a key part of scientific research for many years. I always appreciate the opportunity to review cutting-edge data and clinical results, as I believe we have an obligation to patients and other key stakeholders within the ecosystem. Active and vigorous peer review keeps the fields in which you work intellectually honest and rigorous, and the act of serving as a reviewer returns the service from which authors have benefitted.

Vijesh Kumar I participate in the peer review process for several journals. My goal is to contribute my best to science, and critically reviewing scientific work is part of the process. Additionally, it helps me hone my skills to critically look at the data generated by other researchers.

Hemant Dhamne I frequently participate in the peer review process for various journals, conferences, and editorial platforms. My primary motivation stems from over 15 years of experience in CGT, bioprocessing, and drug development, spanning both academia and industry. It is a privilege to leverage my expertise to evaluate and contribute to the quality of research and review articles.

Q In your opinion, what is the primary purpose of peer review, and why is it so important in science publishing?

Atul Goyal The primary purpose of peer reviews is to uphold the quality, credibility, and integrity of published research. Peer review serves as a critical checkpoint—a first line of validation for scientific methodologies, findings, and conclusions, ensuring that only scientifically sound work is shared with the community. This practice fosters trust in published data, which is essential for scientific progress.

Alexis Cockroft I believe that for an article to be valuable, it must be accurate, well-balanced, and thoroughly researched. While authors typically have these intentions already, peer reviewers often bring knowledge or perspectives that the author may not have considered, which adds to the value of a paper. Furthermore, peer review ensures accuracy, which is particularly crucial in this day and age, especially with the rise of artificial intelligence, which makes it possible to auto-generate content.

Panteli Theocharous The peer review process adds another layer of rigor and ensures the data has been thoroughly vetted by experts in the field who can provide a critical, yet collaborative, viewpoint on the data. The feedback is crucial in enhancing the quality of the final publication. Ultimately, peer review introduces a level of scientific and medical vigour that instils confidence in the publication once it is made publicly available.

Vijesh Kumar In my opinion, the primary purpose of peer review is to get the manuscript critically analyzed by other experts in the field; that way, it can be improved before it gets published. A few years back, when I started my career, I could not reproduce many of the published studies due to a lack of thoroughness in the work. I believe that having an independent researcher critically analyze the work before publication can enhance the confidence of new researchers in the quality of the journal and the manuscripts it publishes.

Hemant Dhamne The primary purpose of peer review is to help ensure that the published work serves as a reliable reference for future studies and reviews. Through this process, critical suggestions regarding experimental design, cited references, analytical tools, and interpretations are carefully evaluated and incorporated, thereby strengthening the credibility and impact of the research.

Q How can the peer review process be improved to better serve authors, reviewers, and the scientific community?

Atul Goyal To serve all stakeholders—authors, reviewers, and the scientific community—the peer review process could benefit from enhanced communication tools. For instance, it would be helpful for reviewers to know which articles are being published and which ones are being rejected. Additionally, streamlining the feedback mechanism and providing recognition or incentives for reviewers would encourage greater participation and commitment from other reviewers.

Alexis Cockroft The current approach to peer review seems sensible, as it is more about finessing the paper and giving constructive feedback rather than completely changing it. I believe it is essential to have the right people with the right experience reviewing the work—those who possess the knowledge and expertise to contribute meaningfully to the review. Ideally, there would be a selection of peer reviewers who bring different perspectives to the process.

Panteli Theocharous One idea that comes to mind is enhancing the interaction between reviewers and editors. For example, having live sessions where we go through the publication, capture feedback and comments in real-time could be beneficial. This could also be done in a group setting with other reviewers to stimulate discussion and debate around the subject matter.

This could create a valuable opportunity for fostering more scientific and clinical interactions, allowing experts to debate and discuss cutting-edge data. Such a format could help elevate the scientific and clinical value of publications.

Hemant Dhamne The peer review process can be improved by fostering greater transparency, efficiency, and collaboration. For authors, providing detailed and constructive

feedback with clear suggestions for improvement can make the process more valuable and less daunting. For reviewers, offering better recognition, such as formal acknowledgments or incentives, can help maintain their motivation and engagement. Streamlining the submission and review timelines through efficient tools and communication can enhance the overall experience for everyone involved. Additionally, incorporating more diverse reviewer panels can ensure a broader range of perspectives and expertise, ultimately benefiting the scientific community as a whole.

Q What are the most critical qualities of a good peer review?

Atul Goyal A good peer review should be constructive, objective, and thorough. It should provide clear and actionable feedback to the author rather than ambiguous comments such as, "This should be corrected." If reviewers have specific feedback, they should be direct and, where possible, offer suggestions on how the article can be improved. This approach not only helps the author but also positively affects the scientific community as a whole.

I also believe that reviewers should approach the process with professionalism and a commitment to improving the overall quality of the work. They should clearly highlight both the strengths and weaknesses of the manuscript. While reviewers can suggest improvements, authors should have the freedom to incorporate these suggestions in their own way, using their own terminology, to make the article better.

Alexis Cockroft For a peer review to be good, the reviewer must have sufficient knowledge in the area and a solid understanding of the current landscape, so they can provide meaningful insights into the work. It is also important to be able to identify inaccuracies, which is why having multiple peer reviewers with varying knowledge and experiences is beneficial. In essence, I believe accuracy and balance are key, and multiple peer reviewers can contribute to ensuring both.

Panteli Theocharous Peer review must be fair and balanced. It is important to think about what the review panel looks like and whether there is an opportunity to gather input from experts across different segments. If the review reflects input from experts and key stakeholders, it allows for adjustments and fine-tuning that ultimately enhance the quality of the final article. In essence, a good peer review adds real value, giving the publication greater credibility by showing that the data has been reviewed by experts in the field.

Vijesh Kumar Most importantly, the reviewers should have significant experience in the work they are reviewing in order to understand the nitty-gritty of the manuscript and be unbiased.

Hemant Dhamne The most critical qualities of a good peer review are thoroughness, objectivity, constructiveness, and clarity. A thorough review ensures that all aspects of the manuscript, including methodology, data interpretation, and presentation, are carefully examined. Objectivity is crucial to provide fair and unbiased evaluations, focusing solely on the scientific merit of the work. Constructive feedback helps authors improve their manuscript by offering actionable suggestions rather than merely pointing out flaws. Finally, clear and concise communication ensures that the feedback is easily understood and effectively implemented by the authors, fostering meaningful improvements in the research.

Q Finally, what would be your message to people who are considering participating in peer review but are perhaps unsure due to time commitment or other reasons?

Atul Goyal I would encourage everyone who is able to participate in peer review to give it a try. It may not work for everyone due to their timeline, but I would still urge everyone to take part because the process is highly rewarding in terms of expanding your scientific knowledge, expertise, and sharpening critical thinking. Personally, I have learned a lot from participating in the process. While it does require a commitment of time to complete, the overall experience can be a valuable opportunity for professional growth and staying current with the latest scientific advancements. Finally, it benefits the scientific community for which you are providing feedback and creates an opportunity to give back.

Alexis Cockroft From my perspective, it is important to give back to the community, and peer reviewing is a valuable way to do so. I strongly encourage people to participate, even if it is just one peer review a year—every contribution counts. By sharing their knowledge and experience, reviewers help authors who are trying to present scientific arguments or illustrate important information to the community. While reviewers may not receive monetary compensation for their work, that does not mean there is no reward in the process.

Panteli Theocharous Firstly, the ability to write a good peer review is a valuable skill and experience that contributes to one's professional profile. Being asked to peer review is a recognition of one's achievements and accomplishments over the course of their career. From my perspective, this is why I participate in peer review processes—I feel I can support authors and their work. Ultimately, we all have an obligation to ensure that data in the public domain is robust and has undergone a rigorous review process before being published. I would encourage other stakeholders to consider joining the process as well, since it is also a way to give back to the scientific community, leveraging skills and experiences that have accumulated over one's career.

Hemant Dhamne Participating in peer review is an invaluable opportunity for emerging thought leaders to contribute meaningfully to the advancement of science while enhancing their own knowledge and expertise. While the time commitment and lack of direct compensation may seem challenging, the rewards go far beyond these considerations. Peer review allows you to stay at the forefront of cutting-edge research, refine your critical thinking skills, and gain insights that can inform your own work. Moreover, it is an essential part of our collective responsibility to uphold the quality and integrity of scientific literature. By engaging in peer review, you are not only supporting fellow researchers but also contributing to the greater mission of advancing science for the benefit of society.

BIOGRAPHIES

Atul Goyal is a skilled research scientist leveraging training and insights to strengthen purification process development for glycoconjugates vaccines against the disease caused by the bacterium *Streptococcus pneumoniae*. With research focus areas in the fields of process development and analytical assay development, Atul thrives in identifying and improving options for outcomes. Known for research and curiosity, he remains up to date through extensive training and professional development opportunities.

Alexis Cockroft an independent regulatory affairs consultant, specializing in CMC regulatory affairs (CMC RA) for cell and gene therapies (advanced therapy medicinal products). She has worked in CMC RA for more than 19 years and has focused solely on advanced therapies for the last 13+ years. She has also worked in various science-based roles.

Panteli Theocharous is a seasoned leader with over 35-years of experience driving groundbreaking translational research, clinical development, and commercial strategy in the healthcare sector. With an established focus on haematology/oncology, cell and gene therapy, Dr Theocharous has led the development and market introduction of multiple transformative cancer therapies, contributing significantly to advancements in patient care. His extensive career includes roles in several private and public biotech companies, and in prominent organisations such as Janssen Oncology and PPD (now part of Thermo Fisher Scientific), where he was instrumental in fostering a culture of innovation, building high-performing global teams, and delivering impactful therapeutic solutions. Trained as a clinical immunologist at the Royal Free Hospital, London, UK, Dr Theocharous specialised in haematopoietic stem cell transplantation, and cell and gene therapies, gaining early expertise in the translational applications of cutting-edge scientific research. In senior executive roles, including his current position as Chief Therapeutics Officer and Head of Medical and Scientific Affairs at Garuda Therapeutics and previous CMO appointments, Dr Theocharous has demonstrated a strategic approach to business growth and commercialisation. His board-level expertise and collaboration with CEOs have added depth and credibility in interactions with investors, venture capital, and banking partners.

Vijesh Kumar is a Principal Scientist at Spark Therapeutics, Inc., Philadelphia, PA, USA leveraging his expertise in protein chemistry, protein purification, and chemical engineering to tackle complex protein manufacturing process challenges. His mission is to enhance the quality and efficiency of therapeutic products through innovative purification schemes and cost-effective methods. With over 15 years of experience in developing purification techniques, primarily for monoclonal antibodies and adeno-associated viral vectors, Vijesh has successfully demonstrated the effectiveness and simplicity of his proposed purification schemes and modeling. He is committed to advancing the fields of biotechnology and gene therapy and contributing to the development of groundbreaking, life-saving treatments.

Hemant Dhamne is a seasoned cell and gene therapy expert (PD/MSAT/CMC) with over 15 years of experience delivering scalable solutions from preclinical to commercial stages, achieving improvement in production efficiency and leading FDA-approved innovations. Currently, Hemant is serving as Associate Director for Process Development with Autolus Ltd, London, UK. Prior to this he has worked with Kings College London, Immuneel Therapeutics, Intas Pharmaceuticals, and he has his PhD and Post Doc from ACTREC, Mumbai, India. Besides work, Hemant is a flute player and photography enthusiast.

AUTHORSHIP & CONFLICT OF INTEREST

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