CELL & GENE THERAPY INSIGHTS

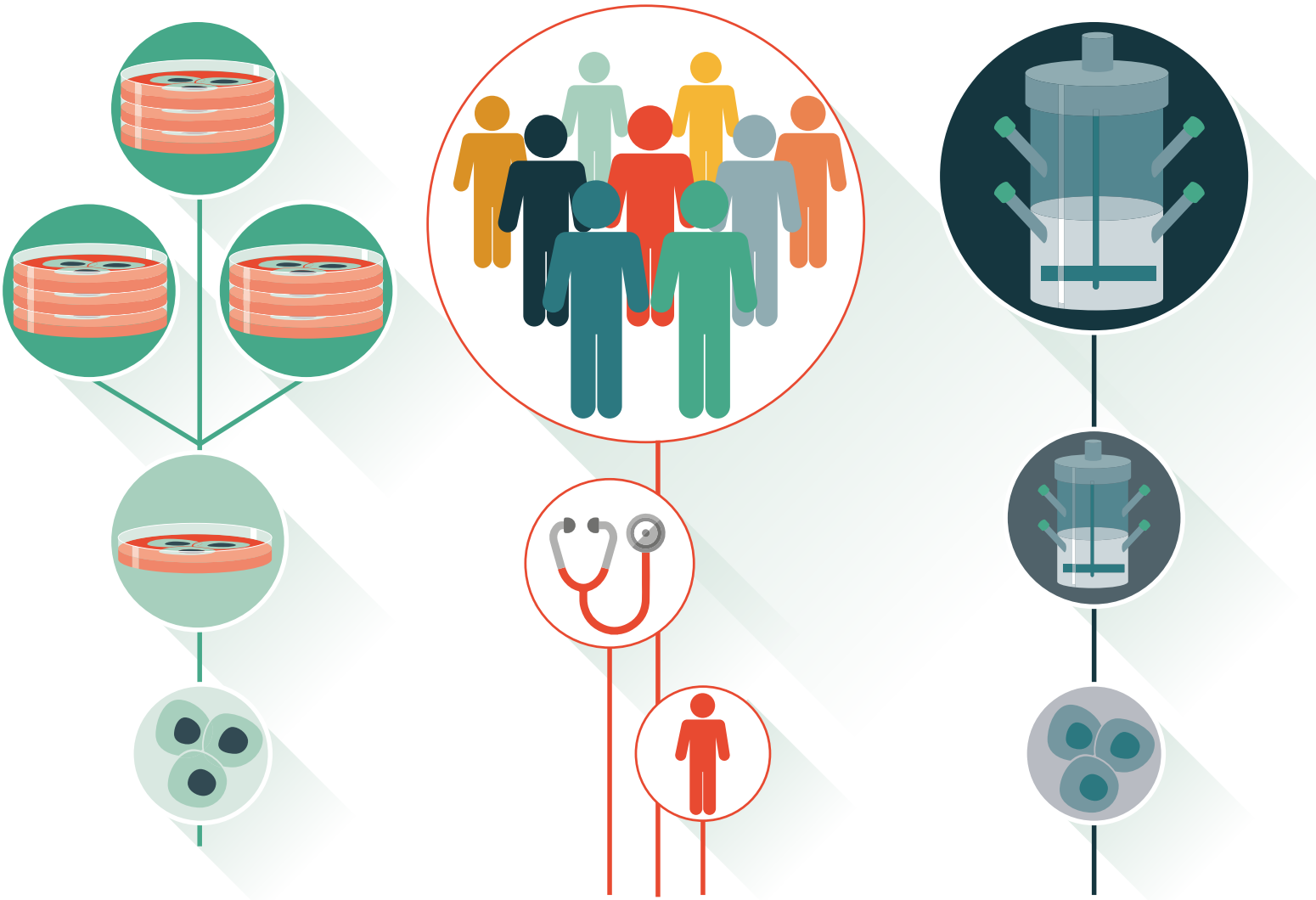
SPOTLIGHT ON:

STRATEGIES FOR SCALE-UP & SCALE-OUT

Sponsor

FRESENIUS KABI

caring for life



CELL & GENE THERAPY INSIGHTS

GUEST EDITOR:
PROFESSOR NIK WILLOUGHBY, HERIOT WATT UNIVERSITY

INTERVIEW

Biopharma Scale-Up Lessons to Learn for Cell & Gene Therapy
Guest Editor: Nik Willoughby

1157–1164

EXPERT INSIGHT

The Long Road to Affordability: A Cost of Goods Analysis for an Autologous CAR-T Process
Katy Spink & Andrew Steinsapir

1105–1116

EXPERT INSIGHT

Back to the Future – Where Are We Taking Lentiviral Vector Manufacturing?
Hanna Lesch

1139–1152

EXPERT INSIGHT

Translating Cell and Gene Therapies to the Clinic
Patrick Hanley, Hema Dave, Devin Saunders & Nan Zhang

1159–1172

RESEARCH PAPER

Scale-Up of a Perfusion-Based Dendritic Cell Generation Process
Andrew Kozbial, Shashi Murthy, Hope Weinstein & Jennifer Rossi

1117–1130

INTERVIEW

Critical Considerations for Allogeneic Cell Therapy Scale-Up
Sharon Grimster

1081–1086

INTERVIEW

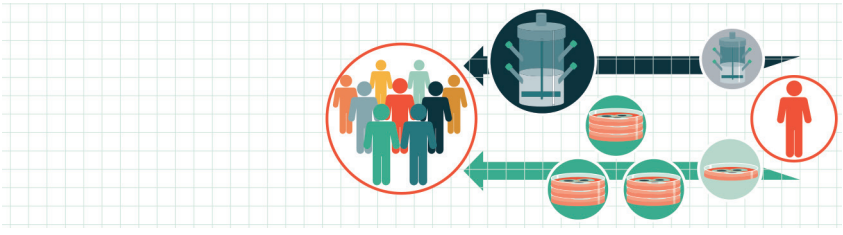
Towards Commercial Allogeneic Cell Therapy Manufacturing
Maria del Pilar Redondo

1153–1156

INTERVIEW

Autologous T Cell Immunotherapy Scale-Out Manufacturing Models - the Biotech Perspective
Nina Kotsopoulou

1073–1076



STRATEGIES FOR SCALE-UP & SCALE-OUT

SPOTLIGHT

Biopharma scale-up lessons to learn for cell & gene therapy

PROF NIK WILLOUGHBY is Professor of Bioprocessing and Deputy Director of the Institute of Biological Chemistry, Biophysics and Bioengineering at Heriot Watt University. Nik carried out his PhD at University College London in Biochemical Engineering, and his research interests focus on applying engineering principles to biological challenges. Primarily working in downstream processing, Nik has moved the focus of his research over the past 20 years from protein therapeutics to more complex cellular targets. His research group have worked with a wide range of cell types but primarily have focused on red blood cells, working with the NovoSang consortium – with the ultimate objective of developing passive, scalable, label-free solutions for separation of heterogeneous cell populations manufactured from multipotent and pluripotent cell sources.

Nik has spent time in both industry and academia, in the former managing the Protein Purification group at Metris Therapeutics as well as working for Lonza Biologics in cell culture development, and in the latter helping to establish the Innovative Manufacturing Research Centre for Bioprocessing at UCL before setting up the Cellular Bioprocessing Group at Heriot Watt.

Q Your current research interests focus on applying engineering and scale-up principles to real-world biological challenges – can you give us some examples that relate specifically to the cell & gene therapy world?

NW: For me, coming from an engineering background, allogeneic platforms will be key if cell & gene therapy is to reach and benefit a really wide patient population. The larger the scale at which you can manufacture, the better your cost of goods and the better your chances of making therapies available to a large number of patients. So I tend to focus on the challenges of large-scale allogeneic cell therapies.

As a rule of thumb, the more scale of production increases, the more the "engineering" aspects need to be factored in. Meeting oxygen demands for mass transfer, for example – even when these are minimal – is a lot simpler in a T flask or cell factory than in a large stirred tank. Similarly, in separation and purification operations, as scale increases current gold standard purification techniques may become less appropriate and other approaches are necessary – approaches that are more likely to exploit the mechano-physical properties of cells as well as the biological or chemical properties. Development in these areas requires close cooperation between biologists, clinicians and engineers to fully exploit different areas of knowledge and innovation. Whilst the idea of a truly large-scale allogeneic therapy may still be some way off, it must be seen as a key objective to fully realise the huge potential of these therapeutic approaches.

Q Much of your background and current activity is focused on proteins production – a field to which the maturing cell & gene therapy manufacturing sector looks for strategic guidance, enabling technologies and increasingly, for personnel. What are the key elements or learnings that the cell & gene therapy field should take, for you?

NW: This is something that has been the subject of many conversations within the advanced therapy manufacturing community over the past few years. There's been this long-running debate: how are advanced therapies going to align with current manufacturing and regulatory models for established biological therapeutics? And to an extent, there's been an opportunity which has been taken by a number of trailblazing companies moving through clinical trials towards commercialisation in this regulatory space, to get a little bit of a say in how these things will work.

The advanced therapeutic product manufacturing model is likely to take aspects from the more established therapeutic protein model, along with aspects of clinical models such as transplantation. Those two models sit somewhat at two extremes – protein therapeutics being all about large scale 'factories' while the transplantation side can see protocols differ from one individual hospital to another.

At the same time, there will of course be aspects that are unique to the cell and gene products themselves. With advanced therapies, we are talking about products that are by several orders of magnitude more complicated and more heterogeneous than proteins. So it will be interesting to see how

DOI: 10.18609/cgti.2018.112

> "There's been this long-running debate: how are advanced therapies going to align with current manufacturing and regulatory models for established biological therapeutics?"

the advanced therapy regulatory manufacturing model continues to take shape.

From the therapeutic proteins side, the link is the concept that the process is the product – so the focus is on consistency of manufacturing and reproducability, which alleviates the need to do a huge amount of QC for every single batch. This has got to be critical for cell & gene therapies, particularly those where time constraints mean that the amount of time available for release testing is limited. But then again, with the fact that cells as products are always going to involve a significant level of heterogeneity, it's going to be pretty difficult to define your process as consistent.

Overall, from a manufacturing and development point of view, I think there are two big things we can learn from the lessons of protein therapeutic development and the challenges we had to overcome there.

The first is to always consider all aspects of the process from day one; how your upstream will affect your downstream, and how both will affect formulation or delivery. It is imperative to develop these stages in parallel, not sequentially. And it is also critical to maintain communication throughout! For a while in monoclonal antibody manufacture, every other office had a cartoon up which had upstream on one side and downstream on the other, and a massive wall in between with people just throwing stuff over it, demonstrating the failure of the two to communicate with each other.

To give a really classic example, when we were driving towards really high cell numbers and high titre expression systems in recombinant protein bioprocessing, the mantra for upstream was more product is best. But as you drove that up, you stressed the cells more and more and they became leaky and weak. So you basically ended up in a situation where you got twice as much protein, but it was four times as hard to purify – so all that extra protein was then lost in downstream.

The same thing could conceivably be the case with cell therapies as we move forward. For example, there might be a situation where it may be that driving your cell differentiation up to 90% efficiency over 80% efficiency is not the best solution, because the 10% of unwanted cells you end up with are harder to remove or are otherwise more problematic than the 20% you had before. It's a matter of making sure that there's proper communication, proper understanding, and that everyone works together across different areas – there's any number of skillsets and disciplines involved in the development of a good therapy.

Secondly, I would highlight the importance of avoiding assumptions that current conventions will always stand. In terms of mammalian cells and proteins, we went through assumptions like 'you'll never get those cells in suspension' and 'you'll never achieve higher than X cell density' and 'titres over Y are impossible'... And yet all of those conventions fell over time. Looking at it now, the idea that protein therapeutics weren't developed in suspension from the start must seem insane, but they weren't. It's surely inevitable that similar things will happen with cell and gene therapy – in fact, there's a very similar debate going on right now in cell therapy regarding the use of suspension systems.

Q Just picking up on the personnel side of things, there is clearly an influx from the protein therapeutics community, which is putting the importance of staff training firmly in the spotlight for cell & gene therapy organisations. What is academia doing at the moment to prepare the future workforce in cell and gene manufacturing?

NW: I come back to my earlier comment about understanding that there are a lot of different disciplines and skillsets required to develop this kind of product properly. They must work together, and they must do so from day one. What you don't want is a situation whereby you start off with brilliant biologists and brilliant clinicians working on an idea, and then you bring in some manufacturing specialists, some engineers, as the process goes along. It must not be sequential, it must be done in parallel.

> "Academia is doing a very good job of driving the interdisciplinary collaboration and communication that's necessary."

Academia is doing a very good job of driving the interdisciplinary collaboration and communication that's necessary.

For one thing, it's very difficult to secure research funding in this space if you don't demonstrate true 'interdisciplinarity'. If you can't show that you're working with the right people in different areas and that you've got them properly engaged, then finding research funding is problematic. And that's good because it's probably reasonable to say one of academia's traditional weaknesses has been the 'ivory tower' issue: that everyone is an expert in their own field and they don't always accept the validity or relevance of others' work to their own. The funding bodies, universities and various collaborative projects have been working very hard to break that down, telling

DOI: 10.18609/cgti.2018.112

researchers, 'You're brilliant at what you do, but what you do is only a small area of this and we need to bring you together with other people'. You can see evidence of this in some of the articles in this spotlight edition – there are comments about understanding the need to involve people from different areas early on in the development process. I think that's probably the single biggest thing that academia does well in this regard.

Q In this month's edition, we have articles focusing on scale-up challenges at both early stages of development and at pivotal trial /commercial stages – starting with the early stages, what should be the key initial strategic considerations and priorities for advanced therapy bioprocess developers?

NW: Always think about your process and your ultimate goal, even from day one, which means starting with the assumption that what you are working on will be a commercially viable clinical product at the end.

It's vital to understand the "what ifs?" regarding the early stage science – what if this becomes a success? Is what we are doing compatible with a manufacturing environment? And where practical, collaborate as early as possible with people who understand the process challenges – otherwise you may well end up with parts of your process that are difficult or even incompatible with scale-up, GMP, etc.

I've worked with protocols in the past in which there were steps that were simply impossible to translate across to anything even closely resembling a manufacturing environment. We've had situations where we've moved protocols from one lab to another and it's not worked well in the other lab. And the immediate response from the lab that moved it across is, 'Well you're simply not good enough at the biology.' But you must be mindful that once this product goes into a manufacturing environment, it's going to be dealt with by a manufacturing engineer – it's not going to be handled by one of your best post docs. If it doesn't work in that environment, then you haven't written a protocol which is transferable.

"You shouldn't put a process step in for which there is no larger scale equivalent."

You also shouldn't put a process step in for which there is no larger scale equivalent. As an example from the protein therapeutics world, I worked on translating a process for a start-up with a chromatography step which

included a heparin affinity chromatography column. Heparin affinity chromatography columns are almost impossible to use at process scale – they must have incredibly slow flow rates through them because the kinetics are very poor for getting things to stick. And there were other problems… the heparin chromatography resin they were using did not have an FDA drug master file. That would have led to a huge delay and significant costs, but more importantly, the reason it didn't have a master file was because it was fundamentally incompatible with process scale production.

It is vital to have someone involved from early stages who will be able to tell you that 'it's not going to be great if you do it like this', or 'it's not going to translate well at scale'. But it is equally important to look at things from a positive point of view – assuming from the beginning that this is going to be a clinical success, so that's how you need to think about it. As opposed to the negative viewpoint one sometimes encounters that the manufacturing people will always come along and ruin everything!

Q And as commercial manufacture of cell & gene therapies increasingly becomes a reality, what do you see as the key remaining issues relating to scale that need to be addressed?

NW: Working across a whole process, media has always been a particular concern of mine. Most commercially available cell culture media for advanced therapies are, out of necessity, focused on small processes. GMP media are still not fully developed and there are components in most media, even in GMP manufacture, that actively hinder the development of novel purification techniques (which brings me back to the key issue of communication in process development!)

While these commercially available media might be perfectly compatible with current gold standard, state-of-the-art cell separation protocols like FACS and MACS, they're not necessarily compatible with the more interesting passive scalable technologies we're now seeing being developed. And there is little appetite on any side to fund or drive improvements in this area.

Beyond this, I think understanding the effects of the microenvironment during large scale suspension culture of advanced therapies is key to understanding the limitations of scale and suspension growth options. This is an area where a lot of work has been done, but arguably, we still need a greater understanding of the difference between the microenvironment around a cell in a t-flask, versus in a spinner, versus in a WAVE bag, versus in a big stirred tank bioreactor. There has been great deal of research on this area regarding cells in general, but not necessarily around cell therapeutics on

DOI: 10.18609/cgti.2018.112

large scale. That is perhaps because there's an assumption we won't get to very, very large-scale manufacture - even the recent successes like CAR-T, or the potential allogeneic therapeutic products in the current pipeline, are probably unlikely to be manufactured at 20,000 litre scale. However, I do think that down the line, there will be advanced therapies that get to that kind of scale, and we need to understand how the microenvironment around the cell differs in terms of nutrient uptake, metabolite removal, oxygen uptake, carbon dioxide and so forth.

And finally, of course, I think there is still a pressing need for development of more scalable, passive, very high throughput cell purification techniques that do not rely on things like surface markers – techniques that will most likely complement current gold standard processes, but which potentially can exist as standalone solutions.

We also cover both scale up and scale out – for instance, of the next wave of autologous cellular immunotherapies. What are your expectations regarding the autologous cell & gene therapy field?

I think it's inevitable that autologous or patient-specific (or at least, narrow range specific) models are likely to dominate this space for some time. As I've said, I would love to see a truly broad, large scale cell or gene therapy and I do believe we will in time, but for the moment, a model of focusing on more tailored therapies is likely. Within this space I believe the purification challenges, whilst still considerable, are not as extreme as faced by, say, an allogeneic cell therapy, so the focus is likely to be on reproducible processes using single-use disposables, and time is likely to be a big driver – rapid, safe processes are critical. I am sure there is likely to be development of platform-style scale-out processes from key manufacturers, which hopefully are translatable across broadly similar products to reduce the regulatory challenge for the industry as a whole.

Q Finally, what particularly excites you in the way of emerging enabling technologies with the potential to alleviate some of the issues relating to large scale manufacture of advanced therapy products?

NW: I am particularly fascinated with the downstream side of things - any new technique that looks to offer a viable alternative (or to complement) surface marker-based separations.

I have been very impressed by some of the progress in hydrodynamic sorting techniques, some of which are approaching commercial reality. What's compelling about them is their ability to separate and move cells around without applying anything other than flow forces.

For instance, I'm very interested in separation through inertial focusing. The basic principle is you can separate cells based on certain aspects of their physical properties, but you can do that simply in a fast-flowing channel. What I find interesting is that people have generally assumed that hydrodynamics tends to rely on cell size and density, and a little bit on cell shape. But going back to the heterogeneity of cells, those are all areas where there can be overlap between the desired and the unwanted cells – for example, if you were look at the size range of finished, enucleated red blood cells and compare it to the size range of cells that still contain the nucleus, there's an overlap. So by definition, anything that separates by size will not have a very high efficiency. However, one of the things you can do with hydrodynamic techniques is you can also bring in cell deformity or elasticity. That changes things considerably because you can now consider all of these different properties and play them off against each other.

The other thing that interests me quite a bit about that area is that you can get cells to transfer between different aqueous media solely through hydrodynamics, which means you could theoretically do media changes just through hydrodynamic flow.

In other areas, whilst I think they are more "established" than "emerging", the importance of systems like the Ambr or the DASGIP for improving understanding and prediction of large-scale conditions for cell culture should not be underestimated. Whilst I think there's a lot of really, really cool new stuff out there, we should still keep in mind the importance of some of tools that might not be considered really cutting-edge. The information we might be able to glean from implementing such established technologies could be critically important as we try and learn more about large scale conditions

AFFILIATION

Prof Nik Willoughby
Professor of Bioprocessing & Deputy Director of the Institute of Biological Chemistry, Biophysics and Bioengineering,
Heriot Watt University, UK

DOI: 10.18609/cgti.2018.112

CELL & GENE THERAPY INSIGHTS

STRATEGIES FOR SCALE-UP & SCALE-OUT

SPOTLIGHT

EXPERT INSIGHT

The long road to affordability: a cost of goods analysis for an autologous CAR-T process

Katy Spink & Andrew Steinsapir

With the 2017 FDA approvals and launches of the first gene-modified cell and pure-play gene therapy products to gain licensure in the United States, increasing attention has been paid to the high cost of this emerging class of therapies. Although currently approved therapies are for orphan indications, prices similar to those charged today will be unaffordable for products marketed for larger indications. Using public information, we constructed a cost of goods model for an autologous gene-modified cell therapy product, evaluated the relationship of estimated manufacturing costs to list prices of CAR-T products, and investigated the potential impact of various factors on manufacturing costs. Our findings highlight in particular the importance of maximizing employee productivity, leveraging automation and technology, and accurately forecasting capacity needs to achieve the manufacturing cost improvements that will likely be required to drive broad adoption of autologous gene-modified cell therapies.

Submitted for peer review: 8 Oct 2018 ▶ **Published**: 18 Dec 2018

INTRODUCTION

After decades of development in which Cell and Gene Therapy (C&GT) products struggled to demonstrate clinically compelling efficacy, fund development efforts, and bring new products to market, the field appears to have passed an inflection point. Compelling demonstrations of efficacy by multiple products have ushered in a new era in the field, led by the first FDA approvals for gene-modified cell therapies (Kymriah®, Yescarta®) and a pure-play gene therapy (Luxturna™)

product in 2017. (Throughout this article, we refer to products delivering a gene therapy directly to patients by way of a viral vector as 'pure-play gene therapies', and those using genetic modification of cells as 'gene-modified cell therapies'. We recognize this language differs from the current FDA convention, however we find it a useful framework for describing the unique manufacturing considerations of these two very distinct classes of therapies). Among other comparable deals, the $11.9 billion acquisition of Kite by Gilead, and the $9 billion acquisition of Juno by Celgene have caught the attention of investors, triggering an unprecedented level of financial investment in the cell and gene therapy space. According to the Alliance for Regenerative Medicine, at least $7.9B in funding was raised by Regenerative Medicine companies during the first 6 months of 2018, and 977 Regenerative Medicine clinical trials were ongoing worldwide as of June 2018 [1].

However, despite palpable excitement for the tremendous clinical advances that have been made in recent years, there has been increasing controversy over the high prices of approved therapies. Each of the three products approved in 2017 command US list prices of between $373,000 and $850,000. In the UK, Novartis's Kymriah® was approved by the National Institute for Health and Care Excellence (NICE; the national arbiter of reimbursement decisions based on analysis of cost effectiveness) for its pediatric ALL indication, but at a price steeply discounted from its $475,000 US list price [2]. Both Kymriah® and Kite/Gilead's Yescarta® were originally rejected by NICE for the larger adult lymphoma indication, although Kite/Gilead later struck a deal with NICE to enable discounted Yescarta® access through the Cancer Drugs Fund. In the USA, the Institute for Clinical and Economic Review (ICER) issued a report suggesting that the $850,000 Luxturna™ list price was as much as 2- to 4-fold above cost–effectiveness standards [3]. Beyond the impact of high list prices for these products, the costs of such therapies are further increased by high ancillary costs associated with product administration (e.g., need for delivery under hospital admission, monitoring for and control of side effects such as cytokine release syndrome, etc.).

We aimed to investigate the relationship of manufacturing costs to high prices for gene-modified cell products, and to determine the highest value routes for potential investment in cost reductions. To this end, we constructed a cost of goods sold (COGS) model for a hypothetical chimeric antigen receptor T-cell (CAR-T) product using publicly available information about the manufacturing process for Yescarta® as our guide.

METHODS: COGS MODEL

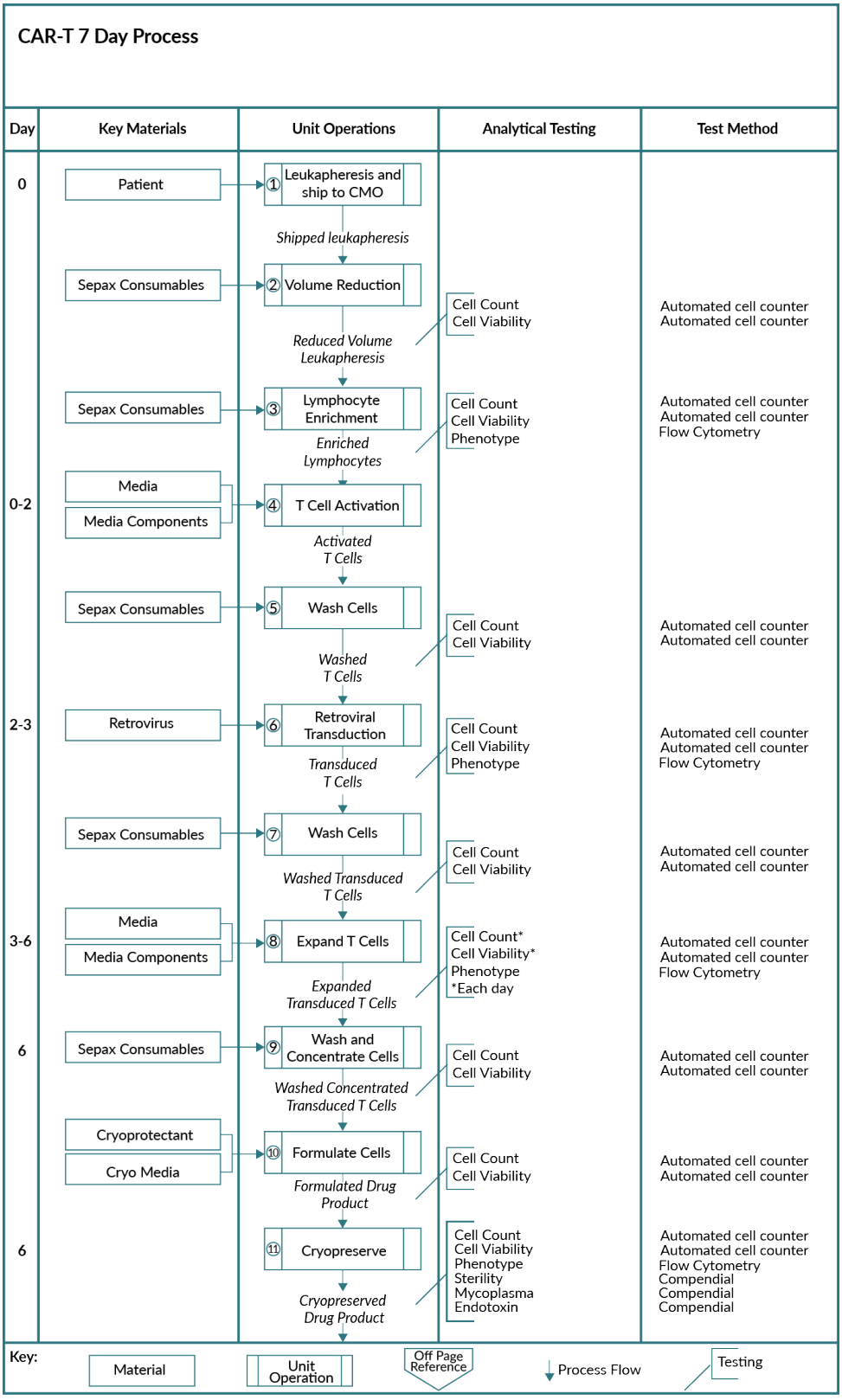
Using a published patent application [4] and journal article [5], LinkedIn profiles of employees, and published articles on Kite's El Segundo manufacturing facility, we constructed a process flow diagram (PFD) describing the unit operations, materials and equipment utilized in a Yescarta®-like manufacturing process (Figure 1).

Labor hours associated with each processing step were estimated based on previous Dark Horse Consulting (DHC) experience

DOI: 10.18609/cgti.2018.108

FIGURE 1

Process flow diagram for a CAR-T manufacturing process.



with the conduct of similar unit operations in a GMP environment and used to calculate projected employee numbers and labor costs for in-house manufacturing of a similar product. For the purposes of this analysis, it was assumed that all in process and release testing was performed in house. Based on these methods, we estimated just over 200 labor hours per lot, inclusive of manufacturing, QA, QC and logistics/supply chain management. Departmental management overhead was estimated at 20% of operating hours, and headcount-driven support functions (facilities, IT, HR, etc) at 10%. Estimated employee numbers based on these calculations (~400 FTE required for 1500 lots per year) were benchmarked against publicly available information (LinkedIn) for current employees and open job requisitions at Kite's El Segundo manufacturing facility (332 employees and 49 open requisitions as of March 2018). Good agreement was seen between both methods.

Facility construction costs were estimated based on the $26M in leasehold improvement costs reported in Kite's SEC filings from 2015 and 2016. These costs were compared to benchmark estimates of cost per square foot for GMP facility construction by ISO classification, and found to be broadly within agreement for both methods. Facility validation expense was estimated at 20% of construction costs, or just over $5 million. Facility construction and validation expense was amortized over an estimated 15-year useful life.

Rent was estimated at $1.6 million per year based on reported numbers for the El Segundo facility in Kite's SEC filings. Other ongoing operational expenses were estimated based on prior DHC experience.

Required equipment per manufacturing line were estimated based on DHC experience. Where appropriate based on frequency of use (e.g., cell counters, BSCs, controlled rate freezers) equipment was assumed to be shared between lines. Required equipment lists were used to calculate equipment costs to fully equip facility for a range of target lot numbers per year. Equipment IQ/OQ/PQ expenses were estimated based on previous DHC experience. Calculated equipment costs using these methods totaled $12 million, which was reasonably close to the $18 million in equipment expenses reported in Kite's 2015 & 2016 SEC filings when it is considered that equipment was likely also purchased for other purposes and facilities during this period. Equipment purchase and setup expenses were amortized over an estimated 5-year useful life.

Apheresis costs were estimated based on procedural costs plus costs of shipping using a cGMP compliant courier service. Materials costs for retroviral vector were estimated based on benchmarking of viral vector CMO production costs per lot, assuming production at the 200L scale, with sensitivity analyses testing upstream viral yields ranging from 6E5 to 1.5E7 IU/mL [6], downstream purification yields in the range of 25–75%, and an MOI of 3 [5].

Sensitivity testing was performed to test variability in COGS within the full extent of reasonable input assumptions for each variable. Except as described for the 'Compounded Effect' scenario below, sensitivities were tested using modification of a single variable at a time for simplicity.

DOI: 10.18609/cgti.2018.108

FINDINGS

Estimated cost of goods is in line with biopharmaceutical industry standards

Our base case estimate of COGS was $58,200 per dose, with an estimated range of between $48,000 and $106,000 per dose based on sensitivity analyses of key input assumptions (Figure 2). At 13 to 28% of the Yescarta® list price of $373,000, these estimated COGS are in line with biopharmaceutical industry standards in the range of 15–25% [7,8]. Therefore, we conclude that, in order to offer similar therapies at more affordable prices without disincentivizing biopharmaceutical and/or venture investment in C&GT products, it will be necessary to effect dramatic reductions in costs of manufacturing.

Labor Costs are the Largest Contributor to COGS

By far the greatest contributor to COGS was labor, at an estimated 71% of manufacturing costs (Figure 3). Just under half of labor costs (48%) were for manufacturing personnel, with the remainder divided between quality control (QC; 16%), quality assurance (QA; 16%), supply chain management (SCM; 11%) and other functions (project management, facilities, etc.; 9%).

Materials costs represented 18% of COGS, with the largest components coming from apheresis, disposables, and virus. Although manufacture of a large lot of virus can represent a significant upfront effort and expense, using published scalable methods to estimate vector production costs and yields, viral vector costs were estimated to represent

FIGURE 2

Cost of goods estimates – sensitivity testing.

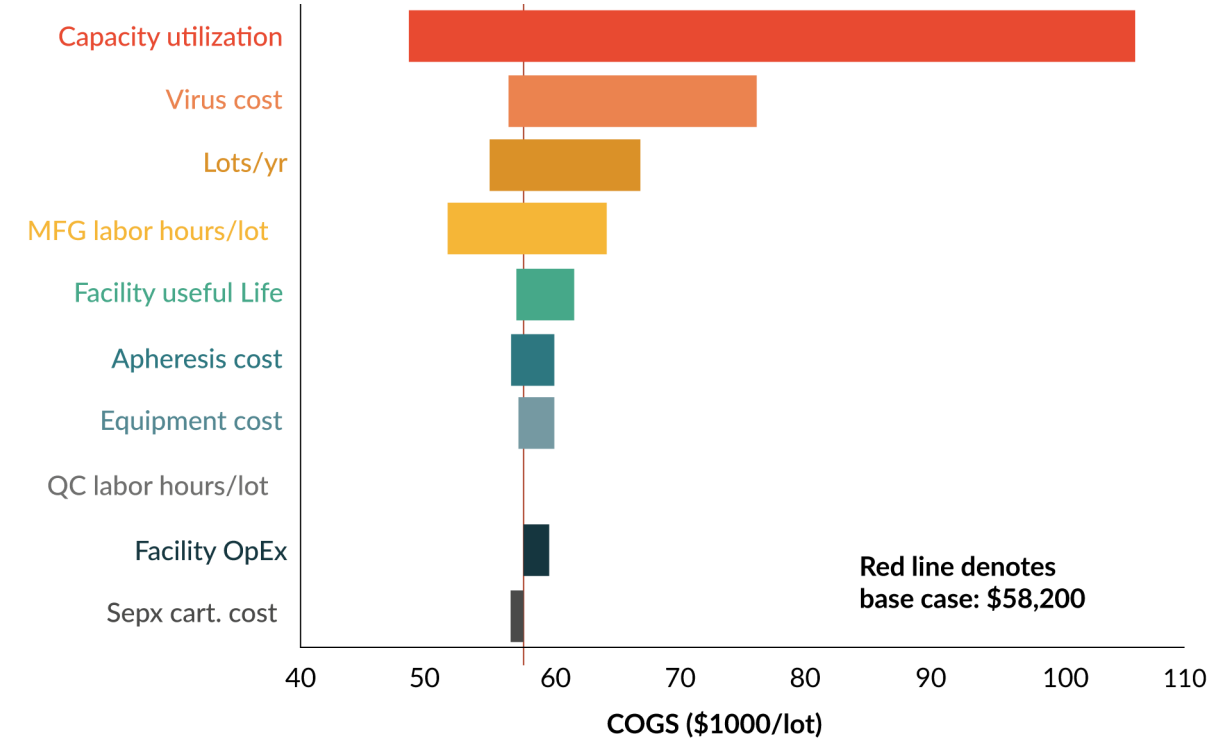
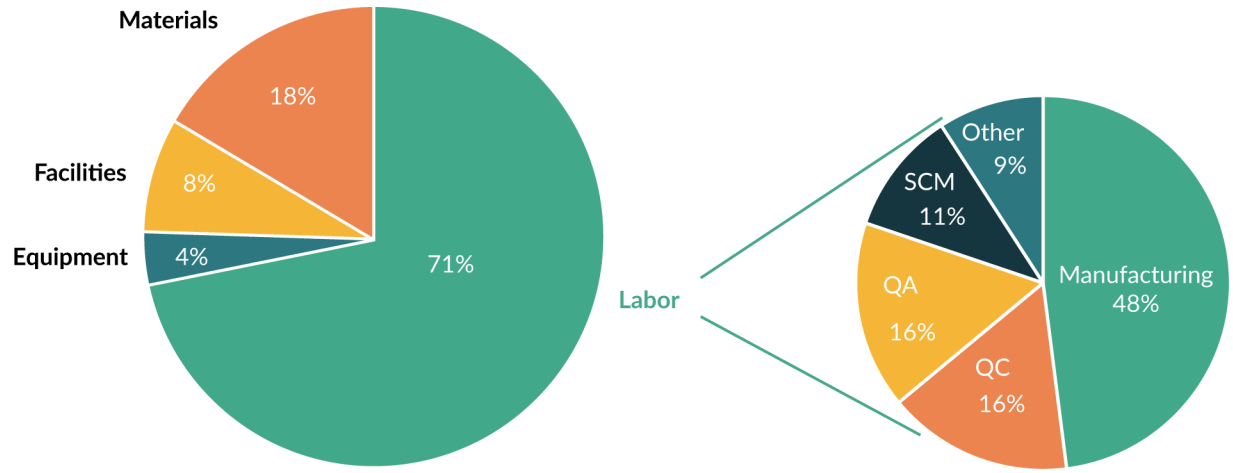


FIGURE 3

Breakdown of cost of goods by component.



only 3–4% of COGS in our base case scenario. However, the model was fairly sensitive to assumptions of less scalable methods and/or poor viral vector process yields (Figure 2), with virus costs reaching as much as 26% of COGS under 'worst case' assumptions for viral titer and purification yields, emphasizing that employing scalable viral production is a critical step in establishing a commercially viable COGS.

Facilities and equipment expense represented significant upfront investments for construction, equipment procurement, and validation, however they were a relatively minor component of per lot cost of goods at just 8% and 4%, respectively, when amortized across their estimated useful lives.

Duration of T-cell Expansion Post-Activation Significantly Impacts COGS

Our base case model assumes a process of 7 days in duration, including 3 days of T-cell expansion post-activation (Figure 1). However, published process descriptions allow for an optional additional 3 days of T-cell expansion if necessary. To explore the impact of process duration on COGS, we modeled this longer process as well. The impact of an extended process was not insignificant. As a result of increased labor hours and longer facility and equipment cycle times, cost of goods increased by 16% to $67,600. This change was driven by both increased labor costs and reduced facility and equipment cycle times associated with the longer process duration.

Autologous Nature of Product & High Labor Costs Limits Benefits from Economies of Scale

Although the high list prices of Kymriah®, Yescarta® and Luxturna™ have attracted much attention, the fact that these products are currently approved only for a small handful of orphan and ultra-orphan indications make a near term affordability crisis unlikely. The prices of the one-time treatments Kymriah® and

DOI: 10.18609/cgti.2018.108

Yescarta® are similar to the annual costs for many enzyme replacement therapies for orphan conditions (e.g., Shire's Elaprase, BioMarin's Naglazyme and Sanofi Genzyme's Myozyme) [9]. Furthermore, even if these products achieved 100% market penetrance of their approved indications at list price (highly unlikely, even for products addressing orphan conditions with few treatment alternatives), the total combined cost to the system for purchase of these three products (not including ancillary care costs) would equate to $3 billion annually, which is less than 1% of annual prescription drug spend, and less than 0.1% of annual healthcare spend in the USA [10].

However, with many similar therapies in development for more and larger indications, it is easy to see the near-term potential for the availability of highly effective C&GT products to rapidly eclipse society's ability to pay for them. According to the Alliance for Regenerative Medicine, there were 314 clinical trials ongoing for gene-modified cell therapies during Q2 2018, including 166 Phase 2 and 14 Phase 3 trials [1]. Among C&GT products in clinical development are many products for far more common indications such as breast and lung cancer, stroke and heart failure. Unless these therapies can achieve manufacturing costs that enable prices well below those of any C&GT product currently on the market, an affordability crisis seems inevitable. As shown in Table 1, adoption of a new therapy priced at $350,000 by just 10% of incident cases for these four common conditions would amount to an additional $72.6 billion in annual drug spend. This equates to 22% of the total 2016 US prescription drug spend across all product classes and disease indications (Table 1). Although some of the additive costs would likely be offset by reduced costs in other categories of healthcare spend, it is clear that any such savings would be inadequate to fully offset the additive drug costs. For example, the annual cost of $213 billion for adoption by 10% of incident stroke cases is is approaching the estimated total annual cost of stroke care in the US ($34 billion) [11].

We investigated the potential impact of economies of scale on cost of goods by modeling COGs at annual production volumes ranging from 500 to 5,000 lots per year. Not surprisingly, given the autologous nature of the product and the high contribution of labor costs, economies of scale were limited, with only a 22% reduction in cost of goods anticipated from a 10-fold increase in production volume. We conclude that further cost reduction levers will be critical to enabling broad adoption of C&GT therapies in prevalent diseases.

TABLE 1

Annual prescription drug spend for $350,000 product adopted by 10% of incident patients.

Indication	US annual incidence	Annual cost at 10% adoption (US$ billions)	% of 2016 US prescription drug spend [10]
Stroke	610,000 [11]	21.3	6.5%
Heart failure	1,000,000 [11]	35.0	10.6%
Breast cancer	266,000 [12]	9.3	2.8%
NSCLC	199,000 [12]	7.0	2.1%

Challenge of Accurately Estimating Product Demand Introduces Significant Risk to COGS

In contrast to the relatively modest impact of economies of scale, we found that efficient use of established capacity was a critical factor influencing cost of goods. Given the long lead times necessary to hire and train qualified staff, build and validate a manufacturing facility, and purchase and validate equipment, it is necessary to predict capacity needs well in advance of the date that capacity needs to be online. Once the decision is made to target capacity to manufacture a given number of lots per year, expenses such as personnel, equipment and facilities become fixed costs that cannot be easily adjusted for changes in anticipated demand. Reimbursement uncertainties and lack of clear precedents to follow in modeling commercial adoption of these therapies make such capacity planning particularly challenging in the case of novel cell and gene therapy products. Therefore, we wanted to model the impact of overly optimistic commercial assumptions on per lot COGS.

To do this, we modeled the COGS per lot for a facility constructed, equipped and staffed to deliver 1500 lots/year (our base case model assumption) if operated at 100%, 80%, 60%, or 40% of capacity (Figure 4). Given the significant fraction of costs that cannot be adjusted quickly for changing demand, the impact of capacity underutilization on cost of goods was highly dramatic – COGS per lot at 40% capacity utilization was $106,000, or nearly twice that of our base case scenario.

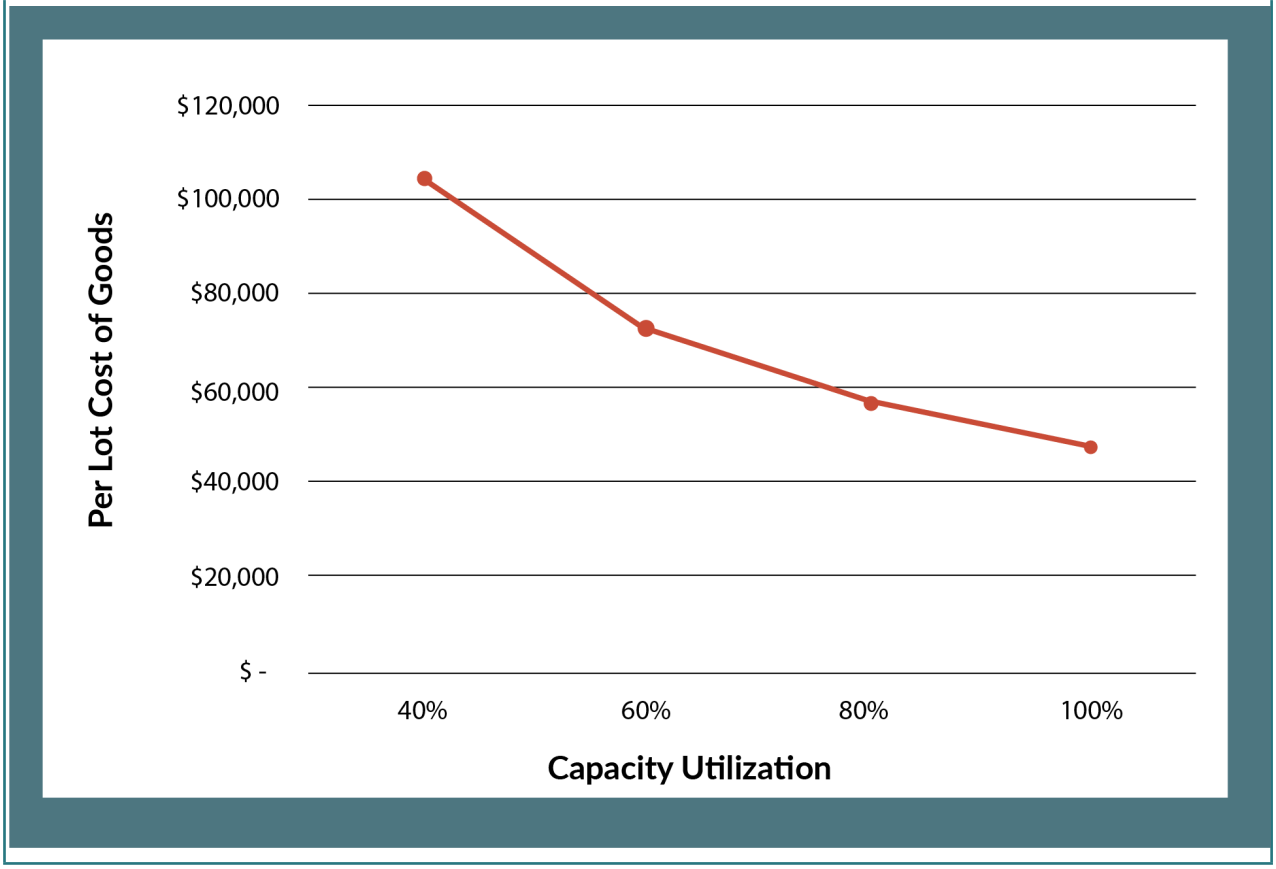
The need to predict capacity requirements well in advance to optimize cost of goods creates a difficult challenge for the field. On the one hand, overestimation of commercial adoption clearly has major, adverse consequences to COGS. On the other hand, given the 'on demand' nature of manufacturing of these products, it is critical to ensure adequate capacity is on hand to address demand. Given the high potential for rapid progression of the patient populations currently treated by these types of products, one could argue the need to even plan for some surge capacity to avoid delays in product turnaround due to peaks and valleys in demand. This assumption was built into our base case model, which assumed an average of 80% capacity utilization to allow for some variation in weekly demand.

The implications of this finding are that, while our estimate of steady state COGS is in the range of typical biopharmaceutical COGS as a percent of list price, the level of risk associated with COGS is substantially higher for autologous cell therapy products than for traditional biopharmaceuticals. For this reason, it will be critical for companies to invest in developing thoughtful and realistic commercial models that appropriately factor in considerations like adoption curves, potential reimbursement challenges, and the impact of competition. Products targeting conditions where rapid turnaround is less critical may be able to better manage their capacity through use of incoming frozen apheresis products and/or building in process hold steps to allow for optimized process scheduling across the 'peaks and valleys' of demand. The clear rationale for developing therapies for very serious conditions should also be balanced against the

DOI: 10.18609/cgti.2018.108

FIGURE 4

Impact of capacity utilization on COGS (at target capacity of 1500 lots/year).



very obvious benefits of addressing conditions in which immediate product delivery is less critical to enable such scheduling advantages to be captured. The ability to share risk surrounding commercial adoption curves and demand peaks and valleys is also highly attractive in this context, and suggests that manufacturing consortia or CMOs offering flexible overflow capacity could be interesting models for further exploration.

Finally, the need to avoid carrying excess capacity highlights the value of developing expedited training programs and adopting process automation and other productivity improvements to enable more nimble adjustments with respect to the single largest cost component driving capacity risk (labor).

Improvements in Productivity Not Only Reduces COGS, but Also Risks Associated with Product Demand Uncertainty

Given the high contribution of labor costs to overall cost of goods, we next looked at the potential impact of a variety of productivity improvements on cost of goods. As significant fractions of labor costs came from each of manufacturing, QA, QC and supply chain, we evaluated potential mechanisms to increase productivity across each of these functions.

We first evaluated the potential to improve cost of goods through productivity improvements in the largest single component of labor costs, manufacturing labor. One study in

the literature reported potential labor savings of up to 72% from use of automation in manufacturing of a similar autologous T-cell product [13]. Case studies on use of Lean manufacturing strategies in pharmaceutical and medical device manufacturing have reported labor savings ranging from 20-50% [14,15]. Novartis scientists have reported reductions of up to 80% in flow cytometry sample preparation and analysis time through use of an automated flow cytometry analyzer [16], and case studies of Manufacturing Execution Systems (MES) report reductions in documentation issuance of 60–75% [17]. We further posited that use of electronic batch records and digital supply chain management systems could deliver similar efficiencies in materials release, batch record review, and supply chain management, and that process validation could be used to reduce skilled labor touchpoints as processes mature. We therefore modeled the COGS impact of labor productivity improvements of up to 70% across manufacturing, QC, QA and supply chain management functions through the combined impact of validation and automation improvements.

Not surprisingly given the significant contribution of labor costs, the impact of productivity improvements on COGS was substantial (Figure 5). For example, in our 'base case' assumption of 80% capacity utilization, a 50% increase in labor productivity across manufacturing, QC, QA and supply chain management functions led to a 35% decrease in overall product COGS from $58,200 to $37,600 per patient. Additionally, the reduction of labor costs had a compounding effect on COGS reduction, reducing both cost per lot and risk of COGS increases due to capacity underutilization (as shown by convergence of the lines at the right end of the chart in Figure 5).

Compounded Effect: A 'Best Case' COGS estimate

We next evaluated the compounded impact of multiple improvements to determine a 'best case' COGS estimate for a highly optimized process. For this analysis, we assumed: a target capacity of 5,000 lots per year; 100% capacity utilization; implementation of automation, lean manufacturing and digital technologies to achieve 70% productivity improvements across manufacturing, QA, QC and supply chain functions; a 50% drop in facility cost per lot due to more efficient facility utilization through automated manufacturing; and a 30% drop in materials costs due to economies of scale and negotiation of bulk purchasing discounts. The compounded effects of these many improvements brought COGS down to $21,400 – a substantial improvement, but still predictive of a price above $100,000 at typical biopharmaceutical gross margins. We conclude that, while significant improvements in COGS are feasible, ultimately more substantial changes such as transitioning to allogeneic platforms may be necessary to make C&GT products affordable for very large indications such as stroke, heart failure or diabetes.

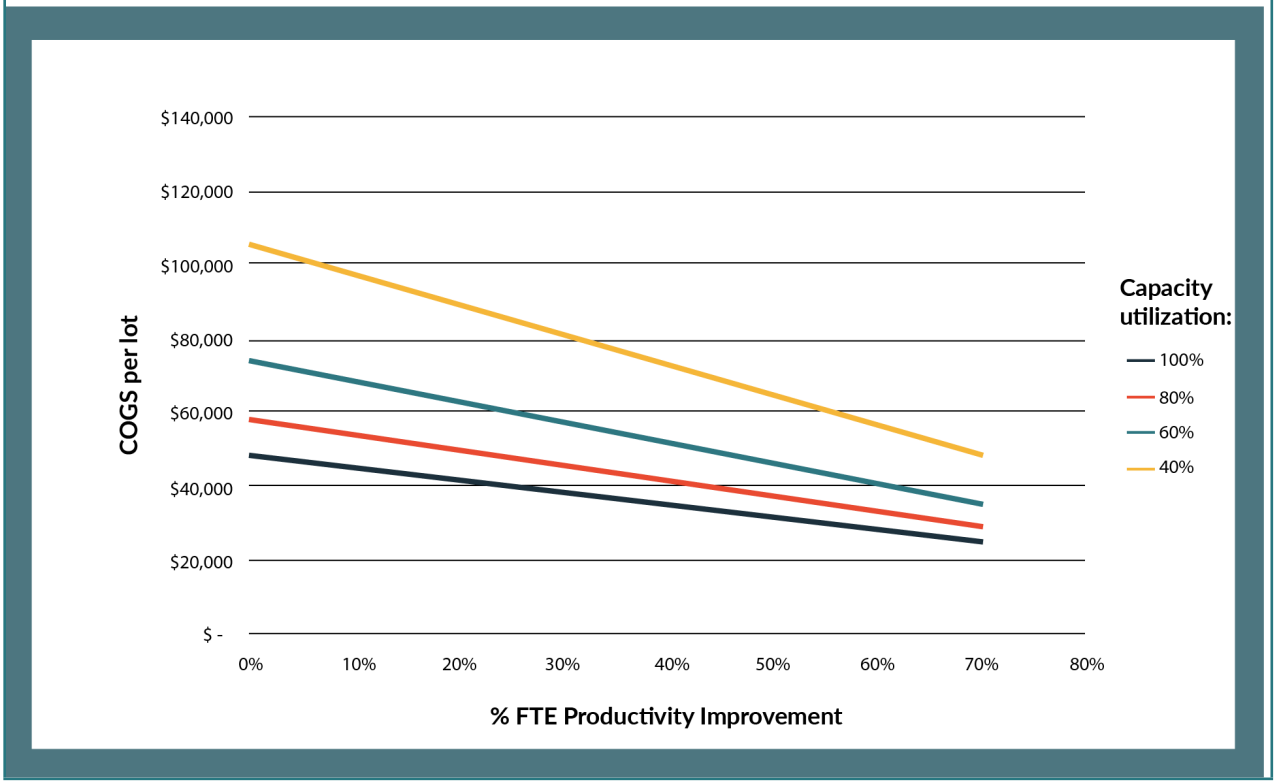
CONCLUSIONS

Although the rare indications of currently marketed gene modified cell products make an immediate affordability crisis unlikely, it will be critical to bring down costs substantially

DOI: 10.18609/cgti.2018.108

FIGURE 5

Cost of goods as a function of productivity improvement and capacity utilization (at target capacity of 1500 lots/year).



in order to affordably address more prevalent indications. By our calculations, current cost of goods for gene modified cell products leave little room for price reduction without disincentivizing investment in this important class of therapies. Furthermore, companies take on significant margin risk due to the need to build capacity well in advance of achieving clarity regarding commercial demand. Economies of scale are unlikely to deliver sufficient COGS improvements to enable affordable manufacturing of autologous gene modified cell products for larger indications. We predict that significant COGS improvements are achievable, but that innovation on multiple fronts will be required to achieve the level of dramatic COGS reduction required to make autologous gene modified cell therapies substantially more affordable than they are today. We propose that the keys to reducing both COGS and COGS associated risk are efficient capacity utilization, use of productivity enhancing technology solutions to reduce fixed labor costs, and establishment of rapid and efficient training programs to enable a more nimble response to evolving demand forecasts.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The authors are employed by Dark Horse Consulting, a provider of consulting services to the Cell and Gene Therapy field. No writing assistance was utilized in the production of this manuscript.

REFERENCES

1. Alliance for Regenerative Medicine, Quarterly Data Report, Q2 2018.
2. Reuters, UK deal makes Novartis cancer cell therapy available to kids, September 5, 2018.
3. Institute for Clinical and Economic Review, Voretigene Neparvovec for Biallelic RPE65-Mediated Retinal Disease: Effectiveness and Value, Final Evidence Report, February 14, 2018
4. Better M, Feldman SA, Rosenberg SA. Methods for Producing Autologous T cells Useful to Treat B cell Malignancies and Other Cancers and Compositions Thereof. US Patent Application 14/614,400.
5. Better M, Chiruvolu V, Sabatino M. Overcoming Challenges for Engineered Autologous T cell Therapies. *Cell Gene Ther. Ins.* 2018; 4(4): 173–86.
6. Wang X, Olszewska M, Qu J *et al.* Large-scale Clinical-grade Retroviral Vector Production in a Fixed-Bed Bioreactor. *J. Immunotherapy* 2015; 38(3): 127–35.
7. Gyurjyan G, Thaker S, Westhues K, Zwaanstra C. Rethinking Pharma Productivity. Latest Thinking (McKinsey), January 2017.
8. Basu P, Joglekar G, Rai S, Suresh P, Vernon J. Analysis of Manufacturing Costs in Pharmaceutical Companies. *J. Pharm. Innov.* 2008; 3: 30–40.
9. Luzzatto L, Hyry HI, Schieppati A *et al.* Outrageous prices of orphan drugs: a call for collaboration. *The Lancet* 2018; 791–4.
10. Centers for Medicare and Medicaid Services, National Health Expenditure Data, 2016.
11. American Heart Association, Heart Disease and Stroke Statistics – 2018 Update.
12. National Cancer Institute, Surveillance, Epidemiology and End Result Program, Cancer Stat Facts: https://seer.cancer.gov/statfacts
13. Morrisey JB, Shi Y, Trainor N. End-to-End Cell Therapy Automation: An Immunotherapy Case Study. Biopharm. International, September 2017; 10–8.
14. Boppana C, George D. Improvement of manufacturing operations at a pharmaceutical company: A lean manufacturing approach. *J. Manufacturing Tech. Manag.* 2011; 23: 56–75.
15. Marchwinski C. A Case Study: Sustain your Lean Business System with a 'Golden Triangle'. Lean Enterprise Institute, April 2014.
16. Reitz R. Designing Technologies to Meet Manufacturing Needs. Presentation at CBI Conference: Optimization of Cell and Gene Therapies Production, Washington DC, March 26, 2018.
17. Emerson Syncade marketing materials: https://www.emerson.com/documents/automation/biotech-firm-automates-manual-document-management-tasks-saving-time-money-while-improving-compliance-en-82174.pdf

AFFILIATIONS

Andrew Steinsapir,

Consultant, Dark Horse Consulting

andrew@darkhorseconsulting.us

Katy Spink,

Chief Operating Officer and Managing Partner, Dark Horse Consulting

katy@darkhorseconsulting.uss

DOI: 10.18609/cgti.2018.108

CELL & GENE THERAPY INSIGHTS

STRATEGIES FOR SCALE-UP & SCALE-OUT

SPOTLIGHT

Back to the future: where are we taking lentiviral vector manufacturing?

Hanna J Lesch

From more than 200 clinical trials involving lentiviral vectors, only a handful of products have reached marketing approval. One reason for this may be the technical bottleneck in large-scale lentiviral vector manufacturing. Today there are several upstream and downstream technology solutions, which claim to support clinical manufacturing at large scale. These still have several limitations, such as a complex production methodology and the relatively high cost of the goods. The fragile nature of the vector further causes its own challenges. No one knows yet where the future will take us. This insight covers an overview of the current technology and discusses the possible future solutions for lentivirus manufacturing.

Submitted for Peer Review: 18 Oct 2018 ▶ **Published**: 19 Dec 2018

ROUGH ROAD FROM RESEARCH TO COMMERCIAL ADVANCED MEDICINE

Although Kymriah™ and Yescarta™, two advanced cell therapeutic products where T cells are *ex vivo* modified with lentiviral vector (LVV) to express chimeric antigen receptors (CAR), have achieved marketing approval, few other LVV products have even managed to reach late stage clinical trials. A major challenge holding back progress in the field is related to technical difficulties with the manufacturing of LVVs. Up to now, production for research purposes has been based on the co-transfection of the adherent Human Embryonic Kidney (HEK) 293 variant, 293T cells with different plasmid constructs [1,2]. As a straightforward method, this provides an attractive small scale manufacturing strategy for early clinical trials, but it is not suitable for commercial stage production,

where plasmid transfections at GMP manufacturing scale, small batch sizes with relatively low titres, high cost of goods, the fragile nature of the lipid-enveloped virus and safety concerns are some of the challenges still to be overcome. In addition, there is the fragile nature of the lipid-enveloped virus.

SCALE-UP OF AN ADHERENT CELL PRODUCTION SYSTEM

For a long time, most production applications have relied on adherent cells cultured in two-dimensional (2D) flask-type approaches, such as Cell Factories or hyperflasks/hyperstacks [3,4]. However, the controllability in flasks is limited as is the scalability. To expand the production area means multiplying the units, which makes them impractical to handle. Microcarriers dispersed in suspension were considered an option for adherent cell culturing but they did not succeed as a popular technology for viral vectors [5]. Problems were reported in the handling of the carriers, expansion of a large cell mass and the need for labour-intensive operations for their separation from the vector later during the downstream process [6].

Pall brought to the market their iCELLis® technology, a disposable fixed-bed bioreactor with an integrated perfusion, developed for scaling up adherent cell-based production systems (Figure 1). There are two different size bioreactors available: iCELLis Nano, providing a culture area up to 4 m^2, and iCELLis 500, with a culture area from 66 to 500 m^2. iCELLis allows a closed and controlled environment and meets the current GMP requirements. The iCELLis Nano has been used for a range of vector applications, such as for adenovirus [7], retrovirus [8] and AAV [9]. Previously, we evaluated for the first time the iCELLis 500 large-scale bioreactor for the manufacturing of Ad5 vectors [7]. Recently, we developed an adherent cell-based manufacturing process also for LVVs. A small-scale process development was originally done in iCELLis Nano system. More recently we have tested different fixed-bed sizes and compaction, and optimized the cell culture parameters, such as cell density and perfusion and transfection conditions [10]. iCELLis 500 100 m^2 and 333 m^2 scale-up runs for two different constructs were successful with a high yield of vector produced. Compared e.g to Cell Factories [3], the iCELLis500 systemcan provide up to 30 times bigger scale to produce vector per single batch in a controlled manner. Such a system may provide one manufacturing solution for future clinical applications.Nevertheless, the adherent production systems are still not trouble-free. In most cases, adherent cell cultures use animal-derived products, mainly fetal bovine serum (FBS), as the culture medium constituents. The serum provides an attachment factor for adherent cells but it also contains other constituents, e.g. growth factors, hormones, additional amino acids, vitamins, trace elements, fatty acids and lipids [11]. FBS has benefits in virus production. It can enable a remarkable increase in the production of LVV and ultimately, stabilize the produced virus [12]. However, there are number of reasons why the use of FBS

DOI: 10.18609/cgti.2018.109

is problematic, e.g. high lot-to-lot variability, introduction of animal components to the cell culture medium and the risk of potential microbial contamination. The serum can also complicate downstream purification. Serum is very expensive and resources need to be directed to thorough tests [13]. Last but not least, ethical issues need to be considered too. Therefore, the demand to reduce the use FBS or to replace it totally has becoming an ever urgent issue. Future development should explore chemically defined media that can maximally support LVV production. Several commercial serum-free mediums are available for HEK293-derived cells, but most of these have been developed and optimized for suspension cells. Whilst some media may well support cell growth, they do support the productivity and vice versa. Pro293A-CDM (Lonza) is an example of a serum-free chemically defined medium for adherent cells that has shown promise for LVV production with PEIpro transfection [14]. The adaptation of the cell line into serum-free conditions is generally time-consuming and the removal of FBS can decrease the titer. Luckily, virus production can be increased again by adding supplements, e.g. Thermo Fisher Scientific supplies LV-MAX-Supplement and LV-MAX-enhancer to boost LVV production in serum-free conditions. Lipids were shown to be one key serum component during retroviral vector production that could increase the yield and vector stability [12]. Several lipid formulations are currently on the market, e.g. from Thermo Fisher Scientific, GE Healthcare and Sigma-Aldrich. The problem with the serum-free media and supplements is still their relatively high price.

FIGURE 1

iCELLis 500 is a disposable fixed-bed bioreactor for scaling up adherent cell-based production systems.

SUSPENSION CELLS HAVE BENEFITS

HEK293-derived cells might be adapted into serum-free suspension growth as another option for upstream development. For a long time,

suspension bioreactors have been the only option available for closed, scalable, cost-effective production where culture conditions, such as the pH, pO_2 and pCO_2 can be controlled. The first successful PEI-mediated plasmid transfection-based method for producing LVVs in suspension cultures was published by Segura et al. in 2007 [15]. Since then, there have been other publications showing that LVV production in a suspension bioreactor is feasible [16,17]. An important step in the development towards this resulted from [18] cross-talk between scientists in the field and technology suppliers. Today, several suppliers provide serum-free media suitable for 293-derived cells, such as Ex-CELL 293 (Sigma-Aldrich), Pro293S-CDM (Lonza), Freestyle CD293 and Expi293™ (Thermo Fisher Scientific). Many suppliers also provide large-scale disposable bioreactors. Examples are e.g. stirred tank bioreactors from Mobius CellReady (Merck Millipore), HyPerforma (Thermo Fisher Scientific), XCellerex XDR (Ge Healthcare), Biostat SRT (Sartorius Stedim) and Allegro STR (Pall). Another option is wave type bioreactors, called WAVE (Ge Healthcare) or Cultibag RM (Sartorius Stedim) [18,19].

Suspension cells do not require the use of FBS so are a natural choice in the future. They also provide an alternative approach for the transfection as suspension cells can be driven through flow electroporation and transfection without the need for expensive transfection reagents [20]. The suspension mammalian cell electroporators, such as 4D-Nucleofector™ (Lonza) and Maxcyte VLX® (MaxCyte) are interesting options but capacity may become limited in very high cell numbers (eg. >1 $x10^{12}$ cells in 200L bioreactor). Suspension platforms are in the pipeline for many companies in the field, such as Oxford Biomedica [21] and Genethon [22]. This is the direction manufacturing is developing for the future.

Many processes described in the literature are batch or fed-batch mode production. If fresh medium is provided continuously while removing the metabolites from the culture, the cell density and viability can be increased. Up to a certain level, this has a direct impact on the increased productivity. Perfusion technologies integrated into suspension bioreactor based have been based on tangential flow filtration (TFF) or alternative flow filtration (ATF) (Repligen) and conditions needs to be optimized for both producer cells and LVVs as the shear stress could break lipid membrane of the cells or the fragile virus [23].

The transfection step has several process parameters (e.g. cell density, transfection reagent, plasmid amount, reagent versus plasmid amount ratio and complex formation time) that need to be optimized. Micro-scale automated bioreactors can speed up the optimization in a controlled environment and in a very small scale (ambr15, Sartorius Stedim or Micro-24 MicrReactor, Pall) usually in a very cost-effective manner. Rapid characterization of conditions are, of course ideally supported by a proper statistical design of experiments (Doe) [24].

ARE PLASMIDS THE WAY TO GO?

The most commonly used plasmid constructs are third-generation packaging plasmids [2], self-inactivating transfer construct [1,25]

DOI: 10.18609/cgti.2018.109

and the vesicular stomatitis virus g protein as an envelope protein for pseudotyping [26]. Traditional calcium phosphate-based transfection is cheap and practical in the small scale but has limitations in terms of its reliability, repeatability and scalability [4,27]. Polymer-based approaches, such as polyethylenimine (PEI)-mediated, or lipid-based transfections appear efficient, better for scalability, less toxic for the cells and suitable for suspension cells [15,28,29]. Raw material for GMP manufacturing must also be sourced from qualified suppliers with appropriate documentation of the batch manufacturing and quality control. Hence, the use of commercial quality assured transfection reagents, such as PEIpro (Polyplus) or Lipofectamine (Invitrogen), is increasing.

We and others have shown that the large-scale plasmid transfection can be successful as a technique, and it is not that much of a 'showstopper' anymore. However, many issues remain with its use. Large batch production can require hundreds of milligrams or even grams of plasmid. The production of these large quantities of plasmid can be very expensive with all the requirements for the master, working cell banks, large fermentation, thorough purification, fill and finish, quality control and documentation [30]. Furthermore, plasmid DNA can affect vector downstream processing [31] and there is a risk of unwanted plasmid recombination [32]. Furthermore, the human immune system can recognize plasmid DNA and induce an inflammatory response and silencing of the transgene expression, in the unlikely event that CpG contaminants can be part of the LV vector prep [33]. In addition, the use of antibiotic selection pressure during the plasmid production is a major regulatory concern. Antibiotic-resistant bacteria are global health problems. The risk with plasmids is not only the antibiotic itself, but the antibiotic resistance gene might, in the worst case, end up in the patient in a scenario called "horizontal genetic transfer" (reviewed [34]). Major risks have already been seen with β-lactam antibiotics, such as penicillin, streptomycin and ampicillin, whereas antibiotics, such as kanamycin, tetracycline and neomycin, are still accepted. Many players in the field are today using kanamycin-selection in their plasmid constructs instead of the traditional ampicillin. In the future, a serious attempt could be regulated to use mini circles or nanoplasmids to effectively result in antibiotic-free plasmids during viral vector production.

There are different types of antibiotic-free systems available for supporting plasmid replication/selection in bacteria cells without the need for the addition of antibiotics [34]. Many of the approaches, however, have not yet been extensively applied to DNA production for Gene Therapy Minicircle technology (Plasmid Factory) has been proven to improve the quality of adeno-associated vectors. Intramolecular recombination of the parental plasmid leads to minimal plasmid DNA with basically only the gene of interest. With this technology there is a minimal risk for encapsidation of the bacterial backbone sequence, a lack of need for an antibiotic gene and improved transfection efficiency for a small plasmid and improved transduction efficiency of

AAV [35,36]. The development of minicircles, nanoplasmids or equivalent structures for LVV production is ongoing.

STABLE CELL LINES

Stable producer cell lines are naturally desired to facilitate a cost-effective way to produce LVVs and increase reproducibility, quality and safety. Scalability is improved when these cell lines can be cultured in suspension serum-free systems [37]. The toxicity of lentiviral protease [38] and the envelope protein VSV-G [26] has hindered the development of stable cell lines for LVVs and constitutive production has yielded only low titres. The development of stable cell lines is very time-consuming. The most frequently used inducible system is the tetracycline/doxycycline antibiotic system, where transcription is regulated through the tetracycline response element (TRE) either by adding or removing the antibiotics in the cell culture medium (reviewed [6]). Constitutive packaging cell lines provide another option, but the traditional VSV-g envelope is replaced with a less cytotoxic option, such as RD114-TR [39,40]. A recent approach was to introduce a mutation into viral protease to minimize its cytotoxicity for the producer cells [41]. Up to 1×10^7 Tu/ml titer has been achieved with many different producer cell lines [6]. A new approach to create producer cells is genome editing, where the characteristics of the produced virus can be modified in the desired direction, such as when Milani et al. modified producer cells to be free of MHC, which reduced the vector immunogenicity [42].

DOWNSTREAM PROCESS OF LENTIVIRAL VECTORS

The lentivirus is sensitive to high temperature, pH and salt concentration changes and it is well known to be very shear sensitive. The stability of the vector can be strengthened by pseudotyping the vector with other envelope proteins, such as VSV-g. Its fragile nature makes downstream purification very challenging. The traditional way is to use an ultracentrifugation-based process [26], but this is not nowadays considered as a valid option. Typical large-scale purification consists of steps for clarification to remove the cell debris, tangential flow filtration (TFF) to pre-concentrate and diafiltrate the product, capture and polishing by chromatographic methods and final formulation and concentration (reviewed in [43]) (Figure 2).

Clarification by depth filters is a straightforward process step performed using a peristaltic pump and the technique does not need very complicated equipment. In the TTF step, the product is circulated as a retentate through the filter (cassette or hollow fiber), while the permeate goes to waste through the membrane pores. The recovery of LVV can be high (>97%) in TFF as long as the shear stress (pressure and flow rate) are kept reasonably low [44]. Anion exchange chromatography, either resin [45], membrane [4,23], monolith-based [17] or affinity-based chromatography [15] have been developed for LVVs. However, the chromatographic step is still the bottleneck in the field as the recovery of the product is typically very low (<50%). Overall, the downstream recovery for LVVs have been only c. 30% [23], so further process optimization is still needed.

DOI: 10.18609/cgti.2018.109

Today several systems are available to support large-scale purification. Improved technology has involved single-use columns and flow paths, which save time and eliminate the need for cleaning and cleaning validation. Several single-use technologies are available, e.g. ÄTKA™ family (GE Healthcare), Allegro™ (Pall) and Mobius® (Merck Millipore).

Downstream processing includes also the endonuclease step to remove/decrease the level of host cell DNA and plasmid DNA [31]. The standard method to date has been to add Benzonase (Merck Millipore) to the harvested product or later during the purification. Competitors to Benzonase are now emerging with a number of new endonucleases entering the field, e.g. Denarase® (C-Lecta) and SAN High Quality (ArcticZymes), the latter of which is said to be more efficient in higher salt conditions.

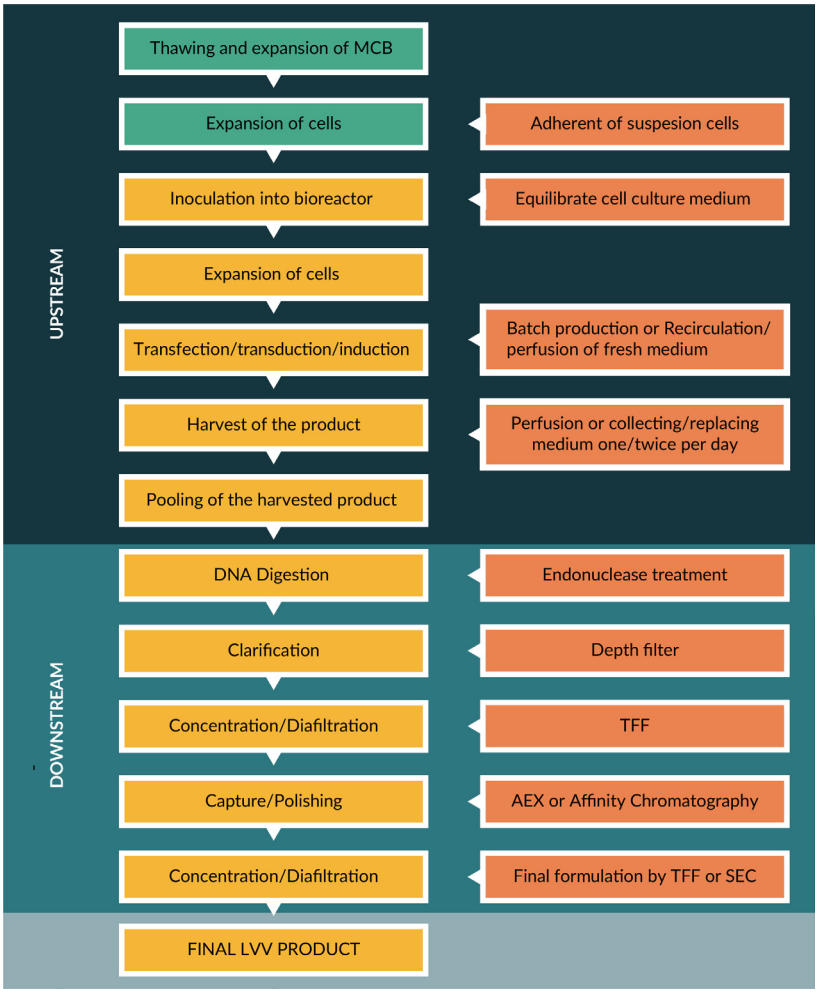
CONTINUOUS PROCESSES

The latest approach in the field of biopharma is a move to continuous processes where the production is long term. The product is harvested continuously, followed by direct downstream processing until the end-product is obtained. The continuous production of monoclonal antibodies can take weeks, even months per batch. However, virus cytotoxicity towards the producer cells is hindering the development of processes taking months for viral vectors. In an ideal system, the whole process would be integrated, fully automated and a product coming from the controlled upstream system would continue directly to purification. This would naturally lead to increased productivity, cost reductions and increased flexibility.

Upstream, continuous production would require monitoring the culture using automated sampling systems or probes and controlling the run based on the monitored parameters. The typical process parameters that are monitored are pH, DO, pCO_2, the number of viable cells and the viability, nutrient availability and waste metabolite concentration and viral titer. pH, DO and pCO_2 probes have been used for a long time in all existing bioreactors. pH measurement is still troublesome as a shifting of the measured value often occurs

FIGURE 2

Process flowchart for lentiviral vector manufacturing.



and thus this can require separate sampling and off-line calibration during runs. Cell growth has been traditionally analyzed by sampling the cells and counting them using a haemocytometer, but improved technology is now providing alternatives. Growing cells with an intact plasma membrane is one option as this acts as a capacitor under the influence of an electric field. This capacitance can be measured and converted to the live biomass reading, typically cells/ml (ABER Instruments). Capacitance can be used not only for monitoring the cell growth but also as a basis for controlling the system, e.g. the perfusion rate based on the cell number [10,46]. Such a system is suitable for suspension cells and adherent cells, though in fixed-bed bioreactors only the top carriers can be monitored. Nutrient availability and the amount of produced metabolite side products require sampling of the medium. This can be done using an auto-sampling system, which takes the samples from the bioreactor and creates the process parameter measurements using external equipment, such as the Bioprofile family (Nova Biomedical) or Cedex family (Roche) equipment. Also glucose and lactate probes directly installed into the bioreactors have been developed. More advanced solutions could also involve real-time systems that would analyze the metabolic activity of the culture (e.g. Ranger technology, Stratophase).

The implementation of a continuous downstream system has been increasing in the recombinant protein and monoclonal antibody fields and might be a future direction in the gene therapy field as well. Traditional TTF operates by circulating the retentate through the filter. Merck Millipore and Pall have launched single-pass TFF systems, which runs at constant operating conditions, and no retentation return is needed as the product is sufficiently concentrated/diafiltrated. The new Cadance™ Inline Concentrator (Pall Life Sciences) utilizes single-pass technology and allows direct flow-through and volume reduction of the in-process product. Single-pass technology reduces the shear damage, improves the recovery, reduces the hold-up volume and is theoretically simple to operate ([47], Merck Millipore, Application note; Pall Life Sciences, Application note). Traditional chromatography is a batch process whereby the product is loaded into one column, so scaling up means using a larger size column. Such process steps can become very expensive and time-consuming. The recovery can be also lower because suboptimal usage of the chromatography column may lead to a loss of important breakthrough products into the waste. Multi-column chromatography using the Cadence™ BioSMB platform is the first scalable, GMP compliant, disposable, continuous multi-column chromatography system (Pall Life Science, Application note). Multi-column chromatography operations are performed in a small-scale column with maximal recovery. The breakthrough virus from the first column is loaded and captured by the second column, while other columns can be for elution, washing, CIP or a regeneration stage for re-use. BioSMB has been used for viral vaccine purification with promising results (Tarpon Biosystems Inc.). A continuous downstream eliminates the hold steps, decreases the process

DOI: 10.18609/cgti.2018.109

time and buffer consumption and reduces the footprint and product costs. This can be also very crucial for LVVs, which are not stable at room temperature [48]. A continuous process would make the operating hours more reasonable than stepwise batch downstream.

LVVs are typically stored at below -70°C. However, the LVV is not particularly stable and its activity decreases during storage or freezing/thawing[48]. Formation of ice crystals inside the virus, pH changes and osmolarity can affect the viral membrane and proteins, and decrease the activity [48–51]. Such decreases can be reduced by optimal formulation of the final buffer. Sugars and salts are the main buffer components [49]. Tris-based buffer has been frequently used for LVV [17,23,28] but problems are related to changes in temperature, which can lead to severe changes in pH. (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES buffer has smaller pH range and thus has been preferred choice in some of the applications [23,49,50]. LVVs for *ex vivo* transduction have often been formulated in the X-VIVO™ medium, which contains proteins and sugars to protect the vector [3]. Nilsson tested different sugars and lyophilization for LVVs [52] and Camro *et al.* showed that recombinant human albumin and lipoproteins were one of the most promising for protecting especially reverse transcription [51]. In Gene Therapy approaches, the human immune response is also a key issue to be considered as some of the components can induce the immune response of the patient, but this is an application-dependent issue.

MODERN ANALYTICAL TOOLS ARE NEEDED

Thorough testing of the vector is done already in preclinical stage. Also the GMP produced final product is heavily analysed by pattern of release assays (Table 1). The characterization and biologic activity of LVV for Gene Therapy follow the regulatory guidelines for other medicinal products, containing also the safety assay for Replication competent lentivirus (RCL) testing. The safety testing will be always unique and based on relevant biology of

TABLE 1

An example of releases assays for lentiviral vectors.

Purpose	Specific explanation
Safety/ sterility	RCL (following infection of a susceptible cell line and detecting amplification of the vector by p24 Gag increase or RTase assay) Insertional mutagenesis (integration site analysis by sequencing) Bacterial endotoxin Fungi Mycoplasma *In vitro* assay for the detection of viral contaminants
Identity	Morphology Identity (PCR) Determination of pH Determination of osmolality Determination of particle size and count
Purity	Host cell proteins (ELISA) Detection of residual host cell DNA (qPCR) Detection of residual plasmid DNA (qPCR) Detection of residual BSA Analysis of process contaminants (e.g. Bensonase ELISA)
Activity	Total particle number (amount of capsid protein taken into account that 1 pg GAG is approximate 10^4 particles or RTase activity or number of LV genomes by RT-qPCT) Transducing activity/integration capacity/incorporation of vector proviral DNA into target cells (qPCR analysis of transduced cells) Transgene expression (ELISA) Ratio between viral particles and functional viral vectors Functionality/potency of the product (application specific assay)

the product [53–55]. Nevertheless, modern technologies are needed to deepen the understanding, robustness and quality control of the process, but also the final product attributes. Scientists involved in the process development should know their process and its demands as the better the understanding you have, the better you can optimize your process. Standard titering, total DNA and total protein measurements are no longer giving adequate answers. Next-generation sequencing (NGS) technologies allow the possibility for greater understanding e.g. viral genome integrity, identification and integration sites in host cells, virus interactions, purity and quantifying transcripts. Equally, recent advances in mass spectrometry together with proteomics are providing broad information on the composition of virions, the structure, viral protein interactions and the effect of the infection on the cellular proteome [56]. Raman spectroscopy provides a structural fingerprint by which molecules can be identified. With TEM imaging and high-throughput quantitative analysis, MiniTEM (Viranova) offers innovative solutions to analyze the morphology, particle integrity, particle size distribution, purity, aggregation and empty versus full virus particle ratio from TEM pictures [57]. Digital-droplet PCR is replacing traditional PCR methods because it quantifies the absolute amount of target DNA and is an excellent method for accurate virus tittering without the need for applying a standard curve [58]. These advanced analytics involve the use of bioinformatics, biostatistics and data management and will be key for the success in this field in the future. For LVVs, a commercial standardized reference standard is not available yet. Well-characterized reference standards are important for a comparison of the internal results and also the results between different sites and can be used for the establishment of appropriate pre-clinical and clinical dosing. LVV transduced cells for the reference purpose have been developed [59] and can be used for standardizing integration copy number analysis. Another worldwide LVV reference standard is under development for standardizing the determination of particle concentration and infectious titer (Lentivirus Vector Reference Standard Initiative - ISBioTech). Nevertheless, the comparison between the different sites can be still troublesome if target cell or purpose varies.

COST OF GOODS

Ultimately, money drives change, and one big challenge in the current production is the very high costs. Production consumables, such as bioreactors, filters and columns, can be tens of thousands of dollars per batch. LVV manufacturing typically still uses plasmids and special reagents, such as FBS, transfection reagents or endonuclease, where the price tag is getting higher and higher as quality standard rise. Good quality control can easily take again tens of thousands more for all the analytics and when one also takes into account the very high overhead costs (facility, supporting actions, human resources), the final price can be suprising. Strimvelis™, a stem cell therapy of ADA-SCID patients, was initially priced at USD 665 000 per treatment. What can be done about this? Costs per dose

DOI: 10.18609/cgti.2018.109

can naturally be decreased by increasing the productivity and maximizing the recovery by efficient manufacturing process development. Automation and optimal raw material supply should theoretically decrease expense. Serum-free production systems would also be the preferred economic choice. Stable cells lines overcome the need for transfection reagents. More supplier and technology options bring increased competition, which has a positive influence on costs. Finding a balance between the optimal batch size and cycle times without forgetting about the risk factors can also create cost reductions. Ultimately, well-known Lean philosophy could bring a significant financial value [60]. This means that cost saving could be achieved by optimizing the procedures and manufacturing processes and making them as simple and straightforward as possible, while at the same time systematically minimizing all the waste from the process. These cost-related issues should always be considered early on in the product and process development pipeline.

CONCLUSION

LVVs can be manufactured today at a clinical scale, but significant effort is still needed to increase the yield and to make the production more cost effective. This will need deep collaboration between the science field and service providers, as manufacturing needs new technologies for the production. A future challenge is also the availability of production facilities. Not every company has the financial capability or knowhow to build its own production suites and so rely on contract manufacturing organizations, booking for production slots months ahead. Success stories have been published showing the technologies are there and the knowhow is there. The industry challenge is to put more effort in to optimizing manufacturing technologies in the future, step by step to get LVV products to phase III and beyond. This may be achievable with existing systems and technologies, but science is often not predictable and who knows what breakthroughs will emerge and radically change the paradigm for LVV production.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The author has no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock options or ownership, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

REFERENCES

1. Zufferey R, Dull T, Mandel RJ *et al.* Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J. Virol.* 1998; 72: 9873–9880.
2. Dull T, Zufferey R, Kelly M *et al.* A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* 1998; 72: 8463–8471.
3. Merten O-W, Charrier S, Laroudie N *et al.* Large-Scale Manufacture and Characterization of a Lentiviral ector Produced for Clinical Ex Vivo Gene Therapy Application. *Hum. Gene. Ther.* . 2011; 22: 343–356.

4. Kutner RH, Puthli S, Marino MP *et al.* Simplified production and concentration of HIV-1-based lentiviral vectors using HYPERFlask vessels and anion exchange membrane chromatography. *BMC Biotechnol.* 2009; 9: 1–7.

5. Wu S-C, Huang GY-L, Liu J-H. Production of retrovirus and adenovirus vectors for *Gene. Ther.* apy: a comparative study using microcarrier and stationary cell culture. *Biotechnol. Prog.* 2002; 18: 617–22.

6. Merten OW, Hebben M, Bovolenta C. Production of lentiviral vectors. *Mol. Ther. - Methods Clin. Dev.* 2016; 3: 16017.

7. Lesch HP, Heikkilä KM, Lipponen EM *et al.* Process Development of Adenoviral Vector Production in Fixed Bed Bioreactor: From Bench to Commercial Scale. *Hum. Gene. Ther.* . 2015; 26: 560–571.

8. Feldman SA, Goff SL, Xu H *et al.* Rapid Production of Clinical-Grade Gammaretroviral Vectors in Expanded Surface Roller Bottles Using a "Modified" Step-Filtration Process for Clearance of Packaging Cells. *Hum. Gene. Ther.* . 2011; 22: 107–115.

9. Powers AD, Piras BA, Clark RK *et al.* Development and Optimization of AAV hFIX Particles by Transient Transfection in an iCELLis ® Fixed-Bed Bioreactor. *Hum. Gene. Ther. Methods* 2016; 27: 112–121.

10. Valkama AJ, Leinonen HM, Lipponen EM *et al.* Optimization of lentiviral vector production for scale-up in fixed-bed bioreactor. *Gene. Ther.* 2018; 25: 39–46.

11. Gstraunthaler G. Alternatives to the use of fetal bovine serum: serum-free cell culture. *ALTEX* 2003; 20: 275–281.

12. Rodrigues AF, Carmo M, Alves PM *et al.* Retroviral vector production under serum deprivation: The role of lipids. *Biotechnol. Bioeng.* 2009; 104: 1171–1181.

13. van der Valk J, Bieback K, Buta C *et al.* Fetal Bovine Serum (FBS): Past - Present - Future. *ALTEX* 2018; 35: 99–118.

14. Guerin-Peyrou G, Vjetrovic J, Cuzange A *et al.* PEIpro®: Optimized PEI-based transfection reagents for production of clinical grade viral vectors. In: *ESACT Conference*. 2017.

15. Segura MM, Garnier A, Durocher Y *et al.* Production of lentiviral vectors by large-scale transient transfection of suspension cultures and affinity chromatography purification. *Biotechnol. Bioeng.* 2007; 98: 789–799.

16. Ansorge S, Lanthier S, Transfiguracion J *et al.* Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. *J. Gene. Med.* 2009; 11: 868–876.

17. Lesch HP, Laitinen A, Peixoto C *et al.* Production and purification of lentiviral vectors generated in 293T suspension cells with baculoviral vectors. *Gene. Ther.* 2011; 18: 531–538.

18. Junne S, Neubauer P. How scalable and suitable are single-use bioreactors? *Curr. Opin. Biotechnol.* 2018; 53: 240–247.

19. Eibl R, Kaiser S, Lombriser R *et al.* Disposable bioreactors: The current state-of-the-art and recommended applications in biotechnology. *Appl. Microbiol. Biotechnol.* 2010; 86: 41–49.

20. Witting SR, Li L-H, Jasti A *et al.* Efficient Large Volume Lentiviral Vector Production Using Flow Electroporation. *Hum. Gene. Ther.* . 2012; 23: 243–249.

21. Miskin J, Biomedica O, Annual A *et al.* Lenti Production , Scale-up and Commercialisation Forward-looking statements and Disclosures. *ASGCT 2017 Annu Meet.*

22. Marceau N, Gasmi M. Scalable Lentiviral Vector Production System Compatible with Industrial Pharmaceutical Applications. 2013.

23. Bandeira V, Peixoto C, Rodrigues AF *et al.* Downstream Processing of Lentiviral Vectors: Releasing Bottlenecks. *Hum. Gene. Ther. Methods* 2012; 23: 255–263.

24. Guy HM, McCloskey L, Lye G *et al.* Characterization of lentiviral vector production using microwell suspension cultures of HEK293T-derived producer cells. *Hum. Gene. Ther. Methods* 2013; 24: 125–39.

25. Miyoshi H, Blömer U, Takahashi M *et al.* Development of a self-inactivating lentivirus vector. *J. Virol.* 1998; 72: 8150–7.

26. Burns JC, Friedmann T, Driever W *et al.* Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl Acad. Sci.* 1993; 90: 8033–8037.

27. Tiscornia G, Singer O, Verma IM. Production and purification of lentiviral vectors. Nat Protoc 2006; 1: 241–245.

28. Kuroda H, Kutner RH, Bazan NG *et al.* Simplified lentivirus vector production in protein-free media using polyethylenimine-mediated transfection. *J. Virol. Methods* 2009; 157: 113–121.

 DOI: 10.18609/cgti.2018.109

29. Tang Y, Garson K, Li L *et al.* Optimization of lentiviral vector production using polyethylenimine-mediated transfection. Oncology Letters 2015; 9: 55–62.

30. Schmeer M, Schleef M. Pharmaceutical Grade Large-Scale Plasmid DNA Manufacturing Process. In: *DNA Vaccines*; pp. 219–240.

31. Sastry L, Xu Y, Cooper R *et al.* Evaluation of Plasmid DNA Removal from Lentiviral Vectors by Benzonase Treatment. *Hum. Gene. Ther.* 2004; 15: 221–226.

32. Cornetta K, Yao J, Jasti A *et al.* Replication-competent lentivirus analysis of clinical grade vector products. *Mol. Ther.* 2011; 19: 557–566.

33. Hyde SC, Pringle IA, Abdullah S *et al.* CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. *Nat. Biotechnol.* 2008; 26: 549–551.

34. Mignon C, Sodoyer R, Werle B. Antibiotic-Free Selection in Biotherapeutics: Now and Forever. *Pathogens* 2015; 4: 157–181.

35. Kobelt D, Schleef M, Schmeer M *et al.* Performance of high quality minicircle DNA for *in vitro* and *in vivo* gene transfer. *Mol. Biotechnol.* 2013; 53: 80–89.

36. Schnödt M, Büning H. Improving the Quality of Adeno-Associated Viral Vector Preparations: The Challenge of Product-Related Impurities. *Hum. Gene. Ther. Methods* 2017; 28: 101–108.

37. Manceur AP, Kim H, Misic V *et al.* Scalable lentiviral vector production using stable HEK293SF producer cell lines. *Hum. Gene. Ther. Methods* 2017; 28:hgtb.2017.086.

38. Haselhorst D, Kaye J, Lever A. Development of cell lines stably expressing human immunodeficiency virus type 1 proteins for studies in encapsidation and gene transfer. *J. Gen. Virol.* 1998; 79 (Pt 2):231–237.

39. Sanber KS, Knight SB, Stephen SL *et al.* Construction of stable packaging cell lines for clinical lentiviral vector production. *Sci Rep*. 5 . Epub ahead of print 2015. DOI: 10.1038/srep09021.

40. Marin V, Stornaiuolo A, Piovan C *et al.* RD-MolPack technology for the constitutive production of self-inactivating lentiviral vectors pseudotyped with the nontoxic RD114-TR envelope. *Mol. Ther. - Methods Clin. Dev.* 2016; 3: 16033.

41. Tomás HA, Rodrigues AF, Carrondo MJT *et al.* LentiPro26: Novel stable cell lines for constitutive lentiviral vector production. *Sci. Rep.*2018; 8: 1–11.

42. Milani M, Annoni A, Bartolaccini S *et al.* Genome editing for scalable production of alloantigen- free lentiviral vectors for *in vivo* Gene Therapy EMBO *Mol. Med.* 2017; e201708148.

43. McCarron A, Donnelley M, McIntyre C *et al.* Challenges of up-scaling lentivirus production and processing. *J. Biotechnol.* 2016; 240: 23–30.

44. Cooper AR, Patel S, Senadheera S *et al.* Highly efficient large-scale lentiviral vector concentration by tandem tangential flow filtration. *J. Virol. Methods* 2011; 177: 1–9.

45. Yamada K, McCarty DM, Madden VJ *et al.* Lentivirus vector purification using anion exchange HPLC leads to improved gene transfer. *Biotechniques* 2003; 34: 1074–1080.

46. Perani A, Gloria B, Wang D *et al.* Evaluation of an online biomass probe to monitor cell growth and cell death. *BMC Proc*. 2011; 5:P16.

47. Dizon-Maspat J, Bourret J, D'Agostini A *et al.* Single pass tangential flow filtration to debottleneck downstream processing for therapeutic antibody production. *Biotechnol. Bioeng.* 2012; 109: 962–970.

48. Higashikawa F, Chang LJ. Kinetic analyses of stability of simple and complex retroviral vectors. *Virology* 2001; 280: 124–131.

49. Baicu SC, Taylor MJ. Acid-base buffering in organ preservation solutions as a function of temperature: New parameters for comparing buffer capacity and efficiency. *Cryobiology* 2002; 45: 33–48.

50. Amorij JP, Meulenaar J, Hinrichs WLJ *et al.* Rational design of an influenza subunit vaccine powder with sugar glass technology: Preventing conformational changes of haemagglutinin during freezing and freeze-drying. *Vaccine* 2007; 25: 6447–6457.

51. Carmo M, Alves A, Rodrigues AF *et al.* Stabilization of gammaretroviral and lentiviral vectors: from production to gene transfer. *J. Gene. Med.* 2009; 11: 670–8.

52. Nilsson SM. Process development of lentiviral vector expression , purification and formulation for Gene Therapy applications. *PhD thesis*.

53. White M, Whittaker R, Stoll EA. A guide to approaching regulatory considerations for lentiviral-mediated *Gene. Ther.* apies. *Hum. Gene. Ther. Methods* 2017; 28: hum.2017.096.

54. EMA. Guideline on Development and Manufacture of Lentiviral vectors.

55. FDA/CBER. Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-Up - Draft Guidance for Industry. Fda.

56. Maxwell KL, Frappier L. Viral Proteomics. *Microbiol Mol Biol. Rev.* 2007; 71: 398–411.

57. Turkki V, Lesch HP, Martin R *et al.* Viral Vector Particle Integrity and Purity Analyses in Early Process Development. *Bioprocess Int.* 2012; 15: 34–37.

58. Wang Y, Bergelson S, Feschenko M. Determination of Lentiviral Infectious Titer by a Novel Droplet Digital PCR Method. *Hum. Gene. Ther. Methods* 2018; 29: 96–103.

59. Zhao Y, Stepto H, Schneider CK. Development of the First WHO Lentiviral Vector Standard: Towards the Production Control and Standardisation of Lentivirus based Gene Therapy Products. *Hum. Gene. Ther. Methods* 2017; 28:hgtb.2017.078.

60. Dos H, Leite R, Vieira GE. Lean philosophy and its applications in the service industry: a review of the current knowledge. *Production* 2015; 529–541.

AFFILIATION

Hanna J Lesch

Dr Lesch is the Gene Therapy Unit Director of Kuopio Center for Gene and Cell Therapy (KCT) in Finland. Her research interest is focused on gene therapy and translational development, including early stage analyticals and the development of scalable, robust manufacturing processes operating under current regulatory guidelines. She also has research and development affiliations with Finvector and FKD Therapies. She has wide experience of all the most commonly used viral vectors (lentivirus, AAV, adenovirus and baculovirus) and a research interest in vascular biology and cancer therapy. Her PhD was in Molecular Medicine, obtained from the University of Kuopio. She followed up her PhD with post doc work at the University of California San Diego UCSD, CA, USA, and the University of Eastern Finland, Kuopio, Finland, concentrating on the understanding of gene regulation and the role of enhancer RNAs in macrophages. She has several patents related to vector manufacturing.

DOI: 10.18609/cgti.2018.109

CELL & GENE THERAPY INSIGHTS

STRATEGIES FOR SCALE-UP & SCALE-OUT

SPOTLIGHT

EXPERT INSIGHT

Translating cell and gene therapies to the clinic

Hema Dave, Devin Saunders, Nan Zhang & Patrick J Hanley

The translation of cell and gene therapy products requires significant resources, knowledge, experience, and infrastructure. Even with vast resources, the path forward is often non-linear, confusing, and cumbersome. Here we review the process by which to take a cell or gene therapy product from discovery into a phase 1 clinical trial in the academic setting.

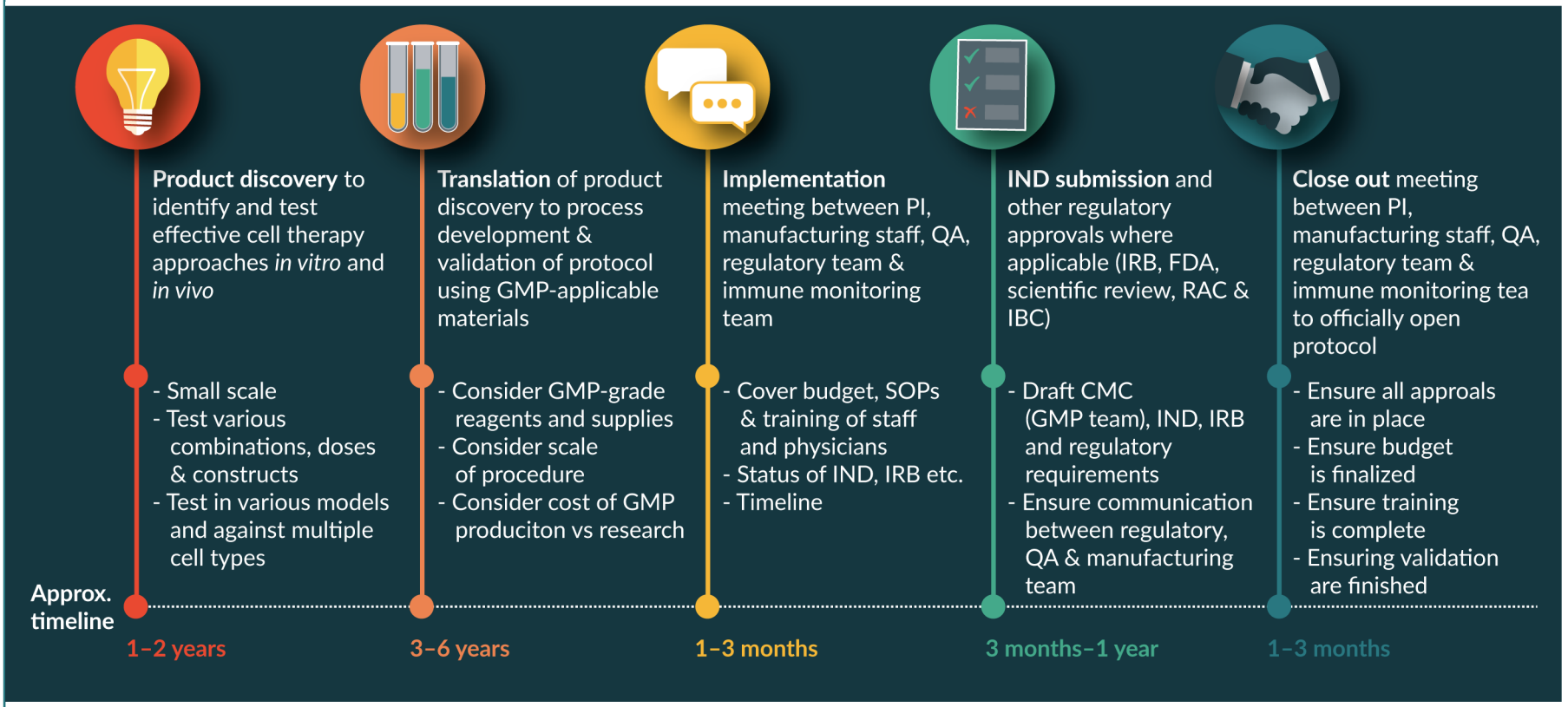
Submitted for peer review: 25 Oct 2018 ▶ **Published**: 19 Dec 2018

Now more prominent than ever, much of cell and gene therapy – beyond bone marrow transplantation – was first developed in the late 1970s and early 1980s by Steven Rosenberg and others at the National Cancer Institute of the National Institutes of Health [1]. In the early 1990s, boutique centers started to develop at individual centers like Memorial Sloan Kettering Cancer Center, St Jude's Children's Research Hospital, Fred Hutchinson Cancer Research Center, and later Baylor College of Medicine. Outside of these premiere institutions, however, the availability, translation, and clinical use of cellular therapies has been impeded by limited resources, experience, and knowledge associated with translating clinical trials from bench to bedside. The recent approval of two Chimeric Antigen Receptor (CAR) T cell products has made these products available to over 60 US centers, with more coming soon worldwide [2,3], but developing investigator-initiated novel therapies to test in clinical trials is still exceedingly challenging and requires an expensive investment in infrastructure along with annual subsidies. This infrastructure typically includes a Good Manufacturing Practices (GMP) facility and dedicated space for testing, storage of supplies, reagents, and products, as well as a multidisciplinary team with medical directors, clinical investigators and nurses, manufacturing staff, Quality Assurance and Quality Control, regulatory staff, preclinical and follow-up researchers, and statisticians. Not all institutions have all of the required team

DOI: 10.18609/cgti.2018.106

FIGURE 1

Overview of what is required to translate a cellular therapy from bench to bedside.



CMC: Chemistry, manufacturing and controls; FDA: Food and Drug Administration; IBC: Institutional biosafety committee; IND: Investigational new drug application; IRB: Institutional review board; PI: Principal investigator; RAC: Recombinant DNA advisory committee.

members, but creative collaborations with other centers and/or industry can help bypass this hurdle. In this manuscript, we will review what is required to translate a cellular therapy from bench to bedside (Figure 1). As an example, we will refer to the translation of allogeneic cord blood (CB) multi-virus-specific T cells (VSTs).

DISCOVERY & IDENTIFICATION OF A CANDIDATE PRODUCT

The first step in translation is to identify the clinical need (e.g., viral infections), the target population (recipients of a cord blood transplant), and evaluate the gaps in current approaches in diagnosis and treatment of that medical condition (the toxicities and inadequacies of current pharmacologic agents).

Product candidate: CB VSTs

Despite several advantages of cord blood transplantation (CBT) as an alternative donor source, it is associated with delayed engraftment and increased risk of infectious complications [4,5]. T-cell immune reconstitution after double or single CBT (with or without serotherapy) [6,7] is significantly slower than peripheral blood or bone marrow as sources of hematopoietic stem cell transplantation and this, along with the naiveté of the infused CB T cells, correlates with an increased risk of viral reactivation or infection from latent and lytic viruses like *Cytomegalovirus* (CMV), Epstein–Barr Virus (EBV) and adenovirus (Ad) in the post-transplantation period. Anti-viral prophylaxis with pharmacologic agents have reduced the burden of early infections, but breakthrough infections can still be fatal and current therapies are limited by toxicities and are often ineffective for many viruses [8].

In the past, we and others have demonstrated that CB VSTs can be generated against CMV, EBV, and Ad and may be protective *in vivo* [9]. CB units are typically cryopreserved in fractions or segments of 80% (used for hematopoietic stem cell transplant) and 20% (for testing, viability, future therapeutic use). Our group demonstrated the ability to produce CMV, EBV, and Ad specific cytotoxic T lymphocytes from the 20% fraction of CB unit using DCs transduced with an Ad5f35pp65-vector in the presence of IL-7, IL-12, and IL-15 [10,11]. A clinical trial using CB-derived multi-virus specific T cells for the prevention and treatment of viral infection after CBT was performed at Baylor College of Medicine (NCT01017705). On this Phase 1 study 9 patients received virus specific T cells (VSTs) generated from the 20% fraction of a fractionated CB unit. VSTs were infused to 9 patients from 63 to 443 days post-CBT [9,12,13]. No infusion-related toxicities or GvHD was observed.

However, reactivation of polyoma viruses further complicates the post-transplant period causing hemorrhagic cystitis (BK virus) and progressive multifocal leukoencephalopathy (JC virus) [14,15]. To date, CB T cells targeting BK virus have not been reported. Furthermore, as detailed below, the previous manufacturing process involved the generation of EBV lymphoblastoid cells (EBV-LCL), which requires infection of B cells

with a laboratory strain of EBV, and also involves the transduction of dendritic cells and EBV-LCL with an adenoviral vector expressing a gene for CMV. Therefore, in an attempt to make the manufacturing process more applicable for late Phase clinical studies, to reduce the

TABLE 1

Considerations for preclinical development

Consideration	Risks	Potential solutions	Current status in the field	Comments
Media	Backorder of media Risk of contamination of 'home brew'	Pre-fabricated media Use of media in bags Chemically defined media	Pre-fabricated media is available but with varying degrees of comparability	'Home brews' are common but have a higher risk of contamination
Scale	Difficulties and delays in commercialization	Bioreactor systems [17–19] Cell factories	Some automated, large scale systems available but not always adaptable	Current scale is difficult to optimize yet difficult to produce at large scale
Gene delivery	Poor transduction efficiencies Vector manufacturing backlog Expensive testing	Non viral vector approaches [20–22] Optimized vectors	Non viral vectors available but non common Second and third generation vectors	Current shortage of available vector production facilities
Live virus	Contamination, mix up, delivery to patient of live virus	Use of overlapping peptides [23,24] Alternative APCs	Peptides available Alternative APCs available	Could be problematic in later phases or with other regulatory agencies
Open culture systems	Contamination Requires isolated incubator space, time and labor intensive	Bioreactors, closed systems	Prodigy, Quantum, WAVE, closed Grex	Open systems require additional quality checks
Labor	Expense Contamination Clean room space Human error	Automated systems	Prodigy, Quantum, Wave	Labor is expensive and shortage of staff with adequate / GMP-compliant training
Origin and quality of reagents	Non –GMP grade can cause delays with FDA/regulatory agencies	Qualified materials Qualified FBS from herds in New Zealand, other approved countries	Most supplies and reagents are available but not all Many antibodies and recombinant proteins contain animal products	'Research grade' can be acceptable for early phase but challenging for later phase studies

APC: Antigen-presenting cell.

DOI: 10.18609/cgti.2018.106

manufacturing time, and to target BK virus [16], the objective was to evaluate whether VSTs could be generated from CB that target BK virus and to remove the use of viral vectors or live virus from the manufacturing process. Here, we will discuss approaches (Figure 1) of bringing novel technologies at the bench to bedside that led to IND submission and favorable review by the FDA of multi-virus VST. At this stage of pre-clinical development, it is imperative to consider what animal studies will be required for a successful IND application.

MOVING TOWARDS GMP MANUFACTURING DURING DEVELOPMENT

Once the concept for a cell therapy product has been conceived and a few pilot studies have been conducted on a small scale, it is critical to start preparing for future clinical validation runs and clinical manufacture. Items to consider during this phase are listed in Table 1 [17–24]. Many of the risks included in this table are acceptable for Phase 1 clinical trials but could significantly delay the commercialization of the cellular therapy product. One example of this delay is identifying suitable reagents. In this case, one of our reagents was genetically modified K562 cells, which were used as feeder cells [16]. Because of the origin of the K562 cells as a chronic myelogenous leukemia cell, it was critical that we were able to demonstrate that K562 cells were not present in the final product and that our irradiation procedure had been sufficiently validated on the K562 cells. These requirements caused us to delay the implementation of this procedure by a few months and also involved identifying an external lab who could help us perform the proliferation assays that the FDA had mandated.

GMP-applicable development of CB VSTs

To induce T-cell activation and expansion, viral antigen-derived peptides must be presented by antigen-presenting cells (APC) expressing class I and II major histocompatibility complex antigens (MHC), as well as co-stimulatory molecules. Previous reports from CB have used EBV-LCL alone [25], dendritic cells and EBV-LCL transduced with an adenoviral vector [10], or CMV lysate as antigens to generate VSTs [26]. There are, however, a few obstacles to extending this approach in the CB setting. These include: (i) the limited numbers of CB T cells available for manipulation; (ii) the naivety of CB T cells; and (iii) lower cytotoxic activity and higher activation-induced cell death than peripheral blood T cells. As a result, the previous manufacture of CB-derived multi-VSTs requires manufacturing times of 10+ weeks [10].

Targeting BK virus: beyond EBV, CMV & Adv

When addressing how to increase the breadth of viruses targeted by the T cells stimulated by our process, we considered two options: i) re-engineer the adenoviral vector to express BK antigens; or ii) switch the entire process to overlapping peptides derived from specific antigens of each virus and add BK virus.

While we had experience with the first option [27], engineering vectors is timely and clinical grade vectors can cost upwards of $300,000. Plus, our goal was to eliminate viral vectors. Therefore, we chose option 2. However, this required working with a peptide company to make the peptides in a GMP-applicable manner (>90% purity) and also involved building the overlapping peptides based on antigens of interest. Identifying the antigens of interest requires knowledge of the lifecycle of the virus, the immune response to the virus, and the conserved regions of the virus. Based on these factors, we chose to target the BK proteins Large T and VP-1 [16].

Reducing manufacturing time & eliminating live virus in culture

The above studies however, still have the lengthy manufacturing time of over 3 months [10]. This delay is mostly due to using EBV-LCL, which take upwards of 1–2 months to culture and also requires the temporary cryopreservation of the CTL product after one stimulation while the LCL expand. There were a number of ways to reduce the manufacturing time and to eliminate live virus. One option was to use artificial antigen presenting cells. However, since the HLA of each line will be different, the APCs had to be autologous (derived from the same CB unit) which limited the options to APCs that could be expanded from the cord blood itself, such as expanded B cells or T cells. To circumvent this, our group used autologous cord blood-derived phytohemagglutinin (PHA) blasts (non-specifically activated T cells) with K562 cells modified to express costimulatory molecules (gmK562) as APCs [28] for second and subsequent stimulation. While this does not entirely eliminate the risk associated with this process since the K562 cells are genetically engineered, they were engineered without the use of a viral vector, which mitigates some of the risks associated with using viral transduction. This simplified the process of generating APCs by eliminating the need for live virus (EBV) for LCL generation, eliminated viral vectors, and also reduced the manufacturing time from 60+ days to 30 days or less [16]. While the process was being improved, it was critical to track relevant in process parameters. Some of these parameters include: DC to T-cell ratio, phenotype of DCs or PHA blasts, ratio of PHA blasts, gmK562, and T cells, and cell density in the Grex device. Likewise, in-process testing was important to further characterize the process. Some of the tests performed include: phenotype of dendritic cells, phenotype of PHA blasts, phenotype of initial cord blood product, and cell count and viability of T cells at each T-cell stimulation.

IDENTIFYING RELEASE CRITERIA FOR EACH CELLULAR THERAPY CANDIDATE

Release testing is a critical aspect of product release; determining safety parameters is crucial to protect the recipient's health and ensure key GMP tenants of product safety, potency, purity, and identity. Once the cell therapy product has been conceived and evaluated preclinically,

DOI: 10.18609/cgti.2018.106

TABLE 2

Examples of release testing of CB VSTs.

Test	Testing facility	Release criteria
Cell product viability	GMP facility	>70% viable
Mycoplasma (using MycoAlert)	Quality Control Lab (ITC)	Negative
Endotoxin (EndoSafe PTS)	Quality Control Lab (ITC)	< 5.0 EU/mL
Cytotoxicity	Quality Control Lab (ITC)	<10% allogeneic killing at an effector:target ratio of 20:1
Flow cytometry	Quality Control Lab (ITC)	<2% CD3-/CD83+ (DCs) <0.1% CD32+/CD83+ within CD3-/CD16_56+ gated pop.(gmK562)
Cell product sterility Aerobic (cultured for 14 days) Anaerobic (cultured for 14 days) Fungal (cultured for 21 days)	Microbiology Lab	 Negative @ 4 days Negative @ 4 days Negative @ 4 days
HLA typing	Immunogenetics Lab	Matched HLA identity between VSTs and donor

the required release testing should be decided in collaboration with the Quality Assurance team based on the risks associated with the product. In the case of the cord blood T cells, since the T cells will technically be allogeneic (donor directed might be a more accurate term, but the cells are derived from the cord blood unit that is also used to reconstitute the patient's immune system), it is important to test the cellular therapy product for alloreactivity using a chromium release cytotoxicity assay with allogeneic (from the patient or a third party donor) PHA blasts as the target (Table 2).

Mycoplasma testing is also required because the cells are in culture for an extended period of time. Likewise, sterility tests for aerobic and anaerobic bacteria and fungus are required, as is endotoxin testing. Given the number of potential products that could be concurrently cultured, there is also the potential for mix up of cell products. We currently employ a number of mechanisms to prevent this risk of cross contamination or mix up, but to test for it we also perform HLA testing of the product and compare it to the HLA of the donor to ensure identity. Another test of identity and purity is flow cytometry. Since dendritic cells are used as APCs, the final product should not contain DCs and should also contain a majority of T cells. Finally, viability is critical to the function of the cells and therefore viability is a release criterion tested before final release of the product. In the case of our program, cell products and supernatant are tested by our Immune Testing & Characterization (ITC) laboratory. They also test product specificity by ELISPOT assay but this is for information purposes only. Potency assays such as the cytotoxicity assay, ELISPOT, or ELISA, though not required until the start of Phase 3, should also

be considered. As discussed above, these tests are often performed in-house but are also offered by commercial entities.

TRANSLATING THE PRODUCT INTO A CLINICAL TRIAL: WRITING THE CMC & IND

Moving the product from the laboratory to the clinic requires proper planning and communication with the clinical development team and various regulatory committees. Every new drug/biologic therapy requires a sponsor (can be investigator or commercial) to submit an Investigational New Drug (IND) application to the Food and Drug administration (FDA). The IND application must include a cover letter with a brief explanation of the intended study and the IND product details and reference to any existing INDs related to the study. Appropriate regulatory forms FDA15721, FDA1572 (Statement of Investigator), and FDA 3674 should be completed. The introductory statement (Section 5) and investigational plan (Clinical Protocol) must include the name of the drug and all active ingredients, the drug's pharmacologic class, structural formula, formulation of dosage form, and route of administration. The investigator must provide broad objectives and planned investigations with a brief description of the overall plan for investigation of the drug in the next year. Good Clinical Practice (GCP) principals must be adhered to in the development, implementation and oversight of the clinical trial [29]. Any pharmacologic and toxicology data with previous human experience must also be included (Section 8). It is also important to note that if the sponsor is also an investigator, there should be a separate and independent monitoring group to manage the conflict of interest.

The Investigator Brochure (IB), required for multi-site studies, contains a detailed description of the product, structural formula (if known) and formulation, summary of pharmacokinetic, pharmacological and toxicological effects of the drug in animals, and to the extent known in humans, summary of the safety and effectiveness of the drug in humans. The Chemicals, Manufacturing and Controls (CMC) section must contain information on the product, with labeling information of the investigational drug and environmental analysis or request for categorical exclusion. Much of this information is included in the paragraphs below.

The IND is then submitted to the FDA and assigned to a review team. As of May 2018, commercial IND applications must be submitted using electronic common technical document (eCTD) format. eCTD is optional but encouraged for investigator-sponsored INDs and expanded-access INDs [30]. Once the submission is received, the review team determines in 30 days if the study is 'safe to proceed' or will be placed on clinical hold. If a proposed study is placed on a clinical hold, study participants may not receive any investigational products. Common grounds for imposition of the clinical hold are insufficient evidence to ensure human safety of the product, unsatisfactory qualification of investigators, inaccurate or incomplete investigator's brochure. The investigators can respond to the FDA and the hold is removed if the review team is satisfied with the

DOI: 10.18609/cgti.2018.106

responses. While the IND application is being reviewed (the timing of this can be institution dependent), the investigator must submit the clinical protocol to the institutional IRB simultaneously. This is a rigorous review process by different stakeholders that review the rigor of the science (scientific review committee), statistical plan (biostat review), and ancillary reviews. Review and initial approval of the clinical protocol may take several months to upwards of a year depending on the review process at each site. Funding for the clinical trials must also be obtained simultaneously because once an IND is approved, the clinical investigation must begin else the IND needs to be withdrawn. The US Code of Federal Regulations (CFR) Title 21 contains all the regulations for gene therapy products. In a recent report from the Office of Inspector General, about 87% of all subjects enrolled in recent biologics trials were enrolled outside the USA [31]. When a study is conducted under an IND but is located outside the USA, the study must still comply with all relevant FDA regulations as if it were being conducted within the USA. However, a Sponsor is not required to conduct a foreign clinical trial under an IND in order to use it as support for an IND. In such situations, the FDA accepts foreign clinical data as long as the study was conducted in accordance with Good Clinical Practice (GCP) and FDA is able to validate the data from the study through an onsite inspection. Readers are referred to 21CFR312.20 for further details [32].

For the clinical trial discussed here, we proposed a Phase 1 dose escalation trial to determine the safety and feasibility of the CB VSTs in recipients of CBT. Although we had a previous IND for CB VSTs, we added a new viral target (BK virus) and made other significant manufacturing changes that required us to submit a new IND. The clinical protocol contained adequate rationale for using the product (VSTs). It described possible risks and side effects to be anticipated by pre-clinical data, justification for using the starting dose, route and frequency, and prior human experience with VSTs from bone marrow and CB donors. Considerations were given to the toxicity of the product and a plan for safety monitoring and grading of adverse events using Common Terminology Criteria for Adverse Events (CTCAE) criteria for the patient population. The primary endpoint was determining the safety of the multi-virus VST and the maximum tolerated dose. Statistical considerations were given to the primary and secondary objectives with well-defined stopping rules.

REGULATORY APPROVAL

As indicated above, regulatory approvals from the institutional IRB and IND from the FDA are required before the trial can proceed. For any investigation, an informed consent must be obtained for each human subject to whom the investigational drug is administered, except in circumstances where clinical emergency research may be conducted without informed consent as described in Guidance for Institutional Review Boards, Clinical Investigators, and Sponsors: Exception from Informed Consent Requirements for Emergency

Research [33]. Depending upon the type of cellular therapy product, additional reviews may also be required such as institutional biosafety committee (IBC, for studies with deliberate gene transfer or potentially biohazardous agents), and the Recombinant DNA Advisory Committee (RAC), which reviews protocols using recombinant DNA. Of note, the IBC is at a local level and often interprets deliberate gene transfer to include genetic modification of antigen presenting cells or other intermediaries, even if they are not in the final product. In our experience, the IBC process can cause unexpected delays and these potential delays should be taken into account when building the expected timeline for the protocol.

FINAL CLOSE OUT & PROTOCOL IMPLEMENTATION IN THE MANUFACTURING FACILITY

Once the IND is approved, the sponsor can start manufacturing the product. The clinical study must be registered with clinicaltrials.gov within 21 days of the first enrolled patient. Before this happens, our manufacturing team will hold a close-out meeting with all relevant parties to ensure that all validations are finished, the Standard Operating Procedures (SOPs) have been released, staff have been trained, and there is a budget for the protocol. Once all of these components are present, the PI, facility director, and quality assurance sign off on the close out form and the trial may proceed. All co-investigators and staff should also undergo appropriate protocol-related training prior to patient enrollment.

MONITORING EFFICACY OF INFUSED PRODUCTS: IMMUNE MONITORING & FOLLOW UP TESTING

Immune monitoring

After product infusion, follow-up whole blood samples are collected from patients at set time points indicated in the protocol. In the case of CB VSTs, these samples allow investigators to monitor: i) T-cell effectiveness by analyzing the decline in viral load over time; and ii) the long-term functionality and persistence of the infused T cells [34]. To address these variables, investigators can use a variety of techniques. Molecular diagnostic assays such as PCR are highly sensitive, making them a key tool in determining the baseline viral load prior to treatment and for detecting minute changes in viral load in response to treatment. Meanwhile, T-cell functionality and persistence can be tracked by several standard laboratory assays. Flow cytometry can be utilized to detect the longevity of infused cells and changes in phenotypical variances, while ELISPOT can be used to track a gain or loss of T cells specific for a given antigen [13,35]. Other assays to consider include deep T-cell receptor sequencing [36], Luminex multi-plex assays for profiling known or novel disease biomarkers or proteins, and novel PCR techniques for the detection and quantification of known genes.

Documentation of patient samples

Documentation is integral to FDA compliance and traceability from the donor to the product and to the patient and back. It is an important

DOI: 10.18609/cgti.2018.106

part of all release testing and Quality Control, result reporting, and follow-up processes. It is also applicable in sample labels, worksheets and result reports, follow-up sample paperwork, product folders, as well as SOPs, standardized lab protocols, and inventory tracking. Formal policies and procedures help ensure compliance and also provide a blueprint for how samples will be processed and tested, labeled, and stored. Specific examples of standardized documents include result reporting forms, protocols for handling and processing follow-up blood samples, and experimental protocols (i.e., molecular sequencing, ELISPOT, flow cytometry, etc). Importantly, laboratories should also include a follow-up blood sample acquisition form that includes the identity of the staff member receiving the sample, patient information (coded if necessary), date and time of delivery and timepoint, sample integrity, and confirmation that proper labeling was included.

Standardized documents such as these are fundamental in clarifying universal language used within the laboratory setting, as well as process and sample tracking requirements. These documents are used to capture unique patient identifiers and data in a consistent format, as well as provide a means to train lab members in an accurate and uniform manner. Before implementation, standardized documents should be reviewed and approved by Quality Assurance to ensure they meet quality standards.

Labeling & processing patient samples

Sample labels should include at a minimum: the patient's unique identifier, the time point, the vial contents (i.e., plasma, PBMC, etc.), and the processing date. Other details, such as the sample's intended usage (i.e., Luminex, PCR, etc.) and cell count, can also be included according to needs. Each sample should also be documented on a processing worksheet that includes: the patient's unique identifier, the time point, at least one copy of each unique sample label, the storage location of the vials if freezing, where and how many fresh cells are used if not freezing, and other necessary information. After processing, all samples should be documented in the follow up sample inventory, and all processing paperwork can be kept collectively in individual patient folders. All information on the sample labels and the processing paperwork should be corroborated by the inventory; any discrepancies should be immediately reconciled to ensure good clinical practice compliance. Importantly, these databases contain sensitive patient information and appropriate precautions must be taken to prevent access to Patient Health Information; using files without strong password-protection or transferring these data to flash drives or personal computers is risky and should be avoided if possible. Institutional and protocol-specific policies must also be followed.

CONCLUSION

The recently approved cell and gene therapy products Yescarta® and Kymriah® were the result of collaborations between a small number of academic centers and industry partners. With the successful commercialization of these drugs, the number of academic institutions and

small biotech companies pursuing cell and gene therapy has increased rapidly. Despite this rapid growth, the number of entities performing this work is outpacing the number of qualified personnel, from trained physician scientists to GMP facility directors to Quality Assurance staff. Additionally, these cell and gene therapy protocols require unique infrastructure that can often strain existing institutions.

While many antigen-specific T cells have an established safety profile [37], CAR T products have been associated with cytokine release syndrome [38], which requires exquisite care by trained clinicians. Hence proper planning and communication within the clinical development team as well as consultation with bench scientists and regulatory is essential. A realistic timeline must be implemented without forgoing any of the steps required for the safe and ethical conduct of the cell and gene therapy trials. Given the novelty and uncertainties of upcoming new cell therapies, a full GMP-grade and regulatory-friendly product with a validated potency test may not be feasible in early stages of development but should be considered throughout the process. What's more, investigator initiated trials are often iterative in nature [39,40]. The first Phase 1 might demonstrate safety with little sign of efficacy, but often there are biomarkers or hints of success that direct the next Phase 1 study. This sort of development can take as long as 10 years and is common in the cell and gene therapy field, where cells can target multiple epitopes, be transduced with activating or inhibitor receptors, and can even be combined with other therapies. The FDA will of course be present through all stages of this development and encourages early communication for prospective INDs.

In summary, by disseminating knowledge gained during our experience we hope to help other sites avoid similar mistakes and delays, resulting in better products being delivered to the clinic faster.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Catherine Bollard, CCIR director and mentor, for her guidance. PJH would also like to acknowledge funding from the Children's Cancer Foundation and the Be the Match Amy Strelzer Manasevit award.

CONFLICT OF INTEREST DISCLOSURE

PJH is the co-founder of Mana Therapeutics and serves on their board of directors. He has intellectual property related to the use of cord blood virus-specific T cells.No writing assistance was utilized in the production of this manuscript.

REFERENCES

1. Staff P. Steven Rosenberg. People. 1985.
2. Find a KYMRIAH Treatment Center 2018: https://www.us.kymriah.com/treatment-center-locator/
3. Where Can YESARTA Be Received? 2018: https://www.yescarta.com/treatment-centers
4. Munoz J, Shah N, Rezvani K *et al.* Concise review: umbilical cord blood transplantation: past, present, and future. *Stem Cells Transl. Med.* 2014; 3(12): 1435–43.
5. Oran B, Shpall E. Umbilical cord blood transplantation: a maturing technology. *Hematology Am. Soc. Hematol. Educ. Program* 2012; 2012: 215–22.
6. Lucchini G, Perales MA, Veys P. Immune reconstitution after cord blood transplantation: peculiarities, clinical implications and management strategies. *Cytotherapy* 2015; 17(6): 711–22.
7. Saliba RM, Rezvani K, Leen A *et al.* General and Virus-Specific Immune Cell Reconstitution after Double Cord Blood Transplantation. *Biol. Blood Marrow Transplant.* 2015; 21(7): 1284–90.
8. Ljungman P, Ribaud P, Eyrich M *et al.* Cidofovir for adenovirus infections after allogeneic hematopoietic stem cell transplantation: a survey by the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant.* 2003; 31(6): 481–6.
9. Hanley PJ, Melenhorst JJ, Nikiforow S *et al.* CMV-specific T cells generated from naive T cells recognize atypical epitopes and may be protective *in*

DOI: 10.18609/cgti.2018.106

vivo. *Sci. Transl. Med.* 2015; 7(285): 285ra63.

10. Hanley PJ, Cruz CR, Savoldo B *et al.* Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes. *Blood* 2009; 114(9): 1958–67.

11. Hanley PJ, Lam S, Shpall EJ, Bollard CM. Expanding cytotoxic T lymphocytes from umbilical cord blood that target cytomegalovirus, Epstein-Barr virus, and adenovirus. *J. Vis. Exp.* 2012; 63: e3627.

12. Hanley PJ, Bollard CM, Brunstein CG. Adoptive immunotherapy with the use of regulatory T cells and virus-specific T cells derived from cord blood. *Cytotherapy* 2015; 17(6): 749–55.

13. Naik S, Nicholas SK, Martinez CA *et al.* Adoptive immunotherapy for primary immunodeficiency disorders with virus-specific T lymphocytes. *J. Allergy Clin. Immunol.* 2016; 137(5): 1498–505 e1.

14. Gilis L, Morisset S, Billaud G *et al.* High burden of BK virus-associated hemorrhagic cystitis in patients undergoing allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 2014; 49(5): 664–70.

15. Franzen J, Ramqvist T, Bogdanovic G, Grun N, Mattson J, Dalianis T. Studies of human polyomaviruses, with HPyV7, BKPyV, and JCPyV present in urine of allogeneic hematopoietic stem cell transplanted patients with or without hemorrhagic cystitis. *Transpl. Infect. Dis.* 2016; 18(2): 240–6.

16. Dave H, Luo M, Blaney JW *et al.* Toward a Rapid Production of Multivirus-Specific T Cells Targeting BKV, Adenovirus, CMV, and EBV from Umbilical Cord Blood. *Mol. Ther. Methods Clin. Dev.* 2017; 5: 13–21.

17. Zhu F, Shah N, Xu H *et al.* Closed-system manufacturing of CD19 and dual-targeted CD20/19 chimeric antigen receptor T cells using the CliniMACS Prodigy device at an academic medical center. *Cytotherapy* 2018; 20(3): 394–406.

18. Nankervis B, Jones M, Vang B, Brent Rice R Jr, Coeshott C, Beltzer J. Optimizing T Cell Expansion in a Hollow-Fiber Bioreactor. *Curr. Stem Cell Rep.* 2018; 4(1): 46–51.

19. Somerville RP, Devillier L, Parkhurst MR, Rosenberg SA, Dudley ME. Clinical scale rapid expansion of lymphocytes for adoptive cell transfer therapy in the WAVE(R) bioreactor. *J. Transl. Med.* 2012; 10: 69.

20. Bishop DC, Xu N, Tse B *et al.* PiggyBac-Engineered T Cells Expressing CD19-Specific CARs that Lack IgG1 Fc Spacers Have Potent Activity against B-ALL Xenografts. *Mol. Ther.* 2018; 26(8): 1883–95.

21. Ramanayake S, Bilmon I, Bishop D *et al.* Low-cost generation of Good Manufacturing Practice-grade CD19-specific chimeric antigen receptor-expressing T cells using piggyBac gene transfer and patient-derived materials. *Cytotherapy* 2015; 17(9): 1251–67.

22. Singh H, Huls H, Kebriaei P, Cooper LJ. A new approach to gene therapy using Sleeping Beauty to genetically modify clinical-grade T cells to target CD19. *Immunol. Rev.* 2014; 257(1): 181–90.

23. McLaughlin LP, Lang H, Williams E *et al.* Human parainfluenza virus-3 can be targeted by rapidly ex vivo expanded T lymphocytes. *Cytotherapy* 2016; 18(12): 1515–24.

24. Gerdemann U, Keirnan JM, Katari UL *et al.* Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. *Mol. Ther.* 2012; 20(8): 1622–32.

25. Sun Q, Burton RL, Pollok KE, Emanuel DJ, Lucas KG. CD4(+) Epstein-Barr virus-specific cytotoxic T-lymphocytes from human umbilical cord blood. *Cell Immunol.* 1999; 195(2): 81–8.

26. Park KD, Marti L, Kurtzberg J, Szabolcs P. *In vitro* priming and expansion of cytomegalovirus-specific Th1 and Tc1 T cells from naive cord blood lymphocytes. *Blood* 2006; 108(5): 1770–3.

27. Hanley PJ, Shaffer DR, Cruz CR *et al.* Expansion of T cells targeting multiple antigens of cytomegalovirus, Epstein-Barr virus and adenovirus to provide broad antiviral specificity after stem cell transplantation. *Cytotherapy* 2011; 13(8): 976–86.

28. Ngo MC, Ando J, Leen AM *et al.* Complementation of antigen-presenting cells to generate T lymphocytes with broad target specificity. *J. Immunother.* 2014; 37(4): 193–203.

29. Guideline for Good Clinical Practice: Consolidated Guidance (ICH E6). June 10, 1996.

30. Providing Regulatory Submissions in Electronic Format – Certain Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications Guidance for Industry. 2018.

31. Levinson DR. Challenges to FDA's Ability to Monitor and Inspect Foreign Clinical Trials. In: HHS OoIG, editor. 2010.

32. Requirement for an IND. 312.20 - CFR - Code of Federal Regulations Title 21.

33. FDA. Guidance for Institutional Review Boards, Clinical Investerigators, and Sponsors. Exception from Informed Consent Requirements for Emergency Research. 2013.

34. Leen AM, Myers GD, Sili U *et al.* Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat. Med.* 2006; 12(10): 1160–6.

35. Miller HK, Hanley PJ, Lang H *et al.* Antiviral T Cells for Adenovirus in the Pretransplant Period: A Bridge Therapy for Severe Combined Immunodeficiency. *Biol. Blood Marrow Transplant.* 2018; 24(9): 1944–6.

36. Chapuis AG, Desmarais C, Emerson R *et al.* Tracking the Fate and Origin of Clinically Relevant Adoptively Transferred CD8(+) T Cells *In Vivo*. *Sci. Immunol.* 2017; 2(8).

37. Cruz CR, Hanley PJ, Liu H *et al.* Adverse events following infusion of T cells for adoptive immunotherapy: a 10-year experience. *Cytotherapy* 2010; 12(6): 743–9.

38. Giavridis T, van der Stegen SJC, Eyquem J, Hamieh M, Piersigilli A, Sadelain M. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat. Med.* 2018; 24(6): 731–8.

39. Barth MJ, Chu Y, Hanley PJ, Cairo MS. Immunotherapeutic approaches for the treatment of childhood, adolescent and young adult non-Hodgkin lymphoma. *Br. J. Haematol.* 2016; 173(4): 597–616.

40. Bollard CM, Aguilar L, Straathof KC *et al.* Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *J. Exp. Med.* 2004; 200(12): 1623–33.

AFFILIATIONS

Hema Dave[1,2,3], Devin Saunders[1,3], Nan Zhang[1,3], Patrick J. Hanley[1,2,3]

[1]Center for Cancer and Immunology Research

[2]Center for Cancer and Blood Disorders,

[3]Program for Cell Enhancement and Technologies for Immunotherapy, Children's National Health System and The George Washington University, Washington, DC, 20010

Corresponding Author:

Dr Patrick J Hanley

PHanley@childrensnational.org

DOI: 10.18609/cgti.2018.106

CELL & GENE THERAPY INSIGHTS

STRATEGIES FOR SCALE-UP & SCALE-OUT

SPOTLIGHT

RESEARCH ARTICLE

Scale-up of a perfusion-based dendritic cell generation process

Andrew Kozbial, Hope Weinstein, Shashi K Murthy & Jennifer M Rossi

Scale up of dendritic cell production is a critical challenge that is infeasible with current static culture systems such as well plates, T-flasks, and bags. We have developed a fully enclosed, sterile cell culture system, called EDEN, that allows for continuous perfusion of fresh differentiation medium into the cell culture cartridge and simultaneous removal of depleted medium. EDEN generated *ca.* 25 million immature dendritic cells (iDCs) per run with a yield, relative to seeded monocytes, of 20-30%. Immunophenotyping showed that EDEN generated iDCs were phenotypically similar to 6-well plate generated iDCs. Maturation of EDEN iDCs using a standard maturation cocktail was successful with upregulation of CD80/83/86 and downregulation of CD209. Computational fluid dynamics simulations aided the EDEN cartridge design to ensure proper differentiation medium perfusion. These results indicate that EDEN successfully generates clinically relevant numbers of iDCs in a single cell culture cartridge with fewer manual interventions compared to standard culture techniques.

Submitted for peer review: 11 Oct 2018 ▶ **Published**: 18 Dec 2018

INTRODUCTION

Generating clinically relevant numbers of monocyte-derived dendritic cells (MO-DCs) for therapeutic use can be challenging for both for research and clinical scale production. Standard well plate and T-flask culture is a cumbersome process with many manual steps that expose the cell culture to the outside environment. Each manual step requires exposing the cell culture to the outside (aseptic) environment and requires intervention by a highly

trained technician, consuming valuable time and resources in the rapidly expanding field of cell therapy production. Although the manual steps are performed aseptically in a laminar flow hood, there are numerous safety and contamination concerns such as patient sample mix-up and misidentification, exposure to unknown contaminants inside the laminar flow hood (*e.g.,* particulates and bacteria/fungus resistant to standard sterilization techniques such as 70% ethanol), and accidental exposure of culture to a septic environment [1]. Furthermore, numerous well plates are required to generate sufficient DCs for a single therapeutic dose. Alternative DC generation vessels include T-flasks and bags which reduce the number of culture vessels compared to well plates but also have the same inherent issues above as well as low DC yield in bags. Immature DC yield in static culture vessels ranges from *ca.* 4–41% in gas-permeable bags when MOs are positively selected. This range is also expected for well plates and T flasks and is dependent on culture conditions and donor [2–7]. Scale-up of manual DC generation techniques is generally not feasible aside from adding more culture vessels to the workflow.

Dosing regimens for DC vaccines vary widely between the type of study being conducted and the targeted disease; however, most DC vaccine regimens require >100 million autologous DCs per patient. Each therapeutic dose is administered at least 3 times, thus requiring 30–50+ well plates or numerous T flasks for a single patient. It is difficult to ascertain the exact number of well plates or flasks required for generating DCs from a single patient because this is dependent upon precursor cell (peripheral blood mononuclear cell (PBMC) or MO) seeding density, cytokine concentration, and final yield of generated DCs which are often times not specified. It is also well known that generating DCs from PBMCs or MOs of cancer patients often times leads to lower DC yields than generating DCs from healthy donors.

Carreno *et al.* investigated a melanoma DC vaccine regimen of 135 million DCs in the priming dose followed by two additional doses of 45 million DCs. The DCs were cultured from peripheral blood MOs for 6 days in tissue culture flasks followed by maturation for 24 hours in new flasks [8–11]. Mitchell *et al.* investigated a glioblastoma DC vaccine regimen of four bi-weekly doses followed by at least six subsequent monthly doses of 20 million DCs per dose. The DCs were cultured from peripheral blood MOs for: (a) 5 days in tissue culture flasks followed by 3–4 days of maturation in the same flasks or (b) 7 days in tissue culture flasks followed by 16–20 hours of maturation in new flasks [12,13]. Additionally, these protocols typically involve supplementing the cell culture with fresh differentiation medium multiple times during DC generation. It's important to note that these are two examples of clinical DC dose regimens and other regimens have been evaluated [14,15].

To address sterility, contamination, and workflow issues associated with DC generation, we developed the MicroDEN system for smaller scale DC generation. This automated cell culture system continuously perfuses fresh medium into a culture vessel while simultaneously removing depleted medium [16]. The aseptic design of MicroDEN

DOI: 10.18609/cgti.2018.110

allows for fresh complete medium (base medium + cytokines) to be added into an inlet bottle that feeds to a peristaltic pump, through the culture vessel, and out into a waste bottle. Aseptic medium addition to the inlet bottle is achieved using Luer activated valves (LAVs) and stopcocks that are simply wiped clean with a standard alcohol wipe; this technique is heavily utilized in intravenous (IV) lines and anesthesia administration. Medium refresh is achieved using the same aseptic procedure. Aseptic cell seeding and harvesting is also incorporated to ensure sterility and minimize contamination sources of the final DC product. MicroDEN was designed to generate DCs on the scale of well plates and T-flasks, but scaleup is generally not feasible.

Using MicroDEN technology as a basis for workflow, we have developed an automated cell culture system for aseptically generating therapeutically relevant numbers of immature DCs (iDCs) in a single cell culture cartridge, called EDEN (Figure 1). A peristaltic pump provides continuous perfusion of fresh medium into the culture vessel at 8 μL/min per inlet along with removal of depleted medium into a waste reservoir. Similar to MicroDEN, transfer of fresh medium, removal of depleted medium, cell seeding, and iDC harvesting are performed aseptically. The stopcocks on the cartridge allow for air exchange when the cartridge is being seeded with cell solution or harvested. Stopcocks on the bottles allow for sterile transfer of differentiation medium to fill the inlet bottle and remove the waste from the outlet bottle. This setup allows for the tubing and cartridge system to remain sterile from setup to harvest without having to break the sterile seal of the system. EDEN was designed as a completely enclosed, sterile iDC generation system for producing iDCs on a clinical scale, effectively eliminating the need for numerous well plates (or T-flasks/bags), ensuring a sterile and particulate free culture system and reducing technician time in maintaining cell culture.

MATERIALS & METHODS

PBMC isolation & monocyte enrichment

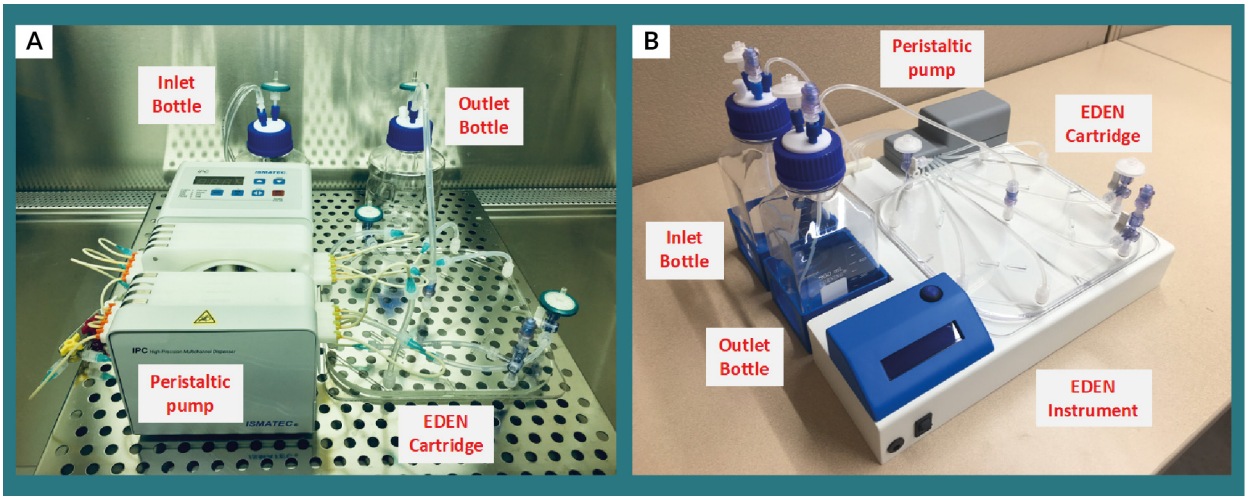
Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (GE Healthcare) from whole blood purchased from StemExpress. The whole blood was drawn and processed on the same day. Isolated PBMCs were cryopreserved at 50–60 million PBMCs/mL in CryoStor CS10 and remained in cryopreservation for at least 7 days prior to resuscitation. Monocytes (MOs) were enriched from resuscitated PBMCs using Miltenyi CD14 MicroBeads and two LS column passes to obtain a MO purity >95%. Enriched MOs from a single donor were suspended in 122 mL differentiation medium and seeded into the EDEN cartridge. Each experiment used MOs from a different donor.

Differentiation medium

RPMI 1640 (Gibco 11875119) was supplemented with 10% heat inactivated-fetal bovine serum (HI-FBS; MilliporeSigma F2442), 1% penicillin-streptomycin (P/S; Gibco 15140122), 500 U/mL IL-4 (R&D

FIGURE 1

The EDEN automated fluidic system generates monocyte-derived immature DCs (MO-iDCs).



The inlet bottle supplies fresh differentiation medium which is continuously perfused by the pump into the EDEN cell culture cartridge at 8 µL/min/inlet. Waste medium from the cartridge is perfused into the outlet bottle. **(A)** Functional EDEN prototype. **(B)** 3D printed EDEN instrument with EDEN cartridge and associated consumables which include inlet and outlet bottles, tubing, and cartridge.

Systems 204IL), and 500 U/mL GM-CSF (R&D Systems 215GM).

EDEN cartridge & fluidic system

The EDEN cell culture cartridge was fabricated from commercially available polystyrene and acrylate cut using an Epilog Zing 16 laser system and assembled using 3M 966 Adhesive Transfer Tape. The polystyrene base was plasma treated. The cartridge has an internal surface area of 383.6 cm^2, volume of 122 mL, and measures 21.0 cm x 21.0 cm x 0.317 mm (length x width x height). Table 1 shows the number of MOs seeded. Eight inlet ports around the perimeter allow fresh differentiation medium to perfuse into the cartridge and a single outlet port at the center allows depleted medium to be removed from the cartridge.

The fluidic system consisted of an inlet bottle for fresh differentiation medium, peristaltic pump, and outlet bottle for collecting effluent from the cartridge. An Ismatec IPC-N peristaltic pump was used with PharMED BPT tubing to maintain continuous perfusion of fresh differentiation medium at 8.0 µL/min/inlet. Silicone tubing was connected between the peristaltic tubing and cartridge inlet to facilitate gas exchange between the medium and ambient environment maintained at 37°C and 5% CO_2 inside a Thermo Forma incubator. Silicone tubing was also used at the outlet port where perfusion flow rate was 64 µL/min. Effluent collected in the waste reservoir was centrifuged to determine if cells were washed out of the cartridge due to perfusion; no cells were observed in the effluent indicating that generated iDCs remain inside the cartridge and perfusion flow rate is not high enough to resuspend cells residing at the polystyrene base. 285 mL of fresh differentiation medium was added to the inlet reservoir at

DOI: 10.18609/cgti.2018.110

TABLE 1

Differentiation data for iDC generation in EDEN and 6-well plates.

Culture Vessel	MOs seeded ($x10^6$)	Seeding density (MOs per cm^2)	Cells harvested ($x10^6$)	Viable $CD45^+$ cells	iDCs $CD209^+$ $CD14^-$	Viable iDCs harvested ($x10^6$)	iDC yield
EDEN 1	114.3	300,200	26.7	98.3%	94.9%	24.9	21.8%
EDEN 2	78.3	205,700	25.8	96.2%	94.9%	23.6	30.1%
6-well plate 1	3.48	366,000	1.17	95.4%	96.8%	1.08	31.0%
6-well plate 2	1.74	183,000	0.47	94.1%	97.8%	0.43	24.7%

Phenotype data is shown in Figure 4.

startup (Day 0) and Day 3 to maintain perfusion throughout the 6-day differentiation. Cells were harvested by collecting the cell solution and washing the cartridge 2x with cold DPBS. Adherent cells after the two DPBS washes were not collected.

6-well plate control

A Corning Costar 6-well plate (3516) was used as a static control for iDC generation. Each well contained 2.5 mL differentiation medium and empty wells were filled with 3.0 mL DPBS. Table 1 shows the number of MOs seeded. 1 mL fresh differentiation medium was added to each well on Day 3. Cells were harvested by collecting the cell solution and washing each well 2x with cold DPBS. Adherent cells after the two DPBS washes were not collected.

Immature DC maturation

Maturation was conducted on the MicroDEN system at 3.5 μL/min perfusion using a small version MicroDEN cartridge that was 17.4 cm^2 and held 5.5 mL maturation medium. Maturation medium consisted of RPMI 1640 supplemented with 10% heat inactivated-fetal bovine serum (HI-FBS; Millipore Sigma F2442), 1% penicillin-streptomycin (P/S; Gibco 15140122), 2 ng/mL IL-1β (BD Biosciences 554602), 1000 U/mL IL-6 (BD Biosciences 550071), 10 ng/mL TNF-α (MilliporeSigma 11088939001), and 1 μg/mL PGE2 (MilliporeSigma P6532). Immature DCs from the EDEN 1 experiment were seeded at 422,200 iDCs/cm^2 and allowed to mature for either 1 day or 3 days in an incubator at 37°C and 5% CO_2. The cells were harvested using 2 cold PBS washes as described in [16].

Immunophenotyping

An ACEA Biosciences NovoCyte flow cytometer was used for immunophenotyping of harvested iDCs. Panel A tested viability (LIVE/DEAD Fixable Green Dead Cell Stain; Invitrogen L34970), CD209 (R&D Systems FAB 161P100), CD14 (Abcam ab157312), and CD45 (R&D Systems FAB1430A). Panel B tested CD80 (BD Biosciences 557226), CD83 (BD Biosciences 556855), CD86 (BD Biosciences 561128), and CD45; viability

was not included due to limited detection channels. Panel C tested CD80, CD83, CD86, and CD209 (R&D Systems FAB161A). Gates were set using a CD209 isotype control (IgG2b-PE R&D Systems IC0041P and IgG2b-APC R&D Systems IC0041A) and fluorescence-minus-one (FMO) controls.

Flow cytometry gating strategy

Large cells were gated in the SSC-A/FSC-A plot followed by single cells in a FSC-A/FSC-H plot. Panel A: viable/CD45$^+$ cells were gated then CD14/CD209 was plotted to determine MO or iDC percentage. Panel B: lymphocytes were gated on a CD45 histogram. Then CD80/83 and CD80/86 was plotted to determine iDC phenotype. Panel C: DCs were gated on a CD209/80 plot followed by a CD83/86 plot on either the CD209$^+$/80$^+$ or CD209$^+$/80$^-$ cells.

RESULTS & DISCUSSION

Computational fluid dynamics (CFD) simulations

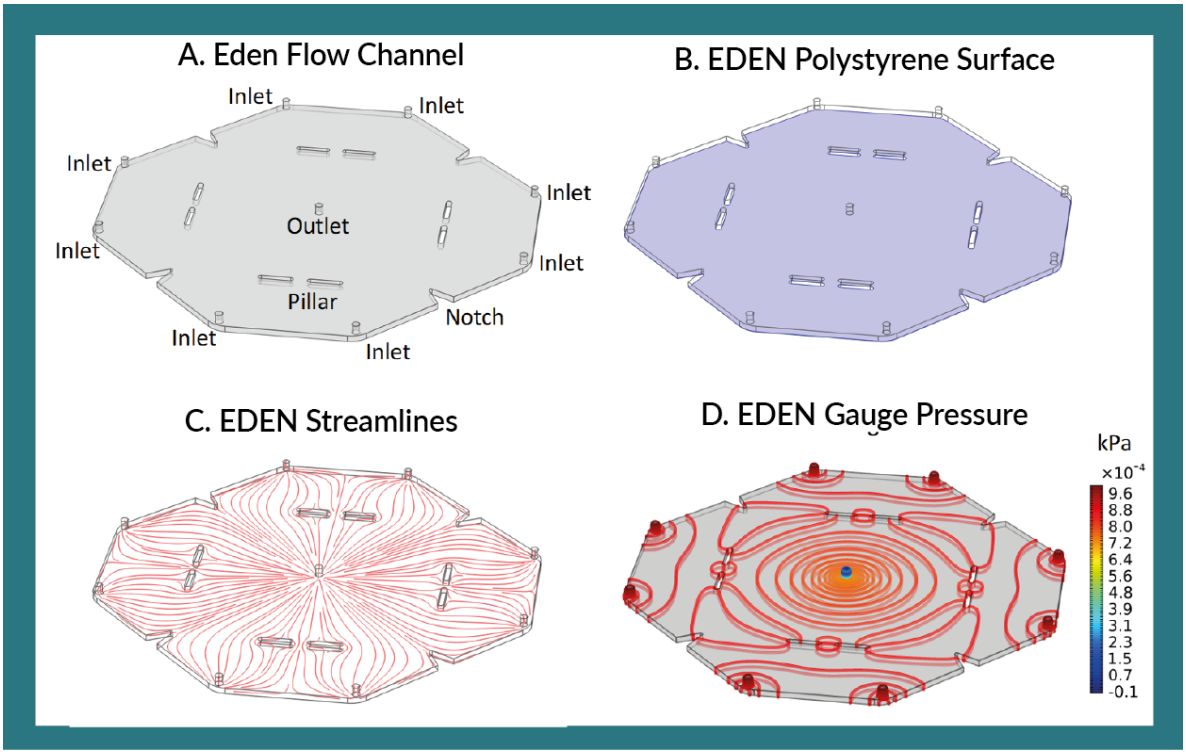
Computational fluid dynamics (CFD) simulations in COMSOL Multiphysics software were utilized in designing EDEN to understand how medium flows within the cartridge. Water at 37°C was used to simulate differentiation medium. The cartridge was initially filled with plain water without cytokines. In practice, the cartridge is filled with differentiation medium containing cytokines; however, initially filling the cartridge with plain medium (water) allows cytokine convection to be visualized since cytokine diffusion is extremely low (9216 μm^2/day) [17–19] and convection is the driving force behind the cytokine gradient. Water containing 1.16 mol/m^3 (500 U/mL) R&D Systems IL-4 was perfused into the cartridge at 8.0 μL/min/inlet and exited through the outlet at the cartridge center. Cytokine consumption/depletion was not factored into this analysis since we were interested in determining optimum medium flow of fresh differentiation medium. Figure 2A shows the cartridge flow channel, which describes the volume within the cartridge that medium flows. IL-4 cytokine concentration was modeled on the lower polystyrene surface of the flow channel where the cells reside on the cartridge base, as depicted by the purple surface in Figure 2B. Streamlines and gauge pressure due to perfusion are shown in Figure 2C and Figure 2D, respectively. IL-4 concentration gradient is shown in Figure 3 for each 24-hour period of perfusion.

These CFD data were critical in designing a cartridge which sufficiently allowed perfused medium to spread throughout the cartridge. Cytokine concentration and streamline data shows that at 8.0 μL/min/inlet laminar flow, the cartridge is split between eight regions. Each region is replenished with fresh differentiation medium after *ca.* 4 days. Initial CFD simulations indicated that dead zones formed at the location of the v-shaped notches, thus these notches were added to eliminate the dead zones and facilitate desired fluid flow. The eight cylindrical pillars within the cartridge support the upper acrylic surface. Before these were added, slight sagging of the acrylic was observed and

DOI: 10.18609/cgti.2018.110

FIGURE 2

EDEN fluid flow simulations.



(A) The EDEN cartridge flow channel. (B) The polystyrene surface (purple) at the base of the EDEN cartridge where the cells reside. (C) Streamlines due to perfusion within the EDEN cartridge. (D) Gauge pressure due to perfusion within the EDEN cartridge.

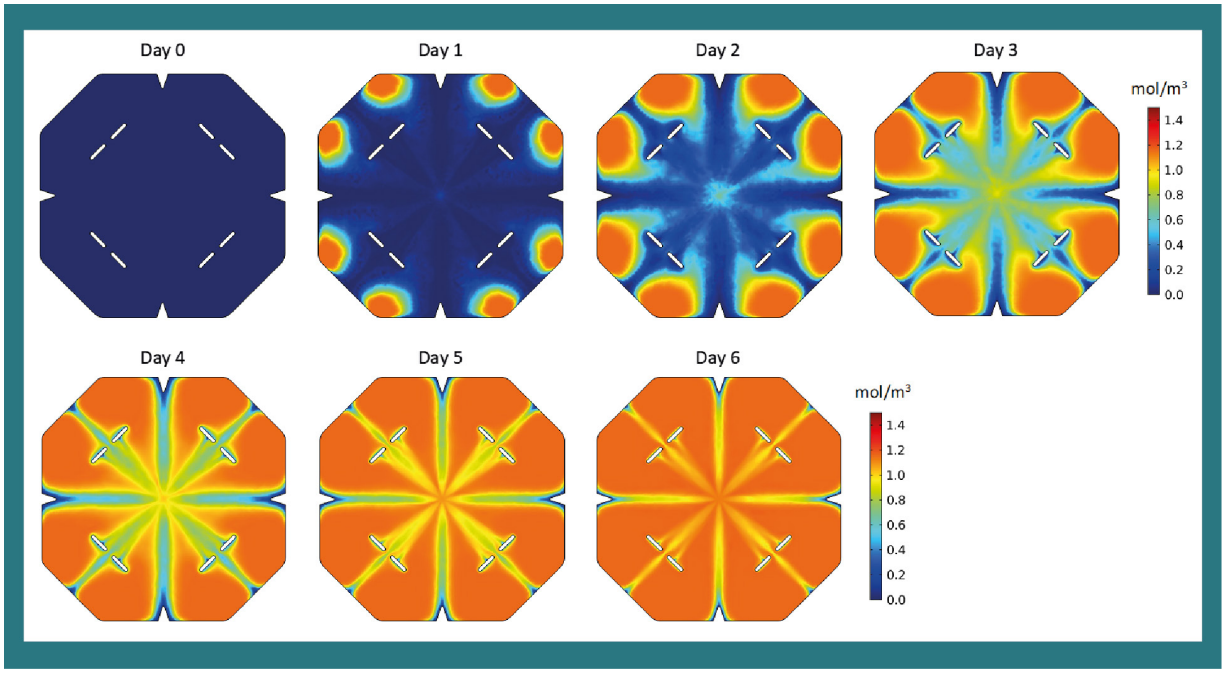
the acrylic was supported by medium within the cartridge which would cause unnecessary pressure within the cartridge that may affect the cells. Thus, these features, *i.e.,* the notches and pillars, were added to alleviate the dead zone and pressure concerns resulting in the final EDEN cartridge design that sufficiently aided perfused medium to flow within the cartridge without causing undesired pressure gradients.

Immature DC generation

Two iDC generation experiments were conducted in which 114.3 million and 78.3 million MOs were seeded into the EDEN cartridge. After 6 days differentiation, 24.9 million and 23.6 million iDCs were harvested from each cartridge. The number of viable iDCs harvested was calculated by multiplying total cells harvested by viable/$CD45^+$ cells by iDCs ($CD209^+/14^-$). IDC yield (normalized to the number of MOs seeded) was calculated as the number of iDCs harvested divided by the MOs seeded and was 21.8% and 30.1% for the two EDEN experiments. 6-well plate controls show that iDC yield was similar to EDEN, where the well plate had a higher yield than EDEN in experiment 1 and a lower yield in experiment 2. Titration of MO seeding density is necessary to optimize iDC yield in EDEN. Tabulated data are shown in Table 1.

FIGURE 3

Cytokine perfusion into the EDEN cartridge.



The cartridge is initially filled with water (medium) without cytokines. Cytokines perfuse into the cartridge at the 8 inlet ports at 1.16 mol/m³ (IL-4), flow through the cartridge driven by perfusion, and flow out through the outlet port at the center. In practice, the EDEN cartridge is filled with medium containing cytokines. The data is taken at the lower surface of the flow channel as indicated in Figure 2B.

Immature DC phenotype

Immunophenotyping of generated iDCs are shown in Figure 4. EDEN and 6-well plate generated iDCs are phenotypically similar after 6 days of differentiation. The iDCs are CD209 (DC-SIGN)$^+$, CD14$^-$, and exhibit low expression of CD80/83 as expected for MO derived iDCs [20–22]. CD86 expression on EDEN 2 iDCs was unexpectedly high as this level of expression is typically expected on mature DCs. Dissolved proteins in fetal bovine serum (FBS) supplemented into the base medium may be a possible explanation for this irregular expression since FBS is animal derived and its composition cannot be strictly controlled [23]. Additionally, contaminating proteins in the cartridge, since it was hand built in the lab, could also explain this high expression [24]. Greater than 99.7% of the cells were CD45$^+$ in the Panel B histogram (not shown). This protein expression profile for EDEN generated iDCs demonstrates the efficacy of EDEN in generating clinically relevant numbers of DCs that are phenotypically similar to well plate generated iDCs.

Immature DC maturation

Immature DCs generated in EDEN 1 were subsequently matured in a MicroDEN cartridge for either 1 day or 3 days. 7.31 million iDCs were seeded into each MicroDEN cartridge (422,200 iDCs/cm^2) and 5.7 million (1 day maturation) and 3.4 million (3 day maturation) mature DCs (mDCs) were harvested,

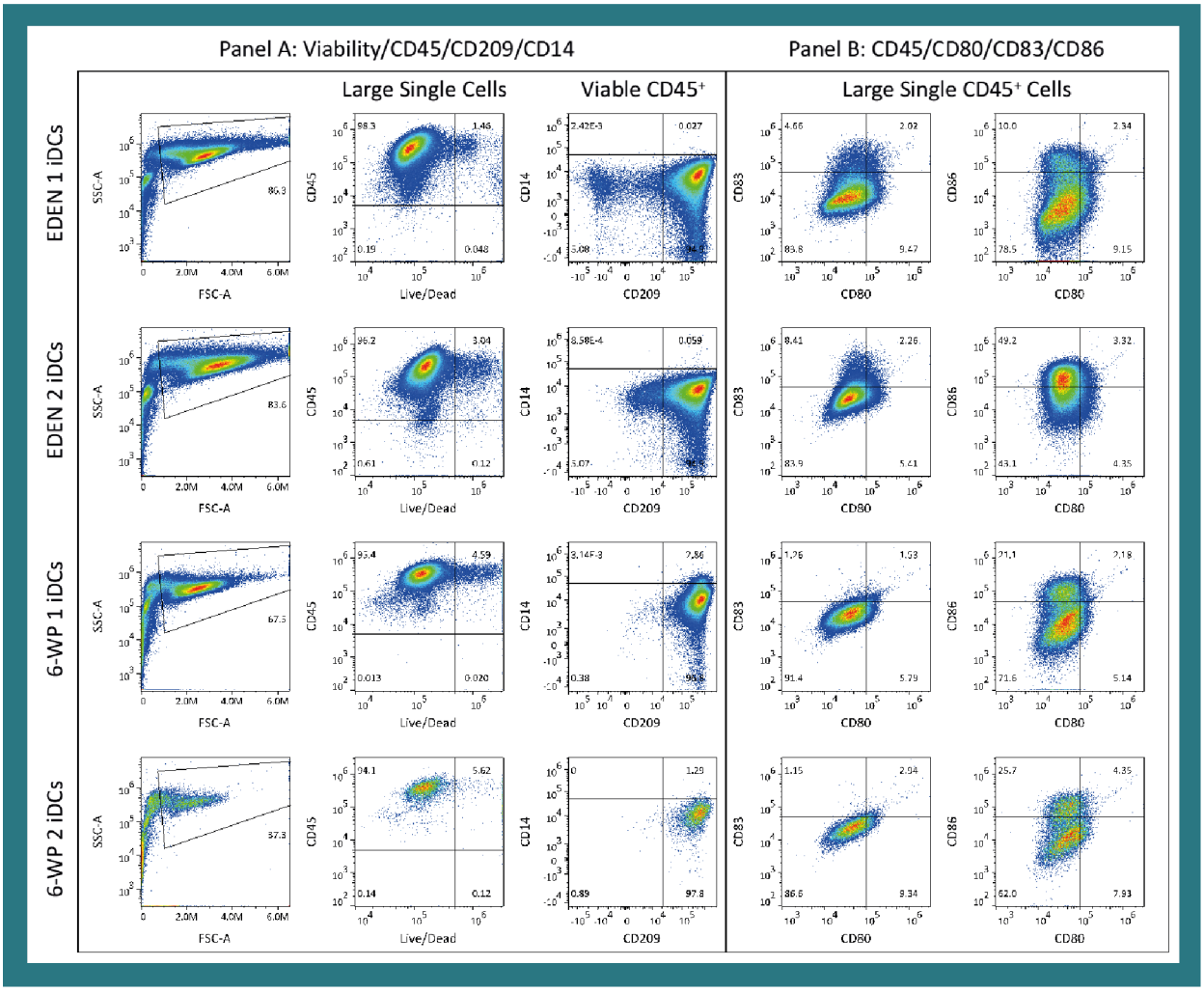
DOI: 10.18609/cgti.2018.110

for a yield of 77.8% and 46.5%, respectively. Yield was calculated as the number of seeded iDCs divided by the number of harvested mDCs. Maturation results are tabulated in Table 2 and immunophenotype is shown in Figure 5.

Interestingly, both CD209 markers (Panel A and Panel C) showed a slight decrease of CD209 expression after 1-day maturation of iDCs and a significant decrease of CD209 expression after 3 days maturation. The low mature DC (mDC) yield for 3-day maturation was due to the decrease of CD209 expression of these cells. The $CD209^-$ population decreased from *ca.* 5% for iDCs and 1-day matured mDCs to 30% for 3-day matured DCs. CD80 expression increased from *ca.* 10% for iDCs to 44% for 1-day maturation; whereas, 3-day maturation yielded 56% $CD80^+$ cells of which only 20% were also $CD209^+$. CD80 expression is generally low on iDCs and upregulated on mDCs, indicating successful maturation [20–22,25]. Both $CD80^+$ and $CD80^-$ mDCs strongly expressed CD83 (>90%) after 1-day maturation. After 3 days maturation,

FIGURE 4

Phenotype of EDEN and 6-well plate generated iDCs differentiated from MOs for 6 days.



Labels above the figures indicate the gates from which the plots derive. WP: Well plate.

TABLE 2

Maturation data for EDEN 1 generated iDCs.

Experiment	iDCs seeded (x10^6)	Seeding density (iDCs per cm^2)	Cells harvested (x10^6)	Viable CD45$^+$ cells	mDCs CD209$^+$ CD14$^-$	Viable mDCs harvested (x10^6)	mDC yield
1-day maturation	7.31	422,200	6.24	96.3%	94.6%	5.7	77.8%
3-day maturation	7.31	422,200	5.30	92.1%	69.6%	3.4	46.5%

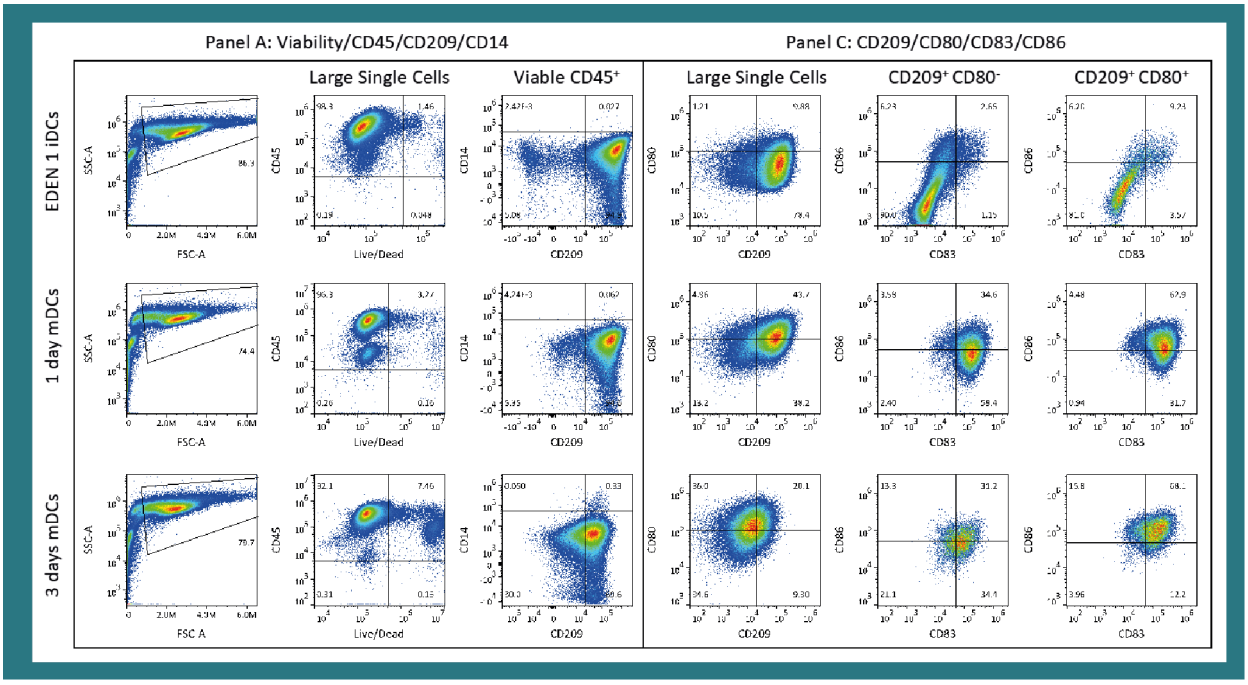
Maturation was performed in a small version MicroDEN cartridge. Phenotype data is shown in Figure 5.

CD83 was expressed on 80% of CD80$^+$ mDCs but only 65% of CD80$^-$ mDC. CD86 expression was greatest for CD80$^+$ mDCs for both 1- and 3-day maturation with 68% and 84% of the mDCs expressing CD86. CD80$^-$ mDCs significantly lower level of CD86 expression, *ca.* 40% for both 1- and 3-day maturation. Collectively, these results indicate that maturation duration significantly affects phenotype and yield of mDCs for the experimental conditions studied. We caution against concluding that 1-day maturation is optimal since function of matured DCs and cytokine excretion should be evaluated in a mixed lymphocyte assay (MLA).

Maturation of iDCs can take place in the same EDEN cartridge as MO differentiation by perfusing maturation medium into the cartridge; however, we decided to

FIGURE 5

IDC and mDC phenotype from EDEN 1.



Immature DCs were generated in EDEN then seeded into MicroDEN for 1 or 3 days maturation. Labels above the figures indicate the gates from which the plots derive.

DOI: 10.18609/cgti.2018.110

harvest iDCs from the EDEN cartridge to allow for counting and immunophenotyping of generated iDCs. One option is to perfuse maturation medium directly into the EDEN cartridge without harvesting the iDCs. A second option is to harvest the iDCs, resuspend in maturation medium, and reseed into the EDEN cartridge. A third option, the path we chose for this work, is to harvest generated iDCs from the EDEN cartridge, resuspend in maturation medium after counting cells and removing 2 million DCs for phenotyping, and seeding into a smaller MicroDEN cartridge for 1 or 3 days maturation while perfusing maturation medium. The desired workflow will depend on user requirements, *e.g.*, obtaining iDC cell count, tailoring maturation cocktail to the number of iDCs, and iDC concentration/seeding density for maturation.

Production of therapeutically active DCs in EDEN

Bespoke production of therapeutically active DCs follows the same general outline regardless of the targeted disease. The exact protocol will depend upon the desired characteristics of the DCs and EDEN is designed to be easily integrated into current vaccine production protocols. The MicroDEN can be used to optimize differentiation, maturation, and peptide pulsing conditions before advancing to larger-scale therapeutically active DC generation in EDEN. It is recognized that monocyte enrichment (*e.g.*, via elutriation or magnetic beads) must precede DC generation in EDEN and some manual handling is required to perform the DC generation process in EDEN; however, with sterile transfer-compatible consumable design, the entire process can be carried out in a closed format.

The following is an example workflow incorporating EDEN for generating therapeutic DCs. **Figure 6** shows how EDEN is used in the three major steps alongside immunological markers for iDCs and mDCs:

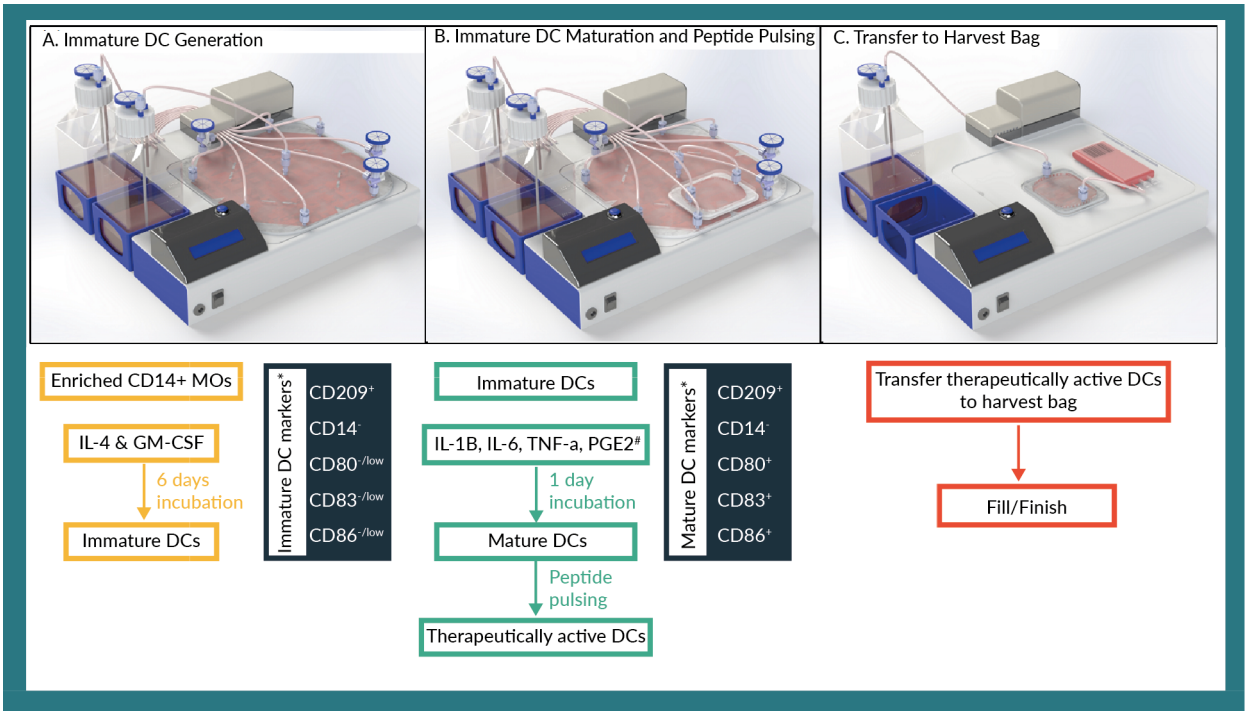
1. $CD14^+$ MOs are cultured with IL-4 and GM-CSF for 5–10 days to generate iDCs. These iDCs will be $CD14^-$ and CD209 (DC-SIGN)$^+$. Few cells will express CD80/83 while a greater number of cells will be $CD86^+$;

2. Immature DCs are either (a) harvested from EDEN, resuspended in maturation medium, and seeded back into the EDEN cartridge or a MicroDEN cartridge depending on the desired cell concentration during maturation or (b) left within the EDEN cartridge (*i.e.*, not harvested) and maturation medium is perfused into the cartridge;

3. Immature DCs are matured for typically 1–2 days in maturation medium followed by peptide pulsing of typically 2–24 hours [8,13,26]. The mDCs express CD80/83/86 and have lower expression of CD209 compared to iDCs. Once the mDCs are pulsed with the target peptide(s), the DCs are considered therapeutically active. **Figure 6B** depicts cells being transferred from an EDEN cartridge to a MicroDEN cartridge for maturation and peptide pulsing;

FIGURE 6

EDEN workflow for generation of DC immunotherapy.



(A) Enriched MOs are cultured in differentiation medium for 6 days in EDEN to generate immature DCs. **(B)** Immature DCs are transferred to a MicroDEN cartridge and cultured in maturation medium for 1 day to generate mature DCs. The mDCs are pulsed with targeted peptides to generate therapeutically active DCs. **(C)** The therapeutically active DCs are transferred into a harvest bag then transferred to Fill/Finish for quality control and preparation before patient infusion. Immature and mature DC markers shown were used in this study and are traditional DC identifying proteins. There are other markers which can be used to phenotypically classify peripheral blood monocyte-derived DCs.
*CD1a, CD11c, HLA-DR, CD45 are expressed by both immature and mature DCs.
#There are numerous other cocktails that can be used for maturing iDCs.

4. The therapeutically active DCs are transferred from either the MicroDEN cartridge (Figure 6C) or the EDEN cartridge into a harvest bag.

CONCLUSIONS

EDEN was developed for GMP production of therapeutically relevant numbers of iDCs in a single cell culture cartridge that is fully enclosed and unopen to the outside environment. Computational fluid dynamics simulations aided the design of the EDEN cartridge to ensure that perfused medium flowed properly throughout the cartridge and cytokines were sufficiently replenished. Fresh differentiation medium was continuously perfused into the cartridge and depleted medium was concomitantly removed. Phenotype expression and yield of MO-iDCs was similar between EDEN and 6-well plate controls. Immature DCs were subsequently matured in a MicroDEN cartridge and exhibited standard upregulation of CD80/83/86 and downregulation of CD209. These results show that EDEN successfully generates 20–25 million iDCs with a 20-30% iDC yield at the conditions tested and demonstrate that EDEN is a viable option for scaling-up GMP production of therapeutically active dendritic cells.

DOI: 10.18609/cgti.2018.110

ACKNOWLEDGEMENT

This material is based upon work supported by the U.S. National Science Foundation under Grant No. 1819306.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

AK is an employee and shareholder, HW is an employee, SKM is a founder and shareholder, and JMR is President & CEO and a shareholder of Flaskworks LLC which is commercializing the EDEN system. No writing assistance was utilized in the production of this manuscript.

REFERENCES

1. Roh KH, Nerem RM, Roy K. Biomanufacturing of therapeutic cells: State of the art, current challenges, and future perspectives. *Annu. Rev. Chem. Biomol. Eng.* 2016; 7: 455–78.
2. Tuyaerts S, Aerts JL, Corthals J *et al.* Current approaches in dendritic cell generation and future implications for cancer immunotherapy. *Cancer Immunol. Immunother.* 2007; 56: 1513–37.
3. Babatz J, Röllig C, Oelschlägel U *et al.* Large-scale immunomagnetic selection of CD14+ monocytes to generate dendritic cells for cancer immunotherapy: A phase I study. *J. Hematother. Stem Cell Res.* 2003; 12: 515.
4. Dietz AB, Padley D, Butler G *et al.* Clinical-grade manufacturing of DC from CD14(+) precursors: Experience from phase I clinical trials in cml and malignant melanoma. *Cytotherapy* 2004; 6: 563–70.
5. Felzmann T, Witt V, Wimmer D *et al.* Monocyte enrichment from leukapharesis products for the generation of DCs by plastic adherence, or by positive or negative selection. *Cytotherapy* 2003; 5: 391.
6. Garlie N, Timler A. Dendritic cell generation from cryopreserved monocytes enriched using the Elutra versus the CliniMACS cell separation systems. *J. Immunother.* 2005; 28: 613.
7. Meyer-Wentrup F, Burdach S. Efficacy of dendritic cell generation for clinical use: Recovery and purity of monocytes and mature dendritic cells after immunomagnetic sorting or adherence selection of CD14+ starting populations. *J. Hematother. Stem Cell Res.* 2003; 12: 289.
8. Carreno BM, Magrini V, Becker-Hapak M *et al.* A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. *Science* 2015; 348: 803–8.
9. Carreno BM, Becker-Hapak M, Huang A *et al.* IL-12p70–producing patient DC vaccine elicits Tc1-polarized immunity. *J. Clin. Invest.* 2013; 123: 3383–94.
10. Linette GP, Zhang D, Hodi FS *et al.* Immunization using autologous dendritic cells pulsed with the melanoma-associated antigen gp100-derived G280-9V peptide elicits CD8+ immunity. *Clin. Cancer Res.* 2005; 11: 7692.
11. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* 1994; 179: 1109.
12. Mitchell DA, Batich KA, Gunn MD *et al.* Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature* 2015; 519: 366.
13. Nair S, Archer GE, Tedder TF. Isolation and generation of human dendritic cells. Current Protocols in Immunology Chapter 7: Unit7.32-Unit37.32; 2012.
14. Garg AD, Vara Perez M, Schaaf M *et al.* Trial watch: Dendritic cell-based anticancer immunotherapy. *OncoImmunology* 2017; 6: e1328341.
15. Saxena M, Bhardwaj N. Re-emergence of dendritic cell vaccines for cancer treatment. *Trends Cancer* 2018; 4: 119–37.

16. Kozbial A, Bhandary L, Collier BB, Eickhoff CS, Hoft DF, Murthy SK. Automated generation of immature dendritic cells in a single-use system. *J. Immunol. Methods* 2018; 457: 53–65.

17. Gammack D, Doering CR, Kirschner DE. Macrophage response to mycobacteriumtuberculosis infection. *J. Math. Biol.* 2004; 48: 218–42.

18. Pigozzo AB, Macedo GC, dos Santos RW, Lobosco M. On the computational modeling of the innate immune system. *BMC Bioinformatics* 2013; 14: S7–S7.

19. Su B, Zhou W, Dorman KS, Jones DE. Mathematical modelling of immune response in tissues. *Comput. Math. Methods Med.* 2009; 10.

20. Mo-DC generation toolbox: https://www.miltenyibiotec.com/_Resources/Persistent/898185d33bc556eb522f23f625388dfa0729cd36/DS_130-093-568.pdf

21. Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. *Immunology* 2013; 140: 22–30.

22. Dudek A, Martin S, Garg A, Agostinis P. Immature, semi-mature, and fully mature dendritic cells: Toward a DC-cancer cells interface that augments anticancer immunity. *Front. Immunol.* 2013; 4: 438.

23. Zhang R, Becnel L Li M, Chen C, Yao Q. C-reactive protein impairs human CD14+ monocyte-derived dendritic cell differentiation, maturation and function. *Eur. J. Immunol.* 2006; 36: 2993–3006.

24. Sansom DM, Manzotti CN, Zheng Y. What's the difference between CD80 and CD86? *Trends Immunol.* 2003; 24: 313–8.

25. Bender A, Sapp M, Schuler G, Steinman RM, Bhardwaj N. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods* 1996; 196: 121–35.

26. Shinde P, Fernandes S, Melinkeri S, Kale V, Limaye L. Compromised functionality of monocyte-derived dendritic cells in multiple myeloma patients may limit their use in cancer immunotherapy. *Scientific Rep*. 2018; 8: 5705.

AFFILIATIONS

Andrew Kozbial[1], Hope Weinstein[1], Shashi K Murthy[2] & Jennifer M Rossi[1]

[1]Flaskworks LLC, Boston MA 02118, USA

[2]Northeastern University, Department of Chemical Engineering, Boston MA 02115, USA

DOI: 10.18609/cgti.2018.110

INTERVIEW

Critical considerations for allogeneic cell therapy scale up

SHARON GRIMSTER joined ReNeuron in 2013 and was subsequently appointed as General Manager of the Wales facility in September 2014. Sharon has significant experience in pharmaceutical development, and she has particular expertise in ATMPs, project management, biologics development and manufacturing. Prior to working at ReNeuron, Sharon held senior team roles at Celltech, Antisoma and F-star, and has had responsibility for a range of development functions, including project management, regulatory affairs, manufacturing, quality and general operations. Sharon qualified as a coach at the Henley Business School and has a BSc from the University of Leicester and a Diplomas in Immunology and in Management Studies. She is also a Fellow of the Royal Society of Biology and an active member of a number of significant industry committees.

Q What are some of your key priorities for the year ahead at ReNeuron?

SG: ReNeuron is going through a very exciting stage at the moment. We've got three key product platforms in our portfolio.

One is CTX, a conditionally immortalized cell line, which is currently being investigated in a Phase 2b study in stroke.

The second programme is our human retinal progenitor cell product, which is a multi-donor product being used in a clinical programme for retinitis pigmentosa.

And thirdly, we have exosomes, which is a platform that is expanding rapidly at the moment. Exosomes are extracellular vesicles, which are secreted by stem cells. They have interesting characteristics of their own: I've heard it said about exosomes that it's the way cells talk to each other – we just haven't learned to listen yet, and I think that's a very helpful way to

look at it, the biological knowledge in this area is expanding rapidly. There's huge potential in exosomes and we have a lead position in the field. The CTX cell line is a very good producer of a consistent exosome population, and so we are able to produce large amounts of exosomes just by virtue of harvesting the spent media from our CTX cell production. We have also developed the purification process and the accompanying analytics, so we are in a great position to further exploit this technology.

So we have three platforms and we're very active in the clinical trials stage at the moment, treating more patients than we've ever treated before in the 20-year history of the company. Clearly, that puts a lot of demand on the CMC team, both in terms of making materials for the current studies and also preparing the cell banks and the Drug Product and the data packages for the next phase of clinical trials. We are fortunate to have an excellent experienced team at ReNeuron who are delivering on this.

Q Cell and gene therapy manufacturers adopting methods, technologies and strategies from the wider biopharma sector can sometimes polarize opinion. As someone who worked through those formative years for the mAbs field, what are the key elements of that experience that you bring to bear at ReNeuron?

SG: I certainly do bring a lot from my background into what I do, and I think one of the things that attracted me to the cell and gene therapy side was that need to work from first principles – to maybe borrow elements from prior experience with other biologics, but without taking a 'cookie-cutter' approach, as ATMPs are very different from traditional biologics and there is much less of a platform approach in the industry. The differences are usually driven by the science behind the technologies.

I think applying the key requirements and concepts of pharmaceutical development and GMP is important, but applying these basic principles whilst making them specific to your process is what really matters. It's often the things that appear simple that end up complex for ATMPs like aseptic manufacturing, supply chain control, practicalities such as mixing and cell counting, as well as operator training, and reducing the potential for human error.

"There's huge potential in exosomes and we have a lead position in the field."

But perhaps it's the fact that I was involved in the very early stage of biologics which makes me feel more comfortable being involved in the

10.18609/cgti.2018.113

"It's often the things that appear simple that end up complex for ATMPs..."

early stages of cell therapy. My experience has been at the front end of new technology more than in very large-scale, established technology areas. So I think that's probably why I feel we're using the same basic toolbox to think through what we're trying to do from first principles, and to design programmes, processes and analytics accordingly.

I do think, for this reason, that it's a significant challenge for CMOs in this space. There are a limited number of good CMOs currently and many of these are expanding rapidly and they are bringing in people with biopharma experience, who are often more used to defined platforms, and the ability to aseptically filter products at the end of the process.

One vital learning to borrow from early biologics is that it's key to have a very close relationship with your contract manufacturers, and to treat it as a partnership as far as you can, notwithstanding the fact that the two parties have different commercial needs. For example, our Chief Medical Officer will visit our contract manufacturers to talk to them about the programmes from the clinical perspective: how the team in the CMO has the potential to benefit patients. At the early stages of development, it's about partnering with the CMO to achieve the delivery of products to patients to establish whether the product is efficacious, The partnership then develops to minimise Cost of Goods, which will become a more significant aspect at a later stage.

Q With ReNeuron's lead CTX platform therapeutic candidate now approaching late-phase clinical development, what are the key specific areas of focus for you in scale-up terms?

SG: We are just completing a work programme that was funded by an Innovate UK grant, which we called CelltoSell, and that's been about developing a platform approach as far as we can for our CTX and hRPC processes as they move into later stage development. We worked with Loughborough University, Roslin Cell Therapies and the Cell and Gene Therapy Catapult as part of the grant.

Both the CTX and hRPC platforms are allogeneic, so we're focused on scaling up to large batch sizes of the same cells. We've looked at what steps the two processes have in common – at what we could combine in a sort of 'semi-platform' approach, albeit one involving two very different technologies. We took this approach because it will make the manufacturing of the portfolio more efficient on a number of levels both while we are working with CMOs and also when we establish our own manufacturing facility.

We've been exploring the extent to which we can get the analytics, supply chain and some of the cell growth steps to be similar, and yet optimised for each distinct process.

That work under this grant has gone very well and is just now coming to a close. In addition to the bioprocess steps and the analytical toolkit, we've been able to explore other elements such as the supply chain – the dual source and qualification of different materials, for example.

So we've make some great achievements in terms of reducing Cost of Goods, improving analytics and scaling these processes. We looked at both 2D and 3D, and 2D gives us sufficient scale to satisfy commercial demand, so this is our approach going forwards.

We do also have some good data with 3D, but that's going to take a little bit longer. We see that as a follow-on option to potentially further reduce Cost of Goods in the long-term – we will continue progressing 3D in the background as part of our technology development programme.

With comparability, again, we take an appropriately holistic approach – relating to comparability in terms of all aspects of the process and the analytics. We think through the preclinical evidence we have for these products and how we expect that to translate into meaningful clinical endpoints for patients to determine what analytics can best predict that clinical response, and how we can make that into assays that can be validated.

"The current trend with facilities is for well sized, empty rooms and flexible space and I think that's key..."

For potency assays, regulators need to see that link from preclinical to clinical with assays that can be validated. From a practical product point of view, it is good to have that as early on in development as possible.

What we've learnt from our CTX cell line, we apply broadly to our hRPC product, which is in earlier stage trials – so we're very much learning from one product to the other. And above all, we recognise that we must take a holistic and comprehensive approach. I've heard it said that comparability is like looking through the windows into someone's house and trying to find out all about their life from that single snapshot. You only get a snapshot of each view, each method, each approach you take, so you have to look across everything – supply chain, analytics and process.

Q Can you go a bit deeper on ReNeuron's approach to ensuring manufacturing of your allogeneic cell line, CTX, is scalable up to the sort of commercial levels necessary for a lead indication (stroke) with such a large global patient population?

10.18609/cgti.2018.113

SG: Unlike the majority of cell and gene therapy products to have reached the market to date, our products are allogeneic, which means our medium to long term objectives relate to scaling up to get larger batch sizes and a process that can operate efficiently at commercial scale in a GMP facility. On one level, this is appears easy, but the practicalities are of course challenging as you go along.

Again, this comes back to that basic toolbox from established biologics. Above all, it's the control of each step as you scale up and establishing the critical control parameters early on, in terms of the media, the cell health, the volume you're dealing with, the mixing, the time in the processing, the temperature… all the features that need to be controlled as you scale up and work with larger volumes and cell numbers. That's why we need to concentrate on understanding critical control parameters and critical quality attributes earlier on in the development process.

So I would say it's both difficult and not difficult at the same time… it's challenging, but it's good for us that we can get to full commercial scale without substantially changing technology at the moment: this helps us keep control, and demonstrate comparability.

Q What are some of the other challenges you have encountered so far – and anticipate meeting in future – relating to scale up?

SG: Every time you change scale, you find things that you don't completely expect. Having relevant in-process analytics is essential to achieving process control during scale up – having ways to understand and predict the health of the cells is essential.

Working at scale is a challenge because while there's a certain amount you can do in scale-down multifactorial experiments, there are some things which you will only find when you work at scale. And of course, once you work at scale, the consumables become quite expensive – which means you simply can't do every experiment at scale. Similar to mature biologics, it's about the predictive power of the multifactorial scale-down experiments – how that translates to what you then see at large scale, and when you tech transfer to different GMP facilities.

Training is so very important, too. Understanding the cells, what they look like, and how to handle them quite precisely is key. And coming back to CMOs, you can't expect them to have the same very specific and deep knowledge of your platform as your own in-house people do. They will have good teams, but their skills are broader and they're not working solely on your product. This is much more of an issue in cell therapy than in other

biologics because at the moment some aspects of early stage cell therapy manufacturing is still based on an individual's skill, and this will be the case until processes are more fully closed and automated. This leads back to the importance of making sure you are very close to your CMO at the operator level in order to get the best performance from them.

Q You have to design a new cell therapy manufacturing facility tomorrow – what will be the key factors that are most critical for you?

SG: Firstly – and this might sound a bit old hat – but start with the end in mind, both in terms of eventual scale and technological and possible future regulatory requirements, the recent revisions to Annex I of the EU GMP guidelines is an example. The current trend with facilities is for well sized, empty rooms and flexible space and I think that's key: making sure you're going to be state-of-the-art, that you have access to all the services you will need, and that you have as few constraints as possible in terms of size and space. That all comes at a cost, of course, but you must design for the future – so take a view on what the future is and stay flexible in your design.

Then think about what else might change in the future. A company's gift is knowing what scale they expect to be at in future, but what are things that might move around them in terms of regulations, and the picks and shovels of the industry in terms of the equipment, and the supply chain of consumables and materials over the coming decade?

Equipment changes over that sort of timeframe will take the form of improvements but may also result in some technology becoming obsolete and no longer supported by suppliers, so you need to think forward and stay ahead of the game given how long it takes to introduce changes. . On the regulatory side, as with technological evolution, there's a limit to what you can predict, but be mindful of what you know about current and likely future trends in this area, and the fact that not all territories are completely aligned in this regard. Take a risk-based approach, in short.

And I think location is important, both in terms of making sure you can attract the right staff and having suitable local infrastructure available to support your supply chain and logistics needs. I don't think this is substantially different from any biological facility, in that quality has be built in from concept to completion This being aseptic manufacture, it has to be right.

AFFILIATION

Sharon Grimster
ReNeuron

10.18609/cgti.2018.113

INTERVIEW

Towards commercial allogeneic cell therapy manufacturing

MARIA DEL PILAR REDONDO holds an MsC in Pharmacy, specializing in Pharmaceutical technology and analysis and control of drugs. Although she began her career at Pfizer within the area of Quality, she later focused on Pharmaceutical Development. After incorporation into the Pharmamar group in 2005, she became in charge of pharmaceutical development and production of investigational medicines for the treatment of Alzheimer's disease. Pilar joined Tigenix SAU in June 2013 as Senior Director of Technical Operations. Her responsibilities included the overview and management of activities in the areas of Pharmaceutical Development (CMC/Industrialization) and Manufacturing of the stem cell platform.

Q What are TiGenix's priorities for the year ahead in manufacturing terms?

PR: For manufacturing, our main priority over the coming months is to make sure we establish a stable supply for the commercial rollout we're planning across Europe – to secure the availability of products to patients in Europe.

Q What have been the chief challenges that you have had to address in scaling up TiGenix's allogeneic cell therapy platform(s) and what has been your approach to addressing them?

PR: I think the main challenges we have faced relate to establishing a robust team. As we approach commercialisation, we need

"It is key to be able to grow your team while ensuring the relevant expertise is on hand to ensure this consistency in quality."

experienced staff to keep our product quality consistently high, and comparable to quality achieved throughout the development phase. It is key to be able to grow your team while ensuring the relevant expertise is on hand to ensure this consistency in quality.

We established a gradual onboarding plan, which has run in parallel with the ramp-up of manufacturing. This was designed to provide for a sufficient qualification and training period with the new staff – to give them the necessary amount of time to achieve the level of expertise required.

The second challenge I would highlight is access to raw materials and reagents. Both adequate quality and consistent supply are important for accommodating scale-up of the final product.

Essentially, what we are doing here is to approach our materials suppliers and establish agreements with them – to be quite transparent with those that are critical to our process, sharing our expectations and making sure that we can work together throughout the course of the project and product lifecycle.

Raw materials are a central part of current discussions in the field: how to bring them up to what we could call 'pharmaceutical standards', both in terms of quality and supply continuity. I think we need to grow together with suppliers in this area towards this deep understanding of quality. The existence of some guidelines and technical documents from different bodies and associations throughout Europe, the United States and other territories is quite helpful for studying the minimum expected levels of quality and helping the alignment between materials suppliers and cell therapy manufacturers.

Q TiGenix is one of a mere handful of advanced therapy companies to have experienced operations at the commercial level, in the shape of ChondroCelect. What do you take from that experience to apply to the manufacturing scale up for other products, such as Alofisel?

PR: We had a fantastic learning experience with ChondroCelect to help guide this current project. I think one of the most important learnings we took from it was realising the paramount importance of being able to count on an experienced, highly-qualified team. As I mentioned earlier, the staff that is taking care of Alofisel is a combination

DOI: 10.18609/cgti.2018.114

"We have developed a very specific tool that enables and ensures direct communication between the hospital and the manufacturing plant, providing visibility for all on the status of a specific patient's product order along the length of the process."

of newcomers and very experienced people who have been involved in the development of the product. Again, the expertise and the qualification of all staff are crucial for manufacturing success, and also on the laboratory side with quality aspects.

A second learning from ChondroCelect which we applied during the development of Alofisel relates to interactions with local regulatory authorities, who of course visited us for inspections prior to licensure of the manufacturing facilities. Cell therapy being a relatively new business, it is especially important to have an open interaction with inspectors, sharing with them limitations, discussing their expectations, and generally having a very fluid dialogue. I have to say, it was a very positive and constructive experience for us.

Q How is the manufacturing organization at TiGenix evolving now that a big pharma company like Takeda has become involved as parent company? For instance, how will you seek to leverage the wealth of experience and resources within the larger organization?

PR: I think that the situation we have now is extremely interesting for all of us. TiGenix is focusing on bringing specific technical expertise into Takeda. We are bringing the experience of developing and manufacturing cell therapy products in an agile manner - experience inside the culture rooms, with the analytics and so on. At the same time, we're now enjoying having Takeda supporting us by providing systems and procedures that we need for further development as the project grows - Takeda is already responsible for the commercialization of Alofisel and realizing the product's full potential - so I think it's a very complementary collaboration of manufacturing through to commercialization.

Q What is the single tool or technological innovation which has made the greatest positive difference to TiGenix's bioprocessing and supply chain activities in recent times?

PR: I would definitely highlight our recent development of the supply chain. Looking into commercial roll-out, it was clearly necessary to evolve from the model we employed during the clinical trials. I have to say that it was a very nice joint effort, which was driven by Takeda's supply organisation. We have developed a very specific tool that enables and ensures direct communication between the hospital and the manufacturing plant, providing visibility for all on the status of a specific patient's product order along the length of the process – from the moment the hospital raises an order for a patient, through production and shipping, to administration of the final product to the patient. I think this is the one development that has made the greatest change in our situation, helping us to address our patients' needs more appropriately.

Q Allogeneic cell therapy – within both the stem cell and cellular immunotherapy realms – is firmly back in the spotlight. Do you feel the time has finally arrived for this field to capitalize on its obvious potential and inherent commercial advantages?

PR: My feeling is that we are now seeing our experiences materialise into something tangible. And I have to say that manufacturing and supply aspects are key drivers for this ongoing 'confirmation of expectations' relating to the allogeneic cell therapy field: they are really the final test for these expectations.

I do think there are still some issues to solve – we have touched on some of them previously, but I think the one I would highlight here relates to the harmonisation of regulations across different territories.

I think in the pharmaceutical industry at large we have already seen the advantages of harmonisation initiatives. And if I had to select one area for allogeneic cell therapy where I think harmonisation across different territories will become clearly necessary in the future, it would be requirements for donor eligibility and donor qualification. We are still seeing a lot of differences country-to-country and region-to-region in this area - between Europe and the US, for example. Progress in this area would provide for real improvements in the speed and depth of market access for allogeneic cell therapy products on a global basis.

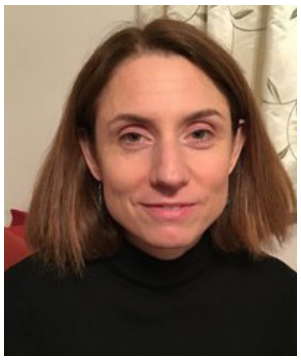
AFFILIATION

Maria Dei Pilar, Senior Director of Technical Operations, Tigenix SAU

DOI: 10.18609/cgti.2018.114

INTERVIEW

Autologous T cell immunotherapy scale out manufacturing models – the biotech perspective



DR NINA (EKATERINI) KOTSOPOULOU is Vice President of Process Development at Autolus. She joined the UCL spin-out CAR-T company in 2015 and set-up the vector and CAR-T production process and analytics, including delivery of the first clinical patient batches. Nina has a BSc in Chemistry from the University of Athens and a DPhil in lentiviral vectors for gene therapy from Oxford University. Following her PhD, she briefly worked in her supervisors' company, Oxford BioMedica, and went on to do 2 post-docs on haematopoietic stem cell biology, at Harvard Medical School and the University of Cambridge. She then joined GSK, where she initially led cell line development and associated process research for monoclonal antibodies and derivatives thereof. She then led Process Development for GSK's Cell and Gene Therapy portfolio, including working with the inspirational team at TIGET, and completing the MAA submission for the first product, Strimvelis, for the treatment of ADA-SCID.

Q What are some of the key priorities for the year ahead for you personally, and for Autolus?

NK: Autolus is a clinical stage company, so the primary focus for the year ahead is delivering on our existing clinical trials: we have a broad clinical stage pipeline, with four product candidates in five haematological indications, and one solid tumour program.

At the same time, we're also preparing for larger scale manufacturing to support future studies and, assuming future approval of our products, commercial launch. Finally, we are planning delivery of next-generation products for both haematological malignancies and solid tumours, with three next-gen versions of our lead programs.

"...the primary focus for the year ahead is delivering on our existing clinical trials: we have a broad clinical stage pipeline, with four product candidates in five haematological indications, and one solid tumour program"

Q What are some of the particular issues relating to manufacturing scalability of technology platforms in the T cell immunotherapy space, and how have you sought to address them?

NK: Designing and fixing a commercially viable process as early as possible is very important.

It's key because we want to minimise comparability risk when we introduce future process changes. And we want to be ready in time for commercial launch, because with this type of product, compelling efficacy can be apparent from early clinical studies which offers the opportunity of accelerated approvals, meaning very short development timelines.

So what we're trying to do – what we have implemented to date - is essentially a process that is commercially ready from the beginning of our clinical trials. We've designed our processes to be fully closed and semi-automated, operated in a low-grade cleanroom, and we've also ensured we have frozen apheresis in and frozen finished product out. This means we can manage the challenges of scheduling and optimising the throughput of products through our manufacturing facility to efficiently get the product to the patient.

Q When it comes to scaling out of autologous T cell therapies there have been some interesting developments from the commercial trailblazers as they look to establish production in different regions of the world. How is Autolus looking ahead to a potentially global commercial scenario in this regard?

NK: We've designed and built our process based on what we feel is the right model for us, which is for semi- centralized manufacture – modular manufacturing established on a regional basis, with a small number of medium-sized facilities in key geographies.

DOI: 10.18609/cgti.2018.115

"What we will do initially is to establish a launch facility for about a thousand patients/products per year in the UK, and our first commercial facility for about 5,000 therapies per year in the US."

Having an automated process that is easily transferable allows us to build the number of facilities over time, based upon demand. What we will do initially is to establish a launch facility for about a thousand patients/products per year in the UK, and our first commercial facility for about 5,000 therapies per year in the US.

I also think that given the number of products that need to be made for the indications we're going for, it actually makes sense economically to go with this regional, central production model: not only do you have more consistency in terms of your testing, your operator experience and the overall quality systems, but using the facilities at full capacity and releasing products rapidly – we are talking about probably releasing a product every 45 minutes to an hour– means that the cost of the investment, the equipment and the labour is reduced due to economies of scale.

Q Looking further ahead, where do you see the long-term future of commercial autologous T cell therapy production in terms of the full spectrum of centralized-de centralized strategic manufacturing models?

NK: I do personally feel that pursuing a semi- centralized manufacturing model is more favourable really, because this model will provide a more consistent approach to quality and economic benefits as well. This is especially true right now, when we (as a field) have neither sufficiently advanced processes nor adequate product understanding, but even looking to a future where those issues have been resolved, I think I would still favour control of manufacturing in a small number of locations rather than at the patient's bedside. But we shall see what the future brings!

Q In technological terms, where are the greatest current shortfalls, or missing pieces, in fully enabling optimal scale-out of these products, in your view?

Everyone acknowledges that our industry is still quite immature and many people have said it's a similar scenario to the monoclonal antibodies space a few decades ago. Although we have seen amazing efficacy in the clinic, and marketed products are now doing great things for patients, the

"Everyone acknowledges that our industry is still quite immature and many people have said it's a similar scenario to the monoclonal antibodies space a few decades ago."

processes we have today have essentially come out of academic labs, and the supply chain that comes with them is not that developed, either. So the consumables and equipment suppliers are lagging behind: there's lots of single-use material, lots of bespoke, high-priced options that have been designed for research and not for routine, large-scale manufacture. I think as demand increases, the suppliers are going to help solve these problems.

The other shortfall is not having deep enough product understanding. What we and everyone else has been doing to counter this is to look to fully characterise our products in order to generate that deeper understanding, and to improve control strategies, so that we have the appropriate release analytics. In tandem - and due to the fact that we are working with one patient-one batch - high throughput analytics are also very important.

Away from the manufacturing system and the process technologies themselves, the system to deliver the cells to and from the manufacturing facility, and the capacity of the clinical centres to deal with the number of products that potentially need to be stored in liquid nitrogen, are also areas that are being addressed and so will mature over time.

Q Autolus was the first company to come on board with manufacturing at the new Cell & Gene Therapy Catapult facility in Stevenage, UK. How important is it to develop and maintain such public–private partnerships for GMP manufacturing?

NK: We feel we have benefited very greatly from collaborating with the Cell and Gene Therapy Catapult. It helped us as a company to build up future clinical trial capacity without having to invest a lot of capital upfront. Instead, we've been able to focus on getting our processes and technologies in place to generate clinical data as quickly as possible. And, at the same time, it's also giving us the opportunity to test our systems and design in a suitable environment before we finalise our own facilities' design.

Having a good quality agreement in place from the beginning, which very clearly states the responsibilities and accountabilities of both parties, is absolutely key for the success of collaborations like this one.

AFFILIATION

Dr Nina (Ekaterini) Kotsopoulou
Vice President, Process Development, Autolus Ltd, UK

DOI: 10.18609/cgti.2018.115