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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:
Preclinical/translational tools & strategies

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CONTENTS

Spotlight

Preclinical/translational tools & strategies

CELL & GENE THERAPY INSIGHTS

Volume 7, Issue 1

Latest articles

Report

Business Insights

CELL & GENE THERAPY INSIGHTS

FOREWORD

Preclinical/translational tools & strategies

BRUCE A BUNNELL is a professor and Chair of the Department of Microbiology, Immunology and Genetics at the University of North Texas Health Science Center in Fort Worth, TX. Previously, he served as Director of the Tulane Center for Stem Cell Research and Regenerative Medicine and Professor in the Department of Pharmacology in the Tulane University School of Medicine. Dr. Bunnell obtained his PhD in Microbiology from the University of Alabama at Birmingham School of Medicine. He then pursued Postdoctoral Fellowship research at the Howard Hughes Medical Institute in the School of Medicine at the University of Michigan and the National Human Genome Research Institute at the National Institutes of Health in Bethesda, MD. Dr. Bunnell was an Assistant Professor at the Nationwide Children's Hospital Research Institute, part of the Ohio State University School of Medicine prior to joining the faculty at Tulane University in 2002. Dr. Bunnell's research program is focused on both the basic science and translational applications of adult stem cells isolated from the bone marrow and adipose tissue. Dr. Bunnell investigates use of mesenchymal stem cells (MSCs) isolated from the bone marrow or adipose tissue as a therapeutic intervention for both multiple sclerosis (MS) wound repair, lung injury and bone repair. He is particularly interested the interactions of MSC with the immune system and how the cells elicit robust anti-inflammatory effects *in vivo*. He is currently working towards a human clinical trials for the treatment of osteoarthritis, traumatic brain injury and MS with these cells. He has served as a reviewer of stem cell, regenerative medicine and tissue engineering grants for the National Institutes of Health, Department of Defense and several state funded programs including Maryland, New York, Virginia and Pennsylvania. He has also served on grant review panels for several foreign countries including Denmark, Ireland, Poland, Germany and Spain. Dr. Bunnell serves as an Editorial Board Member for several journals, including Stem Cells, BMC Genomics, and Regenerative Medicine.

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As a scientist who has spent the majority of my 30 years career in academia focused on the development and application of novel gene and cell therapy strategies, it has been my honor to serve as the guest editor for this spotlight issue focused on innovative tools and novel strategies aimed at the continued successful development and human application of gene and cell therapies. To see the progress made from the early days, which were focused on developing strategies for harnessing viruses and converting them into gene delivery vectors and understanding the basic biologic properties of stem cells, to where the field stands now with several cell and gene therapies approved for human use, has been a tribute to the hard working research teams that have made those visions of three decades ago a reality. In the past 5 years alone, cell therapies including CAR-T cells such as Kymriah® (Novartis) for the treatment of acute lymphoblastic leukemia, and Yescarta® (Kite Pharma) for the treatment of non-Hodgkin lymphoma, and gene therapies including Luxturna® (Spark Therapeutics) for rpe65 mutation-associated retinal dystrophy, and Zolgensma® (AxeXis) for spinal muscular atrophy, are transforming medical treatment for devastating diseases. That being said, the development of cell and gene therapies is still in progress for numerous additional devastating disorders. The successful development of additional clinically approved therapies will require further innovations - the central theme of this issue, which is comprised of a series of Expert Insight review articles and Commentary pieces from, and Interviews with, leading preclinical and translational R&D experts from the global cell and gene therapy field.

While HSC-targeted gene therapies may be a cure for multiple diseases, their progress to human application can be full of hurdles and issues that have limited their success thus far. Dr. Leoni and colleagues reflect on key learnings from their experience in advancing HSC gene therapies through the development and regulatory pathways for human applications. Their commentary is focused on definition of the necessary preclinical research and models that are essential for the clinical translation of HSC therapies as well as identifying translational gaps and how these can be filled.

One of the primary issues with the development and application of cell therapies, both preclinically and clinically, is the limitations surrounding tracking the cell dispersion, migration and persistence of the cells upon *in vivo* administration.

The clinical application of T cell-based therapies, including CAR T cells, is advancing rapidly. However there are still key challenges and issues that must be addressed for the continued expansion of applications for these cell therapies to a broader range of diseases. Gary Waanders, Silke Raffegerst and colleagues from Medigene AG discuss the preclinical and translational R&D challenges faced by T cell therapy as it targets more complex diseases.

Dr. Paul J. Fairchild and Charlotte Cossins of the Oxford Stem Cell Institute provide insights on the application of induced pluripotent stem cells (iPSCs) for modeling late-onset diseases. The primary challenge for modeling late-onset disease is the protracted time frame over which they normally manifest *in vivo*. The article focuses on whether iPSCs can be used for modeling late onset diseases and barriers that need to be overcome.

The preclinical testing of cell and gene therapy strategies has almost always required the application of animal models of disease. Drs. Plata-Salamán and Plata from ESTEVE provide their thoughts and insights on the advent of new strategies to enhance the development and application of animal models of disease to more accurately recapitulate human disease and response to gene therapy.

In the field of adeno-associated virus (AAV) vector-driven gene therapy, while there now exists a greater understanding of patient safety, immunogenicity, and dosing regimens, there remains much work to be done to accelerate development paths. Louise Rodino-Klapac

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of Sarepta Therapeutics shares some keys to bridging the translational gap to the clinic for AAV-based *in vivo* gene therapies.

And finally, Olivier Negre, the Founder of Biotherapy Partners, a consulting firm focused on helping with the development of gene and cell therapies, shares his keen insights on the development of gene therapy strategies from preclinical concept to strategies for successful development and application in an interview. Olivier speaks from his extensive experience in the field – especially on the development of Zynteglo®, a novel therapy for transfusion-dependent beta-thalassemia, on which he played a key role.

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CELL & GENE THERAPY INSIGHTS

PRECLINICAL/TRANSLATIONAL TOOLS & STRATEGIES

SPOTLIGHT

EDITORIAL

Accelerating preclinical development for AAV based gene therapies: bridging the gap between discovery and clinical development

"The potential for AAV-based gene therapies to transform the lives of patients with rare diseases is staggering."

LOUISE RODINO-KLAPAC, EVP, Chief Scientific Officer, Sarepta Therapeutics, Inc.

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INTRODUCTION

Gene therapy continues to gain momentum as a promising therapeutic approach for rare monogenic disorders with the capacity to expand to non-rare indications. Adeno-associated virus (AAV) vector-based gene therapies have demonstrated the potential to transform lives, and what once was a field led by start-ups and small biotechnology companies is now fully embraced by large pharmaceutical companies. Tremendous strides have been realized in the field over the past 10 years regarding the manufacturing of AAV that have allowed for systemic dosing of patients at large-scale. We now have a greater understanding of patient safety, immunogenicity, and dosing regimens; however, there is much more to be done to accelerate development. As a newer field, development timelines are often unpredictable due to cost to manufacture product, lack of regulatory precedent, and limited patient numbers. The potential for transformational results in patients implores the need to develop innovative approaches across multiple stakeholders. This includes leveraging AAV platform strategies for delivery, dose, safety, and outcome measures in the context of preclinical and clinical development.

LEVERAGING THE PLATFORM

There are approximately ten AAV serotypes that are widely used as vectors, and thousands more identified. There are some universal qualities amongst AAV serotypes such as packaging capacity; however, key attributes that impact dose selection and safety are inherent to the tropism and resulting biodistribution profile of a particular AAV serotype. The biodistribution and safety profile of a given AAV serotype can therefore be leveraged for toxicology and first-in-human (FIH) studies after demonstration of consistent results between programs. Caveats must be closely considered, and these include but are not limited to the AAV manufacturing process, route of delivery, and transgene/promoter cargo. In a similar way to how data for the serotype can be leveraged, the same principles can be applied to promoters, transgenes and regulatory elements. The more elements that are identical between programs, the more data can be leveraged across the programs and therefore the number of required preclinical studies can be minimized. Finally, the target organ, target indication or class of indications may be leveraged as well, with key examples including blood disorders, lysosomal storage diseases, and muscular dystrophies.

PRECLINICAL DOSE SELECTION PARADIGMS

The traditional paradigm applied to small molecules, which employs dose-ascending studies, is difficult if not impossible to apply to AAV-based gene therapies. With the current state of technology, AAV-based gene therapy is characterized by one-time dosing; thus, there is no opportunity to slowly increase dose within patients or in between patient cohorts. There is a fine line that must strike the right balance of safety and efficacy. Carefully conducted preclinical studies must guide clinical dosing so that patients are provided the opportunity to receive an efficacious dose that does not push the bounds of safety. Although many strategies are in development to allow for redosing, the current standard is the assumption that patients treated with AAV will develop anti-AAV antibodies precluding a second dose. It is critical that traditional pharmacokinetic/pharmacodynamic (PK/PD) studies that inform clinical dosing be modeled in such a way that captures the properties of AAV with regards to plasma and tissue exposure, cellular uptake, and expression. As the collective experience with AAV grows within a given serotype and across AAVs, PK/PD modeling will improve and allow for interspecies scaling to more accurately predict optimal clinical dosing from preclinical studies.

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ACADEMIC & INDUSTRY COLLABORATION & IMPACT ON CLINICAL DEVELOPMENT

With the exponential growth of the AAV field and the investment and confidence demonstrated by large pharmaceutical companies, the potential to accelerate development is tangible. Careful and equal collaboration of AAV experts from academia with seasoned industry drug developers is crucial to success. As many academics transition swiftly to industry, they must rapidly assimilate to the requirements for health authority approval. Conversely, industry veterans must understand that AAVs are vastly different from small molecules. There is no longer the benefit of multi-phase trials and, in the case of rare disease, time is on no one's side.

CONCLUSIONS

The potential for AAV-based gene therapies to transform the lives of patients with rare diseases is staggering. Incredible strides have been made in the field over the past decade, particularly in AAV manufacturing, which has allowed for transformative systemic dosing of patients at large-scale to become reality. We now have a greater understanding of AAV with regards to safety, immune response, effective routes of delivery and corresponding dose. Even with these great advances, there is so much more to be done to accelerate development paths to fully unlock the potential of AAV to impact lives. With authentic partnership across multiple stakeholders including scientific experts, drug developers, manufacturers, patient groups, and regulators, AAV vector-based therapies will truly turn drug development on its head and help patients in desperate need of treatment.

BIO

Louise Rodino-Klapac

Dr. Rodino-Klapac is a gene therapy pioneer who has dedicated her professional life to advancing medicines designed to treat genetically based diseases. With professional experience across industry and academia, she is renowned for her contributions to molecular genetics and gene therapy that have advanced the field. She is author to a vast body of published, peer-reviewed work, the recipient of multiple awards, a National Institutes of Health (NIH) Fellow appointee, and current Board member of the Association for Regenerative Medicine, as well as a member of the American Society for Gene and Cell Therapy, the American Academy of Neurology, and the American Association for the Advancement of Science. She is the former head of the Laboratory for Gene Therapy Research at Nationwide Children's Hospital, established the Gene Therapy Center of Excellence within Sarepta and leads the Company's Gene Editing Innovation Center, directing a team of researchers to discover and develop novel gene replacement and gene editing therapies. She co-founded and served as chief scientific officer of Myonexus Therapeutics, a gene therapy company focused on limb-girdle muscular dystrophies acquired by Sarepta in 2019. She currently serves as Sarepta's Executive Vice President, Chief Scientific Officer. Her work has led to 11 investigational new drug applications and she is the co-inventor of SRP-9001, an investigational micro-dystrophin gene therapy, and the inventor of five investigational gene therapies for limb-girdle muscular dystrophy. She is the inventor of over 50 U.S. and over 70 international published patent applications. She earned her Ph.D. in molecular genetics from the Ohio State University and graduated summa cum laude from Kings College in Wilkes-Barre, Pennsylvania, with a Bachelor of Science degree in Biology.

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CELL & GENE THERAPY INSIGHTS

PRECLINICAL/TRANSLATIONAL TOOLS & STRATEGIES

SPOTLIGHT

EXPERT INSIGHT

Modelling late-onset diseases with induced pluripotent stem cells: a matter of time management

Charlotte Cossins & Paul J Fairchild

The advent of induced pluripotent stem cells (iPSC) has revolutionized *in vitro* modelling of many intractable human diseases. By deriving iPSC from individuals with progressive disease, it has been possible to capture disease-associated genes and study their impact on the downstream function of terminally-differentiated cell types. Nevertheless, late onset diseases pose particular challenges for disease modelling given the protracted time frame over which they normally develop. Such practical issues are accentuated by the propensity for iPSC to produce cell types of fetal origin rather than their adult counterparts and by the rejuvenation of the resulting cells, thereby erasing any physiological evidence of aging. Here we review progress in overcoming these issues and argue that achieving a combination of maturation and aging will enable better recapitulation of all features of late-onset diseases.

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INTRODUCTION

Current estimates suggest that one fifth of the world's population will be over 60 years of age by 2050 [1]. Furthermore, the incidence of age-related diseases is expected to rise, with the resulting burden of disease already impacting healthcare systems in developed countries [2]. For many late-onset diseases there are no

effective treatments, a situation epitomized by amyotrophic lateral sclerosis (ALS), for which hundreds of promising drugs based on animal models have failed subsequent clinical trials [3]. There is, therefore, a strong imperative to better understand disease pathogenesis in order to develop more targeted interventions. Nevertheless, the study of late-onset diseases has proven difficult due to the limitations of disease modelling. Animal models rarely develop age-related pathologies unless induced by genetic modification or pharmacologic intervention [4]. Furthermore, insights from studies of patient tissue are typically only applicable to the final stages of disease, particularly if it is obtained *post-mortem* [5].

The advent of human induced pluripotent stem cells (hiPSCs) has provided a promising new approach to disease modelling: by reprogramming patient-derived somatic cells to pluripotency, cell lines may be generated in perpetuity that preserve the disease-associated genotype [6–9]. Importantly, these patient-derived hiPSCs can be differentiated into cell types affected by the disease, which may be subject to genome editing to investigate disease pathogenesis [10–12].

Although hiPSC have proven successful in modelling developmental disorders [13], recapitulating disease phenotypes of late-onset diseases has proven rather more challenging due, in part, to the fetal profile of cell types differentiated from them [14]. For example, maturation of erythrocytes is characterized by enucleation and expression of adult hemoglobin, however protocols for their differentiation typically achieve enucleation in only a proportion of hiPSC-erythrocytes which persist in their expression of fetal hemoglobin [15]. Furthermore, cellular rejuvenation induced through the process of reprogramming prevents the development of late-stage phenotypes of age-related pathologies in hiPSC-based models. For example, key features of Parkinson's disease (PD), such as neurodegeneration and alpha synuclein aggregation, are only observed if toxic stressors are used to mimic aspects of aging [16–18]. Here we discuss recent advances in addressing these limitations by inducing maturation and physiological aging among the progeny of hiPSC, largely drawing on examples from neurons and cardiomyocytes, and argue that achieving both processes together will enable better recapitulation of all features of late-onset diseases.

THE IMMATURITY OF iPSC-DERIVED LINEAGES

The ability of cell types differentiated from hiPSC to faithfully recapitulate the equivalent adult cell type was first questioned following transcriptomic analysis of progeny spanning all three germ layers which suggested that, regardless of cell type, differentiated cells more closely resemble cells from very early human development rather than their adult counterparts [19]. Subsequent studies have reported similar findings in cell types as diverse as pancreatic beta cells and hepatocytes [20,21], suggesting that the progeny of hiPSC may perform functions essential to the fetus rather than the adult. For example, iPSC-derived neurons have been shown to lack extensive dendrite branching and produce action potentials characteristic of late embryonic stages of development [22]. Furthermore, hiPSC-derived cardiomyocytes (hiPSC-CM) are mononuclear in nature and lack the anticipated ultrastructure of adult cardiomyocytes, such as T-tubules, thereby affecting their capacity for calcium handling [23]. Moreover, a lack of Ik1 expression leads to a fetal-like action potential profile [24,25]. The impact of this immature profile on attempts to restore heart function after myocardial infarction in mice was evident from studies by Liao and colleagues who demonstrated their inability to electrically couple with existing myocardium, resulting in fatal arrhythmias [26]. These findings involving unrelated cell types therefore highlight the need to develop protocols to promote maturation from a fetal to an adult state in order to accurately model adult-onset diseases.

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IMPROVING CELL MATURATION

One approach to promoting the maturation of hiPSC-derived cell types which has enjoyed some success has been to extend the duration of cell culture. For example, long-term culture of hiPSC-CM has been shown to lead to improved maturation, as evidenced by sarcomere organization, calcium handling and action potential amplitudes that are more typical of mature cardiomyocytes [27]. Nevertheless, extended culture periods alone are insufficient to achieve full maturation since the local microenvironment *in vitro* may fail to elicit the necessary signaling pathways, suggesting the need for further forms of intervention (Table 1).

Guiding maturation *in vitro*

In order to address deficiencies in the culture microenvironment, many have sought to supplement the culture medium with small molecules, successfully reducing the time required for maturation in some cases [28,29]. However, with only limited knowledge of the final stages of ontogeny in the human, many protocols use small molecules necessary for successful embryonic development to direct differentiation of hiPSC, rather than promoting maturation into fully adult cells [30,31]. Identification of signaling molecules responsible for the later stages of maturation is therefore essential, for which various candidate genes in the mouse have been identified. For example, Guo and colleagues recently demonstrated the importance of serum response factor (SRF) in supporting cardiomyocyte maturation in mice, its deletion through genome editing disrupting sarcomere expansion, T-tubule formation and mitochondrial biogenesis [32]. Should these findings prove applicable to the human, the addition of SRF to cultures of hiPSC during differentiation may enhance hiPSC-CM maturation.

An alternative approach which may provide a source of signaling molecules, even in cases where they have yet to be identified, has been to recapitulate the complex microenvironment in which cells mature *in vivo* by co-culturing them with accessory cell types. For example, independent studies have demonstrated how co-culture with astrocytes improves maturation of iPSC-derived neurons, including increased expression of neuronal markers [33] and synapse formation [34], resulting in improvements

TABLE 1

Summary of the advantages and disadvantages of different strategies to induce maturation together with examples of studies employing such approaches.

Maturation strategy	Advantages	Disadvantages	Example studies
Extended cell culture	Inexpensive	Limited maturation	[27]
Addition of small molecules to culture medium	Inexpensive Quick	Limited maturation	[28,29]
Co-culture	Provide a 2D environment of multiple cell types.	Limited maturation Limited contact between iPSC-progeny and accessory cells	[33–35]
3D culture	Provide a 3D environment of multiple cell types. Organized structure. Useful in screening compounds. Enhanced maturation	Highly variable. Need for improved vascularization and optimization of differentiation protocols. Time-consuming and expensive. May raise ethical issues	[36,37]
Combining hiPSCs and animal models	Provide a 3D environment of multiple cell types. Interaction with plasma-derived factors by vascularisation. Enhanced maturation	Highly variable. Time-consuming and expensive. Raises ethical issues	[38–40]
Exploiting epigenetic memory of iPSC	Faithfully recapitulates features of adult cells	Success is highly variable. Difficult to access all adult cell types	[43]

in electrophysiology [35]. Furthermore, the advent of organoid technology has overcome many of the physical limitations of 2D culture, such as the periodic depletion of secreted molecules upon routine replacement of the medium, and has enabled the structural complexity encountered *in vivo* to be more faithfully recapitulated in three dimensions. That these structures support maturation of hiPSC progeny is evident from cardiac organoids that display up-regulation of maturation-associated genes, such as those involved in sarcomere structure and calcium handling, with concurrent down-regulation of fetal-associated genes [36]. That such improvements in culture techniques may translate to better modelling of late-onset diseases is supported by the finding that beta amyloid aggregation may be observed in organoid models of Alzheimer's disease (AD) yet is rarely evident in 2D culture [37].

Combining hiPSCs & animal models

Despite attempts to improve culture conditions to promote maturation *in vitro*, hiPSC-derived progeny continue to resemble fetal cells more closely than their adult counterparts, suggesting that it may prove difficult to recapitulate *in vitro* all signaling pathways required to support full maturation. Transplanting the products of hiPSC differentiation into animal recipients may, therefore, provide an *in vivo* environment conducive to maturation (Figure 1). Accordingly, transplantation of hiPSC-derived microglia into neonatal mouse brains enhanced their maturation so that they closely resembled primary human microglia [38]. This strategy may support maturation by supplying organoids with soluble plasma-derived factors, the complexity of which cannot be adequately recapitulated in culture medium. In support of this contention, Liu and colleagues successfully restored some cardiac function in a macaque model of myocardial infarction (MI) by transplanting human embryonic stem cell-derived cardiomyocytes (hESC-CMs), which subsequently showed enhanced maturation, including aligned cytoplasmic myofibrils and cardiomyocyte hypertrophy three months post-transplant [39]. The authors reported that all donor cardiomyocytes electrically coupled to host myocardium, unlike similar studies in the guinea pig, where only 60% electrically coupled [39,40], possibly due to the greater disparity between the *in vivo* microenvironment of the guinea pig and human.

Exploiting epigenetic memory

Induced pluripotency initially generates iPSC with an epigenetic memory reminiscent of the somatic cells of origin [41], although continuous passaging eventually erases this epigenetic signature, leaving iPSC largely indistinguishable from ESC [42]. Although transient, the epigenetic memory of iPSC has been exploited to generate cells of an adult phenotype: terminally-differentiated dendritic cells (DC) from either mouse or man have been reprogrammed to pluripotency and re-differentiated to yield highly-immunogenic DC expressing abundant co-stimulatory molecules and secreting high levels of interleukin (IL)-12 [43]. Such cells are indistinguishable from primary DC, unlike those differentiated from conventional iPSC derived from unrelated cell types such as fibroblasts, which display poor co-stimulatory capacity and a cytokine profile strongly favoring IL-10, undermining their immunogenicity. Although this study suggests, in principle, that the transient epigenetic memory of hiPSC may be exploited to generate mature progeny, the viability of this method remains to be seen for the generation of other cell types: while routine access to adult neurons and cardiomyocytes may prove challenging, attempts to generate mature B cells from B cell-derived iPSC have proven unsuccessful [44], as have efforts to generate mature iPSC-CM using cardiac progenitor cells (CPCs) [45].

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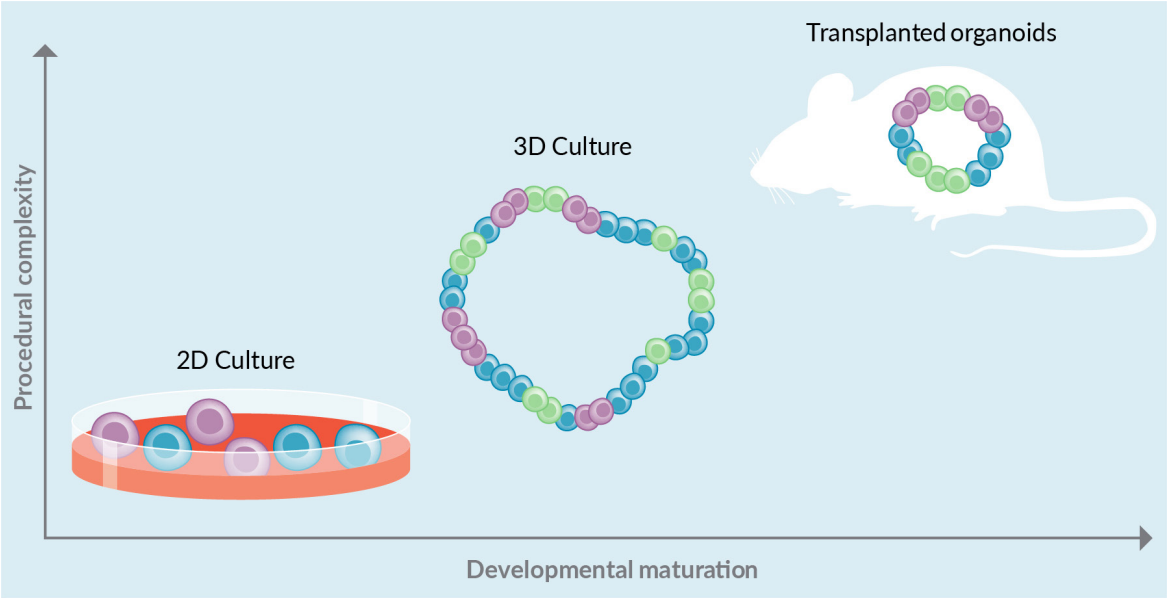
FIGURE 1

Maturation of hiPSC progeny can be improved by combining them with appropriate animal models.



Schematic demonstrating that maturation of hiPSC progeny can be induced *in vitro* with co-culture and organoid technology. However, maturation can be further enhanced by engrafting these cellular aggregates into an appropriate recipient species. Blue cells represent iPSC progeny; purple and green cells represent accessory cells in co-culture and in organoids. The origin and species of these may differ.

REJUVENATION OF iPSC PROGENY

As advancing age increases the likelihood of developing a late-onset disease [46], it is imperative that we recapitulate aging in hiPSC-based models. However, accumulating evidence suggests that the age signature of iPSC is erased upon reprogramming to pluripotency, resulting in restoration of telomere length, mitochondrial function and nuclear morphology, as well as reversal of cellular senescence [47]. Importantly, rejuvenation is not merely a characteristic of iPSC, but also their differentiated progeny [47]. This finding has been actively exploited for certain cell types: cytotoxic T lymphocytes (CTL) are, for instance, prone to exhaustion in response to chronic viral infection by organisms such as HIV-1, losing their capacity to eliminate virally-infected cells. Nevertheless, by reprogramming antigen-specific CTL to pluripotency and re-differentiating the resulting iPSC along the T cell lineage, abundant CTL can be produced which, having been rejuvenated, are able to efficiently kill target cells in an antigen-specific manner [48,49]. Nevertheless, the impact of rejuvenation can prove a significant obstacle to the acquisition of late-stage features of age-related disease, such as the neurodegeneration associated with PD [50]. It is, therefore, necessary to recapitulate physiological aging *in vitro* in order to investigate why aging results in progression of the disease from a compensated dysfunction to a progressive, uncontrollable decline in the elderly.

CAN AGING BE INDUCED *IN VITRO*?

Patients typically present with symptoms of degenerative diseases that reflect the later stages of disease progression. Given that rejuvenated iPSC and their progeny may only provide insight into early disease mechanisms that would likely occur in the pre-symptomatic adult, there is a pressing need to develop protocols to accelerate aging *in vitro* (see Table 2) in order to achieve clinically-relevant disease models [51].

TABLE 2

Summary of the advantages and disadvantages of different strategies to induce ageing together with examples of relevant studies.

Aging strategy	Advantages	Disadvantages	Examples
Toxin treatment e.g. hydrogen peroxide, hydroxyurea, sodium arsenite	Can mimic oxidative stress. Can mimic genomic damage. Quick and inexpensive	Does not induce all age-related markers. Age-related disease models only show earlier aspects of disease	[16,53,66]
Telomerase inhibition	Induce telomere shortening and genomic damage	Does not induce all age-related markers. Age-related disease models only show earlier aspects of disease	[55]
Passaging at pluripotency	More physiologically-relevant. Induces several DNA markers of aging	Does not induce all age-related markers	[54]
Progerin expression	Induces more than one hallmark of ageing. Age-related disease models show later stages of disease	Limited understanding of physiological role of progerin in aging	[50]
Transdifferentiation	Retains age-related markers	Unable to perform genome editing. Highly variable. Cannot divide indefinitely	[64,65]

Manipulating age-related pathways

Attempts to induce aging *in vitro* have generally focused on manipulating specific hallmarks of aging such as mitochondrial dysfunction, characteristic of the later stages of neurodegenerative disease. This has been achieved by treatment of cells with toxins such as hydrogen peroxide, leading to an increase in oxidative stress [16], which has been implicated in both aging and age-related disease [52]. Since genomic damage is also characteristic of aging, the administration of toxins such as hydroxyurea [53] has been shown to recapitulate this aspect of cellular aging. A more physiological approach involves passaging iPSC multiple times prior to differentiation which was shown in a recent study to lead to DNA damage in iPSC-derived neurons, accelerating the aging process [54]. Interestingly, Vera and colleagues exploited the pharmacological inhibition of telomerase to further modify the biological age of hESC-CM and neurons differentiated from iPSC from an individual with PD [55]. In both cell types, they observed telomere shortening and acquisition of some age-related markers while a progressive loss of tyrosine hydroxylase (TH) expression was reported among iPSC-neurons, characteristic of the early stages of PD [55].

While these studies successfully induce some of the recognized hallmarks of aging, they do not promote acquisition of the full spectrum of age-related markers, adversely impacting the ability of iPSC to recapitulate all features of late-onset diseases. Indeed, it is debatable whether it could ever prove feasible to advance the biological age of cell cultures by targeting a single consequence of aging. Instead, it may be necessary to modulate aging at source by targeting pathways that broadly influence all aspects of aging, insight into which may be gleaned from disorders associated with rapid, premature aging, such as Hutchinson-Gilford progeria syndrome (HGPS).

Targeting the underlying mechanisms of aging

In 2013, Miller and colleagues reported successfully modelling the later stages of PD *in vitro* by inducing aging in hiPSC-midbrain dopaminergic (hiPSC-mDA) neurons [50]. Given that the accumulation of progerin, a truncated form of lamin A associated with the inner nuclear membrane, causes cells to

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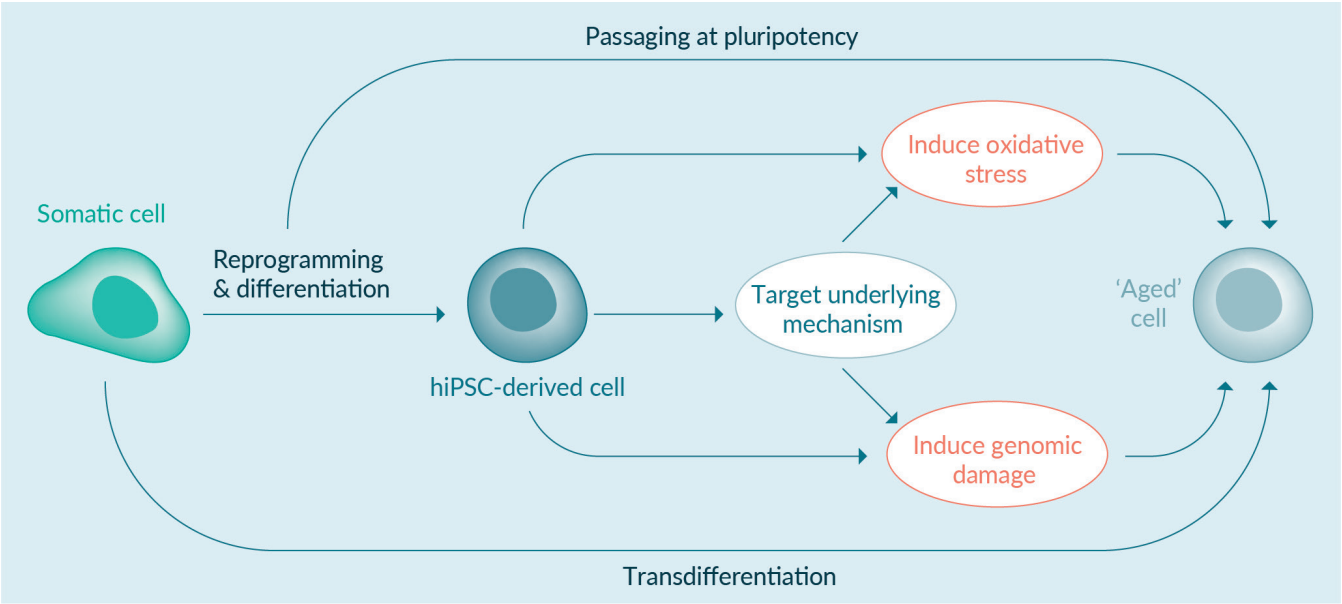
age prematurely in individuals with HGPS, patient-derived iPSC-mDA neurons were genetically modified to over-express the gene. An array of different markers associated with neuronal morphology, mitochondrial function and gene expression were found to be suggestive of accelerated aging. In addition, markers specific to neuronal aging were observed, including loss of TUJ1 expression and neuromelanin accumulation. Increased apoptosis and dendrite degeneration were also observed, both consistent with enhanced neurodegeneration, while the appearance of Lewy-body-precursor inclusions was consistent with the acquisition of a disease-related phenotype. Accordingly, progerin-expressing hiPSC-mDA neurons failed to rescue disease among a subset of Parkinsonian mice, which was attributed to loss of tyrosine hydroxylase (TH) expression, characteristic of early PD [50]. This study demonstrates the need to target the underlying mechanisms of aging in order to better recapitulate the later stages of age-related diseases. Furthermore, given that progerin accumulation is a biomarker of vascular aging [56] and has recently been observed in the hearts of patients with dilated cardiomyopathy [57], progerin-induced aging of iPSC progeny may also provide much-needed *in vitro* models of cardiovascular disease.

Transdifferentiation

An alternative approach, which may circumvent the need to intervene in aging-related pathways, is to exploit the process of transdifferentiation in which patient-derived cells are directly reprogrammed into an alternative cell type relevant to disease progression, thus by-passing the need for pluripotency (Figure 2) [58]. Many protocols have been developed to reprogram fibroblasts into an extensive array of cells types, including neurons [59], osteoblasts [60], endothelial cells [61], leukocytes [62] and cardiomyocytes [63]. These cells might be expected to appropriately model aging, as

FIGURE 2

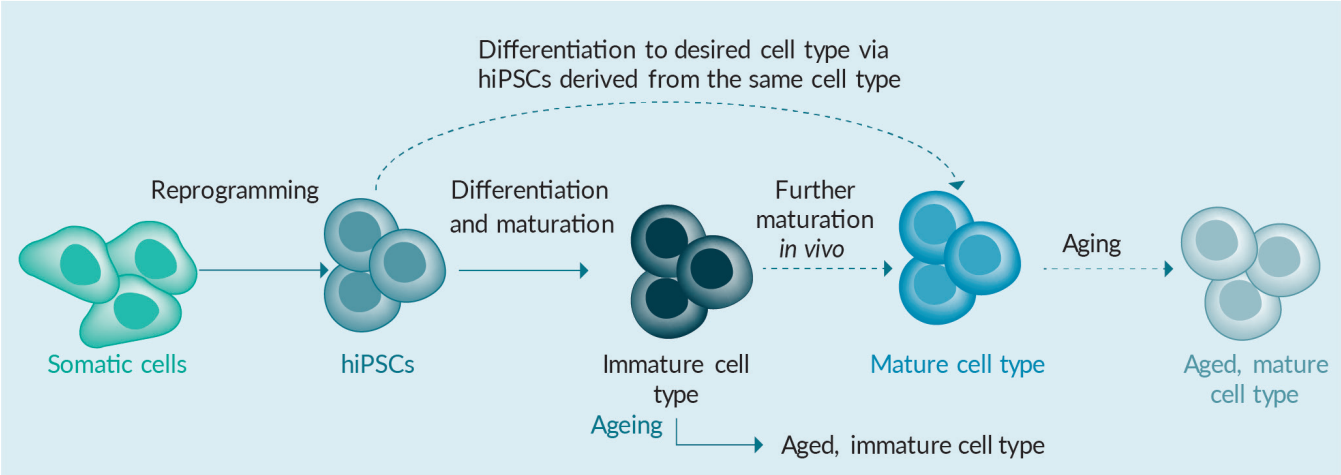
A summary of the different protocols developed to generate aged cells from a somatic cell.



One strategy is to directly convert the somatic cell to the desired cell type, thereby retaining the hallmarks of aging. Alternatively, different methods may be applied to hiPSC progeny to induce rapid aging *in vitro*, such as the induction of oxidative stress or genomic damage, although this approach typically targets a single hallmark of aging.

FIGURE 3

Current and speculative strategies for developing iPSC models of age-related disease.



In order to induce early markers of disease, current protocols induce age in immature hiPSC progeny, however, inducing both maturation and aging in combination among hiPSC progeny may prove a more effective strategy at capturing the later features of age-related diseases that are more clinically relevant. Solid arrows represent established protocols while the dashed arrows denote the alternative protocols discussed.

directly converting fibroblasts into neurons has been shown to preserve the biological age of the parent cells in both mice [64] and humans [65]. Importantly, there is no longer the need to rapidly accelerate aging *in vitro*, which is unlikely to completely recapitulate physiological aging that takes place over a much longer time scale. Developing such overarching strategies that target all age-related markers is, therefore, of great interest when investigating advanced stages of late-onset diseases, although by-passing pluripotency necessarily sacrifices the capacity for genome editing, as well as the scale-up and quality control of differentiated progeny, posing challenges of consistency and reproducibility.

TRANSLATIONAL INSIGHT

While cellular maturation enables cells to perform the specialized functions of the adult cell type, cellular aging is marked by a progressive decline in this ability. That these are distinct processes which would both normally occur in disease, suggests that current models of age-related pathogenesis, in which immature iPSC are induced to age, may be flawed (Figure 3). For example, while over-expression of progerin in iPSC-mDA neurons induced rapid aging *in vitro*, the maturity of the resulting cells was not investigated, raising the possibility that aging had been induced in fetal-like neurons, possibly contributing to the lack of some age-related features in this model [50]. Similarly, the issue of developmental immaturity of hiPSC progeny is raised in a recent study investigating ALS, which is overcome by inducing cellular stress, targeting immaturity with a solution more suitable to cellular rejuvenation [66]. Applying aging strategies to immature cells may contribute to the limited translatability of our current *in vitro* models. In order to address these deficiencies, it may prove necessary in future to combine existing approaches to disease modelling. For instance, the epigenetic memory of early passage iPSC [43] may be exploited to generate organoids consisting of mature adult cell types whose subsequent aging may be induced by transplantation into aging mice. In this context, rapid physiological aging was recently achieved in mice by ablation of a specific neuronal subtype [67], providing an animal model of accelerated aging and an appropriate *in vivo* environment in which to

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investigate the physiological aging of fully matured cells. Whilst hiPSC progeny have the potential to revolutionize modelling of late-onset diseases in the human, it is only through such innovative approaches that it may be possible to address the combined limitations posed by maturation and rejuvenation in order to fully understand disease pathogenesis and develop effective treatments.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

PRECLINICAL/TRANSLATIONAL TOOLS & STRATEGIES

SPOTLIGHT

PODCAST INTERVIEW with:

Charlie Silver, Mission Bio

"...for cell and gene therapy, single-cell approaches provide a much greater level of resolution to better characterize the products..."

On a mission to bring single-cell sequencing to cell and gene therapy

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Q Can you firstly introduce us to yourself and to Mission Bio?

CS: Mission Bio is a very mission-oriented company, and our mission is to help our customers eradicate cancer. We do that by helping drug developers and researchers in translational medicine bring precision drugs to the patients who need them, in the safest and most efficacious way possible. We also support them to reduce the time and cost of bringing these drugs to patients. We are truly very driven by our mission.

Mission Bio is a company we spun out of the University of California, San Francisco (UCSF). We have been on the market for a couple of years now, with the mindset that translational research and clinical spaces really need tools for precision, to understand what is inside cancer biopsies, and to understand with precision how drugs are interacting with patients, and how patients respond to drugs.

We are the only player providing single-cell DNA sequencing. This is a way of assessing the DNA content cell-by-cell, but at high enough throughput that you get a very precise and representative view of every cell in the sample, with great accuracy. Ours is the only platform that does single-cell DNA analysis, and over time we have also added single-cell multiomics, to the point where we can measure both DNA and protein inside every cell that matters.

We started in the space with an offering to cancer centers and drug companies that is used to run large-scale clinical studies, and help assess biomarkers of therapeutic response and resistance by understanding how the clonal architecture of biopsies change in response to treatment. This is particularly applied to clinical trials, where our partners use our technology to understand biopsies on a cell-by-cell basis, and with the depth of resolution and precision of accuracy we provide, they can get exquisite sensitivity to identify every cell in a biopsy that is either responding or resisting therapy.

The platform has come to be adopted by cancer centers everywhere. We sell to the majority of cancer centers here in the US, and many worldwide, as well as drug development companies who use this in support of their clinical trials. We also work closely with CROs and CDMOs that support the whole drug development space.

Q Why the recent move into cell and gene therapy? What can single-cell sequencing bring to this field?

CS: When we came out with the platform a couple of years ago, we had the only product that did single-cell DNA, and we launched with a number of applications in blood cancers and solid tumor profiling.

One of the real advantages that we baked into the platform early on is the ability to build custom content, so we can customize to any gene set that is of interest, for any application. As we were launching the product in the leukemias, it became apparent that there is no one set of content that applies, even within leukemias – and certainly more broadly across cancers. Therefore it made sense to build in this rapid and high-quality customization capability, so we can

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"...our mindset is that it is important to build the toolset that can characterize safety and efficacy of therapy for every patient."

work with our partners and very quickly build in any content, in a way that is purpose-built for their specific application.

Once we had a number of applications across leukemias, we had a lot of partners across the space who were customizing by either adding or subtracting content for their particular disease area or application. Along the way, we had a lot of interest from customers trying to use the platform with its customization capability to assess the quality and underlying characteristics of cell and gene therapy products.

This was not surprising to us. Once you have a technology that is able to measure changes that occur at the genetic level in every single cell that drives cancer, and once you are using that to study biomarkers of disease development within cancer, that technology looks very similar to assessing the gene modifications that we make in these cell and gene therapy drugs. The underlying requirements for the technology are fundamentally the same, just in a different application space.

We kept encountering people who were coming to us and saying "if you have a single-cell approach that does single-cell analysis, we could certainly use that to validate edits and validate integration events for cell and gene therapy products". We recognized that was a clear application set, especially for our customization capability. Over the last year or so we have really started building out applications into that space, to be able to serve the space better.

We know that for cell and gene therapies the burden of characterizing the product comes hand-in-hand with the complexity of manufacturing these drugs. Antibody products on their own are quite complicated to build and manufacture in a robust and repeatable way. When you edit cells, or modify cells, it adds a whole new level of complication when the cell itself is the product.

As a company, our mindset is that it is important to build the toolset that can characterize safety and efficacy of therapy for every patient. When we started with precision medicines, that was very much the mindset – resolution and precision is needed to be able to study the underlying clonality and underlying mechanisms for disease response and resistance for these targeted therapies.

We bring the same mindset to the cell and gene therapy space, where there is an ever-greater need for characterizing the complexity of these products, with a toolset that can untangle the complexity, and characterize it at the most fundamental level. Single-cell analysis is an approach that brings greater resolution in terms of understanding which clones are emerging or evolving as a result of therapy resistance, and understanding at a deep level the clonal heterogeneity of a tumor, and how that changes over time in response to both treatment and other factors of disease evolution.

Similarly, for cell and gene therapy, single-cell approaches provide a much greater level of resolution to better characterize the products, by assessing the critical attributes and characteristics of these products across the whole continuum. It can be applied from R&D development and optimization of the cell and gene product, to the safety and efficacy related to those

"Single-cell analysis is an approach that brings greater resolution in terms of understanding which clones are emerging or evolving as a result of therapy resistance..."

attributes, all the way through to manufacturing release testing and full-on production.

What is really important to this space is the ability to characterize transduction efficiency and vector copy number, which tells you how many integration events have made it into every cell. Then there is the efficiency of that across the entire cell population, hand-in-hand with immune phenotype, which is a way of looking at the surface of the cell and understanding what impact those gene modifications have had on the cell state.

We take the same approach that we have leveraged for biomarkers in the translational space, and apply that toolset to match the complexity of these cell and gene therapies, with an integrated set of measurements that provide greater resolution and greater precision for making these quality control measurements.

Q Can you go into more depth on the specific capabilities and any further applications of relevance to cell and gene therapy, and how they can help address the challenges the field faces today?

CS: Let me step back and give a view of what the challenges are now, to help appreciate what single-cell can provide in terms of overcoming those challenges.

In characterizing a cell and gene therapy product, whether it is an optimization step as you develop the drug, or in release testing once it is developed and you need to qualify the lot to get it back into the patient, what is really important is understanding with great precision what you have done to change the genetic makeup of the cells.

To do that conventionally for the cell and gene therapy space, drug companies are using a large collection of technologies that run a very diverse set of assays. These are combined into a release test that ultimately characterizes dose and toxicity for the product. These technologies can range from qPCR to digital PCR, to flow cytometry, FISH, plus many other genomic technologies, which are all rolled up together at the bulk level to characterize the product.

Typically, up front of those assays there is a step where you sample the product. You take out a number of cells, then plate it up, typically into a 96-well plate. You grow it for 2 weeks, sample what you have grown in every one of the wells, then run your genomics or flow assays. All of these technologies together are fairly labor intensive to run at scale, because on the bench there are a number of discrete assays to run. They are time consuming due to the number of steps, and also because of that up front culture step required to grow the cells for the downstream analysis. As a result, the turnaround time for characterizing the product is typically in the order of several weeks, and usually more than a month.

Single-cell analysis offers a way of directly characterizing the product, and reduces the time to answer, because you don't have that lengthy cell outgrowth protocol. It also integrates a

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number of these assays together, to provide a much more efficient answer on a much more rapid timescale.

This serves as a drop-in replacement for what is conventionally used to characterize these cell and gene therapies. Ultimately what we are trying to do is get these therapies to the patients who need them as quickly as possible, in the safest and most efficacious way possible.

Reducing these several weeks of characterization time down to less than a week, with a single integrated measurement, offers a much more streamlined, efficient, and cost-effective way of running that characterization, in a way that is much more robust. What I mean by that is that conventionally these assays that are typically used can run in bulk, and because they run in bulk, you are inevitably making inferences back to what is happening at the level of the cell. The cell itself is what matters in this case, because that is the drug. So, conventional bulk approaches where you make these inferences are never going to be as accurate or precise as making a true measurement at the single cell level.

For all these reasons, single-cell gives a much more efficient solution for all of these assays that combine together. Rather than having to interfere or interpolate back to the single cell level, you get a true measurement.

That becomes really important for these cell and gene therapy products, because you are trying to understand on a cell-by-cell basis whether, first of all, you have changed the genetic makeup of each cell. Then you need to know if you have inadvertently edited in or modified in anything that might be harmful – in other words, have you added unintentional toxicity to the product? When you look at that on a cell-by-cell basis, you are getting a truer readout of what is happening in the sample. Instead of looking in bulk, which is what is conventionally done, you are truly assessing what every cell is telling you.

What that means is that your bulk measurement might be the result of a few cells that have a totally different vector copy number, or it might be a result of a uniform integration into every single cell, and you just don't know that answer when you make that bulk measurement. Single-cell gives you a much more definitive and robust readout for the same measurement. This gives the space a much more precise view into what cell and gene therapy products are doing, before the product goes back into the patient.

The need across this space is great, and it is really important to begin standardizing how the space is characterizing these cell and gene therapy products. Ultimately, that standardization is best done at the single cell level, because that gives you a true readout of what the cells are doing.

The more you can add in at the front end of a development process for cell and gene therapy to really robustly characterize it, the more your program is set up for success in making it all the way through to FDA clearance. We have seen a number of examples – Freeline were in the news recently, and we have seen some others like Bluebird Bio, BioMarin, and Voyager, where they have started down the path with

"conventional bulk approaches where you make these inferences are never going to be as accurate or precise as making a true measurement at the single cell level."

the best of intentions, and with a set of characterization assays at pilot scale that they then transferred through to clinical trials. Midway through the trials they had to slow down, because they realized there were additional characterization steps that were needed. These types of delays can really have an impact on how long it takes to get these drugs to market.

By reducing the time to answer, by reducing your cycle time in making these measurements, and then by adding in a full suite of characterization attributes, from vector copy number to editing and transfection efficiency through to phenotyping, and providing all of that in an assay up front, you can really enable standardization for these critical attributes that are needed in every stage of the development cycle. This can also alleviate some of the data integration and variability issues that can occur with the plethora of assays that are conventionally being deployed.

We believe that providing the best toolset at the front end of the process, and then enabling that same toolset to carry through to every stage, all the way through to manufacture release testing, gives a much more robust set of measurements to enable these drugs to get into patients within the time that is desired. While also providing a much faster readout, and a much faster characterization cycle time.

Q How easily can single-cell sequencing be implemented into cell and gene therapy developers' existing processes? Are there any challenges in achieving this, and how does Mission Bio address those?

CS: This a good question, because we are leveraging this toolset to replace existing assays. Ease of integration tends to be really important, because folks are used to using these conventional assays, and it is important to cleanly bridge over that.

The real positive of what we are doing is that NGS is our readout. We are doing single-cell sequencing with NGS as a readout, and this is a datatype that the entire space, and particularly the FDA, is very comfortable with. They have had years of working with this type of assay, especially around NGS applications across precision oncology. Technically anyone using these existing assays has a nice clean bridge over to implementing our assays, because it looks very similar.

The ease of integration is also really important because we replace or supplement a number of these different and various technologies and assays with a single integrated measurement. You get a single definitive result without the challenges of integrating multiple assays with bioinformatics, which can be needed to glue together a lot of different assays. It really simplifies the ability to get to a simple and robust answer quickly, and that really makes a difference in terms of integrating with existing cell and gene therapy pipelines.

Again, because it is single-cell analysis, it tends to be much easier to interpret, because you don't have that interpolation step where you have to take a bulk measurement and then try to apply it to what is fundamentally a single-cell product. You are making a true measurement at the single-cell level straight off. That ease of interpretation tends to be much easier to adopt for cell and gene therapy partners. It also provides a much cleaner measurement as they characterize all of the parameters that ultimately add up to the safety and efficacy of the product.

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This single integrated readout is the hallmark of what single-cell does. It is something that as a company, we have got very comfortable with through our years of biomarker work with the pharma industry, and that translates very cleanly over to existing cell and gene therapy workflows.

"Our ultimate goal ... is to move towards a standardized assay that can support every cell and gene therapy player out there."

Q Could you summarize your chief goals and priorities as a company in cell and gene therapy over the next 1–2 years?

CS: As a company we have been working with a lot of partners in the space at the pilot stage, where we will come in and support the front end optimization of their drug development. Again, because of the complexity of these products, that optimization tends to be very important.

For example, for CRISPR edited products, depending on how you set up your CRISPR system, that CRISPR system can have an enormous impact on the efficiency of edits both on-target and off-target, and the functional result of those. So optimizing at the front end tends to become very important. Similarly, for the products that are made using viral transfection, that optimization step in reducing time to answer makes a big difference at the front end of your development pipeline, to make sure you have got the right system to go on to full manufacturing, and ultimately to make sure you don't have hiccups as you scale up your clinical studies. Coming in at the front end tends to be very important with these partners, and that is something we have done a lot of over the last year or so.

Over the next couple of years we are going to continue implementation in our customers' processes, from that early optimization and validation step, through to the chemistry, manufacturing and controls (CMC) process, and ultimately release testing. We will start with the front end, then continue supporting the processes all the way through to release, to help them get their drugs into patients faster.

Our ultimate goal across the space is to move towards a standardized assay that can support every cell and gene therapy player out there. We think that standardization is going to become very important to move the entire field forward. Once we know what we need to test for, and once we have a clean and robust set of measurements that provide both a gold standard but also ground truth for every sample being measured, this will provide a much more accurate characterization method across the entire therapy development pipeline.

There are so many of these drugs in the pipeline right now, and the real bottleneck in implementing them, and getting the therapies to patients, is that characterization time. We believe that by evolving into standards, we will enable the space overall to reduce characterization time, and help get the drugs to market for the right patients, in the right way, as quickly as possible.

A lot of our priorities over the next couple of years are around continuing to move our customers through that pipeline to full-scale implementation at the back end of their clinical

trials. Next, our priority is to support standardization across the space, so that it becomes routine and efficient for every player.

Q Finally, how do you see this technology area evolving further in the future, and what new opportunities and applications might this open up in due course?

CS: The basic mindset behind Mission Bio is that people are complex, and cancer is complex. It is really important to provide tools that simplify, and help us understand that underlying complexity at the level of the disease.

We are fundamentally single cell creatures. Every one of us is made of 30 trillion or so individual cells, but it only takes a genetic change in one of those cells to cause or to drive cancer. We are truly complex, but even within that one cell that would cause cancer, or within the many single cells that constitute a biopsy, every single molecule is important to the disease, from the DNA all the way through to the protein as the functional result. It is important to match that complexity, in order for us to be able to make a difference in cancer, and ultimately enable our customers to eradicate cancer.

We have started with a single-cell DNA product because that is the assay that is needed in the biggest way for the translational, clinical, and production side of the space for real patient impact. When we started with DNA, we started with single-base resolution, which is important for a lot of cancers. We expanded that into copy number, where we can do gene level copy number, which is the foundation of our cell and gene therapy offering. In addition to single nucleotide changes, we can run copy number at large scales across the chromosomes. Every scale of DNA is important, and we have continued to evolve the platform so that we can support everything that is needed from a DNA perspective.

We have also added capabilities for protein, which was a launch that came out last year, and we now have a full toolset. I think of it as book ending the dogma of biology: you can measure DNA, which is the blueprint of life, all the way through to protein, which is the functional result of it. That is what drug development programs are built around, and now you can characterize that entire pathway from DNA through to protein at the ground truth; at the level of the cell.

Over time, the mindset of the company is to continue to fill out every analyte and every measurement that needs to be made at the single-cell level, in order to support bringing these drugs to market faster, and bringing therapies more quickly to the patients that need them, both in a precision medicine setting and also for cell and gene therapies. Over time we are also going to continue expanding the capability of what we can do from a single-cell multiomics perspective.

BIOGRAPHY

Charlie Silver

Charlie is CEO and co-founder of Mission Bio, where he leads a team dedicated to solving complex biological problems with precision engineering, innovative biochemistry, and supported

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bioinformatics. Charlie has dedicated his career to commercializing next-generation hardware technology and scientific instrumentation at emerging ventures in healthcare and semiconductor industries. Prior to Mission Bio, he led R&D and Product at Novelx (acquired by Agilent) and then served in R&D and Marketing at Agilent. Charlie received a joint MBA from UC Berkeley and Columbia University, an MS in physics from UW Madison, and a BA in physics from Columbia University.

AUTHORSHIP & CONFLICT OF INTEREST

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Animal models of disease: preclinical to clinical gene therapy translatability

Erika Matsumoto Plata & Carlos R Plata-Salamán

Animal models of disease provide information on the biological activity and potential toxicity of an investigational gene therapy (IGT) in development. To enhance preclinical-to-clinical translation, animal models of disease must reflect the human pathophysiology as close as possible. This ideally includes the molecular defects, biochemical abnormalities, pathology, functional changes, clinical signs and symptomatology, and the disease´s course of progression. Moreover, the animal model of disease should appropriately respond to an IGT as intended. As efforts continue in the development and characterization of animal models with robust disease phenotypes translatable to human clinical conditions (two examples are presented, Duchenne muscular dystrophy and Sanfilippo A syndrome), significant needs remain. Strategies that can aid in developing new animal models of disease, as well as alternatives to animal models include taking advantage of: i) available genome knowledge in various species; ii) mechanisms and functions that are conserved across species; iii) integrated assessment systems with multiple quantitative readouts; and iv) collaborative approaches between basic scientists and clinicians to ascertain model translatability as well as of testing and evaluation methodologies.

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Gene therapy (GT) is a new therapeutic modality suited for targets and conditions that may not be treatable with standard pharmaceutical approaches. GT strategies comprise diverse mechanisms of action including gene transfer and control

of gene expression. Successful gene transfer (e.g., via genetically engineered vectors or *ex vivo* gene delivery to autologous cells) and expression is directly applicable to monogenic inherited genetic diseases and to well-characterized gene defects [1,2]. Support of GT as a therapeutic modality is strengthened by the number of GTs with diverse modes of action that have obtained regulatory approval for various clinical conditions [3].

Monogenic diseases (which result from modifications in a single gene) are currently estimated to include over 10,000 different human diseases [4]. A key challenge in the development of gene therapies for these diseases is the development of suitable preclinical *in vivo* models of disease with robust phenotype translatability to human diseases. Such models allow for better characterization of biological activity, efficacy, pharmacology and pharmacokinetics-pharmacodynamics (PK-PD) relationships and can also enhance safety assessments. Considerable effort is being channeled towards the development of animal models for specific diseases and new regulatory guidance also provides relevant frameworks that facilitate prioritization of key factors.

For instance, guidance for the development of gene therapies recommends preclinical *in vitro* and *in vivo* proof-of-concept studies to support the scientific rationale for human testing [5]. Data from preclinical studies may guide the design of further preclinical studies including those of regulatory *in vivo* toxicology as well as the first-in-human and early clinical trials [5]. Regulatory guidance highlight the importance of tailoring animal models to specific needs while supporting the "3Rs" (i.e., reduce, refine, and replace animal use in testing when feasible) [5]. Biomedical research into models of human disease gives high priority to these considerations and on identifying alternatives to animal use while focusing on enhancing translatability.

The animal model of disease selected should demonstrate the intended biological response to the IGT product with a similar profile and duration/sustainability of action when compared to the expected response in the targeted human pathophysiology. Data from animal models of disease can also be essential in estimating an IGT starting human dose (as well as the minimally effective dose and optimal biological dose) and PK-PD relationships with biological activity. It can also complement non-clinical safety evaluations and GLP regulatory toxicology studies [6] with focus on identifying doses anticipated to be associated with a positive clinical benefit and favorable risk–benefit [5,7].

Suitable animal models of disease can also better enable the identification and characterization of potential efficacy and safety biomarkers for clinical trial monitoring of clinical response to an IGT, which may encompass clinical, physiological, biochemical, developmental, morphological, and molecular measures and endpoints.

Animal models of disease used in GT investigations have been developed and identified when studying spontaneous mutations. During the last few decades, new knowledge and models have significantly improved preclinical-to-clinical translatability. However, it is still widely accepted and expected that concordance between an animal model of disease and a human clinical condition has to be proven, and although progress has been steady, there remain significant unmet needs.

Although small rodent (in particular mice) models of human diseases continue to be the chief *in vivo* platform for GT investigation and development, these models often do not reproduce human pathophysiology, clinical presentation, and disease progression. Generation, characterization and use of larger preclinical species such as dogs, pigs, and monkeys that better mimic aspects of the human disease have been evolving. In specific cases, larger preclinical species also serve as a bridge between mice and human clinical trials by providing fundamental information [8].

In the case of dogs, multiple spontaneously generated models of disease have been characterized. This is the result of increasing understanding of dog genetics and studies that have identified specific monogenic mutations

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that reflect the pathology of the equivalent disease in humans. Consequently, dogs with monogenic diseases are becoming an important part of our armamentarium for GT development as has been demonstrated in canine models of hemophilia, immunodeficiency, ophthalmological disorders, metabolic disorders, and muscular dystrophy [9].

It is recognized that case-by-case IGT considerations are key to enhancing the probability of technical success. Still, some general criteria apply to translatability aspects. The next sections will cover examples of animal models of disease that have moved ahead of specific fields and a series of highlights regarding requirements and enablers to enhance preclinical-to-clinical translation.

EXAMPLES OF ANIMAL MODELS OF DISEASE BEING USED IN GT DEVELOPMENT

Two diseases that have significantly contributed to the refinement of suitable animal models of disease are Duchenne muscular dystrophy (DMD) and Sanfilippo A Syndrome (SFAS).

Duchenne muscular dystrophy (DMD)

DMD is the most common MD caused by the defect/lack of the protein dystrophin representing one of the most severe forms of inherited MDs [10]. Mutations in the dystrophin gene lead to progressive muscle fiber degeneration and weakness, which clinically manifest as progressive movement difficulties. DMD is also associated with neuropsychiatric-cognitive manifestations and other complications, and death usually occurs due to respiratory muscle weakness or cardiac complications [10–12]. GT-based therapeutic strategies in DMD have been developing [13] and several antisense oligonucleotides for the treatment of DMD specific mutations have been approved [14–16]. Because of the size of the DMD mRNA, adeno-associated virus (AAV) vector GT approaches that have packaging size limitations prevent from carrying the full transcript; Barthélémy and Wein [13] recently reviewed the approaches being used with DMD GT interventions including those being tested in clinical trials such as AAV mini-dystrophins, AAV micro-dystrophins, and various dystrophin exon subtypes skipping.

Existent animal models of DMD include dystrophin-deficient mice, phenotypic double-knockout mice, immune-deficient mdx mice, transgenic mdx mice, clustered regularly interspaced short palindromic repeat (CRISPR)-based models, dystrophic dog strains due to spontaneous mutations as well as rat, cat, rabbit and pig models, which include those generated by specific deletions and spontaneous mutations [17–19].

Dystrophin mutations and deficiencies in various strain of dogs present a clinical phenotype that is more concordant with the human condition when compared to rodent models. In addition to the clinical profile, the progression of canine MD also resembles the human progression and phases of the disease when adjusted for life expectancy. Canine MD also presents heterogeneity of the clinical manifestations and anatomo-pathological abnormalities in the muscles [17,20].

Sanfilippo A syndrome (SFAS)

SFAS or mucopolysaccharidosis type IIIA (MPSIIIA) is an inherited monogenic pathology that manifests as a devastating neurodegenerative disease. SFAS is characterized by the accumulation of the glycosaminoglycan (GAG) heparan sulfate (HS) due to the deficiency of an enzyme involved in the lysosomal degradation of HS: heparan N-sulfatase or sulfamidase [3].

Patients with SFAS appear normal at birth and are generally diagnosed after they reach two years of age. Initial symptoms include developmental delays, behavioral disturbances and learning disabilities, which later evolve into more serious behavioral problems

(e.g., impulsivity and aggressiveness), mental retardation, severe sleep disturbances, skeletal abnormalities, immobility, hepatomegaly, seizures, and swallowing and respiratory problems. The patient is eventually left in a vegetative state and, ultimately, most SFAS patients die in their teenage years [3,21,22]. Due to its profile and progression, SFAS is often associated with caregiver burnout. No effective treatments are currently available.

In the case of SFAS, a mouse model has been developed which closely reproduces the human disease, including the neurodegeneration, neuroinflammation, hepato-splenomegalia, and shortened lifespan [23]. In this model, an IGT using an AAV-9 containing the cDNA of the sulfamidase gene restores sulfamidase activity due to highly efficient transduction and long-term constitutive expression of the therapeutic gene; there is also normalization of GAG tissue accumulation, reversion of neuroinflammation, correction of the pathology in tissues, correction of behavioral deficits, and significant prolongation of survival (lifespan) [23,24]. This IGT was also tested in dogs, resulting in long-term transgenic expression and a stable increase in sulfamidase activity throughout the ~seven year-period of study [25]. Based on the consistent and strong preclinical data in dogs and MPSIIIA mice model of SFAS mimicking the human biochemistry, pathology and aspects of the clinical profile, and since the AAV-9 encoding sulfamidase show the expected biological activity and was associated with long-term expression while being safe in regulatory studies, this IGT proceeded to clinical studies [3].

REQUIREMENTS & ENABLERS TO ENHANCE PRECLINICAL-TO-CLINICAL TRANSLATION

These can be grouped into three general categories. Those 1) associated with the phenotype of the animal model and how this mimics the human clinical condition; 2) related to the GT approach; and 3) associated with the immune system background.

Phenotype

Often, an animal model with a genetic defect that initially seems to mimic a human condition shows a different clinical profile when compared to its human counterpart, with the animal model exhibiting mild symptomatology or different disease progression [8,17,18]. This phenomenon could involve species specificity, activation of biological compensatory mechanisms and/or other gene mutations with modulatory properties in addition to the intended mutation.

Regardless of whether an animal model of disease is identified spontaneously (naturally occurring) or generated in the lab, the following criteria are essential in optimizing preclinical-to-clinical translation in GT testing:

- The underlying biological abnormality and pathophysiology need to be understood and the molecular mechanism of disease (e.g., due to gene defects/loss of function mutations) needs substantiation regarding its clinical implications;
- The molecular and cellular (and tissue and organ) basis, biochemistry, morphology, immunology, and clinical signs and symptomatology all need to be considered when determining the translatability of an animal model of disease to a human pathological condition. Additionally, heterogeneity in human clinical progression and clinical manifestations needs to be considered;
- The magnitude and duration of gene expression need to produce a clinically meaningful and sustainable effect in response to a GT. Although the level of a protein or enzyme required for therapeutic benefit will vary among diseases, in certain conditions, an expression of about 10% of normal levels (inferring full functionality) could be considered sufficient, based on available data on levels associated with mild disease phenotypes. In the examples mentioned above, with the absence of a

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specific protein or enzyme due to loss of function mutations such as in DMD and SFAS, patient data supports that expression at a level of about 20% or less (e.g., 10%) of the normal levels of the protein dystrophin (DMD) [26,27] and 10-20% or less of the normal levels of the enzyme sulfamidase (SFAS) [28,29] could significantly improve the clinical disease manifestations and progression;

- The typology of immune system responses (see below).

Gene therapy approach

There are multiple approaches including:

1. Gene replacement to compensate for a dysfunctional or absent gene e.g., incorporating a functional gene via gene transfer vectors (viral, AAV, non-viral);
2. Gene editing and repair to correct a gene mutation at the DNA or RNA level; and
3. Modulation of endogenous genes not related to the gene deficiency or mutation, i.e., genes that generate products that impact favorably on a disease because i) they have a biological function that overlaps with that of the product of the deficient gene; ii) their deficiency exacerbates the disease condition; iii) their overexpression ameliorates the pathological condition; or iv) their function is to produce a stimulatory or inhibitory factor.

Different approaches may be associated with distinct efficacy and safety profiles.

EFFICACY

An IGT which is robustly effective in preclinical models may show only limited efficacy in clinical trials.

SAFETY

Potential adverse responses to a vector, expressed transgene, and/or *ex vivo* genetically modified cells, and to other GT approaches may result in acute and/or chronic toxicities. Vectors *per se* may have varied safety and toxicity profiles (immunogenicity, integration and the ensuing consequences) e.g., adenoviruses vs AAVs vs lentiviruses vs herpes simplex viruses vs retroviruses vs alphaviruses, etc.

Relevant considerations that could potentially be managed and which merit special attention for enhancing preclinical-to-clinical translatability in efficacy and/or safety and overall data interpretability include:

- Testing method standardization. The design of a study, approaches, measures, readouts, and outcomes that are used between species should be aligned as closely as possible. Gender, age, status of disease progression and control of environmental conditions (such as light/dark cycles, temperature, humidity, noise) are also key considerations. Moreover, stress of experimental procedures in animals is also a key factor since it can affect the endocrine, neurochemical and immune milieu with physiological consequences and potential impact in the assessments being studied. Importantly, randomization treatment allocation, blinding treatment administration and blinded outcome assessments are also powerful approaches in preclinical studies. Furthermore, conducting preclinical studies in different laboratories and contract research organizations under blind conditions with consistency of results also strengthens translatability findings;

- Integrated preclinical measures with accessible computerized systems. These allow determination of relevant information such as functional, motor, sensory, behavioral (including the microstructure of feeding, drinking, and sleep), emotional, cognitive, social and quality-of-life profiles. Videotracking systems that monitor social behaviors, movements, and self-care such as grooming provide complementary information. In motor assessments, many

tools are available to measure locomotor activity, motor coordination and balance, gait characteristics, dexterity, muscle strength, power and endurance, reflexes and other activities (such as gripping, climbing, jumping, etc.) These are examples of measures that can integrate qualitative assessments with quantitative measurements, with a focus on measures that have clinical relevance. Other non-invasive technologies using wireless data transfer frameworks, including with sensors (such as non-invasive telemetry) can also provide moment-by-moment readouts of relevant measures (such as temperature, cardiovascular and respiratory function, aspects of locomotor activity, and biochemical parameters such as glucose). These tools can be implemented for integrated assessments and optimized testing;

- Species- and strain-dependent effects. These need to be assessed and identified early, and it is important to take advantage of the many functions and mechanisms that are conserved across species. We see this demonstrated in neuro-electrophysiology. The basic mechanisms for neuronal excitability and nerve conduction are similar in rodents, dogs, pigs, monkeys and humans, as are the basic ion-related processes. Examples of comparable cross-species neurophysiology mechanisms and tools include the following:

  - Temporal summation which is an instance of post-synaptic integration in which increasing neuronal activity occurs in response to repeated stimulation when subthreshold excitatory postsynaptic potentials sum up to cause suprathreshold excitatory potentials, resulting in the generation of action potentials. Temporal summation protocols which are standardized, repeatable, reliable, and validated provide a consistent translatable interpretation paradigm and are examples of meaningful quantitative assessments that can be associated with specific functional changes that have clinical correlates. In preclinical studies of animal models of disease, the wind-up process, as assessed by neuronal recordings and reflexes, can translate to the components of human temporal summation. The versatility of temporal summation is also shown in that it can use electrical, thermal, mechanical, and chemical stimulation modalities and can be elicited from multiple body structures;

  - By using single neuronal activity recordings, it has also been demonstrated that, in nonhuman primates, the response characteristics of cortical neurons for various functions exhibit neural thresholds and intensity-response functions to specific stimuli that conform well to those reported in psychophysical studies of humans, reinforcing the value of neural models for translational studies in intensity perception [30];

  - Quantitative Sensory Testing (QST), which in the clinic is a standardized clinical sensitivity test, is also an enabler in the design of studies obtaining quantitative measures using stimuli and readouts that can assess, e.g., sensory changes (loss or increase) and various domains (e.g., pain, cold, heat, vibration).

- Genes which are unrelated to the gene deficiency or mutation in question. These may exert regulatory functions with potential therapeutic implications, therefore understanding their *in vivo* mechanisms is key in determining potential translatability. For instance, in DMD, genes that may have regulatory or modifier functions include the secreted

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phosphoprotein 1 (SPP1) encoding osteopontin, β−1 4-N-acetylgalactosamine galactosyltransferase, and follistatin [13]. Moreover, other genes that could be used in DMD GT approaches also include myostatin, utrophin, α7β1-integrin, nNOS, GalNAc transferase, sarcospan, peroxisome proliferator-activated receptor gamma coactivator 1-alpha, and sarcoplasmic reticulum calcium ATPase 2a [13,17];

- Level, location (tissues/organs) and persistence of vector and expressed transgene (and where applicable viral replication) in both target and non-target cells. Vector permanence and clearance data are crucial for safety assessments and determining dosing regimens. Anatomo-histopathology allows evaluation of vector and expressed transgene distribution and assessment of potential deleterious effects on tissues/organs;

- Sustainable transgene expression and translation into protein or enzyme of interest. This is an objective of many GT products. Long-term *in vivo* assessment in animal models aids the understanding of the implications not only for efficacy and sustainability of a desired therapeutic effect, but also for safety. Issues to consider in long-term transgene expression include non-desirable cell (e.g., abnormal cell growth, transformation), immune responses (see below), potential random integration into host DNA (e.g., insertional mutagenesis), with biological implications and risk of oncogenicity. Beyond the patient, the risk and magnitude of shedding, i.e., transmission of replication competent vectors to other individuals is another key consideration;

- The factors considered in the clinical trial plan. These need to include not only the primary outcome measures and endpoint(s) but also multiple pre-defined complementary safety/tolerability, pharmacodynamic/ biomarkers and efficacy domains evaluated at different times [3]. And with these considerations, the design of preclinical studies in an animal model of disease needs to include sufficient animal numbers based on power analysis calculations on effect sizes and robust statistical analysis on the various assessments under investigation. When applicable, the design of a preclinical study should also consider the approach used in clinical trials i.e., one or more assessments should be designated *a priori* as the primary outcome measure(s) while other assessments could also be pre-specified in the analysis plan for evaluating multiple effects (secondary outcome measures). Moreover, to enhance preclinical-to-clinical translation, the primary and secondary measures should be aligned as closely as possible with those that can be used in human clinical trials (e.g., functional tests, biomarkers, imaging, histopathology, etc.) and relationships between outcomes also need to be analyzed.

Delivery procedure/route of administration of an IGT

This has implications for efficacy and safety because the same GT product may induce different profiles, with distinct risk–benefit balances. For instance, an immune response may vary if an IGT product is administered intravenously vs intracerebroventricularly. Different procedures of administration also have dissimilar risks. Regarding efficacy, the target organ should be considered when choosing the method of administration; for example, if the target organ is the brain, a direct brain route of administration may be more suitable [3], but if the target organs are both central (brain) and peripheral (e.g., heart, muscle, liver, kidneys), this should be taken into account.

Immune system background

Cellular and humoral immunity merit special comment. Animal species and strains differ

in immune system modulation, with implications for safety and efficacy (e.g., [31]). As previously mentioned, immune responses also vary depending on the route of administration (e.g., intravenous, intramuscular, subcutaneous, intracerebroventricular).

In addition to different immunomodulation (activation or inhibition), potential adverse immune responses to a vector (e.g., to the capsid proteins), expressed transgene, nuclear material, translated protein, and ex genetically modified cells may differ across animal species.

The immune response (cellular and/or humoral) to an expressed transgene can also have implications for sustained efficacy if the response generates neutralizing factors against the expressed transgene. Neutralizing antibodies elicited by a GT could severely compromise its intended therapeutic objective. Various strategies to overcome this issue are being used, including the administration of immunosuppressants. This approach is partially effective in many cases, but is also associated with adverse effects, potential risks and confounding factors.

OTHER CONSIDERATIONS IN PRECLINICAL TO CLINICAL GT TRANSLATABILITY OF ANIMAL MODELS OF DISEASE

A series of requirements and enablers to enhance preclinical-to-clinical translation have been briefly described in the preceding sections. These reflect approaches to address some of the limitations of animals models of disease for predictability in translational research (of efficacy, bioavailability, metabolism and pharmacokinetics, and/or safety) based on: 1) interspecies differences in molecular, genetic, epigenetic, cellular, biochemical, metabolism, physiological and immunological factors; 2) the complex pathophysiological processes of human diseases; 3) profiles of clinical signs and symptomatology, disease´s severity degrees and pattern of progression in animal models; 4) testing conditions and methodology variability, designs, qualitative and quantitative outcomes; and 5) the intrinsic need of a GT approach to produce a sustainable transgene expression and translation into an intended functional product with acceptable safety and therapeutic application.

There are also further considerations. The first refers to the human disease which often associates with comorbidities and concomitant clinical conditions that result in broad spectrum syndromes and add to the heterogeneity and complexity of individual patients. An example of the potential influence of comorbidities is the experience with X-linked myotubular myopathy (XLMTM) that results from *MTM1* gene mutations and myotubularin deficiency, with many patients developing severe muscle weakness leading to respiratory failure and death, typically at very early age [32]. In a dog model of XLMTM, an AAV-8 vector containing a functional copy of the canine *MTM1* gene and expressing canine myotubularin corrected the skeletal muscle pathology and respiratory function deficiency and prolonged lifespan [32,33]. The AAV-8 GT was well tolerated by the XLMTM dogs, which showed no signs of acute or chronic toxicity and liver function parameters were within the normal range throughout the study in all dogs and the liver histopathology appeared to be normal at necropsy [32,33]. On the other hand, in a Phase 2 trial, three patients that were treated with the AAV-8 vector containing a functional copy of the human *MTM1* gene, were reported to have died; these three patients had signs of progressive liver dysfunction and evidence of pre-existing concomitant hepatobiliary disease [34]. Since other patients that received the higher dose have not experienced the same liver dysfunction, it is proposed that the toxicity is related to the higher dose or the patients' characteristics rather than to a systemic safety issue [35].

The second consideration refers to additional approaches that can be used to enhance predictability and reduce "translational failure" [36]. These include: i) systematic unbiased analyses of the scientific literature

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per therapeutic area and outcomes, statistical methods to evaluate the probabilistic evidence for translational predictivity, and the need for complete reports of high quality studies [36]; and ii) application of integrated systems biology and pathology approaches and enhancing computational models via artificial intelligence and machine learning [37].

TRANSLATION INSIGHT

Rodents (mice in particular) are the most widely used species for animal models of disease. Dogs, on the other hand, offer multiple opportunities for the development of new models. Dogs have many biochemical and physiological similarities to humans and for established examples such as in DMD, they show better alignment with the human DMD condition clinically and pathologically in comparison to mice, allowing integrated assessments of efficacy and safety over time by monitoring multiple parameters and their relationships (e.g., clinical, biomarkers, structural, imaging, pathology). This is also consistent with evidence showing that the severity of the clinical signs in DMD animal models increases with increasing body size across species [19]. However, it is recognized that the logistics associated with breeding, maintaining, fully characterizing, and testing dog models continues to be a challenge. This is where focused initiatives are needed as well as for improving disease models in other species such as pigs, rabbits, cats, etc. Moreover, other models that can enhance preclinical-to-clinical translatability with increasing degrees of complexity, such as nonhuman primates have been identified and are also being developed. An example is the transgenic Huntington's disease (HD) rhesus macaque model [38], which resembles the neuro-psychiatric-cognitive progression of HD patients and with which several potential GT investigations are ongoing [39]. An important consideration in the development of animal models is that they be developed as collaborative efforts between basic scientists and clinicians to ensure that an animal model and the methodologies for assessment are applicable to a human clinical condition.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

PRECLINICAL/TRANSLATIONAL TOOLS & STRATEGIES

SPOTLIGHT

The T cell therapy field faces preclinical and translational R&D challenges as it targets more complex diseases

Gary Waanders, Silke Raffegerst, Petra Prinz-Schulz, Maja Buerdek

Cell-based therapies offer potentially effective treatment options for a variety of diseases with ever-increasing complexity and new technologies being developed aim to increase the applicability of these therapies. Here we outline some of the technical and procedural challenges facing the development and use of T cells as therapeutic agents for the treatment of cancers. There is good evidence that T cells have the capacity to control the progress of cancer in some cases, but efforts to exploit this activity to enable a broader patient population to benefit are hampered by both the intrinsic nature of T cell receptors (TCRs) and the antigenic targets they recognize (processed peptide fragments presented by human leukocyte antigens, pHLA). Assessing the suitability of a given antigenic target for the development of T cell immunotherapies requires careful and extensive mapping of antigen expression across different tissue and cell types at the gene, protein and processed-peptide levels. Once technologies for isolating optimal, fully characterized, cancer antigen-specific TCRs are in place, a significant challenge is testing the activity of TCR-T cells in the preclinical setting to ensure the cells appropriately distinguish between healthy and cancer cells. The absence of suitable animal models requires extensive *in vitro* testing against panels of HLA-typed primary human cells and tumor cell lines healthy tissue. Indeed, the limited availability of human clinical samples (tumor, healthy tissue) is itself a challenge which has driven the development of sensitive technologies for single-cell analysis. Despite the hurdles, progress is being made and the numerous TCR-T clinical development programs now underway begin to generate encouraging clinical responses against solid tumor indications giving us reason to be optimistic that this therapeutic class will ultimately provide benefits to a broader number of patients.

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Cell therapies have been presented as a possible pathway for the treatment of a wide variety of diseases with ever-increasing complexity. Potential indications for cell-based therapies range from those with a single underlying genetic defect such as sickle cell disease, to diseases with unknown or multifactorial etiologies, to neoplastic malignancies, degenerative pathologies of the central nervous system, autoimmunity, and others.

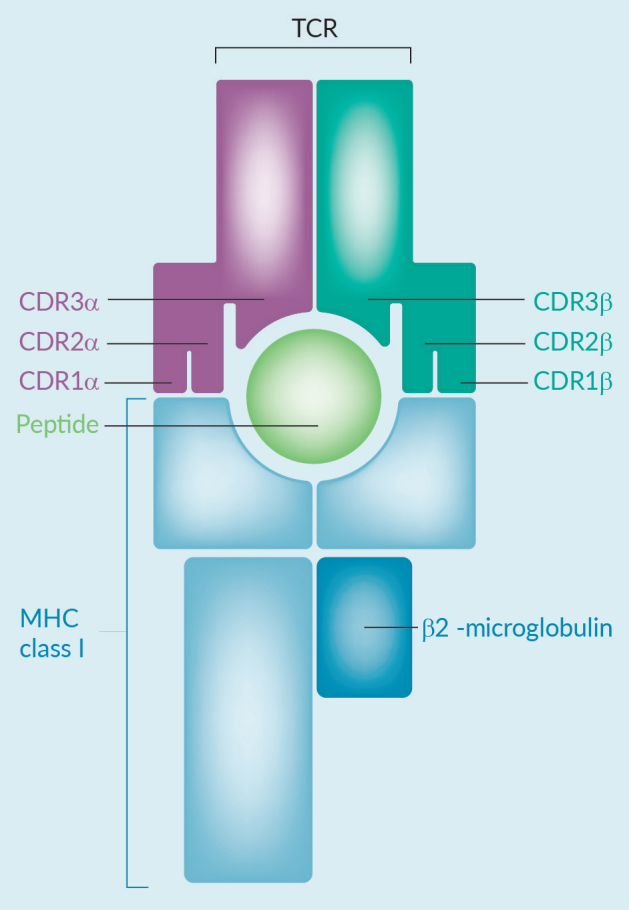
Indeed, certain conditions can be successfully treated or managed using established cell-based approaches such as hemopoietic stem cell transplantation (HSCT) [1] however, the usually limited availability of suitably matched donors and the high toxicity of preparatory regimens often restricts the use of HSCT to a relatively small subgroup and number of patients, globally numbering approximately 84,000 annually [2]. New technologies are being developed to enable greater applicability and tolerability. Here we restrict our discussion to the use of immunological cells, in particular T cells, as therapeutic agents for the treatment of cancers.

T cells detect and destroy diseased tissue both directly through cell killing and indirectly by orchestrating the activity of other components of the immune system through the specific release of chemokines and cytokines. We focus on exploiting the disease/target recognition structures of T cells, the clonally distributed cell surface T cell receptor for antigen (TCR), as a means of specifically redirecting T cells towards characterized antigenic targets associated with cancers [3–5].

The classical TCR is a complex composed of invariant CD3 components (ε, δ, γ and ζ chains) and the paired variant (see Figure 1) TCR-α and TCR-β chains [7]. The TCR-aβ combination specifically recognizes antigen fragments (peptides derived from proteins) that are processed and presented by polymorphic major histocompatibility complex (MHC) molecules [8,9]. MHC proteins, known as Human Leukocyte Antigens in humans (HLA), play a key role in antigen-specific immune responses and are the main immunological targets involved in tissue transplant rejection when mismatches exist between tissue donor and recipient. Each individual inherits two sets of HLA genes, one from each parent, with each set including genes from multiple genetic loci, including HLA-A, B, and C, among others. All the TCRs expressed by a single T cell clone recognize antigenic peptide presented by a single type of HLA complex (e.g., HLA-A or HLA-B, etc.). This 'restriction' pattern is critical to understanding the challenges of using TCRs as the basis for therapeutic interventions. There are more than 20,214 different versions (alleles) of HLA-A, B, and C proteins [10], with some being found more frequently in certain populations e.g. HLA-A2 proteins are present in approximately 50% of Caucasian populations.

FIGURE 1

T cell receptor (TCR) binding to a peptide presented by major histocompatibility complex (MHC) class I [6].



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COMPLEXITY OF TARGETS AND DISEASES

T cell biology

The target specificity of the TCR, requiring the relevant antigenic peptide to be presented by the appropriate HLA type, immediately draws attention to one of the challenges of using T cells as therapeutic entities – so-called HLA restriction or 'tissue-type matching'. In autologous situations, where the therapeutic T cells are derived from the patients themselves, this should be less of an issue because the T cells have been 'educated and selected' during development to recognize antigens presented by the HLA of that individual. Evidence of the effectiveness of this concept has been achieved in clinical settings where tumor infiltrating lymphocytes (TILs) have been isolated and expanded *in vitro* before re-administration to the same patient [11,12]. Similarly, T cells with the appropriate specificity can also be isolated from peripheral blood lymphocytes of patients and used in the same way.

However, the limited clinical success of these approaches may in part be due to using T cells that are not fully characterized in terms of fine target antigen specificity and function, and potentially not using T cells which are optimally active in the different tumor environments. Furthermore, since tumor antigens often represent overexpressed self-antigens [13], TILs and circulating tumor antigen-specific T cells of patients will have undergone negative selection in the thymus and therefore may only exhibit lower avidity TCRs against the target epitopes. To begin to address these issues, it is possible to isolate and sequence the TCRs from fully characterized tumor-antigen-specific T cell clones and genetically transfer these TCRs to either patient T cells or T cells from suitably HLA-matched donors creating so-called TCR-T cells (Figure 2). TCR-T cells have the advantage of using optimal TCRs emerging after significant *in vitro* selections and which are then expanded to large numbers during GMP production.

As a therapeutic class, T cell-based immunotherapies have been investigated for many years in a range of different cancer pathologies including both solid and hematological tumors [5,11]. The greatest successes have resulted in regulatory approvals of alternatively targeted T cell products (CAR-T cells) for hematological cancers, particularly lymphomas of the B-cell lineage. These approved CAR-T cell products, so-called because they carry a Chimeric Antigen Receptor (CAR, which contains an antibody-based target recognition structure specific for cell-surface proteins), target CD19 expressed on both healthy and malignant B cells (Figure 3 and Table 1) [15,16].

It is important to note that the antibody-based CARs often have high affinity and typically encounter high levels of antigen (e.g. CD19) expressed on the cell surface as an intact protein. This may trigger 'over-activation' of the CAR-T cell, which may explain the high rate of cytokine release syndrome (CRS) often seen in patients treated with CAR-T cells [18,19]. Indeed, some CAR-T developers have engineered lower affinity CARs with faster off-rates to try to address the CRS issue [20,21]. The situation

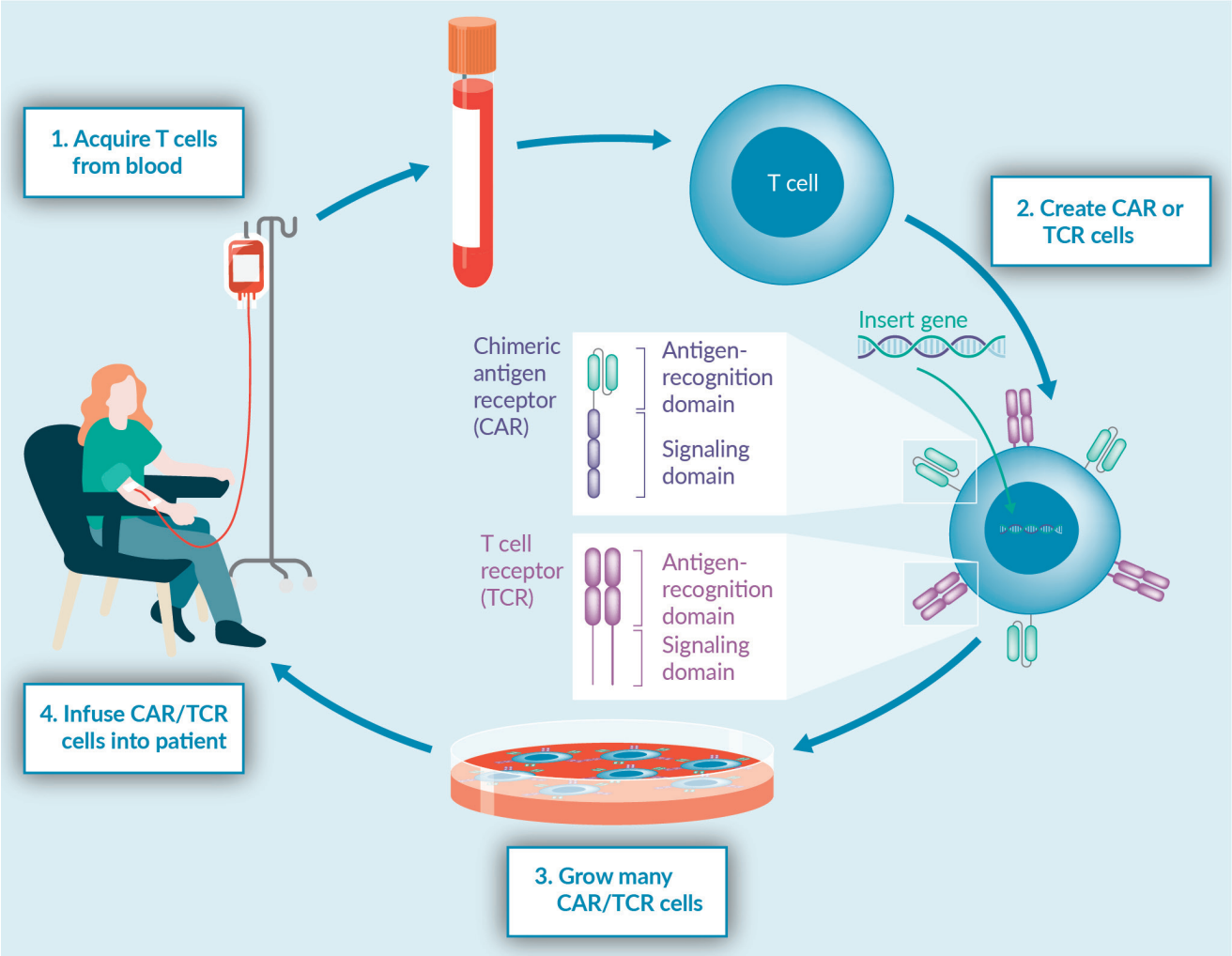
TABLE 1

Comparison of CAR T cells and TCR T cells [17].

Chimeric antigen receptor (CAR)	T cell receptor (TCR)
Surface proteins only	Both surface and intracellular proteins
~30% of human proteome as targets	100% of human proteome as targets
High target density per cell required for effective CAR-T triggering	Very low target density per cell sufficient for effective TCR-triggering
Often toxicity against healthy cells (e.g., B cells)	Many targets available with great tumor cell-heathy cell discrimination
	HLA-dependent recognition aids specificity

FIGURE 2

Production of chimeric-antigen receptor (CAR)/T-cell receptor (TCR) T-cells.



T-cells are isolated from the blood of a cancer patient. A CAR or TCR is then introduced into the isolated T-cells through viral or non-viral delivery. CAR/TCR positive T-cells are then selected and expanded into large numbers before being transfused back into the original cancer patient [14].

with CARs contrasts sharply with the low density per cell of target structures usually recognized by TCRs (peptide antigen plus HLA, pHLA) and the typically lower avidity of the TCR [22] for the respective pHLA (Figure 4).

While CAR-T cells offer the advantage of not requiring HLA expression and therefore not requiring selection of patients on the basis of HLA-tissue type, the availability of suitable targets is limited to a smaller fraction of the human proteome. Target protein antigens that are accessible to CARs are frequently found on both healthy and diseased cells, potentially resulting in higher levels of toxicity. For TCR-T cells, every protein (both cell surface and intracellular) expressed by a target cell is a potential antigen of interest assuming peptides can be presented by HLA. With 100% of the proteome giving rise to possible target antigens for TCR-Ts, selecting the right TCR offers the benefit of discriminating more effectively between healthy cells and cancer cells based on the tissue expression profile of the antigen in question. Good examples of this would be the antigen PRAME [23] or the other 'cancer-testis' antigens such as the MAGE series or NY-ESO-1 [24–26]. A significant risk for TCR-T therapies is the down-regulation of HLA molecules as a means of tumor escape [27,28].

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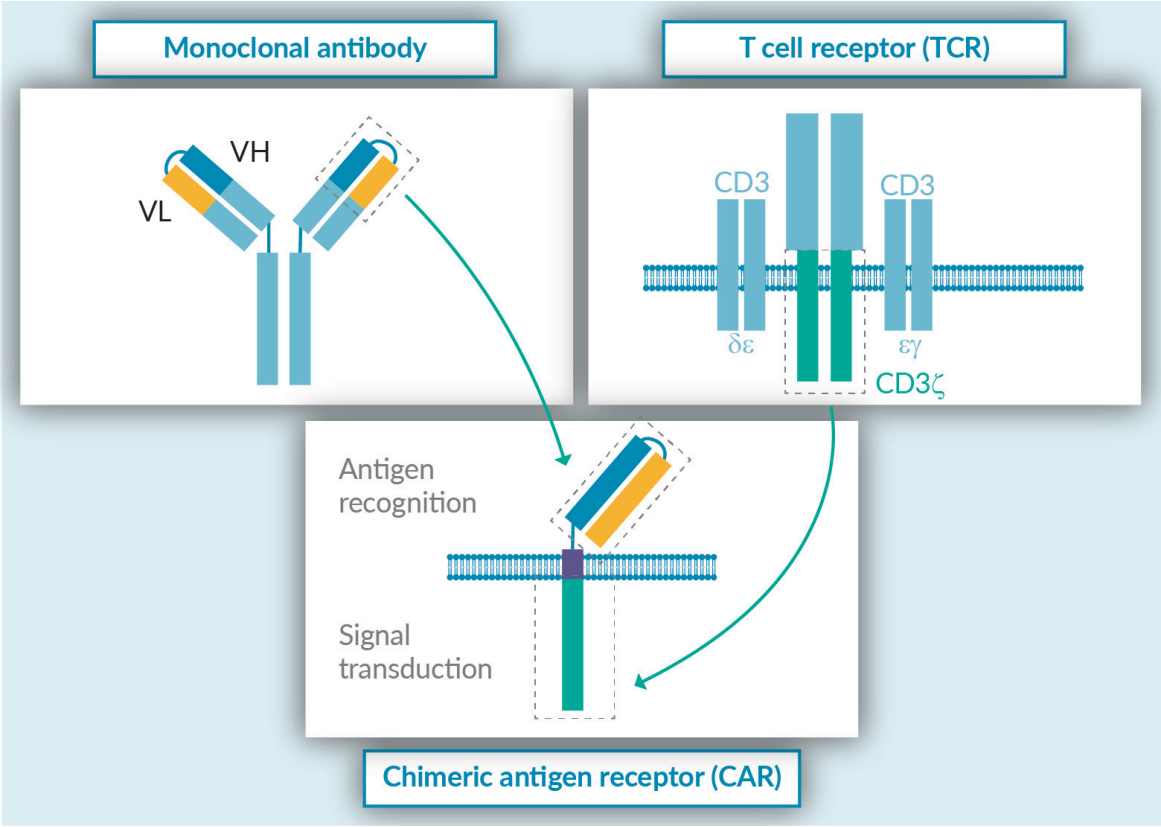
FIGURE 3

Comparison of CAR T cells and TCR T cells



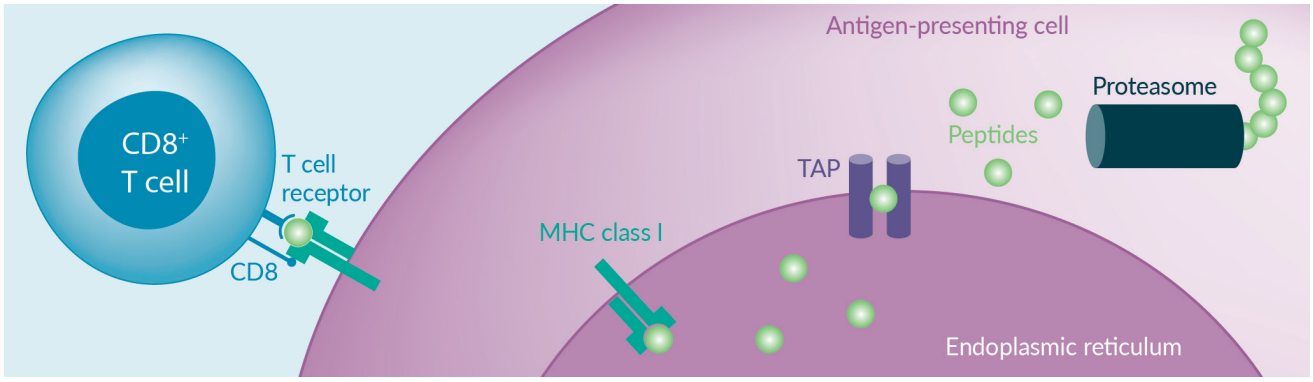
Disease and target biology

The challenges of treating solid tumors in comparison to hematological cancers include the discrete immunosuppressive microenvironment in solid tumors which is known to downregulate T cell activity as well as the overwhelming ratio of tumor cell targets relative to infiltrating T cells [29–31]. Equipping therapeutic T cells (either CAR or TCR) with the functional enhancements needed to overcome this suppression represents an area of active research for all developers of these cell therapies.

Modelling such features of solid tumors also represents a challenge for developers

FIGURE 4

Major histocompatibility complex (MHC) class I antigen presentation pathway for peptides recognized by CD8+ cytotoxic T cells [6].



during preclinical TCR-T development due to the relative paucity of suitable animal models that adequately replicate the ancillary factors (e.g. cell surface checkpoint inhibitors and soluble cytokines) affecting human T cell activity. While some immunodeficient transgenic mouse models and patient-derived tumor xenografts approximate certain *in vivo* conditions allowing the growth and activity of human tumor cells and T cells, there are both molecular and cellular immunological components missing in these mice [32].

Alternatively, both 2- and 3-dimensional *in vitro* culture systems and a choice of tumor cell lines are available to test and develop TCR-T cells. One model system for recapitulating the conditions present in solid tumors has been the use of 3-dimensional tumor spheroids *in vitro*. We have successfully used such a strategy to demonstrate the enhanced performance of TCR-T cells augmented with PD1–41BB switch receptors [33], AACR annual conference 2020, Abstract 3231). Conditions of nutrient starvation, suppressive cytokines and oxygen depletion can also be effectively mimicked in such culture systems. Two-dimensional cell cultures enable short term cell killing and cytokine production to be evaluated down to the single-cell level and represent an effective use of often precious resources such as clinical samples from patients.

CHALLENGES IN PRECLINICAL AND CLINICAL RESEARCH

In an ideal world where clinical samples from patients and donors might be available in abundance one could conceive of studies utilizing tumor and healthy tissue, biopsies and peripheral blood samples, all obtained through different treatment regimens and during disease progression and relapse. The reality of clinical research is usually far more restricted, with tumor samples typically only available at the time of diagnosis, and then generally in a form that is standardized for histopathology analyses (FFPE: formalin-fixed, paraffin-embedded; FF: fresh frozen; and much less commonly, fresh viable). Some methods cannot be used on tissues processed in certain ways e.g. FFPE, so the potential limitations on research activities can already be felt from an early stage.

Commercial tissue banks might be able to provide samples of an array of different tumor types. Still these would rarely include standard information on the HLA-type of the patients, a critical piece of information for researchers investigating targets for therapeutic TCR-T cells.

An additional challenge, peculiar to studying the clinical potential of TCR-T cells, is the absence of suitable *in vivo* models to assess safety, tolerability and toxicity, as no animal model can express the entire human proteome, the ultimate source of all pHLA which a TCR-T cell might encounter in patients. Indeed, this drives the requirement for the extensive *in vitro* testing of TCR-Ts against the broadest possible panel of human cells (normal and tumor) prior to clinical trials.

Other issues to contend with when conducting research on tumor samples include the quantity of material available as well as the heterogeneity of cells within different tumors and biopsies. These two factors affect the types of analyses possible, particularly if analytical methods and technologies are not sufficiently sensitive to work with very small amounts of clinical material.

Similarly, in the ideal world where technical methods and instrument parks are fully funded, automated, robotized and operating according to standardized routine, we could imagine a suite of technologies and tools being used to advance both preclinical and clinical research activities for the development of TCR-T cells.

Our shopping list of desired methods and technologies would include the following:

Panel of human cell lines, primary normal and tumor cells, iPSCs and tissues (FFPE, FF from tumor and normal) – a basic necessity when working with human T cells

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In addition to the standard suite of laboratory technologies such as, ELISA, Flow cytometry (multi-color, single-cell, high throughput analysis), Nucleic acid amplification (dPCR for their ability to amplify signals from minuscule amounts of material), Cytotoxicity assays (e.g. live cell imaging, flow cytometry-based killing assays or classical ^{51}Cr release assays)

Mass spectrometry – for both qualitative and quantitative detection of peptide antigens eluted from HLA molecules. This is important for determining the relevance of a particular target to specific T cell responses (an expressed gene does not always give rise to the appropriately processed antigenic peptide of interest being presented on the HLA of interest). Hurdles for successful MS analysis are the high amount of required cell material and the capacity of different peptides to be efficiently detected in MS.

The development of other tools for easier and more direct detection of respective target peptides, such as soluble fluorochrome-labeled TCRs or TCR-like antibodies, is needed and such tools could also be considered as potential companion diagnostics for patient enrolment.

A number of innovative new technologies such as Single cell Western Blot, Single cell Cytokine, Single cell transcripts and protein assays, Digital Spatial Profiling on mRNA and Protein, High content Imaging (multi color high throughput) and multiplex RNA-In-Situ Hybridization are desirable.

Such technologies, being applicable to single-cell analysis, are well suited to the parsimony required during research on precious clinical samples. They enable a better understanding of tumor heterogeneity and alterations affecting only a sub-fraction of tumor cells, like HLA or antigen loss, which could dramatically impair the clinical efficacy of immunotherapy. These highly complex and costly technologies are not positioned as tools for high throughput research. However, they generate massive amounts of data requiring ancillary technological support in the form of data processing and storage, bioinformatics and potentially artificial intelligence.

TRANSLATION FROM PRECLINICAL TO CLINICAL RESEARCH

As described above, the availability and variability of samples from patients continues to be a major hurdle in systematic research. In contrast to established tumor cell lines, primary patient tumor cells represent a more physiologically relevant model to study TCR-T cell efficacy and to possibly also predict clinical outcome. Primary tumor cells could be used in various *in vitro* assays and for the generation of more relevant PDX (patient-derived xenograft) *in vivo* models. In the discovery research setting, researchers are often limited to tissue samples collected at the time of primary diagnosis. Information about the patient, stage and history of disease and other clinical parameters may not be complete. Furthermore, researchers may not have access to additional material from the initial biopsy or subsequent samples as the disease evolves in response to treatment or progression. This is particularly true in patients treated with IO drugs (such as checkpoint inhibitors), where tumors undergo a process of immunoediting. On the one hand, IO treatment results in the elimination of a proportion of tumor cells, but on the other hand, tumor escape is also observed [34,35] . Since early phase oncology clinical trials target patients that have already been treated with chemotherapy or IO drugs, this would be a highly relevant patient population to be studied in preclinical assays. In this context, it can be challenging to establish full characterization and stratification assays in the R&D phase which can then be transferred to clinical studies.

Translating early research findings and methods of characterization clinical material represent feasibility challenges for critical steps of patient stratification in the design of clinical studies.

FUTURE INNOVATION CONTINUES

Despite the technical and procedural hurdles mentioned, clinical trials are being conducted in the development of TCR-T cell therapies where patients are effectively screened (for the expression of the appropriate HLA, tumor antigen, and potentially also for the presence of the desired antigenic peptide) for inclusion as potential candidates to benefit from TCR-T cells exhibiting the desired specificity and functional traits (ClinicalTrials.gov NCT04044768; NCT03686124; NCT03503968).

Further innovation is still highly important to permit all parameters around the development and use of these advanced cellular immunotherapies to be fully exploited in the field of solid tumors where clinical outcomes to date have been mixed.

For all patients, time is of the essence. The ability to rapidly acquire the whole picture, both clinical and immunological, using highly sensitive technologies and methods would enable product developers to execute clinical trials more efficiently. This will ultimately benefit patients though timely access to the most appropriate therapeutic treatments.

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CELL & GENE THERAPY INSIGHTS

PRECLINICAL/TRANSLATIONAL TOOLS & STRATEGIES

SPOTLIGHT

COMMENTARY/OPINION

Expediting novel *ex vivo* HSC gene therapies to the clinic: a preclinical perspective

Giulia Leoni, Andy Lambert, Charlotte Hudson, Chiara Recchi, Marina Cattoni & Paul Heal

The marketing authorization of a number of *ex vivo* autologous hematopoietic stem cell (HSC) gene therapies approved by the EMA in recent years represents a further milestone in the field of genetic therapies, creating important momentum for more products of this kind and bringing hope to patients and families affected by devastating rare conditions. With more than two decades of clinical experience and compelling data from over 200 patients treated for primary immunodeficiencies, metabolic disorders and hemoglobinopathies, it is beyond doubt that HSC gene therapies hold the great promise to provide a potentially curative, one-time treatment option for these complex and severe genetic disorders. Yet, the pathway from 'bench-to-bedside' is scattered with hurdles and successful navigation requires many years of dedicated cross-functional efforts to reach the destination. Here we reflect on some key learnings from our recent experience in advancing HSC gene therapies through the development and regulatory pathway, with a focus on preclinical aspects and considerations that we believe are critical to enable successful and streamlined delivery of this unique and complex class of products to the clinic and, ultimately, the market. We discuss the necessary preclinical studies and models that are essential to support the clinical translation of these therapies and the strategies that can be implemented to accelerate the preclinical development pathway. We also discuss the current translational gaps and how these can be filled. By addressing these important issues, we hope to provide a perspective to facilitating the progress of similar products through some of the hurdles of preclinical development.

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INTRODUCTION

The marketing authorization of a number of *ex vivo* autologous hematopoietic stem cell (HSC) gene therapies approved by the EMA in recent years [1–3] represents a further milestone in the field of genetic therapies, creating important momentum for more products of this kind and bringing hope to patients and families affected by devastating rare conditions. With more than two decades of clinical experience and compelling data from over 200 patients treated for primary immunodeficiencies [4–8], metabolic disorders [9,10] and hemoglobinopathies [11,12], it is beyond doubt that HSC gene therapies hold the great promise to provide a potentially curative, one-time treatment option for these complex and severe genetic disorders. Yet, the pathway from 'bench-to-bedside' is scattered with hurdles and successful navigation requires many years of dedicated cross-functional efforts to reach the destination.

Here we reflect on some key learnings from our recent experience in advancing HSC gene therapies through the development and regulatory pathway, with a focus on preclinical aspects and considerations that we believe are critical to enable successful and streamlined delivery of this unique and complex class of products to the clinic and, ultimately, the market. We discuss the necessary preclinical studies and models that are essential to support the clinical translation of these therapies and the strategies that can be implemented to accelerate the preclinical development pathway. We also discuss the current translational gaps and how these can be filled. By addressing these important issues, we hope to provide a perspective to facilitating the progress of similar products through some of the hurdles of preclinical development.

FROM BENCH TO BEDSIDE: THE PRECLINICAL JOURNEY OF AN HSC GENE THERAPY

The past decade has seen a rapid increase of novel cell and gene therapies entering the clinical arena [13], with a number of products receiving marketing authorizations in Europe and US [14]. This has led to a paradigm shift in the way this class of products is developed and evaluated, compared to more traditional (and arguably less complex) pharmaceutical products. As the confidence and experience with these advanced therapies mature, major resources are being channeled to address their unique manufacturing, commercial and infrastructure requirements [15]. In parallel, the regulatory landscape has been evolving with improved frameworks and guidelines specifically implemented to help product developers navigate the development pathway while promoting safe and timely access for these therapies to patients [16–18].

From a preclinical standpoint, the conventional approach to safety testing of novel drug products had to be revisited and adapted, at least in some respects, to fit the unique and complex nature of cell and gene therapies. Regulatory standards have also been challenged to enable a more pragmatic and bespoke approach to be applied to the safety evaluation of such therapies [19]. But how does this translate in practical terms? In the case of *ex vivo* lentiviral vector-based HSC gene therapies, our area of interest, preclinical safety evaluation is complicated by a variety of factors. Firstly, there are critical components inherent to the product per se that must be taken into the safety equation, including the type of viral vector backbone and its integration profile within the target cell's genome [20], the transgene of interest and the relative protein that must be produced at levels that are therapeutically relevant in disease target tissues. Secondly, the actual drug product (DP) administered to patients cannot be directly tested in a preclinical setting as by its autologous nature it is specific to each individual patient. While patient-derived cells represent an ideal source to generate material for preclinical testing, they are generally difficult to source also due to ethical considerations. Therefore, healthy donor cells and/or equivalent animal disease model surrogates are generally considered suitable proxies and

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are typically used to develop manufacturing processes and generate relevant material that can be tested in preclinical assessments, respectively. The impact of all these components, along with some elements inherent to the manufacturing of the final DP (e.g., use of novel transduction enhancers), and how cells behave upon infusion (i.e., their engraftment, differentiation, tissue distribution and long-term persistence potential), is critical to how the safety of these therapies is assessed preclinically.

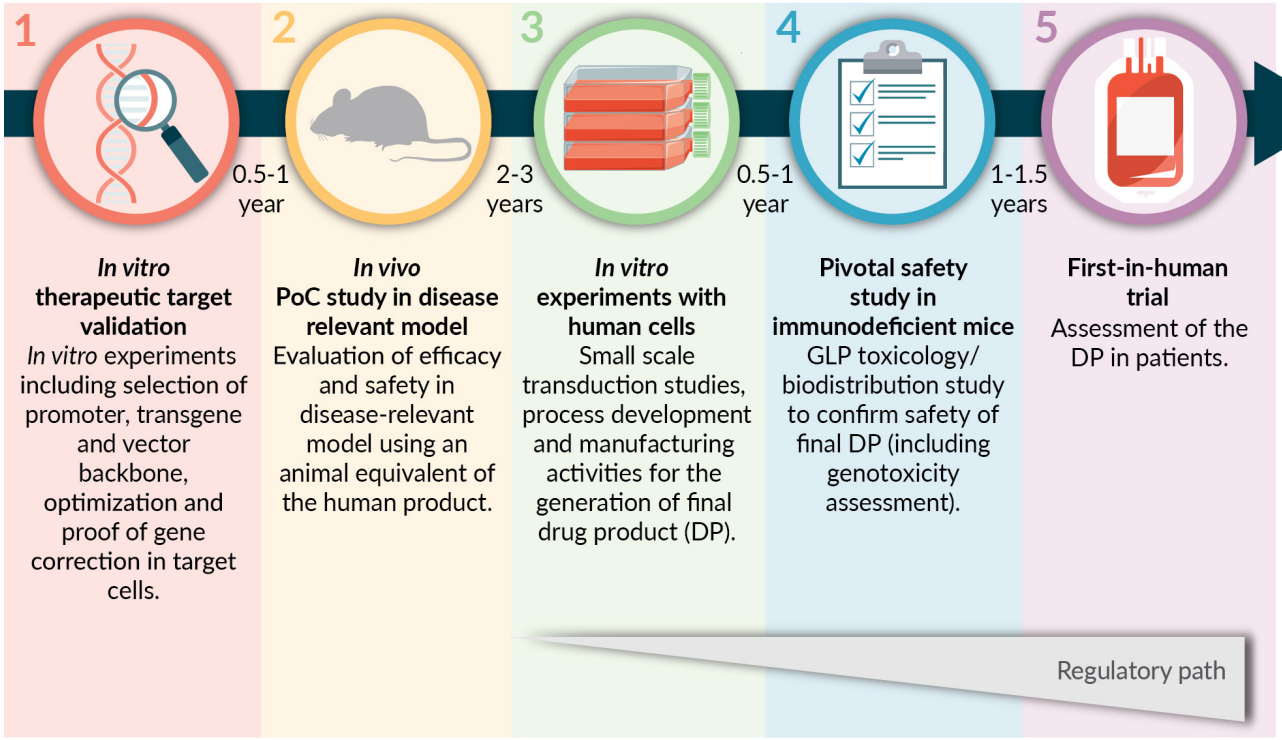
We have been privileged to work on several programs that were originally developed through specialist and academic laboratory partnerships with large pharma and this has laid the groundwork for the strategy supporting our current programs. A wealth of preclinical data has been generated through many years of pioneering research providing the rationale for these therapies. Those early studies have also been instrumental for refining protocols for product characterization and bench-scale cell transduction, as well as for optimizing experimental conditions for *in vivo* testing in specialized animal models. A major step in our preclinical journey was to ensure the relevance and suitability of those early studies to support later regulatory approvals and to identify potential gaps and requirements for additional preclinical work (e.g., bridging studies). To this end, an extensive process of retrospective data review and validation has been required to ensure alignment between early preclinical data and current regulatory expectations.

With novel HSC gene therapies entering the pipeline, efforts have been focused on identifying opportunities to streamline the preclinical development pathway while addressing common translational gaps. As for most drug products, the journey starts with early exploratory and discovery studies (Figure 1), which are typically based on *in vitro* and/or *in vivo* assays to validate the therapeutic target (transgene of interest) and the vector construct (i.e., promoter and other key elements of the vector backbone). These studies provide the foundations for all the subsequent work, including the establishment of optimal transduction conditions, proof of gene expression and correction/mechanism of action in target cells. These are followed by the *in vivo* proof-of-concept (PoC) phase, where a surrogate of the candidate product (e.g., the murine equivalent of the HSCs transduced with a lentiviral vector expressing the human transgene) is assessed in a relevant disease model that, as much as possible, recapitulates the human disease.

The primary goal of a PoC phase is to collect efficacy and pharmacology readouts, for example, proof of expression of the therapeutic transgene in target tissues, correction or amelioration of the disease phenotype/manifestations and other disease-specific readouts supporting the therapeutic benefit of the proposed approach in the target patient population. PoC studies in disease models can also serve to gather valuable data to support the safety and biodistribution profile of the candidate therapy which may be affected by the disease state. This can be achieved by prospective integration in the study design of relevant endpoints, including clinical signs, survival and clinical- and histo-pathology for safety, and analysis of target tissues for the presence of donor cells/vector using validated assays (i.e., FACS and/or qPCR). The value of PoC studies will ultimately depend on a robust experimental design, with adequate control groups and sample size, fully characterized test item(s), well-defined and possibly clinically relevant endpoints and the use of validated assays to enable the collection of meaningful and reliable data. The clinical relevance of animal models of human diseases and surrogate test items must be suitably justified, for instance, by demonstrating that the human and animal cells share analogous phenotypic and functional properties, but they are generally considered powerful tools and, to date, have provided invaluable data for initially supporting progress into clinical studies and ultimately the approval of HSC gene therapy approaches. A perceived limitation of disease models is that they may not recapitulate the

FIGURE 1

Example of preclinical development studies to support clinical use of an HSC gene therapy.



entire pathophysiology of the human disease and therefore may not always be predictive of clinical efficacy of the drug product. While this may be a challenge for diseases presenting a complex etiopathogenesis, transgenic models of monogenic disorders have so far proven to be instrumental for evaluating our therapeutic approach, enabling an overall positive correlation between preclinical and clinical outcomes in support of both safety and efficacy.

Completion of the PoC phase represents an important go/no-go decision point along the preclinical development path as availability of robust and conclusive data is key to support confident transition into the pivotal phase of preclinical studies. These typically include *in vitro* experiments aiming at optimizing/validating transduction protocols in human cells to support scale-up process development and manufacturing activities, followed by an *in vivo* assessment(s) of the human cells transduced with the final clinical process to confirm their engraftment potential and lack of toxicities prior use in patients. Some practical aspects to be considered before entering this pivotal phase include: i) definition/confirmation of the final DP manufacturing process; ii) availability of test material for the pivotal *in vivo* safety study, which must be of sufficient quality and relevant to the final DP, and iii) identification of a suitable contract research organization (CRO) to conduct the pivotal safety study. Regarding the first and second points, these will require a tight alignment between process development/product manufacturing and preclinical activities to ensure the test material used in the pivotal *in vivo* toxicology study is representative of the final clinical product. For instance, material obtained from a pre-GMP process development run can be considered sufficiently suitable for testing in the final toxicology study.

Regarding the third point, outsourcing to a specialized CRO will ultimately facilitate regulatory compliance of preclinical safety packages. Regulatory bodies routinely expect pivotal non-clinical safety studies to be carried out in conformity with the principles

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of Good Laboratory Practice (GLP). This also applies to cell and gene-based therapies, with any deviation from this being explained and the impact of non-compliance assessed [21]. Outsourcing to a GLP capable CRO may therefore provide great benefit, particularly if such capabilities are not available in-house. For HSC gene therapies, testing of human-derived DP in the pivotal non-clinical safety study requires the use of an immune-deficient strain to support engraftment of human cells, such as the NOD SCID Gamma (NSG™) mouse, which is commercially available and can be largely accommodated by most CROs. On the other end, specialized capabilities and expertise are required to ensure appropriate handling, analysis and dosing of these products. The selection of a CRO should therefore be carefully considered and requirements for pilot work to set up specific experimental procedures (e.g., non-canonical dosing methodologies and pre-conditioning regimen) and to transfer and validate product-specific assays discussed well in advance.

Ultimately, successful progression through this final leg of preclinical development will require attentive coordination of cross-functional efforts and expertise. We will discuss in the next section strategies to prevent common bottlenecks and opportunities to expedite preclinical programs.

EXPEDITING NEW HSC GENE THERAPIES TO THE CLINIC: PRECLINICAL CHALLENGES & OPPORTUNITIES

A burning question for any product developer is how long it will take for a new therapy to go from 'concept' to 'market'. There is no straight answer as development timelines, especially for complex products like cell and gene therapies, are driven by multiple factors, including a bit of serendipity on occasion. It is not uncommon for novel and ground-breaking therapies leading the way through the approval pathway to take many years for development. Yet, opportunities to streamline and expedite the route to the clinic and the market exist, especially for novel HSC gene therapies at the start of their preclinical journey. For instance, the use of the same vector backbone and manufacturing process across different indications may help to leverage certain aspects inherent to the safety of these products, ultimately expediting the regulatory approval process.

One open question remains on whether the use of such a 'platform' approach could provide the opportunity in the future to reduce the need for repeated large pivotal safety studies. For instance, a case could be made for indications with similar etiopathology and therapeutic proteins that present key commonalities. For such indications, whilst the impact of the disease status (e.g., tissue inflammation) on cell engraftment and distribution kinetics in disease models will likely remain a variable that requires further evaluation, the test items may be expected to present consistent biodistribution and toxicity profiles in immunodeficient mice. Thus, with the agreement of regulatory authorities, it may become possible to leverage a single definitive study in immunodeficient mice across multiple programs to refine/reduce or even entirely avoid the need for additional animal studies. As we gather more evidence to support this concept, the prospect of leveraging pivotal safety data across different indications under the same 'umbrella', where scientifically justified, has the potential to reduce the need for extensive animal work and to expedite the route to the patient. It is important to reinforce that the suitability of any preclinical strategy, especially if deviating from current expectations and 'standard' practices, should be discussed with regulatory authorities to ensure its acceptability.

Strong academia-industry alliances are also a vital part of the success of these programs. As the early discovery and PoC studies are often conducted in academic settings, establishing these partnerships at the very beginning of the preclinical journey provides the

opportunity to define *a priori* an optimal development strategy while implementing early on appropriate standards and processes in alignment with industry expectations. A good example is provided by the principles of GLP, which have been developed to promote the quality and integrity of non-clinical safety studies supporting human clinical trials [22]. While enforcing GLP or GLP-like practices in non-GLP, academic settings is not practical, some fundamental elements of GLP can be taken to provide an overarching framework within which early development and PoC studies can be planned, conducted and reported to ensure the integrity of the data generated and their readiness to support prospective regulatory submissions. Later, we will briefly discuss some practical considerations to ensure data integrity in a preclinical context.

As mentioned earlier, process development and manufacturing activities should go hand in hand with preclinical development activities, especially when entering the pivotal phase of the studies to ensure the material tested is as close as possible to the final clinical product. For early-stage development and PoC work the gap can be significant as the product tested is typically generated using bench scale, manual processes and research-grade materials. Accurate recording of key product characteristics and transduction conditions is therefore critical to enable better correlation between data collected from the various stages of preclinical development and ultimately ensuring their relevance to the final DP. Novel and more efficient manufacturing strategies may also become available at later stages of product development, for instance during clinical development or post-approval. This often raises the question of whether changes in the manufacturing process may require additional preclinical *in vivo* work to support comparability between the pre- and post- change drug product [23]. This will ultimately depend on the nature and extent of the changes and their overall impact on the predefined product's critical quality attributes resulting in potential significant effects on the product safety and efficacy profiles. When quality data are not sufficient to demonstrate comparability of the final drug product, the requirement for additional *in vivo* assessment should be then carefully evaluated and discussed with regulatory authorities, to prevent potential delays along the approval path.

One more conundrum is the value of early human data obtained under compassionate use, which may build the expectation for accelerating the path to market. Compassionate use programs are strictly intended to treat critically ill patients with an experimental medicinal product under development, where the potential benefit may outweigh the risk for the patient if no other treatments exist. However, such data are rarely considered an enhancement or replacement for preclinical data and may not be suitable to augment a clinical package, given the data are not captured in a controlled manner, the very low number of patients (usually n = 1), the age/disease stage, and the use of manufacturing processes which may not represent the final commercial process. The outcome from these compassionate cases may influence definitive 'go/no go' decisions for a given therapy and be viewed as supportive to regulators, however it is important to reinforce that the basic package of preclinical studies informing the efficacy and safety of a candidate product **(Figure 1)** would be still a requirement for marketing approval.

ENSURING DATA INTEGRITY IN PRECLINICAL DEVELOPMENT

Availability of robust and retrievable preclinical datasets is imperative to ensure a smooth transition to the clinic and prevent potential bottlenecks down the approval path. The requirement for individual animal raw data to be provided as part of a regulatory submission can pose, at times, some practical challenges. For instance, data generated in early investigations may not be easily accessible or may have been lost or not recorded at all,

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such as analytical readouts or husbandry data kept within academic animal facilities that may have been discarded by the time of request or data stored in superseded software programs and not properly archived. Product developers are ultimately responsible for ensuring the quality and integrity of the work produced and used for regulatory submission, whether conducted by academic collaborators, internal or outsourced to selected CROs. In the context of academic-led work, data integrity can be ensured by working alongside and supporting the academic partners in developing the understanding and tools required to meet industry and regulatory standards. In most of the cases, this will involve just small changes in working practices which can go a long way to providing a robust and relevant data package. Some pillars of data integrity are described in Box 1.

MIND THE REGULATORY GAP

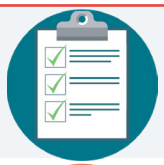
Original guidelines for cell and gene therapies have existed for over two decades. However, only in recent years, these have been updated together with the release of new guidelines specifically developed to keep up with the scientific advances in the field. For most HSC-based gene therapies reaching the later clinical stages of development or being recently marketed, their development will have begun before evolution of the current regulatory requirements for advanced therapies. Fortunately, the more recent guidelines remain consistent with the science-driven, iterative and risk-based approaches generally adopted in the early development of the current wave of advanced therapies, so 'old' preclinical work remains valid to support current regulatory approvals. This is an important consideration given that the ethical implications around animal use in medical research remain a sensitive issue and repetition or duplication of prior work should be avoided. In this context, the rapidly expanding industry at this frontier of science has a huge responsibility and opportunity to reduce, refine and replace (3Rs) animal use by leveraging emergent scientific knowledge and advances to support the evaluation of novel therapies.

BOX 1

The pillars of data integrity in preclinical development.

Training

Appropriate staff training in all technical aspects of the study, including data integrity.



Responsible person

Appointment of a single responsible person to coordinate all study activities in line with study plan.

Supervision

Study oversight by an external study monitor, e.g., Study Sponsor representative.

Documentation

Generation of appropriate study documentation (e.g., study plan, protocol, SOPs, file notes, study amendments).

Data storage

Safeguarding of all study data and documentation in secure storage for long-term traceability and access.

Quality checks

Interim data quality checks by an appointed person to ensure each dataset is consistent, accurate and complete.

Complete data set

Ensure completeness and accuracy of final data package to support prospective regulatory submissions.

Final report

Generation of a final study report with all salient data to enable robust study reconstruction.

Points to consider to ensuring data integrity for preclinical studies intended to support clinical trial approval and marketing authorization.

FROM RARE TO LARGE INDICATIONS: PRECLINICAL CONSIDERATIONS

Thus far, HSC-based gene therapies have shown potential therapeutic benefits in targeting rare, monogenic disorders, including diseases affecting the immune system (e.g., Wiskott Aldrich syndrome [WAS], adenosine deaminase severe combined immunodeficiency [ADA-SCID], and X-linked chronic granulomatous disease [X-CGD]), the metabolic system (e.g., Metachromatic leukodystrophy [MLD] and mucopolysaccharidoses) and hematological disorders (e.g., beta thalassemia and sickle cell disease) [24]. Notably, the therapeutic potential of HSC gene therapies could also be extended to less rare and multifactorial diseases affecting the central nervous system (CNS), such as frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), where specific and sustained expression of potential therapeutic factors (e.g., proteins or small hairpin RNAs [shRNAs] for up-/down-regulation of key targets) could be achieved upon reconstitution of microglia cells by the engineered HSCs [25,26]. Preclinical PoC studies are currently underway, which, it is anticipated, will provide the evidence for their use in these new therapeutic avenues. From a translation perspective, the sole use of murine models has proven successful and been acceptable to regulatory authorities in the context of rare diseases. It will be important to understand whether a similar preclinical strategy will be also applicable to support the clinical translation of HSC gene therapies to more common neurodegenerative disorders, for which novel modalities of cell delivery to specifically target the brain or spinal cord may be required to maximize the therapeutic value [27]. For instance, non-human primates (NHPs) have been long used in neuroscience and brain disorders research due to their highly developed cognitive and motor functions and complex neuroanatomy, including in studies evaluating the potential of novel therapeutic strategies such as *in vivo* gene therapy using adenoviral vectors and cell replacement therapy for Parkinson's disease [28,29]. Investigations in larger species may be warranted to enable a better extrapolation of human-relevant findings including selection of optimal dose as well as evaluation and optimization of the surgical delivery procedure intended for the clinic. However, for autologous HSC gene therapies, the use of larger animal species, including NHPs, may pose some major limitations for translation to an *ex vivo* gene therapy modality, including those associated with the need for immunosuppression to support xenotransplantation, or otherwise for the requirement for an animal equivalent product, which may not be feasible. Ultimately, understanding the potential benefit–risk balance of these therapies in the context of larger neurodegenerative disorders will be crucial to define an optimal preclinical strategy and de-risk their translation to the clinic.

CONCLUDING REMARKS

Smooth seas never made skilled sailors. This certainly applies to any developer of pioneering therapeutics and *ex vivo* HSC gene therapies have been no exception, but we have learned a lot and come a long way since the early human trials initiated over 20 years ago. Opportunities still clearly exist to streamline preclinical programs by capitalizing on platform approaches and by creating strong synergies between experts in academia, industry, laboratory service providers and regulators. As the science matures and experience grows, we gain a greater understanding of the challenges and can adapt to meet the similarly evolving clinical, manufacturing, regulatory, social and commercial requirements that will ultimately enable faster and sustained access of these exciting therapies to patients.

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CELL & GENE THERAPY INSIGHTS

PRECLINICAL/TRANSLATIONAL TOOLS & STRATEGIES

SPOTLIGHT

INTERVIEW

Beginning gene therapy preclinical development with the end in mind: from preclinical to economic modelling

OLIVIER NEGRE, PHD developed his expertise in preclinical research, bioassays, and drug development through more than 20 years of experience in biotherapies. After working on recombinant vaccines with Bioprotein Technologies, he joined bluebird bio (formerly Genetix Pharmaceuticals) and contributed for 15 years to the development of the first approved gene therapy for beta-thalassemia (Zynteglo™). From preclinical studies to marketing authorization, he served as Senior Scientist/Team Leader in France and Director translational Research in the USA. He is currently, co-founder and Partner at Biotherapy Partners, acting Chief Development Officer of a biotech company, a participant in HEC Challenge+ program, board member of the French Society of Gene and Cell Therapy, expert of the Cure Sickle Cell initiative (NIH) and active member of the think-tank Gene and Cell Therapy Institute (G&CTI). Olivier graduated from ENSTBB engineering school and earned a PhD in cell and molecular biology from Paris Diderot University. He contributed to several patents and scientific publications in the field of gene and cell therapy (e.g. *Nature* 2010, *Blood* 2011, *Stem Cells* 2013, *Current Gene Therapy* 2015, *Human Gene Therapy* 2016, *The New England Journal of Medicine* 2017, *The New England Journal of Medicine* 2018, *Science Translational* 2019, *Molecular Therapy Methods & Clin. Dev.* 2020, *BioDrugs* 2020, *The New England Journal of Medicine* 2021).

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Q What are you working on right now?

ON: I am working on the development of gene and cell therapies with a consulting firm, Biotherapy Partners, which I co-founded when I returned from the US. The goal for Biotherapy Partners is to support projects in the field of gene and cell therapies, from preclinical proof-of-concept to clinical trial, and potentially towards commercialization for programs that are providing good results and safety profiles in the clinic.

I am also Acting Chief Development Officer for a biotechnology startup in Paris. I help team members with the programs they have in pre-IND or pre-CTA stages, to move towards clinical trials.

Q You were involved in the preclinical development of a trailblazing *ex vivo* gene therapy product; Zynteglo. Can you talk about how you approached that challenge?

ON: I was involved in the development of Zynteglo for 15 years. It was an amazing journey full of challenges and team achievements. We had many contributors and did a lot of cross-functional work to shape this product, along with work on all the platforms that were necessary to take this from a breakthrough concept to a reality. It was one of the first lentiviral vectors to go to the clinic, and it provided hope of a treatment to many patients with sickle cell disease and beta-thalassemia, which are hemoglobin disorders and relatively common genetic diseases.

At the time, producing vectors for a patient was a whole batch of production, and it was difficult to take this from a proof-of-concept to making this therapy at an industrial level. Those 15 years involved development of the process production, development of all the different assays related to characterization of the product, and set up of clinical sites. I was not involved in the development of all of this – I was mainly playing a role in the non-clinical section of those activities, but I was lucky enough to see the organization of those programs in project teams combining different functions (research, preclinical development, manufacturing, analytics, regulatory affairs, patient advocacy, clinical, and commercial). It was amazing to see both the complexity of the program and the organizational efforts that led this product to market authorization in Europe in 2019. I had the chance to meet with some brilliant mentors during this work, and connect with many gene therapy experts, both in Europe and the US. It was a great experience to work on topics like genetic diseases, gene transfer, hematopoietic stem cells, hemoglobin disorders, red blood cells and preclinical models.

In parallel, I was thrilled to be involved in the development of the company itself. When I joined bluebird bio, known at the time as Genetix Pharmaceuticals, we were just 5 people in France and about 10 in the US. When I transitioned back to France in 2019, bluebird was a pretty big biotech with around 1,000 employees. It has different sites in Europe, a manufacturing site in Durham NC, and two research sites on the East Coast (in Cambridge MA) and the West Coast (in Seattle WA). Watching the development of this field

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was extremely interesting, and I think this company brought a lot of energy. They were developing a new type of therapeutic strategy with the lentiviral platform, and also new products for hemoglobin disorders, adrenoleukodystrophy (ALD) and multiple myeloma, with BCMA CAR-T cells. The company was able to support these projects from the preclinical proof-of-concept towards commercialization, with all the funding and structure that necessitates. I was really lucky to be part of this scientific and industrial adventure.

"We have seen scientific potential become industrial organization, and more recently, become products for patients with unmet medical needs. It has been fantastic to see the different stages of this development."

In 2005, the technology of lentiviral vectors was just coming to the clinic. The vectors were developed for ALD and beta thalassemia, in parallel in different groups. Patient cells were transduced at Necker Hospital in Paris, France. By 2010, we saw those first trials getting good results, and a new management team came aboard and the name of the company became bluebird bio. At that time, the expansion of the company meant it was moving from a small team to a relatively large biotechnology company, with multiple sites and clinical trials.

It was interesting to see the translation of the gene therapy field from the bench to the clinic, with an increasing number of clinical trials. There were just four gene therapy products on the market worldwide in 2010 (Gendicine, Macugen, Oncorine, and Rexin-G). And then, over this journey, we saw the maturation of the field, and now we see many products in clinical trials, and arriving on the market. If we take the examples of genetic disease and cancer, it is amazing to see the number of clinical trials with CAR T-cells and viral vectors being used.

We have seen scientific potential become industrial organization, and more recently, become products for patients with unmet medical needs. It has been fantastic to see the different stages of this development. However, the capacity of production for gene and cell therapies has still to be improved, and the economic models to make them accessible to all are not yet in place.

Q What have lessons you learned from the experience of guiding Zynteglo through development that you take forward in your work today? What guidance would you pass on to others at a similar stage of development with a next-gen cell or gene therapy product?

ON: From the beginning, one of the most important skills was being creative and being able to innovate. It was necessary to make a globin vector able to efficiently modify the patient's stem cells, and produce a high level of therapeutic globin specifically in

erythroid cells. The idea of rational design to create a single-shot curative treatment for patients with unmet need guided the development of this new therapeutic modality..

Before I joined, a lot of work had already been done on the vector design and that was the foundation of this future product. They tested gammaretroviral vectors first, but they were not really efficient at transducing stem cells, and the cargo size of the vector was quite small. Then they came to the lentiviral vectors, which had better efficacy of transduction of hematopoietic stem cells, and the capacity to carry a large transgene, including the regulatory sequences. At the early stages, the tool itself was a condition of success for this gene therapy, and this creativity and innovation was key. Next, it was the perseverance needed to be able to take this idea and industrialize it.

As I mentioned, it was one vector batch per patient at the beginning. Thanks to this perseverance in making it more efficient at the industrial level, it was then possible to produce enough for clinical trials, and many patients, with multiple sites. And at the next level, to produce a sufficient amount to have a commercially available product.

The last key piece after creativity and perseverance is teamwork. The connection between visionary people in the field was crucial, as it allowed them to work together and make this idea a success in the industry and the clinic. The bluebird bio management team added even more potential by developing a company able to support gene therapy programs toward commercialization and to handle the complex manufacturing process and logistics.

The four pillars of success were the science and technology that formed a strong foundation, then the medical expertise of the physicians involved in the clinical trials – the conditioning of the patient was key to obtaining a successful therapy. Then there is the team collaborating to make all of this happen. The fourth pillar still represents a challenge for the field: finding the economic model(s) to make the product available for all patients with medical need.

Q In your work as a consultant, what would you say are the most common errors or misconceptions you see in preclinical development of novel therapies at the moment?

ON: When people start a new program one of the potential errors is to begin too quickly or too early, when a lot is still unknown about the physiopathology of a disease. What we are making is not like small molecules, where you can screen thousands of molecules randomly to find one with a potential therapeutic effect. In gene and cell therapy, you need to have a good understanding of the disease first, in order to create a solution to treat it with what is missing from the cells. If the understanding of the physiopathology of the disease is not sufficient, the product may not meet the characteristics necessary to make it successful.

A second challenge is the limitation of animal models. The key is to know the limitations, and understand what kind of conclusions can be drawn, and what kind of translation can be made to potential clinical applications. For example, we use animal models for thalassemia and sickle cell disease, and of course they have differences from the physiopathology

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"A second challenge is the limitation of animal models. The key is to know the limitations, and understand what kind of conclusions can be drawn, and what kind of translation can be made to potential clinical applications."

of the human disease. By understanding both the differences and the similarities, it was possible to have a proof-of-concept. But you need to know the limitations in order to avoid drawing the wrong conclusions.

Another element in this field is the question of intellectual property. Freedom to Operate is not always easy to find. For example, in the field of gene editing, there are a lot of patents around CRISPR. When you want to design a new gene editing strategy, the way you are going to modify the cell can be constrained by the intellectual property and the patents already in the field. Looking at the existing patents very early in development is key, because that can turn into a blockage.

The last element I see as a potential challenge is the lack of cohesion, or lack of alignment, between the different stakeholders in a product – especially when you are involving a company, industry, hospitals, and so on. You need to have alignment on a program very early; it is key for your success. If you don't, you may face some very big difficulties in getting a group of people with different expertise, and also from different companies or entities, working in the same direction. From the beginning it is very important to have this cross-functional discussion, and ensure all the stakeholders have the same vision. This is not always easy to set up – sometimes it is as difficult as developing the program itself!

Q You mentioned limitations of current animal models – and today we hear more and more about exciting *in vitro* models, organoids, and other non-clinical *in vitro* and *in silico* tools. Are there any particular examples that catch your eye as having the potential to really make an impact ?

ON: There is a trend to reduce the number of animals in the preclinical phase of drug development, both because these models may not be mimicking the effect of the product in the clinic, but also because of rules to reduce the number of animals. It is therefore important to think about alternatives.

I think organoids are very interesting, especially with induced pluripotent stem cells (iPSCs), because you can potentially create tissues from a somatic cell reprogramed to become an iPSC, and then you can re-differentiate into different tissues. It is a very interesting tool because you can create a tissue from the patient themselves, and potentially differentiate what you want to test. If we think about testing the off-target effects of CAR T-cells, you can potentially create tissues that can be used for those kinds of assessments. Of course, it is still early in terms of iPSC development. And even if there are a lot of examples of differentiation in very specialized tissues like neurons or blood cells, there is still a lot of work to do with the

three dimensional organization of the organoids – in addition to the ability to differentiate into different cells, the way they are shaped is important to mimic real tissue. The bioengineering around organoids is likely to be a very interesting field in the future for preclinical studies. Recent improvements in massive production of iPS cells thanks to encapsulation are also promising.

In addition to that, current *in vitro* studies can be useful if they are well described. For example, if you think about genotoxicity – the IVIM (*in vitro* immortalization assay), which is an *in vitro* assay to test genotoxicity of viral vectors, is not really mimicking human cells well, because it is based on mouse cells transduced to perform this *in vitro* assay. But at some point, because the assay is pretty well set up with positive controls , I think it is nice to have this kind of standard to evaluate your vector. It will not precisely predict the potential of oncogenicity, but it will give you an idea on the risk of genotoxicity, in comparison to a reference. The idea is to be able to compare different vectors with different designs with the same reference. If you have that, even if it is an imperfect model, I think it is still useful.

The last piece is of course the *in silico* models. These models are becoming more and more powerful, and I think it is very useful for the field. Connected to that, clinical trials can feed *in silico* models. With clinical trials and additional exploratory assays, other than the end points, you can build large databases, with real data from patients, and inject them into *in silico* models to create much more powerful and predictive analytics software products.

What we see with integration site analysis, for example, is that we learn a lot about the dynamic of the differentiation of the cells. We learn for example about the plasticity and the lifespan of early progenitors. So *in silico* models and artificial intelligence (AI) are clearly a very strong field in development, but they need to be connected strongly to the ancillary studies in the clinic, because *in silico* modelling is really powerful when it has a connection with clinical reality. We see a lot of clinical trials now with ancillary studies, performed in parallel to the assessment of the main end points, and I think it is a good practice.

Q What for you are the strategic and organizational keys to successfully integrating early process development with preclinical translational development of advanced therapies?

ON: In addition to a good understanding of the science, meaning good understanding of the biological mechanisms and also a good understanding of what your drug product is, which is the foundation of development, agencies are highlighting the fact that companies and project stakeholders in general need to have a good understanding of their product during development.

Then the next element is probably the TPP; the target product profile. This is a good way to brainstorm the product the team wants to develop. It is also a way to align on the development, because sometimes people from the same team have a different vision of the product. It is helpful to align the visions, so that everybody is working in the same direction.

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Q Can you briefly summarize your chief goals and priorities in your own work over the next two years?

ON: This year, I would like to continue my activities related to safety assessment and preclinical development of new gene therapy products. This is what I am currently doing with biotechnology companies.

In addition to that, I am thrilled to participate as a board member of the French Society of Cell and Gene Therapy. We are participating in the development of a gene therapy network in France and in Europe, and we are currently working on a heatmap of gene and cell therapy players. The goal is to make connections which will facilitate and stimulate the development of new projects and programs.

"Economic models are key, because if we don't have a way to make products on the market successful and available for patients, then I am afraid that will block the whole process, from the bench to the clinic and on to the market."

It is part of the goals of the European Society of Gene Therapy, but also of the different societies in each country, to be able to strengthen the cross-talk between the different labs specializing in gene therapy, in order to be more efficient when developing a project. So that, for example, you know how to make your vector, that this animal model for your program is well understood, and you know where to do your clinical trial, by accessing a comprehensive database of what every lab connected to the field of gene and cell therapy is doing.

The last element I am planning to really focus on this year is the think tank G&CTI, which is the Gene and Cell Therapy Institute in Paris. The goal is to have cross-functional thinking around gene therapy. Coming from the preclinical world, I am really thrilled to participate in discussion around manufacturing, educational questions and also economic models.

Economic models are key, because if we don't have a way to make products on the market successful and available for patients, then I am afraid that will block the whole process, from the bench to the clinic and on to the market. We need to think about how to make the production of new cell and gene therapies affordable and efficient, and also about how to make economic models compatible with reimbursement.

There is a lot of discussion in different countries within Europe. Structures like think-tanks can help connecting stakeholders and brainstorm on economic models. There is a lot of work going on to make gene and cell therapies accessible to all.

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CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

The importance of a strong collection cell collection network during the COVID-19 pandemic

Cell collection for the development of cell and gene therapies is a complex process. With the additional stresses of a global pandemic, the industry is facing more obstacles than ever before. In spite of this, the advancement of cell and gene therapies for patients cannot stop. Be The Match Biotherapies is facing the challenges posed by COVID-19 by utilizing a robust, well-managed cell collection network in order to continue to support the development of cell and gene therapies, and ensure life-saving therapies reach patients.

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BUILDING COLLECTION NETWORKS

For both autologous and allogeneic therapies, the supply chain originates in the collection of starting materials from patients or healthy donors. Be The Match Biotherapies (BTMB)'s work is central to the ecosystem that supports what is probably the most widely-used lifesaving cell therapy: bone marrow transplant.

Figure 1. US Apheresis and Marrow Collection Center Network.

- Apheresis Center
- Marrow Collection Center
- Combined Apheresis & Marrow Collection Center

The organization manages around 36,000 cell and blood shipments annually. In order to meet the needs of stakeholders and patients, BTMB has developed and maintains a network of around 110 apheresis and marrow collection centers across the US built, around major population centers **(Figure 1)**. Cultivating and managing a network of this scale has taken a sustained effort over many years.

LESSONS LEARNED FROM MANAGING LARGE-SCALE NETWORKS

BTMB has learned that experienced management of a large-scale cell collection network provides benefits to all stakeholders:

- Establishing long-term working relationships provides unique knowledge of each individual center's capabilities
- Specialized knowledge of training, auditing and managing collection centers is gained over time
- Audit fatigue and time away from clinical responsibilities is reduced for clinical staff
- An efficient and proven process for onboarding centers is developed, helping to streamline the process

COVID-19: A NEW CHALLENGE FOR CELL COLLECTION

The ongoing COVID-19 pandemic has brought dramatic new challenges to the industry, particularly when collecting allogeneic materials, as it is difficult to secure an environment where healthy donors feel comfortable and are not concerned that they will be exposed to COVID-19. The risk to staff of bringing in new donors must also be considered. The onus is on BTMB, its cell and gene therapy clients, and the industry as a whole, to ensure the safety of all individuals who interact with collection centers.

Measures taken include:

- Pivoting to collection sites outside of COVID-19 hotspots and focusing on collection centers outside of hospitals.
- Offering virtual onboarding, training, and auditing of cell collection centers.
- Maintaining frequent contact with centers to understand the regional clinical and regulatory issues that are being faced.
- Minimizing donor travel.
- Modifying donor screening and collection processes to minimize risk of exposure to COVID-19.
- Increased use of cryopreserved materials to increase flexibility in collection scheduling and transport.

PUTTING CELL COLLECTION IN EXPERIENCED HANDS

More than ever in these unprecedented times, hard-won experience and knowledge drawn from decades serving the cell and gene therapy arena are essential to bridge the gap between infrastructure and industry, and ensure cell therapy materials are collected and delivered in a safe and timely manner to the people who need them. BTMB has unique strengths in building and managing collection networks, that continue to be invaluable in navigating the complexities of delivering cell therapy materials during a global pandemic.

CELL & GENE THERAPY INSIGHTS

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TRANSLATION INSIGHT

Flow cytometric analysis for cellular therapy products: design and practical considerations

Alison J Thomson, Laura Bailey & John DM Campbell

Flow cytometry is the most commonly used technique for quality control of cellular therapy products. In developing and then validating flow cytometry QC assays for living medicines, these research-originated assay techniques must be made sufficiently robust for manufacturing use. Key to success is truly understanding all the aspects of design and validation that a GMP-compliant process entails. In this translational insight paper, the authors highlight major themes found in designing and applying flow cytometric analysis for cellular therapies and discuss practical solutions to common problems.

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INTRODUCTION

The supply chain and manufacturing processes of cell and gene therapy products are long and complex. In both development and manufacture of these living medicines, it is essential that sufficient characterisation of the starting materials, manufacturing intermediates and final products is carried out. Cellular analysis of identity is therefore vital for the release of an experimental or licensed product (including potency), and is also commonly required for qualifying starting materials; determining that intermediate manufacturing stages are proceeding correctly; and confirmation of stability during cryogenic storage (potentially for years) *inter alia*.

Flow cytometry has been the mainstay of the quality control of cell therapy products for many years. This often reflects the origin of stem cell and immune cell-based

therapies from research labs, where complex flow cytometry is a principal cell characterisation method. Flow cytometry can be used to assess viability, cell count, purity, identity, proliferation and potency. One of the barriers to successful translation to cell manufacture can often be taking these complex assays away from the green fingered-flow cytometry expert in an academic lab who can make the assays "work", and generating a reproducible and robust assay that can be carried out by any trained operator. Key to this is a true understanding of the design and validation that a GMP-compliant QC assay requires. One approach is through the application of Cellular Metrology – systematic capture of defined metrics to fully describe a cellular population. This is a powerful but complex approach and we direct readers to a recent review highlighting the principal areas to be addressed in analysis of cellular products, in a digestible format [1].

THE REGULATORY ENVIRONMENT

There is no single set of regulations regarding the quality control of cellular products, and products may cross geographical and jurisdictional boundaries during the manufacturing process. In this complex supply chain and manufacturing scenario e.g. in the manufacture of CAR-T cells, the leukapheresis starting material may first be simply collected and stored and is thus regulated as a tissue or non-manipulated cellular product. Subsequently, the product undergoes shipping, thawing, expansion, genetic manipulation and cryopreservation. This part of the process is commonly regulated as an (investigational) Advanced Therapeutic Medicinal Product (ATMP), subject to the GMP-regulations within each territory. (Useful links to European and American product definitions can be found here [2,3]). This means that establishment of good practice in terms of analysis is the responsibility of the individual centre and requires thorough validation for the cellular analysis in each step of the process.

Designing cellular analysis to give meaningful, accurate and robust results to support manufacturing is a complex area for the uninitiated. There are a variety of resources available which are helpful, including defining rigour in assays in general, and some cell type-specific guidelines - a selection are highlighted in Table 1. Many of these resources have been developed in the diagnostic flow cytometry field, and the rigour applied here to reagent selection and assay qualification translates well into good practice for cellular therapeutics.

In the first section of this paper we discuss the strategic choices to be made in designing and initiating flow analysis for cellular therapy products, and in the second section we discuss practicalities of acquiring and analysing data, based on the experience within our own labs.

INITIAL STRATEGIES IN DESIGNING FLOW CYTOMETRY ANALYSIS OF CELLULAR PRODUCTS

Flow cytometer choice, set-up & validation

There are no overarching standards for flow cytometers for use in the cellular therapy industry, but any machine chosen should meet a rigorous user requirement specification in terms of specification, safety and reliability. Devices must be validated through manufacturer's Installation Qualification / Operational Qualification (IQ/OQ) and backed up by a regular maintenance schedule. Certification of conformity (CE marking in the EU or 21CFR part 11 / CSA equivalents in North America) can be an advantage in showing that the equipment meets appropriate safety standards such as access -controlled operation and other parameters. The choice of a machine marked as "IVD" (in vitro diagnostic)-compliant may be a good route to identifying a machine with GMP/GLP-features built in but this does not make a machine "GMP", as IVD certification will be tied to specific assays and reagents.

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TABLE 1

Useful resources when embarking on cellular characterization studies.

BSI publicly available specification (PAS)	Characterization of human cells for clinical applications: Guide. BSI publication, 2011; PAS 93:2011 [4]
Extensive suite of documents on manufacture and analysis of ATMPs	Guidelines relevant for advanced therapy medicinal products [3]
Flow Cytometric enumeration of $CD34^+$ cells	Flow Cytometric Enumeration of $CD34^+$ cells USP <127> [5]
Minimal information to collect to describe T cell products	Janetzki S. Britte CM. Kalos. *et al.* "MIATA"—Minimal Information about T Cell Assays. Immunity. 2009; 31: 527–528 [6]
Criteria for standardisation of mesenchymal cells	Dominici M Le Blanc K. Mueller I. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8: 315-317 [7]
Euroflow Consortium publicly available SOPs	A variety of publicly available cell staining and machine set-up protocols. EuroFlow [8]
International Clinical Cytometry Society Resource Centre	A variety of "ask the expert" and more formal learning resources [9]

Multiple-laser devices are now standard in all laboratories, and the majority of "GMP" assays will likely be performed on machines with at least 3 lasers that can interrogate 8-10 different fluorochromes. This does not mean that the majority of assays will examine 8+ fluorescence markers, rather, 4–5 are more typical (Figure 1). However, multiple lasers allow for greater "spacing out" of fluorochromes, using the full bandwidth/spectrum across the lasers and strong fluorochromes reduces spectral overlap and the need for compensation.

Performance Qualification (PQ) of the flow cytometer will involve regular checks on the laser and fluidics and analyse performance over time in validated cellular assays.

Flow cytometers, regardless of manufacturer, require a method for validating that the machine is performing within specification and this is commonly through the use of manufacturer-supplied polystyrene beads. These beads have a range of sizes and fluorescence properties and can be used to measure performance and alignment of the lasers and detectors, comparing the data against baselines and previous runs. Here, IVD-certified calibration beads are of use in validated performance assays. While there are many different programs available for analysis of flow cytometry data, it is important the data generated is in an un-manipulated form which can be electronically stored for extended periods (years to decades). For example, the Flow Cytometry Standard (FCS, current version FCS 3.1) format is commonly used, although some manufacturers use other standards [10].

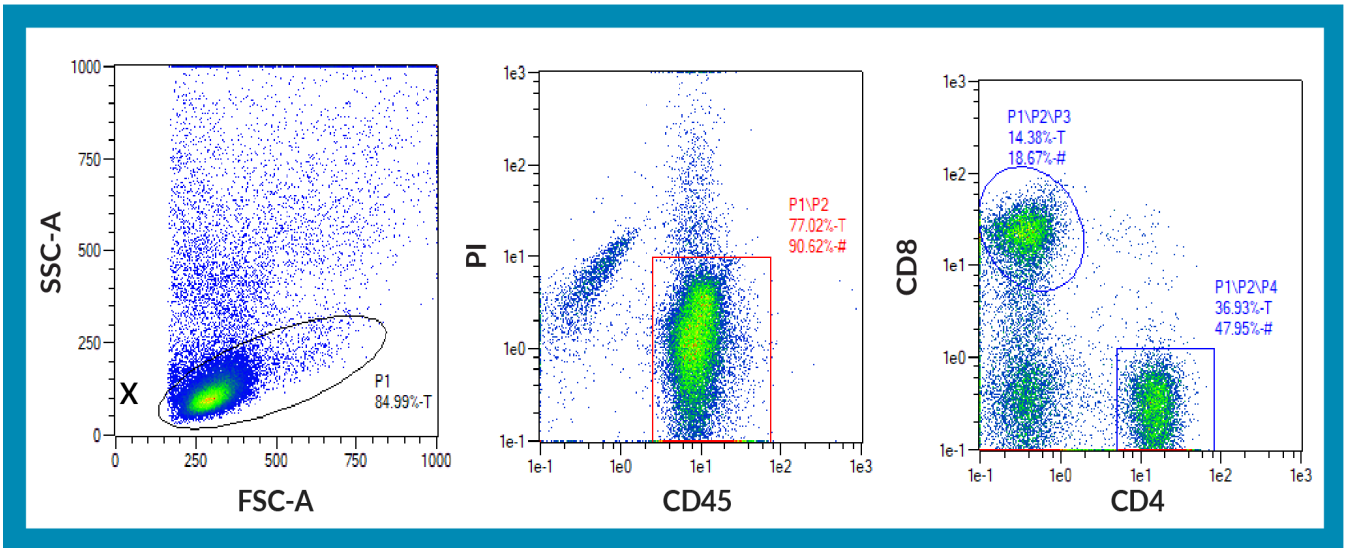
SAMPLE PREPARATION: GENERAL PRINCIPLES

Staining controls

Antigen positive cells can be accurately identified through the use of controls to distinguish from signal caused by instrument noise, spectral overlap, autofluorescence and non-specific antibody binding. The 2 main controls used are isotypes and FMOs (fluorescence minus one). Isotype control antibodies replace test antibodies with an antibody of the same immunoglobulin class and conjugated to the same fluorochrome but that does not recognise any of the antigens present on the cells. Use of such an antibody can determine how much of the fluorochrome signal is due to non-specific binding, and the isotype control is commonly used to set baseline fluorescence levels. This is valid providing that the isotype control has the same number of fluorochrome molecules per immunoglobulin and is used at the same concentration as the antibody it is replacing. It can be difficult to obtain an isotype that meets this requirement (and manufacturers realistically cannot make exact matches for each test antibody)

FIGURE 1

T cells analysed with a debris exclusion threshold marked with "x" in the FSC/SSC box.



Live Leukocytes are then positively identified as $CD45^+$, excluding PI viability dye. $CD4^+$ and $CD8^+$ T cells are positively identified as $CD4^+CD8^-$ and $CD8^+$ $CD4^-$ respectively.

so isotypes are commonly used at matching protein concentration only and so are limited to providing basic background staining information. It is not good practice to set a strict positive/negative threshold based on isotype staining alone.

FMO samples are labelled with all the antibodies of the staining panel except for one. This allows the baseline fluorescence in the context of spectral overlap and background noise to be determined for each fluorochrome. These controls can be used to set the boundaries and gates for positive populations. It is considered best practice to use FMOs rather than just isotype controls to set the boundaries for populations. Once analysis templates are validated, they must be locked to ensure consistency across all samples measured. It is not within the scope of this review to discuss gating strategies, but these must be thoroughly validated before locking down.

SAMPLE PREPARATION PROTOCOLS

When preparing samples for flow cytometric analysis the aim is to minimise both cell loss and alteration to the composition of the cell population being tested. This is particularly relevant where flow cytometry is used as a single platform to enumerate cell counts and viability as well as to determine phenotype. It is therefore beneficial to design strategies to reduce sample processing wherever possible. Whether fresh starting material or cultured cells, flow cytometry assays are robust in terms of staining a small unmanipulated sample with antibodies, and then diluting in a suitable buffer before analysis. An example of this is the clinical enumeration of $CD34^+$ hematopoietic stem cells from whole blood samples [5] for which protocols have been developed that omit any washing step to reduce loss, so called 'Lyse, no wash' protocols [11] (the erythrocytes are removed with lyse buffer prior to running on the flow cytometer to allow discrimination of the leukocytes). We also apply this technique in our laboratory, extending it to use with cultured cells diluted with buffer. This omission of any centrifugation step can facilitate discrimination of cell populations and debris in the cultures (Figure 2). (See also analysis of thawed samples section).

The measurement of culture debris remains a vexed question – it is standard flow

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cytometry practice to set a threshold to only acquire events of a certain size (Forward Scatter – a logarithmic scale may help discriminate debris). Equally, the presence of a large amount of debris in the analysis could indicate manufacturing issues and may represent a contaminant in the final product. Each centre is currently required to set their own policies on thresholding debris – a sensible limit may be <1-2um, for example. Identification of intact nucleated cells and distinguishing them from debris can be aided by the use of a cell-permeable nuclear stain such as DRAQ5 (Figure 2). The viable cell population can be determined using a positive inclusion marker, such as CD45 for blood cells, and a vital dye such as Propidium Iodide (PI), or DRAQ7 (Figure 1).

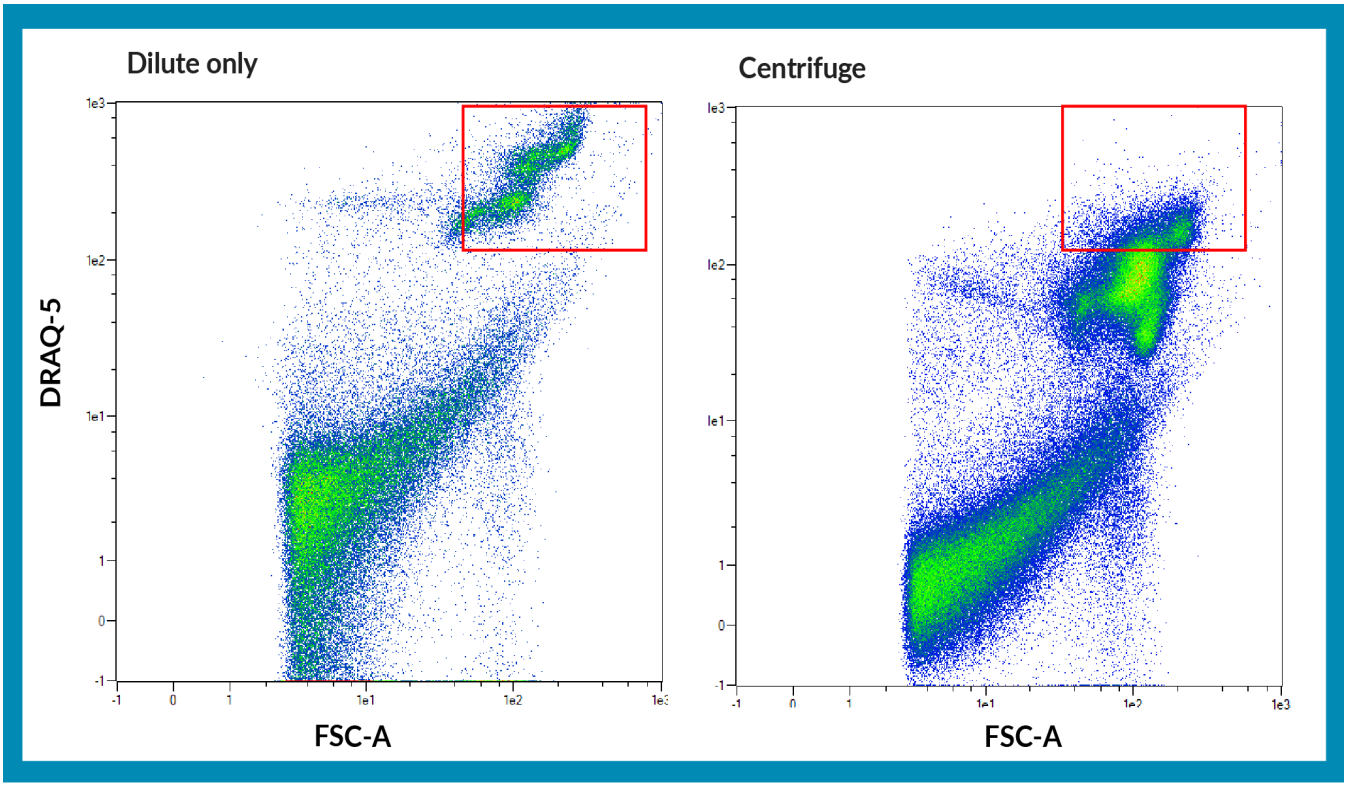
It may be desirable to fix samples after preparation, e.g. to facilitate transport of samples to the analyser, or to allow samples to be analysed in batches. Stability of the stained samples can be extended by treating them with a fixative such as 4% paraformaldehyde. However, fixation commonly alters scatter properties, may not be compatible with some (especially tandem) fluorochromes, and requires the use of fixable live/dead dyes to be added to the cells before fixation. Ad hoc fixation is likely to increase the risk of an out of specification result. If fixation is thought to be regularly required e.g. for intracellular cytokine assays in T cells, the process should be validated with 100% fixed samples.

ASSAY CONTROLS

Release criteria commonly include viability, an accepted range of cell-specific markers and a limit to the number of unwanted cells. Establishing these assays on the flow

FIGURE 2

Complex mixture of cultured leukocytes processed by dilution only, or by centrifugation and resuspension.



Nucleated cells identified by staining with DRAQ5 dye (stains nuclei in intact cells). Dilute only culture shows 5–6 distinct populations of cells based on size – red box distinct from debris. Centrifuged sample shows merging of distinct cell populations and less distinct staining of cells versus debris.

cytometer involves a process of development and optimisation followed by validation. Where available, cellular reference standards can be used (examples of stabilised blood cells or specific T cells [12,13]) to assess the accuracy and precision of the assay. However, there are currently very few available and this is a major limitation in validating the accuracy of flow cytometry assays, reflecting the relative novelty and variety of cell therapies that are entering GMP production. In the absence of reference standards, cells produced either in-house or commercially, that express the epitope(s) of interest can be used as a 'ruler material'. A ruler is a cellular material that is known to express the epitope of interest and can be used to confirm antibody performance. Alternatively, for some situations beads which bind antibodies via Fc receptors can be used to help assay set-up but may not closely resemble staining on cells.

The sensitivity of the assay must also be determined. For instance, if the release criteria are ≤2% of unwanted cells then the assay must be proven to be able detect ≤2%. Detection of other cells usually relies on epitopes that are not present on the cell product, but are expressed on the cells most likely to contaminate. While reference cells can be run separately, it is highly preferable to "spike" the test product with known numbers of ruler material cells. Spiking in of such a cell type or a surrogate that expresses the necessary epitopes can be used to determine the sensitivity of the assay when used on the actual product. Producing a range of spiked samples also allows the assay to be checked for linearity, the amount of contamination detected should be in proportion to the level of spiking.

DESIGNING FLOW CYTOMETRY ANALYSIS FOR CELLULAR THERAPIES

Release assays: identity

In designing a QC assay for final release of a cellular product, it is vital to distil the information required into a minimum data set. This often goes against the instincts of the keen cell biology researcher with powerful analysers to hand. However, it is standard practice to include the minimum number of criteria needed to identify the product and distinguish it from other cells. The identity results will form a large part of the release specification, as part of the target product profile and may describe both the product and the levels of contaminating cell types. Meeting this specification is an absolute requirement and is not open to interpretation – if a product fails to meet specification it cannot be used, thus assays must be as minimal as possible. As an example, a virus-specific memory T cell product can be readily defined as a live leukocyte, expressing CD4 T cell lineage and T cell memory markers. This is easily described using doublet discrimination, a live/dead marker, CD4 and or CD3, CD45RO, as illustrated in Figure 3. This analysis is aided by the clear delineation of the different T cell populations allowing for confident gating of the different populations. This analysis could be augmented by the addition of e.g. CD19 or CD56 to show that unwanted lymphocytes are not present in the culture.

This identification of cells by positive expression of a number of markers, and absence of others is well illustrated in the analysis of mesenchymal stromal cells (MSC). Phenotypically, MSC are required to express CD73, CD90 and CD105, but not express haematopoietic markers such as CD34, CD45 and CD11b [7]. These phenotypic assays are sufficient for day to day analysis but final confirmation of MSC identity also relies on cell culture tests – a cell is only a true MSC if it can be differentiated in vitro into 3 lineages, osteogenic, chondrogenic and adipogenic [7]. This is a complex assay to validate and requires long-term culture in differentiation medium and an immunohistological read out. Developers may choose to rely on passing the flow cytometry tests during expansion of MSC isolates, and to perform the complex culture tests at the end of the process.

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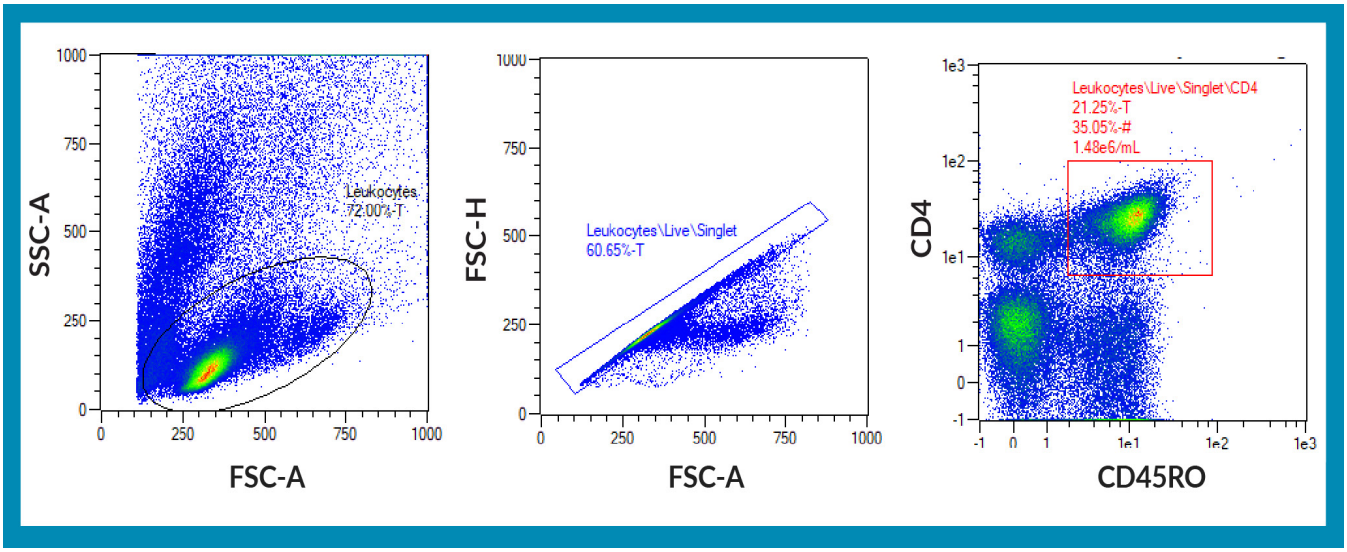
FIGURE 3

Typical T cells analysis. A leukocyte gate is set using scatter, dead cells are excluded (as in Figure 1).



Doublets are excluded from the analysis by plotting FSC area versus height. CD4 memory T cells are positively identified as $CD4^+$ $CD45RO^+$.

Beyond simple phenotyping to potency

Measuring the potency of a cellular product is also part of the release specification, and thorough guidelines have been developed – for example in products to treat cancer [14]. As discussed in this guidance, measuring potency can be done directly – e.g. CAR-T killing of targets expressing the receptor cognate for the transduced CAR, or can be done through the measurement of a surrogate potency marker. This can be through expression of e.g. cytokines as surrogates of cytotoxicity, or through expression of surface molecules that correlates to a biological effect. In our lab we have developed measurement of the cell surface marker CD206 as a surrogate for phagocytic activity in therapeutic macrophages [15], and also measurement of intracellular expression of IL-2, IFN-Gamma and TNF-alpha to assess the potency of T cells [16].

Even when there is a direct potency assay for a therapeutic cell – for example suppression of T cell proliferation by MSC, there are challenges in setting up the culture-based assays: the precise mechanism by which MSCs mediate immunomodulation is not known, the activity of MSCs shows variation across MSC donors and responder donated T cells and there is a lack of reference standards. The ISCT has published guidance around QC assays for characterising MSC potency including assays to directly assess immunomodulation [17]. In one iteration of this assay peripheral blood mononuclear cells (or T-cells or purified T-cell subsets) are labelled with a cell tracker dye, stimulated to proliferate and then co-cultured with MSCs usually for 6-7 days. At the end of the assay the difference in proliferation of the T-cells cultured alone versus co-cultured with MSCs is quantified. There are many parameters to understand and control in this type of assay and a review of these factors can be found here [18]. Setting pass/fail limits on this type of assay must be done pragmatically to take into account the natural variability in the donated test and responder materials.

One critical aspect in the use of these complex culture-based assays is the environment in which they are performed. These assays really make a bid for all of the Ps in GMP - people, premises, processes, products and paperwork. Many functional assays are based on well-known research lab assays, but these are not commonly controlled in a sufficiently rigorous manner. To fully comply

with GMP and/or GLP every reagent, cell line and SOP must be controlled and meet a rigorous set of criteria to ensure that the assay can be properly validated and performance checked. This also extends to all aspects of laboratory equipment used in the assay – controlled space, temperature-mapped fridges and incubators, analysers fully performance monitored etc. This is a real challenge for a developing organisation to comply with, and critical performance assessment of these assays is needed to make them meaningful.

For Information Only (FIO) assays

FIO assays are performed on the final cellular product, but do not form part of the release specification. These assays are a valuable tool in understanding the extended phenotype of the cellular therapy and may be used to build a dossier of information, without addition of extra markers increasing the possibility of failing to meet one or more release criteria. Many more complex FIO assays will eventually become part of the release specification – commonly these are tested extensively as FIO, still building valuable information on the product, before they are incorporated into a release specification.

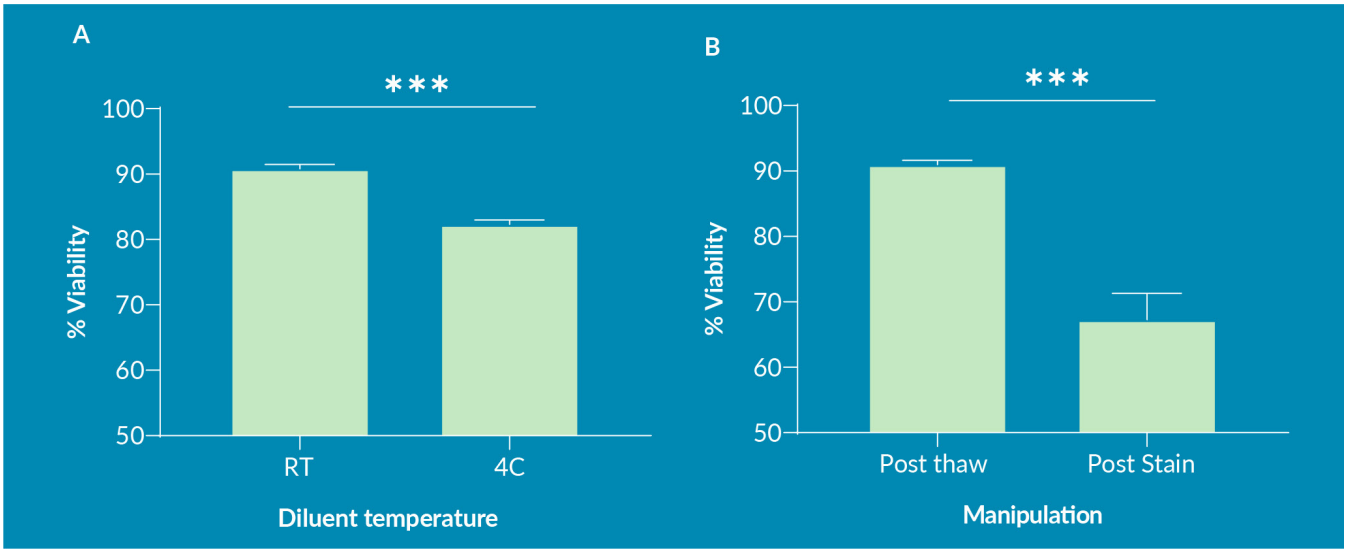
ANALYSING CRYOPRESERVED CELL PRODUCTS

The vast majority of cellular therapies are cryopreserved, as well as starting or intermediate materials during production. These cryopreserved products must continue to meet specification after long term storage and demonstrate proven stability and shelf life. Thus, the original release or acceptance criteria must also hold for the cryopreserved product. This presents a real challenge as no in vitro assay models the effects on the product of thawing and directly infusing into the patient.

When designing post-thaw assays, the principles of minimal manipulation apply again – centrifugation can be particularly damaging to freshly thawed cells. Exposure of cells to cryoprotectant such as DMSO can increase the permeability of the cell membrane and incubating cells with viability dyes post thaw may result in an over-assessment of cell death due to this transitory leakiness. It is also likely that cells which have been damaged due

FIGURE 4

Cryopreserved PBMC were assayed for viability using DRAQ7 immediately after thaw.



Viability was determined with dilution only (no centrifugation) using room temperature or 4°C diluent (A), or after processing for antibody staining (including washes and centrifugation steps at RT) (B). Mean from 4 different cryopreserved donations + standard deviation, *P<0.005.

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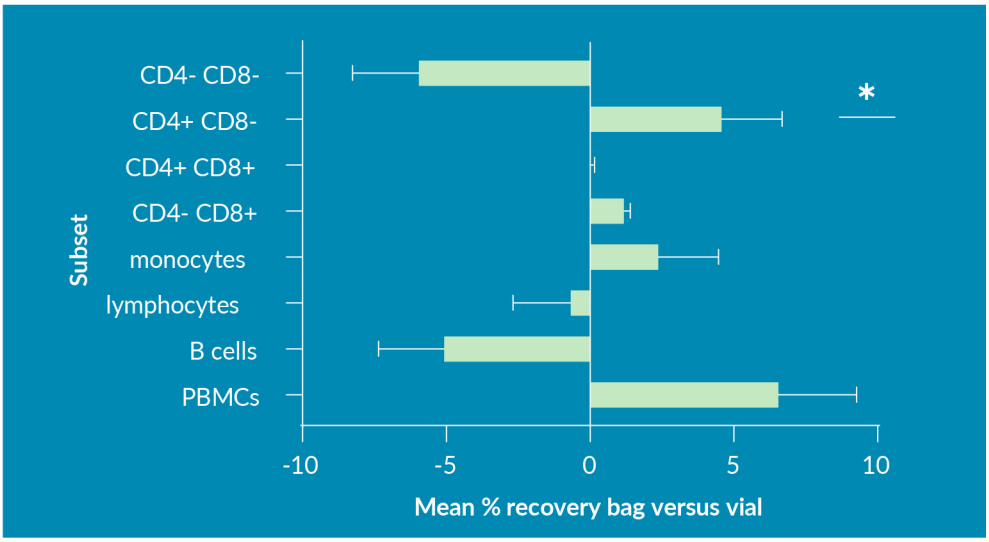
FIGURE 5

Cryopreserved PBMC were assayed for common subsets after being processed for antibody staining (including washes and centrifugation steps at RT) (Figure 4B).



Graph shows mean difference in subset recovery from vials compared to bags (bags = 100%). Mean from 4 different cryopreserved donations + standard deviation, *P<0.05. Only $CD4^+CD8^-$ subset shows a statistical difference.

to freezing or thawing may rapidly die post thaw. In our labs Peripheral Blood Mononuclear cells (PBMC) are sensitive to diluent temperature (diluent at room temperature is preferable to that at 4^0C and post-thaw centrifugation (centrifugation increases measured cell death) Figure 4.

Bags versus vials

A further issue commonly perceived is that QC samples stored in 2ml vials or small pouches attached to the cryobag may not truly represent the stability of the main product in a cryobag. The samples in these pouches or cryovials are processed alongside the cryobags and so experience the same freezing profile and storage conditions, however, the difference in their dimensions to the cryobag can affect physical properties such as rate of heat transfer during both freeze and thaw. This may be addressed by simply sacrificing product bags for QC, but this is not applicable to scarce or small-volume products. In our lab we have carried out analysis of products in bags and vials specifically to address whether these perceived differences are found in the real world. An example analysis on peripheral blood mononuclear cells is detailed in Figure 5. Overall, in carefully controlled conditions, there were few significant differences in recovery of blood cells from bags or vials, with the exception of $CD4^+$ T cells in this case. The true accuracy of vials versus bags will therefore require to be determined on a case by case basis for each product.

FINAL REMARKS

In this review we have highlighted many common aspects of flow cytometry analysis for cellular therapies. Much of what is discussed are based on good practice in flow cytometry and assay validation. The reader is urged to read the in-depth regulatory and policy papers highlighted in the text.

Data sources & research ethics

No new biological samples were collected for this expert insight article. All data shown in this review, as examples of flow cytometric analysis techniques, are from biological samples donated under fully informed consent with explicit consent for research use, and institutional review board ethical approval, as required.

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AUTHORSHIP & CONFLICT OF INTEREST

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PODCAST INTERVIEW

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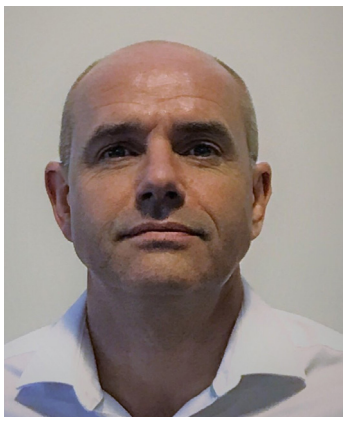

Get in line: developing in-process analytical tools for gene therapy applications

Cell & Gene Therapy Insights 2021; 7(1), 23–32

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Q What is variable length technology, and how is it different from other instruments on the market such as Raman and in-line refractometers?

RS: Variable pathlength technology (FlowVPE) is a subset of UV absorbance spectroscopy. It uses a precision linear stage to change the pathlength of the absorbance measurement, and creates a regression of absorbance versus pathlength.

This allows you to avoid sample prep – namely dilution of samples – and the error associated with it. It also provides an opportunity to take a direct measurement in-line, and use it as a process analytical technology (PAT) tool.

We like to call it slope spectroscopy. Instead of taking a single absorbance reading, the linear regression improves the robustness by taking multiple readings. Our in-line instrument can do this in five seconds, and this gives you a high quality measurement of concentration versus time for any purified material during the process.

It is quite different from things like Raman, refractometers, or densitometers. It is very direct, easy to understand, and simple to implement. Raman is a very powerful technique, and it has the ability to look at many different parameters, but you need to understand it well. Variables such as probe tip geometry, calibration, and how you analyze the data, can affect your measurement and therefore the quality of the data you get. Whereas with absorbent spectroscopy it is very straight forward.

Something like a refractometer, in my opinion, is a bit sensitive for a concentration measurement. Factors such as temperature, density, and other buffer conditions can affect the outcome, and you have to properly calibrate that ahead of time to ensure you get a quality measurement.

JW: A refractive index probe is sensitive to matrix, to the point where if you are doing inline monitoring and the matrix is changing – as it often is in bioprocessing – you cannot obtain highly accurate protein concentration monitoring. It is only accurate, or in other words within 5% of the true value, when the matrix is not changing. This is my understanding as to why RI technology has not widely adopted to this point.

One of the major advantages of VPE is that it requires no specific model and is matrix insensitive. Each protein has its own ideal setting on the VPE to achieve best accuracy, but in general, you can use the platform method and be within 5% of true concentration. That is one of the biggest advantage of VPE: it is very quick to set up for any biologic (protein drug).

Q Jay, with regards to process analytical technologies, and in-process control testing, what led to your team exploring new technologies for protein concentration?

JW: We started using FlowVPE technology several years ago because the technology was analogous to the SOLOVPE, which has been in widespread use throughout the industry for over a decade.

One reason we implemented it was because of a trend in increasing in protein concentration for a drug substances. The previous range of drug substance concentration was 5-50

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g/L. Based on technology allowing for subcutaneous injection the DS concentrations increased manifold, to over 100 g/L. This is a trend where the biopharma industry is pushing the limits of how much protein drug you can put into a single dosage in a small volume and eliminate the need for IV transfusions, at least for some drugs. As protein concentration increases there is less room for error because we are near the point where precipitates can form. The final step in mAb drug substance manufacturing, the downstream ultrafiltration and diafiltration operation, is the process where we have used the FlowVPE as a development tool.

> "Our in-line instrument ... gives you a high quality measurement of concentration versus time for any purified material during the process."

Another consideration was to implement a semi-continuous process for biologics manufacturing. Before final DS formulation an offline measurement is required, and this can take several hours fora QC group using the traditional cuvette and A280 method. It is not truly continuous process, which no one to my knowledge is using for GMP manufacturing of biologics now. Our team created a hybrid process incorporating elements of continuous manufacturing to streamline the process. The cadence between batches can be halved using these improvements along with PAT incorporated into the control strategy.

Q A risk mitigation strategy is key to a control process. How does Bristol Myers Squibb approach the overall strategy, and how does process analytical technology play a role in that strategy?

JW: PAT is a major evolutionary step forward in bioprocessing, and companies are adopting it based on its potential for return on investment. Because of so many uncertainties and unknowns for so long on how much investment is required to get this into a GMP space, change has not been as rapid as first anticipated. . There has to be a really strong reason to make a change that can increase efficiency while also improving batch to batch consistency and quality, before biopharma buys into the promise of PAT

However right now, in terms of data analytics and process monitoring, it is going to be valuable without needing to be used in the control strategy.. The industry consensus is that this inline VPE instrument will be relatively simple to put into GMP, for a PAT technology.

Q At what point of the development process is it important to establish your in-line analytics plan?

JW: In-line analytics really has to be tested extensively at bench scale then in a pilot scale facility before you move to GMP. When you start your validation, you want to have already performed many engineering runs to establish robustness and do a real time risk assessment.

As I've mentioned before, the FlowVPE is that it is a platform tool that can used across an entire portfolio. Therefore the return on investment into development of this instrument can be justified, because it is going to be used throughout the portfolio.

RS: I have seen a lot of these implementations happen, and in my experience there is no real right place to put it. There are areas or times during the development where it is easier and more straightforward to do it, but I have seen people put this kind of tool directly into the commercial process. It does take a little extra work and validation time, but it can be done. Or like Jay said, I have seen people go through development then put it into the clinical process.

With regards to cell and gene therapy, there is an opportunity to put it in, because the modality is pretty new, and a lot of these manufacturing areas are being built. When you are starting the implementation of a manufacturing site, this is the perfect time to look at PAT and bring it into a plant.

Q What validation is required to introduce an in-line analytical measurement system?

JW: We have an established precedent with the SoloVPE, a very similar instrument, and gives a good starting point to design a validation study. However, it requires some creativity to design a validation, because it is an inline instrument.

The hardware validation, specifically of the flow cell, is incredibly important, because the flow cell has to have very low bioburden. The industry now uses mainly single use hardware, so if a single use flow cell can be developed that will make validation simpler.

Another important area of validation is the software. Clearly the software must be CFR-compliant for use in a GMP facility..

Q What team members and experts are involved in the decision making process? Where are the in-line testing strategies developed, and when are they implemented?

JW: It requires a large cross functional team to develop PAT.

PAT implementation into GMP is something that is still evolving, and the entire industry and regulatory authorities are working on the roadmap. The strategy really developed from the bottom up: from bench to pilot to GMP scale. At each point in the process more stakeholders have become involved and sharpened the strategy

Q What process analytical tools or methods show the greatest promise in both upstream and downstream applications?

JW: In-line liquid chromatography has a lot of promise but also complexities. The hurdles are greater because of the validation process and how many moving parts are part

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> "...the FlowVPE is ...a platform tool that can used across an entire portfolio. Therefore the return on investment into development of this instrument can be justified, because it is going to be used throughout the portfolio."

of that PAT technology, including the autosampler. In terms of the potential value of the data to the process, it is high.

There are other in-line probes with high potential for PAT applications, with FTIR and Raman the hottest technologies right now.

Q What BMS learnings should a gene therapy manufacturer take into consideration when developing their in-line testing strategy?

JW: Perform extensive offline analytical testing for comparability, to ensure the inline method is robust and accurate. The hardware has to of be the right dimensions for the process. If you need some custom sizing application, you should start working with the inline probe vendor right away and tell them your needs.

Do not underestimate the validation procedure for flow cells. I would start looking into this early in the process sand figure out your needs. If you do not need single use, it may be more economical to use stainless steel flow cell. But start figuring out the validation procedure to sanitize inline as early as possible, as that can be the most time consuming part of GMP validation.

Q Gene therapy developers are using a lot of the mainstream analytical solutions such as monoclonal antibody solutions, simply because there aren't many analytical solutions specific to gene therapy. Would the same FlowVPE system benefits apply to gene therapy manufacturers?

RS: Gene therapy is an exciting and accelerating field, and it is natural to apply technologies from the mainstream, such as MAB production.

We are still understanding where the VPT, or variable path link technology, can be applied in this area. We have had some good preliminary data. In theory, the FlowVPE, or slope spectroscopy in general, should give all the same benefits in gene therapy manufacturing as it would in MAB manufacturing. The straightforward and easily validatable method, the robustness of the data, repeatability of the method, and the relative ease of use and implementation should all apply.

There are differences I see between MABs and gene therapy. In gene therapy the value of the product is extraordinarily high; it is expensive, and needs to be better tracked. That lends more strength to the argument of using PAT to improve the quality and process time. It is also a new industry where new manufacturing plants are being built, and this is the perfect opportunity to put in PAT. I predict it will make it a little bit easier to validate some of these things, in comparison to the MAB field where they already have established technologies you may have to displace in order to put something new in.

Q Have you seen process analytical technology successfully implemented in a gene therapy process as yet?

RG: The short answer would be not much, if at all. I think this is because there is still a lack of knowledge and characterization of gene therapy products.

PAT is part of the global quality by design initiative. Applying PAT means you have already defined your target product profile (TPP) that is informing on the product's critical quality attributes. You are able to then define the critical process parameters that you would have to measure, monitor, and control, to make sure that your process is running well, and your product quality is kept within the defined acceptable range.

We are just not necessarily there yet with a lot of gene therapy products. The viral vectors injected into the patient, or in the case of CAR T therapies the modified cells infused into the patient, are much more complex products than the recombinant proteins and monoclonal antibodies that have been used now for decades.

A better, or deeper, characterization is required for gene therapy products – for example, the route of administration, the dosage, the tissue specificity, and so on. Then, the implementation of PAT will make sense in the context of the manufacturing processes.

There are many ongoing developments with the hundreds of clinical trials currently underway, and this makes us learning very fast in this context. Along with the experience acquired from previous developments in MABs and recombinant proteins, this will accelerate the learning phase.

We see a major focus on developing robust and reliable analytics in the gene therapy field. PAT will become more and more present in this context, as we learn more and advance into this knowledge space.

"The viral vectors injected into the patient ... are much more complex products than the recombinant proteins and monoclonal antibodies that have been used now for decades."

JW: No I have not, but gene therapy is an emerging field where I anticipate PAT applications will have a significant impact. It is arguably easier to incorporate PAT into new manufacturing processes like those used in gene therapy than fit into an older existing process.

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Q Where is process analytical technology being implemented in the gene therapy workflow? What are the essential process measurements currently identified?

RG: As I mentioned, PAT is not extensively implemented. Mostly in the sense that we see PAT as an in-line tool, for in-line analytics, but in reality, the current measurements that are used for gene therapy processes are almost entirely performed offline. For instance, the qPCR and ELISA used for product quantification, the electronic microscopy used for product characterization, or the flow cytometry used to indicate the product activity – all of these measurements are performed offline. With, I would add, quite low precision and accuracy, or long turnaround times in some cases.

Having said that, there is some PAT used for plasmid and viral vector manufacturing processes. I am thinking here about the standard sensors that we use in the process like pressure, temperature, pH, conductivity, and UV. These are PAT tools that are used in the context of the UF/DF product concentration and formulation steps.

In the purification steps with chromatography, UV spectroscopy is a critical tool to first develop a robust separation and purification process, and is being applied to monitor the process at clinical or commercial manufacturing scale. These are the unit operations where we see some PAT already used in gene therapy processes.

Q René, based on what we have heard from Jay and Ramsey, how do you think that slope spectroscopy technology could benefit gene therapy?

RG: Slope spectroscopy is a UV-VIS spectroscopy method, and I see an obvious use for it where the same, or similar, UV spectroscopy methods are used already.

The obvious question then would be, what for? What would be the added value compared to the existing sensors or measurements? As Ramsey mentioned, the standard UV method is often limited by the saturation of the signal as the product concentration goes up. We see this a lot for plasmid DNA processes. With slope spectroscopy, the user could use the SoloVPE to measure the product concentration without the need to dilute the sample. Then we can think about in-line measurement with the Flow VPE, and being able to plug the sensor in-line with the TFF skid when we do the UF/DF steps in the process. That would be an obvious need.

I see slope spectroscopy as giving us an extra dimension, or I should say an extended dimension, in which to apply UV spectroscopy. It creates a much bigger design space to explore and look at where to use this extended capability. We are working with selected partners to investigate that extra space, and are already collecting some very promising data.

It is too early to communicate extensively on this, but we are at the beginning of the story of implementing spectroscopy in the gene therapy processes. There is a lot to explore, and lot of potential.

BIOS

René Gantier

René Gantier is the Director of Technology for Gene Therapy at Repligen. He has almost 20 years of experience developing production and purification processes for biotherapies. René holds a MSc in Cell Biology from the University of Nantes (France) and a PhD in Biochemistry from the University of Rouen (France). He started his career in the biotechnology industry as a scientist developing long-lasting therapeutic cytokines at Nautilus Biotech (France), before joining Pall Life Sciences as a downstream processing field application specialist, and then expanded his role to lead the Biotech Process R&D team developing end-to-end upstream-downstream continuous bioprocessing solutions for mAbs and cell/gene therapies. In his current role at Repligen, he provides technology guidance to develop high productivity next generation gene therapy manufacturing processes.

Ramsey Shanbaky

Ramsey Shanbaky is the Bioprocess Applications Manger for C Technologies, Inc. He spent the first 8 years of his career working in R&D, Manufacturing and Product Management designing and building fiber optic products for high powered laser delivery in the medical device industry. In the first part of his tenure at C Technologies, he worked alongside his customers to implement the SoloVPE product with biopharmaceutical accounts primarily in the western region. For the last 5 years, he has been partnering with customers to implement the FlowVPE system for inline concentration measurements both in the process development areas and GMP manufacturing areas for bioprocessing. He holds an BSEE from Purdue University and multiple patents and applications.

Jay West

Jay West is Principal Scientist at Bristol Myers Squibb in the Process Development Analytics group in Devens, MA. He obtained his BS in Chemistry from Rensselaer, MS in Chemistry from University at Albany, PhD in Biochemistry from Boston College, and performed postdoctoral research at Northeastern University. He has 8 years pharmaceutical industry experience, having worked at Regeneron Pharmaceuticals precious to BMS. His focus the past three years has been on Process Analytical Technology, particularly in downstream process development.

The FlowVPE System uses patented variable path length technology to accurately measure various targeted concentration in the process stream (in-line). For more information on the FlowVPE, please click here

We hope you enjoyed reading this interview.
You can also listen to the recorded podcast here:

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INTERVIEW

A stepwise approach to process optimization in cellular immunotherapy development

JIM LU is the co-founder and CSO of TriArm Therapeutic Co., a company specializing in developing innovative cellular immunotherapies for unmet medical needs. Prior to joining TriArm Therapeutics, he worked at US FDA as a reviewer in the cell and gene therapy area. He was a recognized expert in this field and co-authored several FDA Guidance for Industries. Dr Lu was also the recipient of the CBER award for Excellence in Regulatory Science Research in 2015. Prior to joining the FDA, Dr Lu worked in the biotechnology industry as a senior scientist, manager and consultant in the development of viral vaccines and of viral vectors for cancer therapy.

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Q Can you briefly introduce us to the TriArm Therapeutics technology platform and R&D pipeline?

JL: Cell therapy has made great strides in recent years. Several products have successfully made it to market, and more are coming along the pipeline. As a company focusing on developing novel cellular immunotherapies, TriArm is striving to solve critical issues facing the whole field: high production costs, limited indications, underlying safety concerns, and

"...we want to create synergies among various platform technologies to reduce the burden of development work, and increase the productivity of developed processes."

more. Our mission is to bring safer, more effective, and more affordable, cell therapies to patients.

Through in-licensing and internal development, TriArm owns several technology platforms covering different aspects of cellular immunotherapy product development – gene delivery, safety improvement, indication expansion, activity prolongation, and relapse prevention. The company has built a rich pipeline based on these platforms, and we are now moving towards the clinic with our first project, a CAR T-cell therapy against B cell malignances which uses a third generation transposon-based gene delivery technology. We now have data demonstrating that CAR T-cells generated with this technology may have better antitumor activities, fewer safety issues such as cytokine release syndrome (CRS), and cells that may survive longer *in vivo* when compared to CAR T-cells generated with conventional viral vector-based methods. More importantly, the new process cut the production time from a few weeks to just a couple of days, which will significantly reduce production costs. TriArm's other projects include CAR/TCR T-cell therapies for blood and solid tumor indications, with unique CAR molecule configurations which can either reduce immune suppression, or better differentiate between healthy and malignant cells.

Q Can you outline the particular bioprocess considerations and requirements for the vector you use?

JL: As a 'live' product with specially designed features, cell therapy brings many challenges. During bioprocess development, we take into consideration regulations and guidelines from regulatory agencies, industry best practices, and lessons learned from our peers. The need for aseptic processing is emphasized throughout our whole process, and closed and single-use process steps are maximized, from bacteria fermentation and virus transduction through to cell culture. When selecting a particular vector for use, the safety, yield, stability and ease of manipulation are considered first. In some cases, procedures for characterization and quality control, GMP compliance, speed and production cost, scalability and reliability also play important roles in our decision-making.

Q Can you go into more depth on some of the key vector bioprocess steps, particularly those where you've been able to bring novel tools and technologies to bear to improve the bioprocess overall – for example, how have you been able to improve plasmid purification and sterile filtration?

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JL: We take a holistic view of process development. To us, the purification of plasmid starts at fermentation, not after. We optimized the upstream process to increase the ratio of plasmid to total biomass, thus reducing the burden on the downstream process. Each step of purification including bacteria lysis, clarification, chromatography and filtration were stepwise optimized. However, process steps and parameters were selected based on systemic outcomes, rather than by best individual step performance. Throughout development, the yield and purity of covalently closed circular DNA (cccDNA) is our focus. An infiltration device from Sartorius helped us a lot in completing the plasmid purification process – it is easy to set up and manipulate, and helped us to increase efficiency and get relatively high recovery.

Q And how have you been able to improve RV vector concentration?

JL: In addition to the considerations of safety and transduction efficiency, yields and concentration – both in terms of physical and biological titers – of virus stocks are important factors in designing a process. We optimized parameters such as timing of virus harvest, and type and concentration of protectants, for better outcome and stability of purified virus stocks. Importantly we found that in our hands, tangential ultrafiltration reduced virus loss and impurities of finished product compared to alternative processes. Now, we have a process that can produce sufficient virus stocks to support our early phase clinical studies.

Q What can you tell us about your preparations to address some of the chief bioprocess development aspects and priorities as your product candidates continue to advance?

JL: At TriArm, we take a long-term view in our operations. Firstly, we want to create synergies among various platform technologies to reduce the burden of development work, and increase the productivity of developed processes. Secondly, we begin with the end in mind when planning our activities. We want to have a process that is in compliance with regulations, scalable, and cost-effective from the very beginning of process development, although it may be realized with a phase-based approach. Currently, we are focused on planning for GMP facility process validation.

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SARTORIUS

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Optimization of AAV process development: transfection matters

Kelsey Wosnitzka, Shandel Pariag, Hélène Trottin, Alengo Nyamay'antu, Mathieu Porte, Malik Hellal & Patrick Erbacher

With the increased number of therapeutic rAAV candidates reaching the clinical trial pipeline, there is demand for innovative technologies to improve process development and facilitate manufacturing scale-up for future commercialization. To this end, Polyplus-transfection has worked hand-in-hand with viral vector manufacturers to develop a transfection reagent specifically for large scale manufacturing in suspension cell systems: FectoVIR®-AAV. FectoVIR®-AAV aims to improve rAAV manufacturing processes by boosting productivity, bringing flexibility and facilitating scalability. Here, we share preliminary data from Allergan Biologics' recent evaluation of FectoVIR®-AAV against their current AAV production platform. Analysis of physical titers revealed a 3-fold increase in both viral particles (VP) and viral genome (VG) per ml of cell culture when using FectoVIR®-AAV transfection reagent compared to PEIpro®.

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RAAVS AT CENTER STAGE FOR GENE THERAPY

Adeno-associated virus (AAV) are recombinant viral vectors used to deliver corrective gene therapies into target cells, a promising approach to address a wide spectrum of monogenic diseases from blood disorders, neurological and ocular diseases. Initially discovered as a contaminant of adenovirus preparation [1], AAVs have been propelled to the center of the stage due to their unique biological and

physical properties. AAVs are 25 nm small non-enveloped and replication-defective viruses that have yet to be linked to any known human diseases [2]. Unlike wild-type AAVs, recombinant AAVs (rAAV) are genetically modified to remove all viral coding sequences (rep and cap) of its 4.8 kb genome, thereby allowing the packaging of a transgene expression cassette of up to 4.4 kb in size [3]. Without its viral coding sequences, rAAVs function is restricted to being a protein-based nanoparticle that carries a DNA cargo into the nucleus of cells. In the absence of the replication gene (rep), the DNA cargo, once delivered into the nucleus, does not undergo site-specific integration into the genome of cells and instead persists as episomal DNA, as long as cells do not actively divide [4]. This latency as episomal DNA which permits long-term expression in cells largely contributes so far to the excellent safety profile and ensuing popularity of AAV-based gene therapy strategy.

The popularity of AAV-based gene therapy is also attributable to the existence of numerous AAV variants with tissue and cell specific tropisms. With the identification of a human cell line that could not be transduced by the first characterized AAV-2 [5], research on the transduction mechanism of AAVs led to identification of a combination of cellular receptors and co-receptors for AAV-2 to gain entry into cells. This led to the identification of presently 13 naturally occurring human and primate AAV serotypes (AAV1-AAV13) and more than 100 AAV variants across animal species [6]. AAV serotypes can be chosen for their tropism with the ability to preferentially transduce a cell or tissue type or their ability to have a broad tropism. For example, AAV2 with a known broad tropism has been approved as a viral vector for the treatment of an inherited form of retinal disease (Luxturna®), while AAV serotypes with a more specific tissue tropism have been approved for the treatment of inherited lipoprotein lipase deficiency (AAV1; Glybera®) and more recently inherited spinal muscular atrophy (AAV9; Zolgensma®).

ADDRESSING RAAV MANUFACTURING BOTTLENECKS: PRODUCTIVITY AND SCALABILITY

With the increased number of therapeutic rAAV applications reaching clinical trial, and the high doses of rAAV often being administered, production of rAAVs in sufficient amounts within acceptable cost limitations has become critical. rAAVs are mainly produced in human HEK-293 cells. This requires the transfection of HEK-293 cells with up to three plasmids containing elements needed for AAV viral vector assembly, with a plasmid carrying the transgene expression cassette, a plasmid carrying the rep/cap genes and a third plasmid containing helper genes, provided by adenovirus or herpes virus [4]. Co-delivery of these plasmids in cells is determinant to produce functional viral particles. The transfection method is therefore critical to ensure efficient co-delivery, in as many cells as possible and last but not least in a reproducible manner to ensure robust production yields irrespective of the manufacturing scale.

Currently, viral vector manufacturers are switching from adherent cell systems to suspension cell systems to reach higher production rates per batch and to develop flexible and scalable manufacturing processes [7]. To support this transition, there is a demand for a transfection reagent that can support scale-up of rAAV manufacturing by fulfilling the following criteria: (i) improve rAAV viral vector yields, (ii) ensure process scalability for large scale manufacturing, (iii) improve batch to batch reproducibility, and (iv) importantly comply with quality and regulatory requirements for GMP manufacturing and commercialization. Polyplus-transfection's latest innovative transfection reagent, FectoVIR®-AAV, addresses many of these requirements [8]: it is a novel class of animal free transfection reagent that is specifically developed for large scale transfection in suspension cell systems. Due to its particular physico-chemical properties, FectoVIR®-AAV can improve

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productivity at large scale, decrease batch to batch variability and simplify scale-up with a large-scale transfection protocol that addresses time and volume constraints [7].

CASE STUDY: ALLERGAN BIOLOGICS' EVALUATION OF FECTOVIR®-AAV

Allergan Biologics, an Abbvie company, is a Centre of Excellence for Biologics R&D, with a focus on the development and manufacture of gene therapy products. Due to their strong interest in the investigation of innovative technologies, Allergan Biologics recently tested FectoVIR®-AAV against their AAV production platform in place. Comparison of FectoVIR®-AAV and another transfection reagent, referred subsequently as "market alternative", was achieved side-by side with PEIpro®. PEIpro® transfection reagent (Polyplus-transfection) is optimized for small to large scale transfection of both adherent and suspension HEK-293 cells, for the manufacturing of viral vectors such as lentivirus, AAVs and virus-like particles. Since the availability of GMP (Good Manufacturing Practices)-compliant PEIpro® in 2018, PEIpro® has become the first PEI-based transfection reagent that can accompany viral manufacturers from process development to commercialization.

For viral manufacturers, the current challenge is to optimize AAV production platforms by reaching high production yields in HEK-293 suspension cell culture systems. In comparison to PEIpro® and the market alternative, FectoVIR®-AAV improved AAV productivity when tested against Allergan Biologic's rAAV production platform. AAV production yields were assessed by measuring physical titer in both viral particle (VP) and genome copies (VG) number per milliliter of harvested cell culture. FectoVIR®-AAV led to approximately a 3-fold increase in physical titers as measured in VP/ml and VG/ml compared to PEIpro®, and was also superior to the market alternative (Figure 1 & Figure 2). In addition, cell viability at the time of harvest was measured following transfection with PEIpro®, FectoVIR®-AAV and market

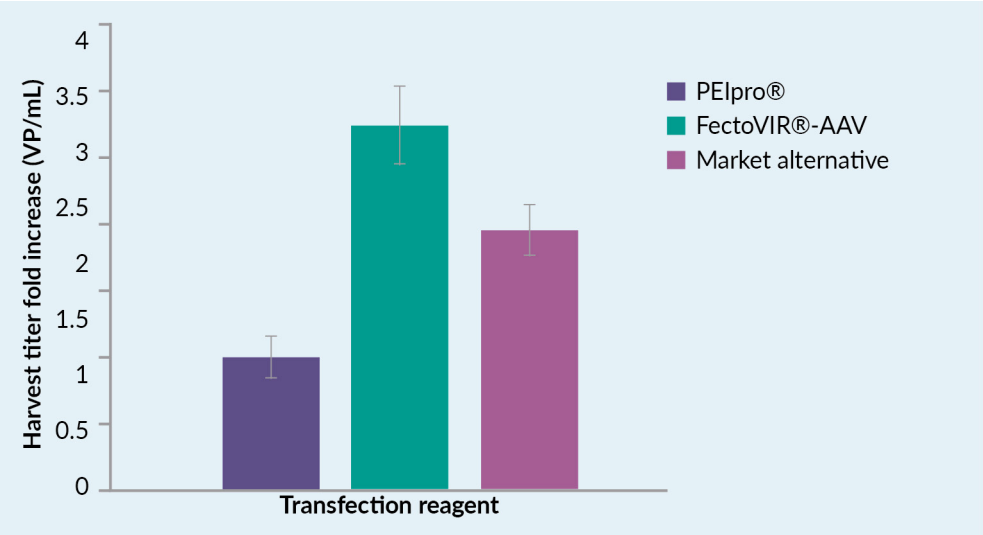
FIGURE 1

Improved harvested physical titer with FectoVIR®-AAV results in approximately a 3-fold increase compared to PEIpro®.

Suspension HEK-293 cells were grown in shake flasks and transfected with a set of three plasmids using either transfection reagent PEIpro®, FectoVIR®-AAV or a market alternative. The number of viral particles per mililiter of cell culture (VP/ml) at harvest is represented in fold increase. The number of viral particles (VP/ml) was assessed before purification using an ELISA-based method. Data are mean ± standard deviation (n = 3) per group.

FIGURE 2

Improved harvested physical titer with FectoVIR®-AAV results in approximately a 3-fold increase compared to PEIpro®.



Suspension HEK-293 cells were transfected with PEIpro®, FectoVIR®-AAV and a market alternative. The viral genome per ml of cell culture (VG/ml) at harvest is represented in fold increase. The viral genome per mililiter (VG/ml) was determined before purification using a qPCR-based method. Data are mean ±SD (n=3) per group.

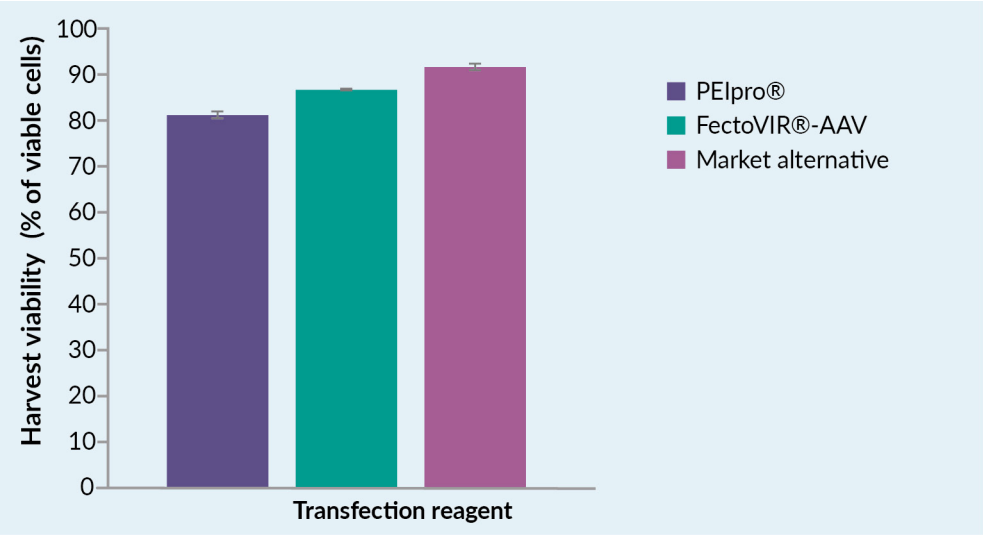
alternative. In all cases, cell viability was above 80% at harvest (Figure 3). A high viability at harvest has the potential to positively impact downstream processing due to an improved impurity profile.

MEETING COMPLIANCE WITH QUALITY REQUIREMENTS

In addition to improving AAV production yields, a transfection reagent also needs to fulfill regulatory requirements as growing

FIGURE 3

Similar viability at harvest with FectoVIR®-AAV compared to PEIpro® and market alternative.



Data are mean ± standard deviation (n = 3) per group.

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manufacturing capacity demands that all materials used in the production of AAV gene therapy vectors meet stringent quality and traceability requirements. Polyplus-transfection is a pioneer in the manufacturing of GMP transfection reagents for gene therapy, with the launch PEIpro®-GMP. The strategy for developing GMP-grade transfection reagent is therefore a fully validated process and FectoVIR®-AAV at GMP grade is expected to be commercially available Q2 2021, concomitantly with a residual test to support process related impurity clearance of transfection reagent throughout AAV manufacturing process.

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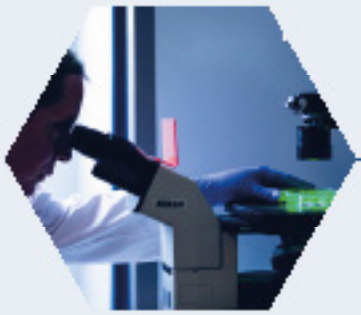

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FASTFACTS

Identify and Select Optimal T Cell Phenotypes

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PERFORMING MULTIDIMENSIONAL, FUNCTIONAL ASSAYS ON SINGLE T CELLS CAN ADDRESS KEY CHALLENGES IN ADOPTIVE CELL THERAPY DEVELOPMENT

When developing an adoptive cell therapy (ACT), identifying and selecting optimal cell phenotypes is a critical step – but identifying which cell characteristics correlate to clinical efficacy is an ongoing challenge for the field. The Berkeley Lights Platform allows the characterization and selection of T-cell phenotypes at a single-cell level, in order to ensure the best chance of developing powerful and effective therapies.

KEY CHALLENGES FACING ACT DEVELOPMENT

While immune checkpoint blockade therapies have greatly improved the survival of cancer patients, many patients still see their disease progress. It is thought that ACTs could be an additional treatment option for patients whose cancers do not respond to checkpoint blockade. However, a lot is still unknown about ACT, and many research groups are working to identify which T-cell phenotypes correlate to clinical efficacy.

Using traditional approaches, different subpopulations of cells are used for cytokine secretion analysis, tumor killing assays, and transcriptional analysis to associate a gene expression pattern with cellular function. This approach does not provide a complete profile of any single cell. However, a study has shown that a single CAR T-cell, and the progeny of that one cell, was able to cure patients with chronic lymphocytic leukemia [1], highlighting that finding the right cell can be the key to a successful therapy.

IDENTIFYING THE BEST T CELLS FOR ADOPTIVE THERAPIES

Important characteristics of T-cells which are likely to be associated with patient response include cells with early, less differentiated memory phenotypes that maintain proliferative capacity in the presence of a tumor cell, providing durable anti-tumor immunity. Polyfunctional cytokine secretion to mediate a wide breadth of immune mechanisms is also important, as is serial killing behavior – i.e., cells which are able to kill multiple tumor cells quickly.

THE BERKELEY LIGHTS PLATFORM

The use of optofluidic chips is a key component of the Berkeley Lights Platform and there are two commercially available instruments: the Beacon® and Lightning™ optofluidic systems, which can perform various automated workflows for cell discovery and development.

MULTIDIMENSIONAL, FUNCTIONAL ASSAYS ON SINGLE T-CELLS

With the Opto™ Cell Therapy Development workflow, a variety of assays can be used to study T-cells and identify the most useful phenotypes:

- Polyfunctional cytokine secretion
- Cytotoxicity
- Antigen-specific proliferation

Layering these assays together makes it possible to identify T-cells with the ideal combination of functions.

FINDING THE RIGHT CELL MATTERS

Using the Opto Cell Therapy Development workflow on the Berkeley Lights Platform, a variety of assays can be performed to measure T-cell characteristics in thousands of individual cells, which can then be recovered for further analysis. This uniquely empowers the correlation of gene expression patterns to multidimensional phenotypes, in order to identify the most effective therapeutic cells.

REFERENCE

1. DOI: 10.1038/41591-018-0010-1

Figure 1. OptoSelect™ chips contain thousands of NanoPen™ chambers, each less than one nanoliter in volume. Using opto-electropositioning (OEP™), single cells are imported and stored. Once assaying and culturing is completed, cells can be dispensed into well plates for downstream expansion or genomic analysis.

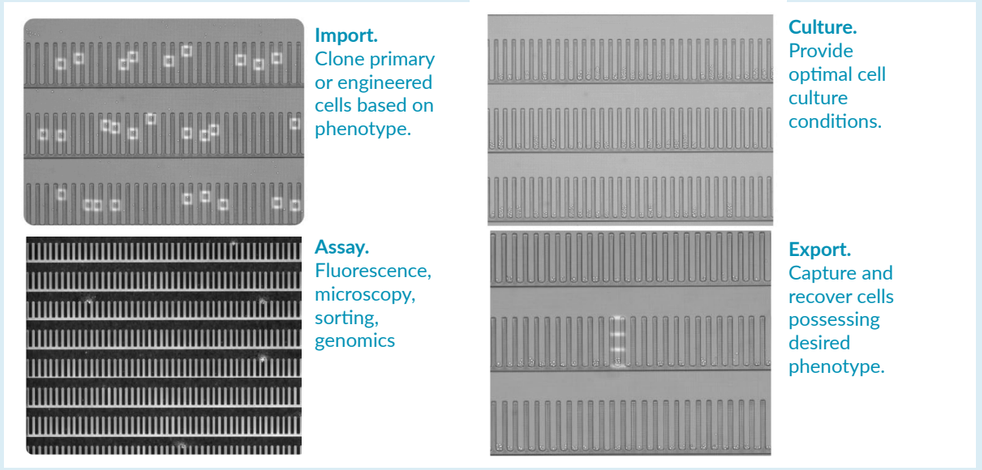


Figure 2. To identify T-cells which are prolific killers, cytotoxicity and proliferation assays were combined to find a subset of cells which were both capable of tumor killing and also proliferative.

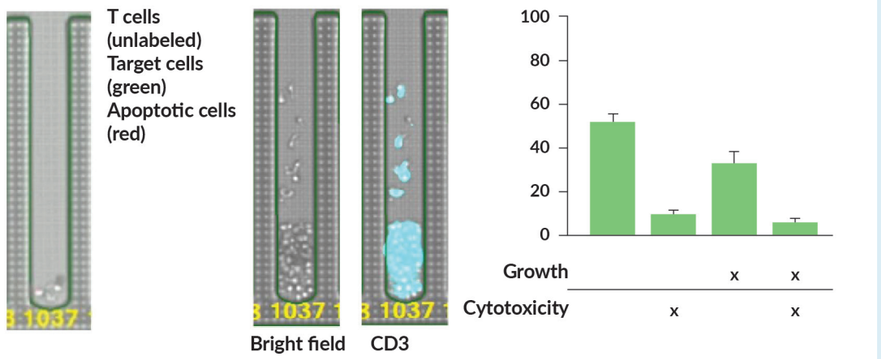
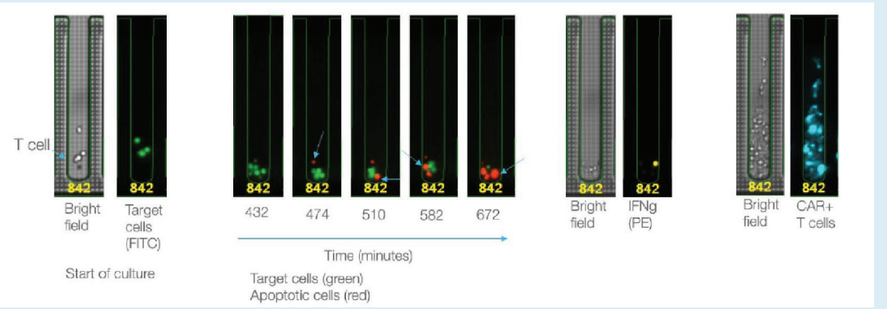


Figure 3. Every function can be studied collectively. A single T-cell with several tumor cells (green) was studied, and 4 separate killing events (blue arrows) were identified. IFNγ secretion from the cell was then measured. After the chip was left to culture for 5 days, a colony of proliferating T-cells was observed. This single T-cell was shown to kill multiple tumor cells, secrete cytokines, and proliferate. The T-cell colony can easily be recovered for downstream analysis.



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European legal issues stemming from the COVID-19 pandemic: regulatory, intellectual property, contract and competition law relevant to cell and gene therapy products

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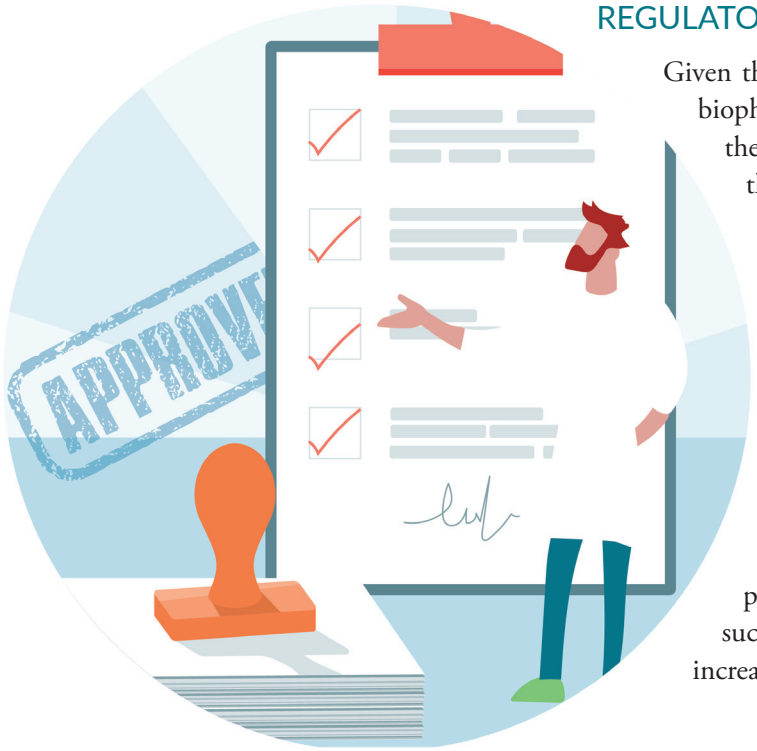
INTRODUCTION

On 30 January 2020, the Director-General of the World Health Organisation (WHO) declared the SARS-CoV-2 outbreak a public health emergency of international concern [1]. At the time, there were 98 reported cases in 18 countries outside China and no deaths outside China. By 11 March 2020, the WHO declared COVID-19 a global pandemic with 118,000 reported cases in 114 countries and over 4,000 deaths [2]. As at 14 January 2021, there were over 91 million global confirmed cases of COVID-19, including over 1.9 million deaths reported to the WHO [3]. The speed and impact of this pandemic has been unprecedented. In this article, we consider some of the legal challenges and considerations that have arisen during the COVID-19 pandemic, with a focus on Europe, and explore how this has impacted the biopharmaceutical industry in its drive to develop vaccines and treatments for COVID-19, with a particular emphasis on the impact to the cell and gene therapy industry.



REGULATORY & PROCEDURAL CHALLENGES

Given the broad nature of regulatory challenges for the biopharmaceutical industry as a whole stemming from the COVID-19 epidemic, this section focuses on the challenges specific to the cell and gene therapy industry. The regulatory environment surrounding advanced therapy medicinal products (ATMPs) is already complex, with a number of interlocking regulatory regimes, a complex global supply chain, and difficult reimbursement environment. There are also particular difficulties in conducting trials in small patient populations suffering from rare diseases. The combination of small patient populations and the ATMP regulatory environment already puts strain on the authorization and launch of such products; the COVID-19 pandemic has only increased these complications.

Clinical trials

ATMPs are often developed to treat small patient populations. This means that randomized controlled trials are difficult to run. Instead, small patient numbers are enrolled in clinical trials, which are often supplemented by post-marketing obligations and/or the collection of real world data. The treatments are also novel, developed to treat rare diseases, meaning a number of precautions are often built into the protocol to monitor unforeseen adverse reactions and ensure the safety of patients. Conducting such trials is already difficult, even without the additional stain of a global pandemic.

At the start of the pandemic, clinical trials in many countries were stopped. The Commission and EMA issued guidance for clinical-trial sponsors on how they should adjust the management of clinical trials during the pandemic to enable trials to continue [4]. For example, it may be necessary for the protocol to be amended to change recruitment practices or to include social distancing measures, quarantine and self-isolation requirements, or mHealth and telemedicine aspects could be incorporated to minimize travel or to reduce interactions between healthcare professionals and patients. While these difficulties have affected all clinical trials, the impact is arguably greater for trials involving small patient populations, spread across the globe, which is often the case for certain ATMP trials. Further, many of the patients involved in ATMP trials may be vulnerable or have been told to shield during the pandemic, meaning their willingness to be part of a clinical trial may have changed.

Logistical difficulties and delays with clinical trials also put a strain on the marketing authorization process for ATMPs. Given the small patient numbers that are involved in many ATMP trials, there had already been pre-pandemic discussions among authorities on the level of data that is required for authorization of these products, and the extent to which gaps in the data can be supplemented post-marketing. Further delays in clinical trials, and reduced numbers of subjects due to COVID may exacerbate these data difficulties and lead to further delays in marketing authorization.

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However, the pandemic has also led to innovation in clinical trials, particularly in the use of digital technologies during clinical trials, and in authorities accepting real world data as part of regulatory submissions. The routine use of mHealth technologies has helped to streamline trials, and in fact increased efficiencies with patient recruitment and communication [5]. Such technologies could also assist with data collection, including of additional supporting data, such as quality of life data that are needed for reimbursement discussions. This could be a welcome unintended consequence of speeding up the adoption of remote monitoring technologies in clinical trials. Similarly, there have been global collaborations on the collection of real world pharmacovigilance data associated with COVID vaccine use [6], and it is hoped that such initiatives will increase the authorities' comfort with accepting such data.

Manufacturing

Certain ATMPs (particularly autologous cell therapies) are manufactured at multiple sites, starting with samples collected from the patient. Further, despite the EU-wide nature of the authorization of such products, the manufacturing processes are still controlled at the national level; where materials travel through a number of countries, different rules in relation to licensing or customs apply.

Transport of people and goods has obviously been impacted over the last months, and while there can be exemptions for treatment, these are not uniformly applied. Similarly, national differences in terms of lockdown, quarantine and travel restrictions causes complications for global products such as these. The manufacturing chain for ATMPs is usually time critical, and so minor delays at customs or due to travel restrictions may lead to samples being unusable or products being spoiled. The EMA has provided guidance [7] to authorization holders on flexibilities that can be introduced to the manufacturing supply chain to ensure continued supply of medicines during the pandemic, for example to ensure the continued validity of good manufacturing practice certificates, and flexibilities around inspections. This guidance also applies to ATMPs.

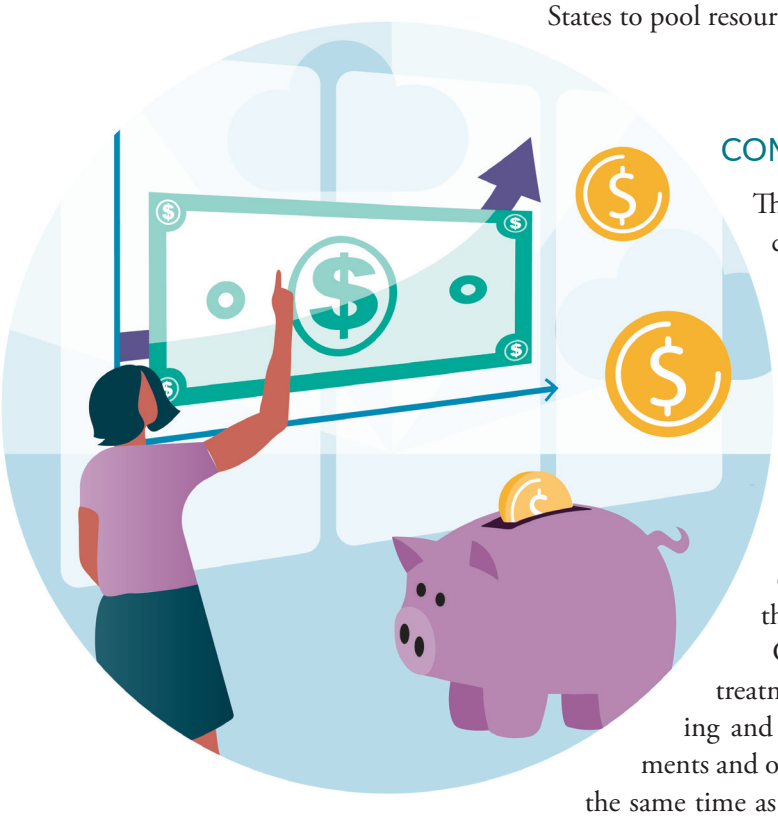
The urgency of the pandemic has shown that quick legislative changes are possible in order to streamline the process across the EU and minimize national differences. While not relating to manufacturing specifically, new legislation has been introduced to facilitate the conduct of clinical trials using products containing or consisting of genetically modified organisms [8]. This is specifically focused on development of vaccines and therapies to treat COVID-19, but demonstrates that rapid changes can be made to streamline the process across the EU. These harmonization measures may expand beyond COVID-19 therapies and address some of the difficulties identified here.

Pricing & reimbursement

Pricing and reimbursement for specialized products has always been difficult. These products are often used to treat a small number of patients at high cost, and healthcare systems have been reluctant to purchase them without large discounts. The pandemic has exacerbated this, as healthcare systems have had to make difficult decisions on prioritizing treatment and allocation of finite resources.

However, the pandemic has increased the use of centralized procurement regimes, whereby the European Commission on behalf of member states (and other countries which are signatories to the Joint Procurement Agreement) has sought to pool buying power. Completed

joint procurements cover PPE, testing kits, as well as vaccines and Gilead's remdesivir. Other planned procurements are intended to cover ICU medicines, vaccine carriers, waste containers, injecting devices, more personal protective equipment (PPE) as well as anesthetic consumables [9]. Centralized procurement is specifically encouraged where serious cross-border threats to public health are involved, which is clearly the case here, but the use of such systems will hopefully encourage authorities to further utilize voluntary agreements between Member States to pool resources and ensure quick access to ATMPs.



COMMERCIAL TRANSACTIONS

The COVID-19 pandemic has led to accelerated commercial transactions and new contracting models for those companies developing vaccines and products to treat COVID-19 patients. Whilst vaccines are not classified as ATMPs, some of the vaccines in development involve gene-based technologies, and the transactions highlight what can be achieved contractually in the development of new pharmaceutical products and vaccines, or in the redeployment of existing products for other purposes when there is sufficient political and societal pressure.

Companies involved in developing COVID-19 treatments have found themselves negotiating funding and supply agreements to enable multiple governments and other organizations to purchase their products, at the same time as developing the products and ramping up their supply chains, all under intense public scrutiny [10,11].

In addition to logistical issues surrounding the sheer volume of contracts required, there have been difficult discussions with payers about who should bear the risk of product liability claims brought by patients who may have been injured as a result of the new products. Companies placed under pressure to develop their new products at speed and then roll them out to millions or even billions of patients have been extremely sensitive to this potentially enormous downstream risk, and many have asked governments to share in the risk of product liability claims.

This discussion has been relatively straightforward in the US, where the 2005 Public Readiness and Emergency Preparedness Act (PREP Act) offers immunity from tort claims for product liability for products supplied in a public health crisis. For developing and least developed nations, the COVAX vaccine scheme co-led by Gavi, the Coalition for Epidemic Preparedness Innovations (CEPI) and WHO is setting up a compensation fund for individuals who might suffer any side-effects from COVID-19 vaccines [12,13].

The position in the EU is less clear cut than in the US. This prompted Vaccines Europe, a division of the European Federation of Pharmaceutical Industries and Associations (EFPIA), to engage with the EU and governments around the introduction of no-fault and non-adversarial compensation systems, and exemptions from civil liability for vaccine developers during the pandemic [14,15]. For now, the Commission is addressing liability with individual manufacturers via the relevant supply agreements on a case by case basis (see, for instance the European Commission's press release regarding its contract with AstraZeneca, which states

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that the Member States will indemnify the manufacturer for liabilities incurred under certain conditions, while liability still remains with the companies [16]).

Designers and manufacturers of ventilators experienced similar issues following requests to scale up and manufacture products to treat COVID-19 patients at the start of the pandemic. In the UK, the government responded by agreeing to indemnify designers and manufacturers of rapidly manufactured ventilator systems not only for product liability claims, but also for infringement of third-party intellectual property (IP) rights, accepting that, as a result of the accelerated process, there was less time to consider the patent and design rights landscape and design-around, license-in, or seek to invalidate third party IP rights [17].

COMPETITION & COLLABORATIONS

Since the start of the COVID-19 pandemic, we have witnessed significant collaborations between companies and institutions engaged in the development of vaccines and therapies to treat COVID-19. Such collaborations, including for the development of vaccines using gene-based technologies, frequently have involved cooperation between competitors and thus must be structured carefully.

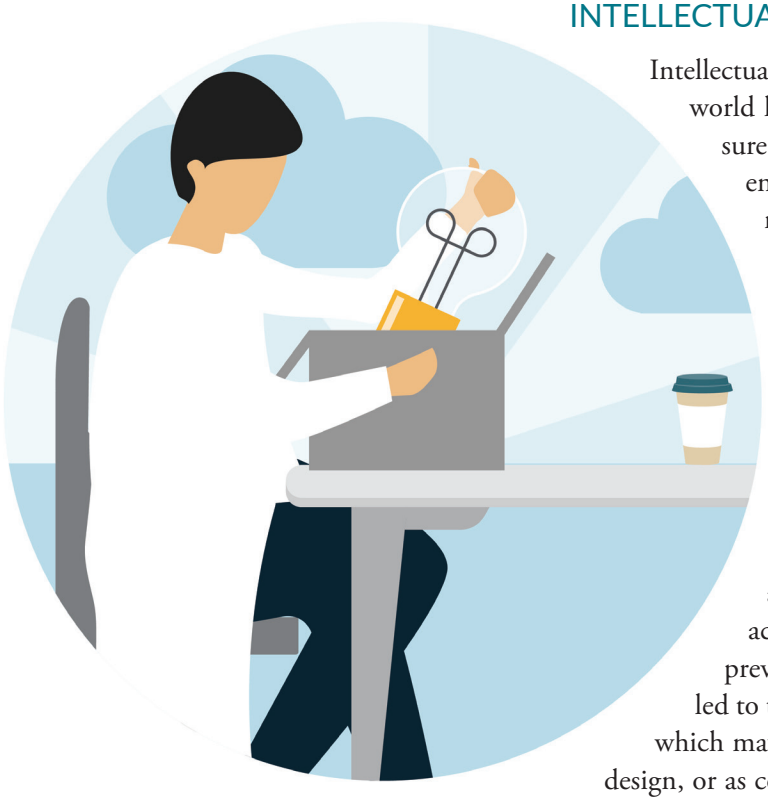
In the early part of the COVID-19 pandemic, the UK's Competition and Markets Authority, the European Commission as well as national competition authorities in other countries responded to growing demands from businesses to provide guidance for parameters of lawful cooperation among competitors seeking to address the challenges posed by the epidemic.

Competition laws generally restrict information exchange and supply chain optimization agreements between competitors. However, a real crisis such as COVID-19, clearly creates an imperative to permit and even encourage such exchanges and agreements. The authorities' guidance sets out areas of permissible cooperation but also the limits of such cooperation. This is particularly relevant for the pharmaceutical sector, where companies are often jointly developing vaccines, tests and therapies, and for those manufacturing and distributing medical devices.

The limits set out in the guidance broadly seeks to ensure that any cooperation is strictly necessary, limited to the duration of the pandemic, that it is proportionate and specifically benefits customers/patients. In addition, the UK government adopted specific orders excluding the application of UK (but not EU) competition laws to certain qualifying activities in the provision of health services to the NHS in England and Wales, again subject to certain requirements and limitations.

The key takeaway is that competition law will continue to be relevant to any form or co-operation between actual or potential competitors and that there is no blanket exclusion or relaxation of competition laws that will obviate the need for specific advice.

INTELLECTUAL PROPERTY



Intellectual property offices and courts across the world have implemented multiple measures to ensure, as far as possible, that the protection and enforcement of intellectual property rights is not adversely affected by lock-downs and the need to work remotely as a result of the COVID-19 pandemic. This has included the extension of deadlines for filing evidence and submissions before registries, the ability to file documents digitally, as well as virtual registry hearings, court hearings and trials.

The COVID-19 pandemic has led to an unprecedented global effort in accelerated research and development efforts across academia and industry to diagnose, treat and prevent infections. These efforts have inevitably led to the creation of intellectual property, some of which may be potentially protectable as a patent, as a design, or as copyright or may be treated as a trade secret. This has, in turn, led to debates as to the extent to which the owners of such IP should seek to protect and enforce their rights. The outcome of this debate may impact the IP position for companies in the cell and gene therapy space, especially as some of the vaccines being developed deploy gene-based technologies.

On 1 June 2020, the WHO called key stakeholders and the global community to commit to take action to, amongst other things:

- Promote innovation and facilitate the sharing of intellectual property for COVID-19 detection, prevention, treatment and response;
- Promote that all COVID-19 publicly-funded and donor-funded research health product outcomes include non-exclusive voluntary licensing and the sharing of IP rights; and
- Encourage that all research outcomes are published under open licenses that allow access free of charge, use, adaptation and redistribution by others with no or limited restrictions [18].

The WHO also called on IP rights holders to voluntarily license their rights on a non-exclusive and global basis, to share relevant knowledge, IP and data to enable widescale and worldwide production and distribution, including by placing it in the WHO COVID-19 Technology Access Pool (C-Tap) [19]. It is notable that of the 40 countries that have, to date, endorsed the WHO solidarity call to action, the majority are developing countries.

On 2 October 2020, India and South Africa sent a proposal to the World Trade Organisation (WTO) requesting a waiver from the implementation, application and enforcement of a number of provisions of The Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS; TRIPS is an international agreement that applies basic in international trade principles regarding IP) until widespread vaccination is in place globally and the majority of the world's population has developed immunity. So far, the proposal has been rejected by the majority of WTO members, including the UK Government, which stated that such an

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"extreme measure to address an unproven problem" would be "counterproductive and would undermine a regime that offers solutions to the issues at hand" [20]. It pointed to existing mechanisms that facilitate the sharing of IP, including the initiatives being developed by the WHO. The proposal is due to come before the WTO Council formally in December 2020.

If a waiver to TRIPS is agreed, biopharmaceutical companies would likely lose some of the protection afforded to them through their ability to control the use of their IP rights. Although the TRIPS waiver proposal is not limited to developing and least developed countries, we anticipate that, if a waiver is agreed, its application is more likely to be limited to such countries. Even if a waiver is not agreed, WTO countries are entitled under TRIPS to allow the use of the subject matter of a patent without the owner's consent in the case of a national emergency or other circumstances of extreme urgency [21].

Even if the TRIPS waiver is not deployed, where there is a national security risk, emergency and/or a public interest need, many countries have specific provisions in their patents laws that provide either a defense to patent infringement, authorize certain acts to be done which might otherwise constitute patent infringement or which enable IP rights to be expropriated from their owners. The UK Patents Act 1977, for example, includes "Crown Use" provisions whereby a government department can authorize the infringement of a patent without the owner's consent to provide services to the Crown and such Crown use includes the production or supply of specific drugs and medicines [22]. Although there was an amount of speculation and hype early on in the pandemic as to whether governments would deploy these emergency measures, we have instead seen a significant amount of commercial deals being struck between governments and companies developing vaccines and therapies to treat COVID-19.

FINAL REMARKS

This article only addresses some of the legal challenges presented by the COVID-19 pandemic and how they may impact cell and gene therapies. At the time of writing, the outlook is looking favorable for at least three of the COVID-19 vaccines in development. The speed of development of numerous COVID-19 vaccines in under 12 months is unparalleled. This has, at least in part, been possible due to flexibility and revised guidance from regulatory authorities, the implementation of emergency legislation, as well as innovative ways of contracting and collaborating between academics, governments and the biopharmaceutical industry. In contrast, research and development efforts for non COVID related cell and gene therapies have been more challenging this year, with companies experiencing delays to clinical trials and caution from investors. Nevertheless, the fact that a number of the COVID-19 vaccines in development deploy gene-based techniques may assist in the long-term by helping to carve a path to market and also increase societal acceptance of such products.

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