OCTOBER 2021 Volume 7, Issue 10

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:

New horizons for cell therapy: emerging platforms

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NEW HORIZONS FOR CELL THERAPY: EMERGING PLATFORMS

SPOTLIGHT

EDITORIAL

Engineering new killers: bringing NK cells to the battle against cancer

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Cell & Gene Therapy Insights 2021; 7(10), 1429–1434

DOI: 10.18609/cgti.2021.188

The ability to re-direct the intrinsic power of the immune system toward cancer has been translated into unprecedented outcomes in the treatment of patients with otherwise incurable diseases. Whether through checkpoint inhibitors or cellular engineering, multiple promising approaches seek to modulate the immune response, enhance effector function, and tailor therapies to the needs of each patient.

In the cell therapy space, T cells engineered to express a tumor-recognizing chimeric antigen receptor (CAR) dominate the landscape with five FDA-approved products currently on the market [1–3]. Though remarkable progress has been seen with CAR-T cell therapy, its autologous nature limits patient accessibility, as many who are candidates for this potentially curative treatment do not possess the required number of T cells that

are sufficiently robust to undergo extensive manufacturing. Furthermore, the manufacturing process is costly, labor-intensive, and results in slow turnaround time [4,5]. In an attempt to address these limitations and increase the breadth of cell therapy application, the field has turned to allogeneic platforms, with the goal of generating products that are available to patients upon need, in an off-the-shelf manner [5].

T cells, however, are not ideal sources for allogeneic therapies due to their HLA-dependent recognition of antigens on target cells and, consequently, their potential for inducing graft-versus-host disease (GvHD). Gene editing of T cells provides an opportunity to reduce the risks associated with T-cell alloreactivity. Strategies focusing on eliminating expression of the endogenous αβ T-cell receptor (TCR) have shown potential for rendering these immune effector cells a viable choice for allogeneic cell therapy [6,7]. Recently, two clinical studies employing an engineered allogeneic T-cell product expressing a CAR targeting CD19 (namely, UCART19) showed the feasibility of using allogeneic gene-edited T cells to treat patients with relapsed, refractory $CD19^+$ leukemia [8].

Natural Killer (NK) cells, similar to T cells, are powerful immune effector cells that possess intrinsic anti-tumor properties and are capable of generating strong cytotoxic response upon engaging tumor cells. Unlike T cells, which to become activated require antigen recognition by the TCR in the context of HLA presentation, NK cell activation depends on the balance between activating and inhibitory signals received upon engaging a target. Furthermore, NK cells recognize their targets in an HLA-independent manner, thus presenting very low risk of inducing GvHD [9]. NK cells can also be activated and expanded *ex vivo* to yield large numbers of cells for downstream applications [10–12]. Given these favorable characteristics, NK cells have drawn great interest as a suitable allogeneic source for off-the-shelf cellular immunotherapy [13].

Many sources of allogeneic NK cells are available, including: NK-92 cell line, peripheral blood mononuclear cells (PBMC), hematopoietic stem cells (HSCs), induced pluripotent stem cells (iPSCs), and umbilical cord blood (CB) [14–25]. Though each source has its advantages and limitations, overall benefits of employing allogeneic NK cells include reduced manufacturing time, increased feasibility of large-scale production, reduced cost, and, most importantly, broad patient applicability [13,26]. Furthermore, allogeneic platforms allow for generation of mature NK cells that respond well to in vitro expansion under defined culture conditions [10–12] and yield large numbers of cells that can then be characterized prior to clinical use.

NK cells can also be genetically engineered to express CAR, and to modulate expression of proteins associated with potency and persistence [21,23,27,28]. Moreover, therapeutic NK cells have been shown to display a synergistic effect when delivered in combination with checkpoint inhibitor therapy, inducing T-cell activation and recruitment in a cooperative manner [24]. In recent years, work by several groups has led to promising strategies for transforming NK cells into cancer treatments. Engineered iPSC-derived NK cell products show high cytotoxicity against solid and hematological tumors and have transitioned into clinical trials where preliminary data show encouraging safety and efficacy profiles [27]. Umbilical cord blood NK cells, likewise, are a great resource for cell therapy. CB-NK cells can be readily obtained, easily purified, and subsequently activated and expanded *in vitro*. Moreover, activated CB-NK cells can be genetically modified to enhance anti-tumor function [21,23,25,28]. Our group has shown that CB-derived NK cells co-expressing a CD19-targeting CAR and interleukin 15 (IL-15) exhibited significant antitumor cytotoxicity in a pre-clinical Raji lymphoma model [21]. When taken into clinical trials, these CAR-NK cells efficiently targeted $CD19^+$ tumor cells leading to an objective response rate of 73% in patients with relapsed and refractory disease [28]. Notably, there was no evidence of GvHD or toxicities,

DOI: 10.18609/cgti.2021.188

and CAR-NK cells were still detectable in patients a year after treatment [28].

NK-92 cells are an NK cell line that has shown potential as a therapeutic agent against cancer. They are likely the most highly scalable starting material for NK-based therapies, and can be easily genetically modified to eliminate inhibitory molecules and to express receptors that recognize tumor antigens, thereby increasing their tumor-targeting efficiency [16–18]. A major limitation, however, is that due to NK-92 cells being an immortalized cell line, they must be inactivated prior to infusion to avoid occurrence of secondary tumors. There are several clinical trials evaluating the efficacy of NK-92 cells against different cancers, and emerging data from these studies will aid in evaluating the overall therapeutic potential of NK-92 cells (clinicaltrials.gov) [17, 29].

Though the landscape of NK cell applications in cancer therapy looks promising, there are challenges which must be considered. Several checkpoint molecules and inhibitory ligands have been found to induce NK cell dysfunction. Examples of these include LAG-3, TIGIT, CISH, TGF-beta, B7-H3, and Siglec-7, among others [30]. Many ongoing clinical trials are evaluating the benefits of incorporating strategies to block these inhibitory signals [31]. Delconte *et al.* identified cytokine-inducible SH2-containing protein (CIS) as a negative regulator of NK cell function through IL-15 signaling. By ablating *CISH*, the gene encoding CIS, they observed increased NK cell anti-tumor function *in vivo* [32]. Others have also shown that CISH deletion using CRISPR-Cas9 technology enhances CAR-NK cell effector function and significantly improves anti-tumor response [33,34].

The effect of metabolic immunosuppression on NK activity has also been evaluated. As shown in work by Woan *et al.* [35], removal of CD38 along with expression of IL-15/IL-15 receptor fusion led to a unique metabolic profile resembling that of adaptive NK cells which appear to have enhanced features that make them attractive for immunotherapy [36,37].

Obstacles to NK cell function also emerge from cell-to-cell interactions. This is especially critical in the solid tumor setting where the tumor microenvironment (TME) consists of various immunosuppressive factors and cell subsets [38]. TGF-β is a widely known cytokine that is secreted by tumor cells and impairs NK cell function in the TME. In recent work, we demonstrated that knocking out the TGF-β receptor, TGFBR2, on NK cells prevented dysfunction and prolonged the anti-tumor response in a glioblastoma model [39].

Additional efforts focus on addressing potency and persistence *in vivo* by engineering NK cells that can provide signals to sustain their own proliferation and to promote strong cytotoxic effect. Our group has demonstrated that CAR-NK cells exhibit longer and more robust function when engineered to produce and secrete IL-15 constitutively [21,28]. Others have shown that endowing NK cells with enhanced receptors boosts their function, as demonstrated by Fate Therapeutics' Phase 1 clinical trial of their leading iPSC-derived NK cell products – FT516 and FT596 – designed for treatment of relapsed/refractory B-cell lymphoma [27]. FT516 is engineered to express a non-cleavable CD16 Fc receptor to enhance NK-driven antibody-depended cell-mediated cytotoxicity (ADCC), whereas FT596 is engineered to co-express CD19 CAR, the non-cleavable CD16 Fc receptor, and an IL-15 receptor fusion to enable persistence [27]. Initial data show encouraging results, with several patients achieving an objective response.

Recently, NK cell engagers have garnered attention as another viable approach to NK-based therapies. These reagents are designed to facilitate NK cell detection of tumor targets and to concomitantly drive activation of anti-tumor response [40]. A typical bi-specific killer cell engager (BiKE) is designed to contain two scFvs linked together – one specific for a molecule on the NK cell (e.g. CD16, NKG2D, NKp30, NKp46 etc.), and the other targeting an antigen on the tumor cell (such as CD30, CD33, EGFR, BCMA,

HER2, CD38 etc.) [41]. Novel designs adding more scFvs (such as TriKEs and TetraKEs) can potentiate the immune response by targeting more antigens or by using cytokines as crosslinking moieties [42–47]. NK cell engagers are capable of eliciting CAR-like responses in patients, as was recently observed in an ongoing trial at MD Anderson Cancer Center. In this study, patients with $CD30^+$ hematological malignancies were treated with non-engineered NK cells pre-complexed with AFM13 (a bispecific engager developed by Affimed and designed to bind CD16a on NK cells and CD30 on tumor cells) and achieved desirable responses [48].

The landscape continues to evolve as many innovative NK-based approaches emerge in academic centers and industry around the world, promising to deliver the next-generation of specialized cell therapies [2,49]. With many developments underway and encouraging initial clinical data, NK cells have risen as a powerful line of defense against cancer, and have earned a place in the immuno-oncology armamentarium. Furthermore, the compatibility with allogeneic platforms makes NK cells an attractive option for expanding the reach of innovative cell therapies to a broad patient population. We look forward to a promising future as novel strategies provide answers to the unique challenges of each cancer, and discoveries transition from the laboratory into the clinic where they can meaningfully impact the lives of patients.

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DOI: 10.18609/cgti.2021.188

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *MD Anderson has licensing agreement with Takeda for CD19 CAR NK program. Dr Acharya is an Affimed stock holder.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 22 2021;* ***Revised manuscript received:*** *Oct 22 2021;* ***Publication date:*** *Nov 16 2021.*

DOI: 10.18609/cgti.2021.188

CELL & GENE THERAPY INSIGHTS

EXPERT ROUNDTABLE

Accelerating time to market for CAR-T cell therapies through translatable workflow

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Dr Rivière joined the faculty of the Memorial Sloan-Kettering Cancer Center (MSKCC) in 1999. She is currently the Director of the Cell Therapy and Cell Engineering Facility where she investigates novel strategies for cell therapies and immunotherapies to increase or retarget the immune response against tumors and treat hematological disorders. Her laboratory has developed multiple processes for clinical-grade gamma-retroviral and lentiviral vector production as well as hematopoietic and T-cell manufacturing.

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Cell & Gene Therapy Insights 2021; 7(10), 1379–1206

DOI: 10.18609/cgti.2021.182

Q What are the key considerations when advancing CAR/TCR therapies to the clinic and commercialization?

TH: When looking to advance manufacturing processes to clinic there are several aspects to consider, and frankly all are important. These start with choosing the right T-cell expansion platform and associated consumables – for example, cell culture media and reagents.

These considerations should happen during process development – for example, when demonstrating consistency in performance while using an automated closed system – or even before it. When selecting your consumables such as the cell culture media, it is critical to ensure it is manufactured by the vendor in alignment with cGMP and that it has all the supporting documentation. It also helps if it has a proven record of use in some commercialized therapies. One has to manage the risks and then validate these consumables pretty early on in development, so that as you scale-up you don't encounter any issues.

It's important to understand the critical quality attributes that you are driving for, and then qualify and validate your process to achieve these critical quality attributes – developing a strategy for cell and gene therapy products as part of process development is crucial. It's also important to understand how you can actually achieve these critical quality attributes, and also (if you do this work prior to filing for an IND) whether it will result in significant benefits when the process and product needs to be transitioned to clinical manufacturing.

All of these aspects, if considered early on, will definitely assist in accelerating the process to clinic and commercialization.

IR: I completely agree. Even in the early stages in academia, when we initiate Phase 1 clinical trials, it is really important to select adequate raw materials and biologics with the right specifications that mean we can avoid having to make changes in critical reagents down the line. We try to be very aware of those reagent specifications so that hopefully, the same manufacturing components can be kept through late-phase clinical and commercialization.

Obviously, because we cannot necessarily automate the process from the early stages and the automation platforms are still quite sparse, there might be changes in the manufacturing process itself at a later stage. But we can at least look to control the raw materials and by doing so, avoid changes that may be really important for the biological properties of the cells that are being manufactured.

MK: I agree with both Tariq and Isabelle and want to add that, coming from a company that develops CAR T cells engineered with mRNA for both oncology and non-oncology indications, product development such as this inevitably involves a lot of unknowns. These unknowns start from what is the real dose with which you may see efficacy with a CAR T cell that is engineered in mRNA. In this kind of example, with many unknowns, one of the key things you want to have in the early stages is flexibility.

Processes like a virally transduced CAR T cell where you are trying to change the intracellular signaling domain may have important clinical outcomes, but at least the manufacturing is very well established, for the most part. This means you can plan the development of this kind of a product with very late-stage amendments included early on – and you should do so, because otherwise you will find yourself in trouble. But in our case, where you do need the clinical data in order to be able to continue optimizing the product, you also need to build in that flexibility.

DOI: 10.18609/cgti.2021.182

I would add that in times like these with the COVID-19 pandemic, when materials become an incredibly challenging thing to acquire, again, you need to retain some flexibility in the materials you use, such that you can switch to one or another if needed. Hopefully, such changes are not going to be frequent, but you have to be ready.

Q What are the major pain-points of CAR-T process that need further development, and why?

MK: That is a broad question because one needs to look at it firstly in the aspect of autologous versus allogeneic – and the pain points could be different between these two processes. For example, at the high level, there are the logistical challenges in producing a patient-specific lot that need to be figured out, including cell shipments and so forth. With an allogeneic product, the amounts of the cell product you manufacture are greater, so cost and materials acquisition become a lot more important. We can then add another layer for autologous therapies in that the pain points for a virally produced CAR T versus a non-viral CAR T are also different. This relates to the main nucleic acid material that you use, as well as the fact that viral CAR Ts have much more established processes than non-viral CAR Ts, as we discussed earlier. Again, there's definitely more process development challenges with non-viral processes.

For all programs, though, it is really important that the manufacturing development is hand-in-hand with the clinical development of the product. For example, with typical drugs, as you go into late-stage trials you know almost for certain that the costs of manufacturing will reduce, because you are working with known materials and there are no equipment changes. But in the CAR-T cell therapy field, there are many updates happening every year with the cell manufacturing platform, from equipment to materials, so that in the later stage trials, you suddenly end up with higher costs. That is a really unique situation to be in.

IR: From the academic point of view, and speaking specifically about autologous products, one of the issues is the lack of scalability of the equipment that we are using to manufacture the CAR-T cells, and the inability to do Design of Experiments where we can test multivariant parameters in order to determine what is the most efficient process. It is very difficult to do this with manufacturing platforms where there is not much scalability. I think we would really benefit from improvements in this area. Some of the new automated manufacturing platforms are taking this into account now, meaning we can better develop and establish these manufacturing processes with Design of Experiments in mind.

In terms of genetic modifiers, in the viral vector field there are issues with production yields and downstream purification – there is still much work to perform there. And the costs of these vectors and purification platforms are still very high. Those are some of the pain points we are facing in the autologous setting right now.

TH: I agree with the points that Metin and Isabelle brought up.

I think Cost of Goods, both for systems and the consumables, is certainly a concern. Especially the cost of viral vectors, for example. The expense and time it takes to get GMP lentiviral vector is a critical pain point in the industry today. One solution to address this pain point is to utilize non-viral transfection methods to modify T cells.

The vendors do recognize that and they are working to provide systems and consumables that meet the demands of the processes, and match the quality that the manufacturers desire.

As Isabelle pointed out, scalability is a particular issue. One needs to create processes that are scalable and that generate a product quickly while maintaining quality. This is regardless of whether the therapy is autologous or allogeneic – these challenges are tied to both areas. But especially for future allogeneic therapies, achieving scalability will require that as the therapy is developed, it is possible to provide cell products that maintain the quality attributes the manufacturer is looking for.

IR: I think that we are starting to see some protocols that are decreasing the dose by basically optimizing the signaling domains – specifically in the field of CAR, we now have better signaling domains that promote less exhaustion and better factor memory phenotypes.

One of the critical aspects that would really improve the manufacturing process will be to be able to plug in the inline monitoring and real-time monitoring of the parameters of the cultures. We could then see in real time if the cultures are behaving in an expected manner and if the specifications during the process are being met. It will also be key to incorporate more performance analytics in QC to decrease the time of the testing, so that we can release the product as quickly as safety permits.

TH: Another thing I want to point out is the variability that is inherent in the starting material. That's just the nature of the cells being used. It's important to understand the difference between healthy donors and cancer patients, and how they can affect *ex vivo* expansion of T cells.

Cell product characterization requirements – specifically, around identity and purity – are key in meeting regulatory expectations for the assessment of process and product stability, reproducibility, and compatibility.

Q What are the advantages and challenges of closed system transduction/transfection platforms?

IR: Obviously, the goal is to completely close the manufacturing system. But these platforms need to remain flexible and the parameters have to be adjustable – for example, in terms of volume or time of incubation – as well as being modellable, because not everyone will necessarily want to use the same sequence of operations. I therefore think it's really important to have operation units for selection of cell subsets, or transduction, or gene editing, or electroporation, or expansion, etc. that are kept independent, but in a way where they can be integrated so that they can constitute a closed system.

Again, we also need to have scalability in these closed systems so that we can design experiments to test multivariant and multiparameter at the same time as the process development, and be able to translate that into full-scale.

MK: In our company, we currently focus as much on allogeneic products as we do on autologous, and for allogeneic, the cell number is always the most important part. Whether you transfect or transduce, in the end, you need very large number of the cells that you want to express your gene of interest.

This has its own unique challenges when it comes to closed systems because a lot of the current systems are still very early on in their development, meaning they can only handle a small number of cells. These systems

DOI: 10.18609/cgti.2021.182

are generally more geared towards autologous products, which have been around longer – allogeneic development is only just being done now, so the technology is trying to catch up. Additionally, just having a large number of cells, whether *ex vivo* or *in vivo*, is in general hard to deal with. It is simply not going to be a trivial equation to solve when you want to do large-scale allogeneic cell transfections.

Again, for our company, this fact becomes incredibly important because we use mRNA, which requires the transfection, freeze, thaw, etc. to be very close together (as opposed to viral transduction, where you typically have some time to allow the cells to recover before you take them on to further stresses at the freeze and thaw stages). And the closed system technologies for this are simply not there yet. They are just being developed now, though, so hopefully in the next few years we will be seeing these kinds of processes where large-scale transfections using mRNA or other approaches will be closed and efficient all the way through freeze and thaw.

> "...hopefully in the next few years we will be seeing these kinds of processes where large-scale transfections using mRNA or other approaches will be closed and efficient all the way through freeze and thaw."
>
> - **Metin Kurtoglu**

TH: Isabelle mentioned the goal of closed manufacturing systems. At Lonza, we recognize this need and have optimized the Cocoon platform, which enables automation or cell isolation, activation, transduction, expansion cell washing, and harvest whilst providing real-time process parameter monitoring throughout the process. That's because we believe it's really important to have all these parameters monitored as well.

The Cocoon platform has integrated multiple unit operations and provides a closed system, and we are implementing the improved electroporation technology (Nucleofector) that was originally introduced to the market in 2001. This technology enables non-viral gene delivery for cell immunotherapy manufacturing in a functionally closed, automated workflow. The 4D-Nucleofector LV unit, and the related consumables, support the use of the system in a GMP environment.

IR: Yes, and I think with these closed, automated systems, the same themes come back: being able to integrate online and inline monitoring so that we are able to monitor in real time and also provide feedback to these cultures if any of the parameters that are being monitored need to be adjusted, so that we can keep the cells in the best possible biological state according to predefined specifications.

Q At what therapy developmental time point do you recommend a). planning scale-up for late-phase trials and commercial stage, and b). selecting and adopting a manufacturing platform for commercialization – and why?

TH: As soon as possible!

As I mentioned previously, developing strategies for cell and gene therapy products as part of process development prior to scale-up is very important, because it will result in significant benefits when you need to take the process and the product into clinical manufacturing.

"...plan ahead and during the process development stage at least, choose and qualify the closed system that you want to use."

- Tariq Haq

So it's important to consider automated tool systems early on, even before the preclinical stage. As you qualify and validate the process, you want to make sure that there are no significant process changes later on – if that does occur and you're trying to change a process later on, then the onus will be on you to perform comparability studies. That will happen on occasion, of course – it does happen that you have to change the process because of some unknown challenges you face down the line – and at Lonza, we have executed comparability studies on several occasions that were successful. But it's certainly not a trivial undertaking in terms of time, cost, and risk. So plan ahead and during the process development stage at least, choose and qualify the closed system that you want to use.

MK: I would repeat the theme of having flexibility, especially in that 'grey zone' between early and late process development, but also being smart enough so that you don't repeat too many things as you change the process.

One piece of good news is that the market has responded to the upcoming explosion we are going to see in cell and gene therapies due to the fact they are very powerful products. And it has responded such that we have seen a lot of interesting automated systems coming online, especially in the past 2 years. When you are in very early-stage development, I would suggest that you spend quite a bit of time really investigating these automated systems right from the get-go. Sometimes it could seem to be a hassle to look at these larger – and especially, automated – systems, but it is worth taking the trouble to cross-compare them because there are some very interesting solutions out there right now. They can make addressing many of the challenges we have discussed a lot more feasible.

IR: Yes, I agree. As you say, I think that thinking about it even in the preclinical setting is really important. That brings me to an additional point, which is that we also know that these therapies have been approved, at least in the past, in the very early stages of development – as early as Phase 2, which is very unusual and is not the regular path for approving other biologics. I think this emphasizes the need to consider automation very early on – potentially, even when these trials are still in the academic setting. And that prompts me to say that it's really important for the technology developers who are developing these automated systems to also consider that there should be benchtop units available that can potentially be implemented at the academic centers - not fully automated apparatus, but a platform that is similar, which could be used to demonstrate proof of principle and provide an easy transition to the later development and commercialization stages.

Q Manufacturing is also about raw materials and GMP ancillary choices, of course. How urgent is it to move to a GMP chemically defined medium for clinical applications, assuming equal performance, and why?

MK: Using chemically defined media makes the process a lot easier in many aspects. For one thing, any kind of human product that goes into the manufacturing

DOI: 10.18609/cgti.2021.182

process involves the asking of regulatory questions, relating to everything from material acquisition to its safety, etc.

Having said that, I think the field has become highly experienced in using these human products in manufacturing. And I also think there are still a lot of unknown elements in the human serum or plasma that are helping these cells in interesting ways.

In summary, I don't think anyone should be scared to move forward, even to the later development stages, using defined media that are supplemented by human products. But of course, if you can get rid of them, it does make the process a little easier.

TH: I think it goes back to the discussion we were having about planning, selecting, and adopting a manufacturing platform for commercialization – that should occur right at the beginning, and the same goes for cell culture medium. Making the right choice of a medium that will support cell growth whilst maintaining functionality is obviously very important.

There has been substantial focus on developing chemically defined media that will provide the same performance as serum-containing media or xeno-free media. So chemically defined media is definitely the future, and the more so because it does not contain animal origin components such as the fetal bovine serum (FBS).

FBS is always in short supply, and there are challenges with it being consistent, and also in terms of tracing its specific origin. And when you really look at it, the way these therapies are increasing, the amount of fetal bovine serum that will be needed is just enormous.

Along with answering this supply challenge of FBS, we are also looking to use a formulation that will meet the regulatory requirements, so it is key to perform proper risk assessment of whatever culture medium you are trying to use, ensuring that it provides robust and consistent performance. Ultimately, that's what is important. And one should evaluate multiple lots of media from the same supplier during process development to ensure it provides the consistency that you're looking for.

IR: I think that is a really important point! I've seen projections that if cell therapies are successful in solid tumor indications, and potentially autoimmune diseases and other indications, there will be a worldwide shortage of these reagents – in particular, FBS, but we will also need more human AB pooled subjects. And potentially, these sources can also be affected by things like pandemics – we have seen that in the past with HIV and potentially with COVID now, although it may be less acute with COVID. So I also think it's really important that we reach the goal of chemically defined components over time.

Q Given the complexities of finding manufacturing slots for viral manufacturing and the costs associated with it, when do you think the field will move solely to non-viral gene delivery methods? Or if you don't think they will, please explain why.

IR: That's the million-dollar question – literally!

I do think that some of the non-viral gene editing methods could be very promising and right now, looking at the CRISPR Cas9 and guide RNA, people are getting extremely excited. These are really highly specific and there are tools to predict the off-target effects, so therefore you can study your off-targets and determine if they pose a safety problem. And they also provide the ability to multiplex and have multiple targets.

However, I think the issue is with some of the reagents that you need to use – for example, if you want to not only knock-out but to also knock-in, then you need to use either an AAV6 technology or another plasmid DNA-based technology – and they are uniformly either very expensive or very inefficient. We don't seem to have found the ideal combination of reasonable price and acceptable efficiency yet. We are still dealing with this conundrum. We know that plasmid double-strand DNA are quite toxic when they are delivered into the cells. And while single-strand DNA might be better in terms of safety, they are still quite inefficient and manufacturing is complex because of their structure.

So unfortunately, we don't have a perfect solution in the field as yet to get rid of the viral vectors that are close to my heart, and which I have studied for many years. I do think they might still be here for some time.

Obviously, I think that DNA (both single-strand and double-strand) and potentially other means of delivery such as lipid nanoparticles will play a role, and will improve over time. But in the meantime, I think there is still room to work on developing much more efficient processes for viral production. We know that currently, post-purification lentiviral vectors or AAV6 yields are somewhere in the order of 20–30%, which is really low and makes these goods pretty expensive.

At the end of the day, we all want new technologies that will deliver better performance and in terms of manufacturing, reduced cost.

TH: The potential for AAV vectors in tandem with the CRISPR Cas system seems limitless, but there are safety and efficacy concerns based on the possibility of the AAV vector genome carrying the CRISPR components integrating into the host-cell genome at the site of a double-strand break.

I believe that viral transduction technologies will persist in the industry regardless of the cost, the long lead times, and other bottlenecks in their production. However, at the same time, I think that other technologies such as electroporation can also enable CRISPR Cas9 gene editing to further modify or improve T cell products. Today's electroporation platforms are optimized to provide high cell viability and still achieve efficient gene transfer. As I mentioned previously, the Nucleofector technology provides an alternative, non-viral gene delivery system, which removes the need for costly, time-consuming viral vector preparations.

MK: I think we need to emphasize that the cell and gene therapy is still in its infancy, if that – perhaps still in its embryonic state right now. Yes, there is considerable experience with viral transduced products such that there are FDA-approved products currently in the market and producing amazing results so far, albeit it in a very small and geographically limited patient population.

As these products continue to grow, viral production and its challenges will need to be resolved, absolutely – that will be important for many products that will be coming even after those that are now on the market. But the field is still just trying to understand the biology of what cell and gene therapy can do, and that involves not only CRISPR Cas and transposons and so forth, but RNA-based technologies, too. We also need to understand that in terms of indications, most of these approaches have only been tried in late-stage cancer to date. There is a lot of preclinical work demonstrating how they can work outside oncology, but it's very early days in that regard. In conclusion, everyone should still be greatly encouraged to continue developing a wide range of both viral and non-viral approaches.

DOI: 10.18609/cgti.2021.182

Q What are the attributes and regulatory requirements for Laboratory Information Management System (LIMS) manufacturing?

MK: This is another area, like the materials we discussed earlier, that is heavily regulated – acquiring the data, whether it is from manufacturing or relating to the labeling of autologous products, involves strict regulatory compliance. And again, we are in the early stages, so there are a lot of things that you need to figure out and there is a balance to find.

When a product is in its developmental stage, there is so much documentation that you are trying to cross-compare. This is where, for example, retaining a degree of flexibility with your release criteria is important because you are still trying to understand your quality measures, your sampling processes, and so on. And all these data will need to be acquired in detail when you scale-up, so you should of course start planning early on.

Again, the sector has responded pretty well in the past few years. We have a lot of automated systems now, all the way from individual patient cell product labeling that can be traced all around the world, to LIMS solutions for acquiring and integrating data from your manufacturing and quality processes without requiring too much manual labor to put them all into a 21 CFR Part 11-compliant system. Good solutions have come up which give you that flexibility you need in the beginning around how you acquire the data, and you can now also integrate automated solutions very early on.

TH: Autologous cell therapy is unlike any other therapeutic technology in terms of how complex it is. For example, the need to compile data for each batch, the traceability data required, the process data, maintenance all the way through to the point of care, the information from the manufacturer, the physician, the technician doing the QCs, etc. etc. All of these things create a lot of complexity. And a lot of vein-to-vein tracking data is needed because there are also lots of touch points.

As Metin pointed out, there are several solutions out there right now that are tackling pieces of this. But I think over time, you are going to see even more need for end-to-end digital solutions that can combine all the different elements – all the different unit operations, data acquisition, device integration, and advanced analytics. All are needed to deliver efficiencies in the cell therapy field.

IR: I agree with the requirement to integrate those vein-to-vein tracking data, and it is key to take the hospital systems into consideration. Some of these systems are really difficult to access by virtue of the fact that all the information is hyper-protected, and hospitals need to provide solutions that can incorporate these parameters into their own databases and LIMS. There is some work being done on this front and I think it will be important to continue to provide that very complex chain of custody and tracking to continue the proper identification of the products.

> "...it will be important to continue to provide that very complex chain of custody and tracking to continue the proper identification of the products."
>
> - Isabelle Rivière

Q The number of genome editing trials using CRISPR/CAS based systems is growing at the moment. Where do you see the advantages/disadvantages compared to transposon-based systems, for example, and what kind of improvement do you expect coming for CRIPSR/CAS systems?

IR: I touched on this a little bit earlier and I mentioned the high efficacy of the CRISPR Cas9 system in terms of enabling the knock-out or deletion of certain undesired genes, or repairing genes. in other diseases, I think they are really easy to design for any genomic target, and they probably provide a simpler approach in terms of targeting than some other solutions out there. We have also seen that some of the CRISPR Cas9 components can be modified so that they actually provide a higher fidelity in terms of cutting points and less off-target effects. So there are definitely improvements that have been seen already with these modified Cas9s.

I think the transposons – Sleeping Beauty, for example – can definitely be useful, but they are less efficient, so they provide less targeting. I think there is also some progress being made along these lines, but we have not seen yet the efficacy reaching that of Cas9.

In terms of the CRISPR technology, what will really need to do be assessed over time will be the off-target effects, and our ability to detect them and their potential impacts with computational biology. I think the prediction of those off-targets is quite good currently, but not perfect. There remains actual benchwork to do using next-generation sequencing technology to determine these off-targets.

Time will tell if genome editing platforms are as safe as, or safer than, our non-targeted lentiviral or retroviral vectors, depending on the sites that are being targeted and how many off-targets there are for each of these sites. However, the key advantage for me is that you can really multiplex, so you can have products made in a single step with these multiplexing approaches.

Q Finally, can you each give us your thoughts on the future of CAR therapies?

TH: The future is great. This is just the beginning, and it's gratifying to see successful work in this new field in the form of the first commercially available therapies.

I believe that CAR-T holds great promise for revolutionizing cancer treatment. It does deliver long-term value. The autologous approach has been successful and as challenges to the allogeneic approach are solved, these therapies are going to expand even further, and we will see more successful approvals.

To aid the success of a new generation of therapeutic products, we are seeing a lot of new technologies coming through and now being implemented. Together with existing innovations, these will help make CAR even more successful – implementable, as well as accessible and consistent. So I strongly believe that CAR is here to stay.

IR: As we gain more knowledge from the ongoing clinical trials, hopefully we will also gain some more insight into the biology of those cells that are really the active therapeutic ingredients within the mixture of cells.

DOI: 10.18609/cgti.2021.182

I think we have already made some progress in terms of defining some of the parameters that make these cells more persistent and more active with reduced side effects. We have seen cell doses from the hundreds of millions going down to around 10–20 million cells infused, but still providing a therapeutic effect. So, I think the biological understanding will continue to improve and that will lead to smaller and smaller doses, which will in turn reduce manufacturing cost of goods, allowing us to really attack the field of solid tumors.

On that note, one of the challenges we are facing is all the way back upstream with the targets, because there are a limited number of targets that are specific to solid tumors. But there are companies out there that are working on identifying some new and hopefully specific tumor targets, which will help us overcome the challenges presented by the immunosuppressive tumor microenvironment.

And hopefully, we will all move to allogeneic cell therapies at some stage – I think the investors are already there, really believing that we should be making faster progress! We are moving towards these allogeneic platforms, but there are still some hurdles to overcome – specifically, in terms of preventing the early rejection of these products, as well as avoiding these products attacking the recipient. Promoting these two properties is a delicate balance to find.

There is also a growing interest in developing approaches for *in vivo* gene delivery. That is an area where we are seeing more activity, delivering the genetic cargo through lipid nanoparticles or by other means. It remains to be seen if they can achieve the necessary degree of specificity and efficacy, but one would think that if we do succeed in delivering them on-target, they would represent the most economical approach of all.

MK: As we have discussed, the CAR-Ts are doing amazing stuff, but they are not too safe, meaning they still need to be administered in a very carefully monitored setting. As the field progresses, doses are being reduced, and the safety component is improving, but nevertheless, there is a cost associated not only with the product itself, but also with the monitoring associated with the product's safety. That needs to change and it is being changed – I do think that in near future, we will hopefully be able to see the provision of CAR T cell therapies in the outpatient setting, which would substantially help with the cost of this treatment.

We've talked a lot about tumors, but engineered cells are very powerful products that can basically eliminate their target. And eliminating the target in a non-oncology indication – for example, in an autoimmune disease, removing a B cell or a plasma cell – could be incredibly important and may provide very good clinical benefit. Of course, that relies on the product being both incredibly safe and cost-effective. But I'm hoping that in near future, we will be seeing CAR-T cells, and cell and gene therapy in general, providing a lot of products for non-oncology indications. We are already seeing a lot of promise on that side – for example, in COVID-related ARDS with mesenchymal stem cell-based products.

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Rivière has received grants from Juno/BMS, Takeda, Atara and Fate. She has received consulting fees from Fate and Mnemo Therapeutics; is on the board for SAB, Flodesign/Millipore, Akron and C3; and is a stock holder in Mnemo Therapeutics.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *This article is based on a recorded roundtable, which can be found here.*

Roundtable recorded: *Sep 14 2021;* ***Publication date:*** *Nov 22 2021.*

DOI: 10.18609/cgti.2021.182

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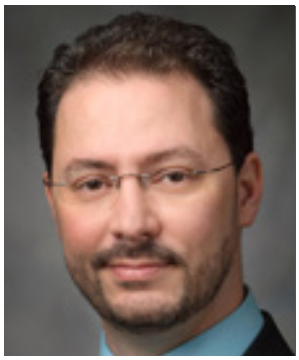
SPOTLIGHT

TECHNICAL UPDATE: EDITORIAL

A model for where we live



"As we start understanding how the tumor immune microenvironment functions in the primary tumors, we can extrapolate that information to these metastatic tumors as they likely have the same linchpin."

JARED K BURKS, Professor, Co-Director, Flow Cytometry & Cell Imaging Core Facility, The University of Texas MD Anderson Cancer Center

Cell & Gene Therapy Insights 2021; 7(10), 1513–1519

DOI: 10.18609/cgti.2021.186

PROMISE

With the advent of many burgeoning imaging technologies (Hyperion Imaging Mass Cytometry, CODEX®, CyCIF, etc.) allowing researchers to visualize classical pathology tissues with higher number of antibodies targeted to structural, phenotypic, and functional protein markers we are gaining an understanding of the cellular architecture of healthy tissue and the ravages that disease takes to remodeling said tissue. With these technologies we can understand how different cells types interact and how differently that appears from a diseased tissue. How that is performed and how it is different from what we have been doing is all in the details, but it boils down to the same issues we face when we are determining where we want to live, but I digress. Let's start with where we have been.

HISTORY

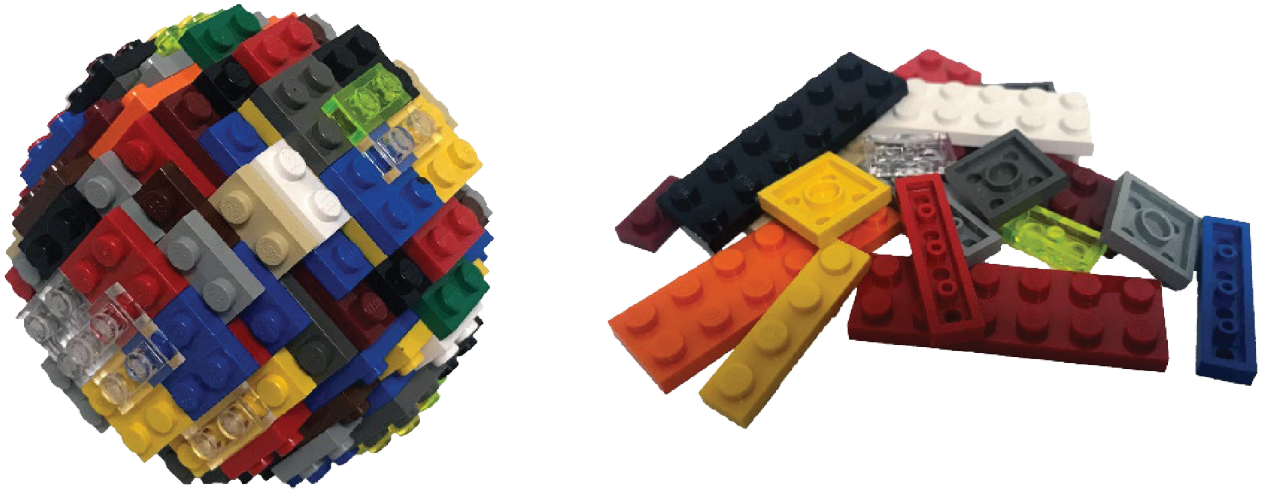
Immunology has for years been developing a very dynamic technology that came to the fore front with the HIV/AIDS epidemic in the 1980s. Flow Cytometry was able to quickly show a decrease in the $CD4^+$ T-cell population, which was of specific benefit in the diagnosis of HIV/AIDS. This example showcases the power of flow cytometry, but also the limitation. Flow cytometry assays require cells to be in suspension, which is fine for a bloodborne illness, but complicated by solid tumors. For solid tissue and tumors, we must disaggregate the tissues we study to employ flow cytometry. In order to perform this disaggregation, we mechanically, enzymatically, and/or chemically dissociate the cells from one another. In Figure 1, the sphere composed of LEGO elements has been dissociated from each other in the image on the right. This model demonstrates the ideal perfect dissociation; however, this is rarely the case when using tissue.

BIAS

The final step in these disaggregation/dissociation protocols is to filter the resultant cells to remove any cell clumps, where cells might not have completely separated into single cells. This action is taken for the benefit of the instrument as we don't want the flow cytometer to clog. Can you see where I am driving? We are performing a selection based on the method of dissociation used and following that with a filtration. For the dissociated cells we are subsequently performing a compositional study, merely counting them. While that has immense power to understand what is going on in a disease, as mentioned for the HIV/AIDS study above, it doesn't allow an understanding, at the single cell level, of how cells are either positively or negatively affected by the disease.

Why is a construction method important to these studies? In the example above, if given the LEGO elements and asked to reassemble the sphere, how many of us would be able to do so? This challenge is furthered if the colors must appear in exactly the same order as in the original model. Now, consider that certain LEGO parts, hopefully at random, are missing from the filtration. This model gets to the very nature of how flat elements interact to create a round object, but also the order in which they are colorimetrically organized speaks to the specific reason that certain functional markers may be up or down regulated. A construction method is needed to solve this puzzle as cells do not have a method of ranged cell killing and most cell signaling

FIGURE 1



DOI: 10.18609/cgti.2021.186

is also within cell-cell proximity. Cells lack cell phones, they need to be adjacent to their communication targets or the cell they intend to kill.

SPATIAL DATA

Why does spatial organization matter? How do we think about disease progression? And lastly, how do we consider these data? We need to understand the niche or neighborhood we want to image. Let's consider cancer more specifically. In Tammel *et al.* [1] and Lim *et al.* [2] they demonstrated when a cancer cell divides it can produce two different types of cells, a tumor cell and a supportive cell that helps to structure the tumor niche. This implies that the tumor cell recognizes the importance of the neighborhood and, for its benefit, restructures the neighborhood to meet its needs.

This understanding is furthered when one examines how specific immune cell – tumor cell interactions, and even the distances between cells, correlate with and/or predict which patients will survive their cancers for longer periods. This was found in Carstens *et al.* [3] and extended in Gartrell *et al.* [4] where the distance between the cytotoxic T-cell and the tumor cell was found to predict longer survival. Furthermore, these relationships go beyond T cells to include $CD68^{+}$ macrophages. Macrophages complicate these relationships, as these cells generally drive the cytotoxic T-cell-tumor cell interaction in a negative direction for the patients. One can hypothesize as to why the macrophages complicate this process. These sorts of relationships were placed in the spotlight clinically in Lu *et al.* [5] where the authors retrospectively looked at the diagnostic accuracy of anti-PD-1/PD-L1 therapy. They reviewed 8135 patients across 10 solid tumors comparing the diagnostic accuracy of using single color PD-L1 IHC, tumor mutational burden, gene expression profiling, individual and combined versus multiplex immunohistochemistry/IF. The only method that retains the spatial data and compares various cell types to tumor cells is the multiplexed IHC/IF assay and given the information above it was no surprise that this one method outperformed the other three and even when these three were combined for diagnostic accuracy. Spatial data reveals single cell biology not considered before because we were unable to perform these types of complex studies. Proximity speaks to cellular activity, which in turn has clinical diagnostic value.

Clearly cellular organization of these tissues affects clinical outcomes. Given this knowledge how do we consider these data as we revolutionize clinical pathology?

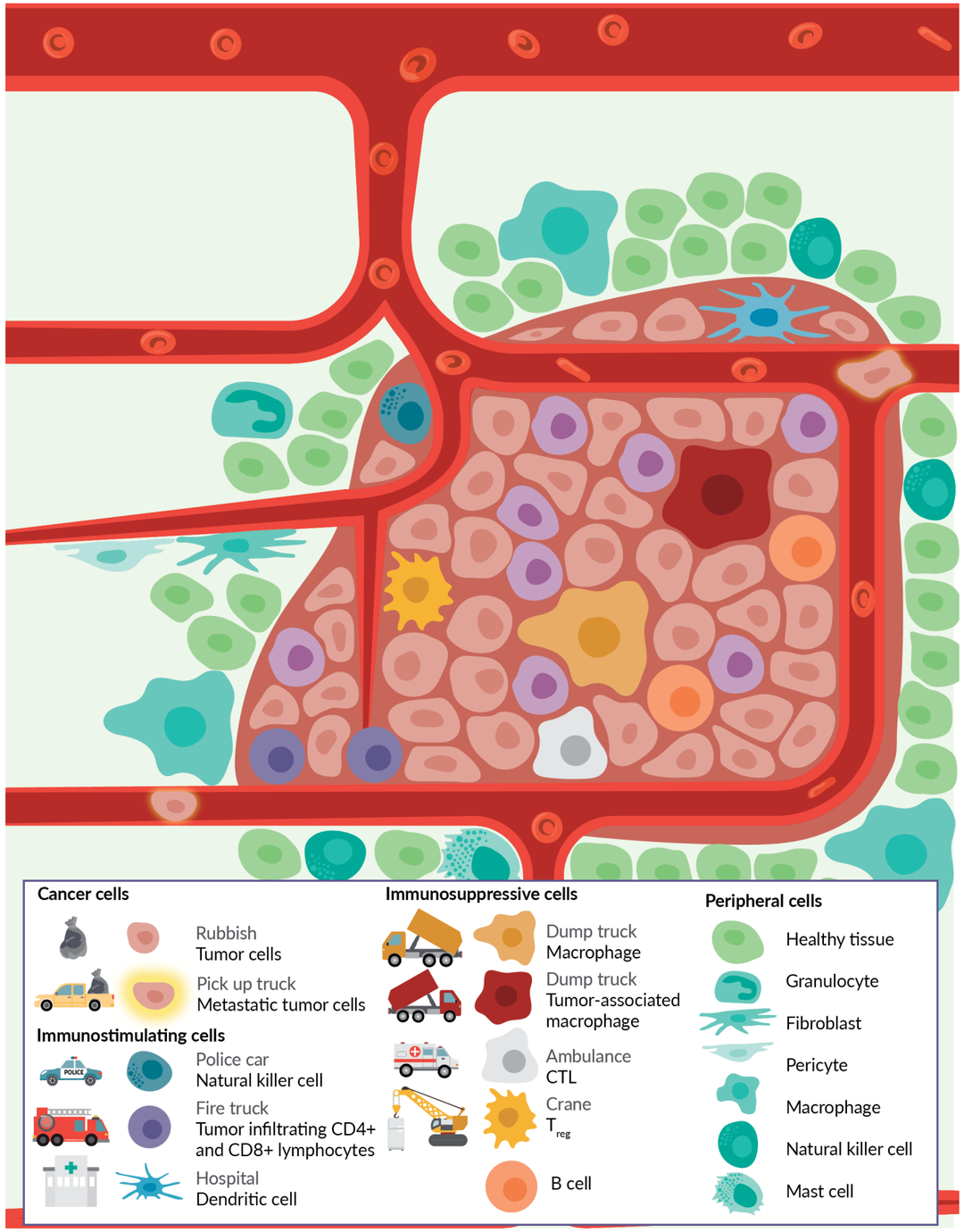
MODEL

Well, this is when we look at our own behaviors for a model system. How do we decide where we want to live **(Figure 2A)**? Who among us would be willing to choose a location with no knowledge of the surroundings? What or who is next door? A quick Google search for "Where to buy a house," turned up a lovely Rocket Mortgage page [6], which points out some interesting thoughts that we should consider for our studies including the points below:

- Cost of living: nutrients and resources (metabolomic/lipidomic/O_2)
- Cost of transportation: cell migration (cytoskeletal)
- Commuting and public transportation: vasculature
- Consider the climate: advantageous or not (immunosuppressive)
- Research the school district: immune cell health
- Scope out the area: meet the neighbors

FIGURE 2A

FIGURE 2B



Plan for the future: growth/metastasis

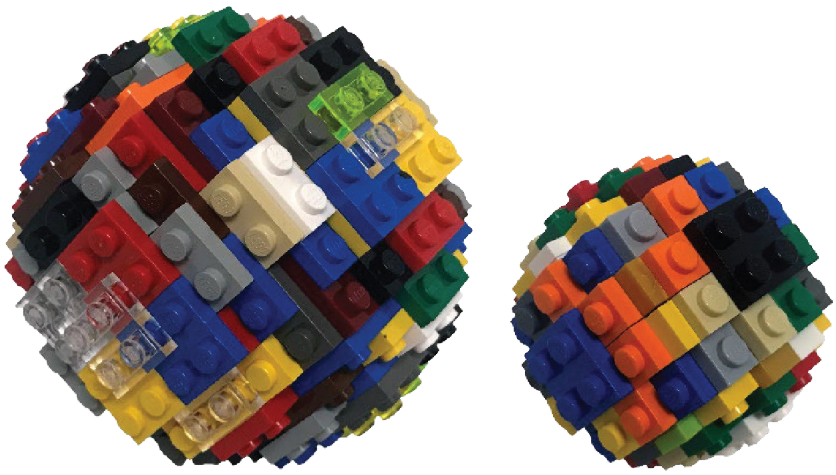
We likely go beyond this simple search when we consider crime rates, proximity to restaurants, entertainment, and other city infrastructure. Where we live would be interesting without an understanding of the neighborhoods, and the Lu *et al.* study shows the flaws we have been experiencing in the methods we have been using since the 1960s when single color IHC became the pathology method of choice. These flaws were furthered when we disaggregated our neighborhoods and merely counted cells via flow cytometry. Just as cells find their place in the TIME (Tumor Immune Microenvironment) using their cues, we use residential information such as city infrastructure (police, fire department, trash pickup, schools, grocery stores, etc.) **(Figure 2A and B)**. The cues we use can serve as cellular homologs (T cells [police and fire], macrophages [trash], B-cells [schools], metabolites [grocery stores], roads [vasculature], etc.) for the TIME. We need to consider all these metrics when understanding how our diseases progress and what helps patients survive and ultimately what does not.

These new modalities can get rather complex. In Jackson *et al.* [7] the authors identified 27 cellular metaclusters or niche environments and, using the clinical outcome data, they stratified these according to the hazards they represent for breast cancer. This is the dawn of a new understanding and these images represent the first of their kind. We don't immediately appreciate their meaning and testing them is challenging. We can attempt to get more functional details by layering on transcriptomics, which is forecasting the development of the niche neighborhoods. This layering requires placing those transcriptomic studies into context with the spatial proteomics, or we have lost the cell-to-cell putative interactions that have been occurring and are, therefore, stepping backwards. This study direction was showcased in Carstens *et al.* [8] and in Zhu *et al.* [9] and others where the authors performed single cell RNA sequencing or microdissection transcriptomics on tissue adjacent to the high plex imaging. New technologies are expanding these further, such as MerScope with spatially resolved single cell details at higher resolutions.

With higher-plexed imaging and the promise of spatial transcriptomics we can now characterize where the cells are (spatial proteomics) at a moment in time and we can hypothesize where they are going (spatial transcriptomics). This is the best sort of real-estate forecasting available, but we need to keep those base questions in mind: cost of living, cost of transportation, climate, school district, neighbors, and plan for the future. These points bring us back to another aspect, tissue heterogeneity. This term has been broadly applied for years without the support of imaging studies that can truly classify the diversity of content accurately. We are now creating bias based on this 'feeling of heterogeneity' that has developed over the last 40 years of two-color/low-plex imaging. The information gathered from these low-plex methods is driving experimental decisions about where and how much tissue to interrogate using these new high-plex technologies. At issue is the lack of understanding of the cellular patterns and the sizes of these niches or neighborhoods we are now seeking to understand. This creates the question of how much tissue must be imaged to capture the presence of the cellular patterns described above. We need to recall what we have learned from genomics and proteomics, and the patterns we have uncovered in genes and proteins: evolution plays a role and there are homologies to these patterns across organs and even systems. This means we don't need all – mathematically, we hit redundancy quickly. We need to spot-image the various regions in the tissue (tumor/stroma, necrotic, margin/invasive front, and 'healthy tissue'), which again mirrors the city concept (residential, commercial, hybrid residential/commercial, industrial, ports) [10]. The cellular patterns we are discovering vary by the ability of the cells to

DOI: 10.18609/cgti.2021.186

FIGURE 3



rapidly or not rapidly move (i.e. the mode of transportation: think about how easy it would be to differentiate modern Houston from 1900s New York). Tissues have either fast or slow movement of cells; fast movement creates larger expanses (urban sprawl – city designed on use of the car), and slow movement requires more density (vertical building – city designed based on foot traffic/horse and buggy). Perhaps that T cell in Houston can 'patrol' a larger region and thus we have lower T-cell involvement – however, in New York, the cells are so tightly packed it requires more T-cell involvement or disease can get a foothold. Ultimately, cells have specific tasks in our bodies and more specifically, in our tissues. These roles have been determined through evolution and conserved across species. This means that if a cell or cell type is in the incorrect location, or if there is a specific cell type missing, abnormality will occur. This also means that cancers can use these abnormalities to modulate the environment to further support the growth or metastasis of the cancer.

To this final point, I bring up a new LEGO sphere, built out of unique elements save one, which is constructed with the same patterns (a homologue of the original sphere) (Figure 3). If we are using the LEGO elements as stand in for cells and the underlying structure is conserved, this new sphere could also be susceptible for the same disease or cancer that affected the primary sphere. I ask, what could we learn if we had taken the first sphere apart piece by piece instead of disaggregating it? What does this homology mean? Generally, in my mind it predicts putative metastatic sites. If this is the environment the cancer needs, it will find or create other similar environments to which it can migrate [11,12]. Again, circling back to how we choose where to live, characteristics of the metastatic niche can be thought about in this manner: hypoxia and nutrients, cell to cell crosstalk (tumor, stroma, and immune), and finally, homeostatic imbalance [13]. What this means is that the primary tumor supports the development of the metastatic tumor, but we are in luck here. As we start understanding how the tumor immune microenvironment functions in the primary tumors, we can extrapolate that information to these metastatic tumors as they likely have the same linchpin. If we described tumors based on their cellular environments, I believe we could treat patients more effectively as we could target specific cell subsets and take advantage of the local resources to help in the fight.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Burks has received consulting fees from Fluidigm and Bethyl Laboratories; and a keynote fee from Digital Pathology Association.*

Funding declaration:*This research was performed in the Flow Cytometry & Cellular Imaging Core Facility, which is supported in part by the National Institutes of Health through M. D. Anderson's Cancer Center Support Grant CA016672, the NCI's Research Specialist 1 R50 CA243707-01A1 (if Dr Burks was involved), and a Shared Instrumentation Award from the Cancer Prevention Research Institution of Texas (CPRIT), RP121010 (if Mass Cytometry was used).*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 28 2021*; ***Revised manuscript received:*** *Oct 21 2021*; ***Publication date:*** *Nov 25 2021.*

DOI: 10.18609/cgti.2021.186

CELL & GENE THERAPY INSIGHTS

NEW HORIZONS FOR CELL THERAPY: EMERGING PLATFORMS

SPOTLIGHT

TECHNICAL UPDATE: EDITORIAL

Full spectrum flow cytometry: a powerful cytometric analysis to benefit cell therapy

"Using full spectrum flow cytometry to diagnose and predict individual responses to therapy is just around the corner. In the near future, not only science, but especially patients, will benefit from this advanced flow cytometry technology."

ANA LEDA FIGUEIREDO LONGHINI, Scientific Assistant Director, Flow Cytometry Core Facility at Memorial Sloan Kettering Cancer Center

Cell & Gene Therapy Insights 2021; 7(10), 1401–1404

DOI: 10.18609/cgti.2021.184

Cell therapy can be defined as the transfer of cells into a patient to ameliorate or cure a disease. The simplicity of the definition hides how broad the field can be in terms of cell type, genetic manipulation, specificity, treatment approach, etc. Although cell therapy is a vast field, there is at least one common and important aspect, the need to predict the treatment outcomes by monitoring patient response. This is especially important when the therapy relies on the immune system to fight the disease, as in immunotherapy. The identification

of subpopulations of cells, their activation/exhaustion state, and the functional response can be determined for each individual during the course of treatment, by using flow cytometry.

Flow cytometry is a technology that analyzes thousands of cells in seconds. Each cell in the solution flow passes the laser/s beam and is analyzed for visible light scatter and one or multiple fluorescence parameters originated from conjugated antibodies that recognize specific markers on the cell surface or intracellularly. The complexity of the immune system and the number of markers needed to define subpopulations of cells, has forced the flow cytometry field to evolve accordingly. This includes signal sensitivity, number of simultaneous parameters that can be measured, and high-dimensional analytical approaches.

Although conventional flow has served cell therapy since the beginning, there are few limitations that are still present, such as compensation. Compensation is the term applied to the process of correcting fluorescence spillover. The adjustment of the overlapping dyes has always been challenging. Currently, with antibody panels expanding to 30 colors or more, adjusting compensation and choosing the right combination of fluorochromes for each antibody can be even more difficult. In the past few years, another technology called mass cytometry brought some relief to the cytometry field, by allowing the analysis of nearly 50 parameters using antibodies conjugated to metals. The use of metals instead of fluorochromes bypasses the challenges of compensation. The downsides are that the signal intensity is not as strong as fluorescence and the batch effects can be more concerning compared to conventional flow cytometry, a fact that can be difficult to overcome when performing longitudinal studies [1].

A NEW ERA

A new fluorescence-based approach to cellular analysis is called full spectrum flow cytometry. Although this new functional spectral flow cytometry was first presented in 2004 [2], it has only been recently well accepted in the flow cytometry community. The increasing number of articles in the literature is evident, encouraging new users to design and perform high-dimensional experiments.

The advantages of full spectrum compared to conventional flow are:

1. As opposed to conventional flow cytometry that uses a limited number of filters/detectors for each different fluorochrome, full spectrum uses a broader combination of filters, or diffraction gratings, that capture all fluorophores which emit light across UV to infrared range. The beneficial outcome is the freedom to use any commercially available fluorochrome, or newly developed fluors, without the need to modify or buy new instruments. Also, different labs and institutions will be able to use standardized panels and compare results, facilitating collaboration between centers.

2. Cells emit autofluorescence that will be captured by the light detectors. When using full spectrum flow cytometry, the autofluorescence signature can be measured as an additional parameter. An added advantage is that the autofluorescence signal can be subtracted from the light emitted by each fluorochrome. Subtracting autofluorescence can improve data resolution as it decreases background. This feature is especially useful when analyzing highly auto fluorescent cells, such as cells dissociated from tumors.

Although full spectrum captures the whole emission of the fluorochromes, allowing the use of fluors that highly overlap, it does not eliminate the challenge of building antibody panels. Many considerations and expertise are required to create a reliable combination of markers and fluorochromes. The flow cytometry community is putting significant effort to educate and facilitate this task for less experienced users [3], but not much automation

DOI: 10.18609/cgti.2021.184

has been employed. Software capable of predicting best combinations, based on the level of cell marker expression, would be a game changer for research and clinical labs. Indeed, it is anticipated that AI technology will be a necessary and useful component as full spectrum cytometry evolves.

Despite the advantages, there are a few challenges that are yet to be overcome. Full spectrum flow does not use compensation to correct spillover, instead, a mathematical algorithm unmixes the signatures to resolve each fluorochrome present in the sample [4]. At the present, there is not a consensus about the best pipeline to unmix the fluorescence. The lack of standardization in the unmixing packages developed by different companies can lead to variability in the analysis and impact data reproducibility.

Another aspect that is equally challenging in full spectrum flow cytometry, compared to conventional flow and mass cytometry, is data analysis. Choosing controls and navigating between manual analysis and 'unsupervised' analysis, by using dimensionality reduction and clustering algorithms, cannot be done in an unordered manner. Every protocol must be well planned in order to have the right controls and analysis strategies to ensure that the complexity of the data does not affect reproducibility and lead to wrong scientific conclusions [5]. Close collaboration between physicians, scientists, instrument vendors, and bioinformaticians was never so important in order to ensure good quality data.

WHAT COMES NEXT

In parallel with the field becoming more confident with the technology and experiencing the advantages of full spectrum flow, commercial entities are working to launch full spectrum flow cytometry instruments with cell sorting capability. The separation of cells based on high parameters, 45+ markers, allowing downstream experiments such as functional assays and/or genomics, brings an exciting future for immuno-oncology and cell therapy researchers.

Although clinical labs will take much longer to employ full spectrum cell sorting, on account of the implications of managing cells in a non-GMP compliant instrument, they can still benefit from full spectrum flow analysis. Using full spectrum flow cytometry to diagnose and predict individual responses to therapy is just around the corner. In the near future, not only science, but especially patients, will benefit from this advanced flow cytometry technology.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 22 2021;* ***Revised manuscript received:*** *Oct 7 2021;* ***Publication date:*** *Nov 10 2021.*

DOI: 10.18609/cgti.2021.184

CELL & GENE THERAPY INSIGHTS

NEW HORIZONS FOR CELL THERAPY: EMERGING PLATFORMS

SPOTLIGHT

TECHNICAL UPDATE: EDITORIAL

Mass cytometry for comprehensive immune profiling in CAR T cell clinical trials

"Evaluating multiple clinically relevant timepoints, from apheresis through post-infusion timepoints, will be invaluable for identifying the most desirable and functional CAR T cell populations and may provide insight into potential approaches to optimize CAR T cell therapies for the future."

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Cell & Gene Therapy Insights 2021; 7(10), 1465–1470

DOI: 10.18609/cgti.2021.194

Chimeric antigen receptor (CAR) T cells are a powerful tool to treat patients with cancer, providing previously untreatable patients with a chance for cure. These advances have resulted in an increasing number of FDA approvals, first for CD19-directed CAR T cells, but now also for CD22 and BCMA CAR T cells [1-4]. Despite these advancements, a subset of CAR T cell-treated patients will unfortunately relapse. Moreover, CAR T cells have demonstrated limited activity in patients with solid tumors. A crucial next step in optimizing CAR T cell therapies for patients will be understanding the potential drivers or predictors of CAR T cell success and failure.

To date, analysis of patient apheresis and CAR T cell product samples, primarily by flow cytometry, have identified characteristics of T cells associated with patient responses. For example, T cell exhaustion has been linked to dysfunctional patient response [5] and the presence of naïve or early memory T cells has been correlated with improved clinical response [6,7]. These studies illustrate the value of understanding T cell phenotypes in the context of clinical response, but are limited by the number of parameters evaluated in any given sample. Consequently, these assays mostly target a limited set of cell types, with each test costing both time and additional clinical samples. In clinical trials, especially pediatric trials with limitations in sample volumes, high-dimensional single-cell assays will be essential to evaluate a wider array of cell compositions and to identify rare populations that may drive or predict clinical response.

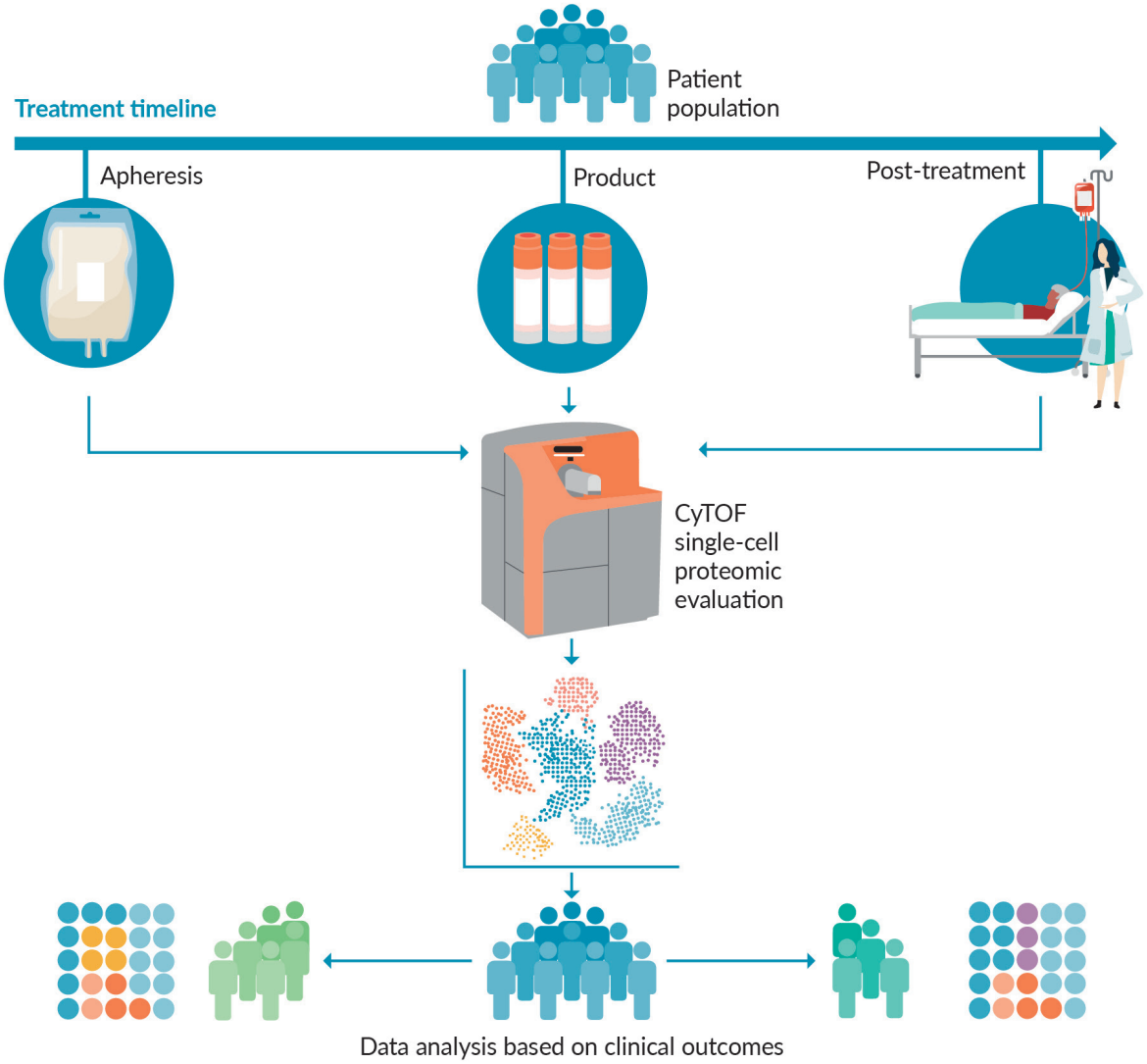
Fortunately, the advent of immunotherapy has been paralleled by significant innovation and advancement of single-cell technologies. These technologies can provide key insights into the biology of immunotherapies by interrogating the proteomic, transcriptomic, and epigenetic landscape at a single-cell level. Such approaches will facilitate a more comprehensive evaluation of immune cell composition and activation, enabling novel insights into the correlation of these populations to immunotherapeutic response.

High-dimensional proteomic platforms have developed by expanding upon the principles of flow cytometry. One such technology is mass cytometry or cytometry by time-of-flight (CyTOF), which combines the core principles of flow cytometry with elemental mass spectrometry, allowing simultaneous assessment of more than 40 parameters at a single-cell level with negligible signal overlap, lower background, and minimal signal compensation [8–10]. CyTOF requires rigorous validation and optimization techniques and data analysis requires immunology, biology, and computational skills [11]. With thoughtful implementation, these assays can provide high-quality and clinically meaningful data to understand CAR T cells as a living drug in patients.

When applied to CAR T cell clinical trials, CyTOF can both characterize the CAR T cells themselves and identify various immune populations that may enhance or inhibit CAR T cell functionality over time (Figure 1). The technology has already begun to identify key populations associated with response. In 25 adults with relapsed/refractory B-cell lymphoma treated with CD19 CAR T (Axicabtagene ciloleucel or Axi-cel), CyTOF evaluation at day 7 following CAR T cell infusion identified that $CD57^{+}Tbet^{+}$ T cells associated with complete response and $CD57^{-}Helios^{+}$ T cells associated with progressive disease at 6 months [12]. A BCMA CAR T cell trial for patients with refractory multiple myeloma evaluated baseline and post-treatment timepoints, identifying that $CD45RO^{+}CCR7^{-}CD28^{-}CD95^{+}$ remained the main subtype of persistent CAR T cells [13]. Another BCMA-CAR T cell trial combining scRNAseq with CyTOF analysis revealed that overexpression of TCF7 and TIGIT was associated with response, while $CD14^{+}$ myelo-monocyte subsets enriched within the bone marrow of patients correlated with disease progression

DOI: 10.18609/cgti.2021.194

FIGURE 1



Analyzing multi-parametric assays of patient samples from clinically relevant timepoints in the context of clinical outcome will enhance insight into the features of CAR T cells that predict or drive patient response.

[14]. These results demonstrate the power of multi-parametric single-cell analyses to yield predictive biomarkers of response and relapse.

To optimize the potential of CyTOF in clinical trials, standardized and consistent sample collection can enhance quality of inter-patient and intra-patient comparisons over time. To date, most CAR T cell analysis platforms have retrospectively evaluated pre-CAR T cell manufacturing apheresis samples and CAR T cell products. Apheresis and product samples are generally abundant in research sample availability, thereby allowing for consistently adequate cell numbers. These samples can provide pre-treatment predictive value. However, post-treatment timepoints can characterize the trajectory of CAR T cell therapy following CAR administration. To integrate CyTOF for post-treatment samples, trials must prospectively collect, process, and store samples at clinically relevant timepoints. To ensure optimal CyTOF data acquisition, especially of rare populations, samples must meet a threshold cell number for processing and can be barcoded to enhance efficiency of sample acquisition and reduce antibody variability between samples [15–17]. In CAR T cell therapy, where peak CAR

expansion often coincides with immunologic nadir from lymphodepleting chemotherapy, it is essential to collect an independent sample specific for CyTOF to optimize cell numbers. Additionally, to be able to best integrate and assess samples across a clinical trial, CyTOF samples benefit from batched evaluation to reduce batch effect and enhance data integration and analysis.

One important limitation of CyTOF is the need to know and integrate the specific markers of interest into the CyTOF panel. To harness the full potential of CyTOF, it is necessary to identify the key markers of interest and integrate these into the platform with titration and validation studies to ensure quality of results. By integrating markers of both different cell types and subtypes, CyTOF can provide both breadth and depth of analysis of immune populations. CyTOF panels have been developed to characterize immune phenotype/proliferation [18], signaling [19], chromatin state [20], and metabolic regulome [21]. Each of these platforms provides unique insight into immune biology. The optimal CyTOF panel to evaluate CAR T cell-treated patient samples should be determined based on the lead hypotheses of mechanisms underlying CAR success or failure.

Thus far, CyTOF has been primarily developed and implemented in independent laboratories. However, CyTOF panels have been harmonized across institutions with validation of the assay at many sites [15,22,23]. Harmonized CyTOF panels strengthen assay integrity and allow for analysis of CyTOF data both within a clinical trial and between clinical trials. As the field integrates CyTOF analyses into clinical trial assessments, the true power of these types of analyses is the ability to superimpose phenotypic, functional, and even metabolic characteristics of cell populations at a single-cell level. Combining multi-dimensional analyses has only just begun to be explored in hematologic malignancies [12,18,24,25]. As these combined datasets are developed, integrating clinical parameters up front will allow for downstream clinically relevant data analysis.

With CAR T cells coming into the therapeutic forefront, integration of innovative multi-parametric analysis platforms will enhance insight into the features of CAR T cells that predict or drive patient responses and outcomes. Evaluating multiple clinically relevant timepoints, from apheresis through post-infusion timepoints, will be invaluable for identifying the most desirable and functional CAR T cell populations and may provide insight into potential approaches to optimize CAR T cell therapies for the future. Such studies will expand understanding of CAR T cell biology in patients, improve predictions of clinical response or toxicity, and inform the next generation of CAR T cells, with the ultimate goal of providing every patient with the chance for cure.

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DOI: 10.18609/cgti.2021.194

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author is the recipient of Hyundai Hope on Wheels Young Investigator Grant, which supports author and author's research The author received no other financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 28 2021*; ***Revised manuscript received:*** *Oct 22 2021*; ***Publication date:*** *Nov 17 2021.*

DOI: 10.18609/cgti.2021.194

FASTFACTS

Effective integration of adherent cell culture and process characterization using a perfusion/circulation platform

Ann Rossi Bilodeau PhD, Corning Life Sciences

The life sciences are in a race to produce more cells to meet increased demand for vaccines and acceleration of cell/gene therapy programs. The challenge is to meet rising demand while reducing cost and increasing production, namely by improving cell densities and cell numbers per batch of operations. This poster details how the Corning® CellCube® culture system can address these challenges.

Cell & Gene Therapy Insights 2021; 7(10), 1293
DOI: 10.18609/cgti.2021.172

CORNING® CELLCUBE® CULTURE SYSTEM

The Corning CellCube system is a perfusion/circulation platform for attachment-dependent cells. The sterile, ready-to-use closed system tubing sets and adapters eliminate the time and complexity of assembling in-house, and the scalability of the CellCube modules allows transition from process development to manufacturing. During cell expansion, the parallel-plate design allows for reliable distribution of nutrients and oxygen across all cells. These features make the CellCube system appropriate for use in vaccine, recombinant protein, and cellular therapeutics manufacturing applications.

The CellCube system offers a high surface area in a small footprint, allowing flexible use of facility space. Notably, four CellCube 100-layer modules are equivalent to 400 roller bottles **(Table 1)**.

The CellCube module is paired with a single-use bioreactor (SUB) that provides a reservoir of conditioned medium (temperature, pH, dissolved oxygen) to feed the closed system **(Figure 1)**. Level sensors in the SUB or a scale can be integrated to equip the system for perfusion. The circulation tubing allows a peristaltic pump to draw conditioned medium from the SUB, which is flowed through the CellCube module and returned to the bioreactor for re-conditioning.

PROCESS CONTROL TO CHARACTERIZE CELL EXPANSION

During the course of cell expansion, probes in the SUB measure and record the medium conditions, gas, and bicarbonate delivery. Conditioned medium is also monitored with daily offline sampling from the SUB. Overall, stable pH and dissolved oxygen (DO) are observed during cell expansion.

For HEK293T and vero cell expansions, glucose depletion and lactate accumulation indicate when cells are ready for harvesting. Importantly, whereas growth in a static vessel might slow as medium acidifies, the active medium conditioning in the CellCube system supports continued cell growth. The CellCube 100-layer module achieves a total yield of greater than 10 billion HEK293T and Vero cells with high viability using the simple circulation approach. Furthermore, with the CellCube system, it is possible to perfuse fresh medium to eliminate the accumulated waste and boost glucose to extend the expansion period.

Several cell types have been used in the CellCube system **(Box 1)** with the general method being similar regardless of cell type. To date, much of our work has been on a small scale; however, it is possible to manifold multiple vessels to be fed from a single bioreactor. In addition to the Corning CellCube Culture System Clean Room Cart, custom automation for handling large CellCube system manufacturing processes is available from third-party automation specialists.

The Corning CellCube 100-layer module provides high-density yields of more than 10 billion cells. Its compact design, efficient use of media, process monitoring and small manufacturing footprint make it an ideal system for large-scale adherent cell culture.

Table 1. Comparison of the manufacturing footprint of the CellCube system and other cell culture systems.

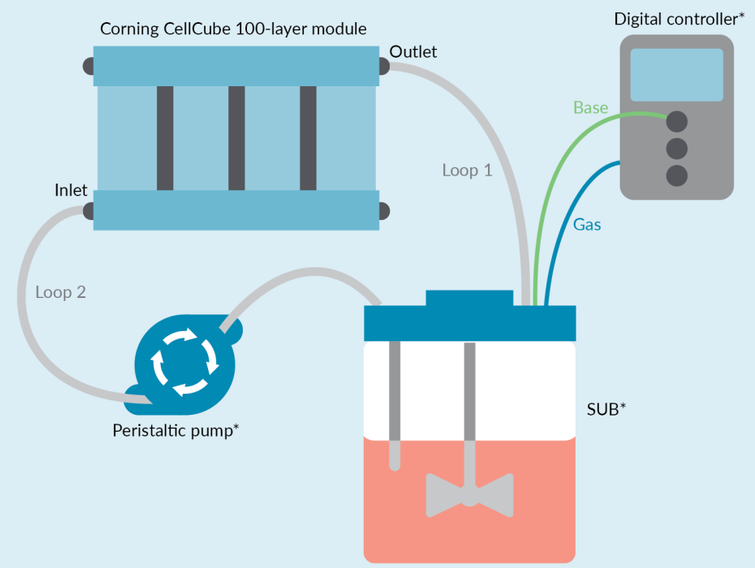
Platform	No. of vessels	Total surface area (cm^2)	Media volume (L)	Media-to-surface area (mL/cm^2)	Required equipment
Corning® CellCube® 100-layer module	4	340,000	32*	0.09	Controller, oxygenator, warm room
Corning HYPERStack® 36-layer vessel	20	360,000	78	0.22	Incubators
Corning CellSTACK® 40-chamber	14	356,160	38 to 45	0.11 to 0.13	Incubators or warm room, manipulator
Corning CellSTACK 10-chamber	54	343,440	38 to 45	0.11 to 0.13	Incubators or warm room
Roller bottle	400	340,000	51	0.15	Racks, warm room

Box 1. Cell types tested.

HEK293T*	CHO
Vero*	MRC-5
Serum-free Vero	COS M6
Bone marrow	SKNMC
MSC	TE Fly GA18
BHK21	Phoenix Frape-1/3
LMH	
MDBK	

Corning has generated data for the cell types listed in green; cell types listed in black have been cited in technical literature.
*Corning data supporting expansion in warm room and at ambient temperature with heating blanket.

Figure 1. Corning CellCube closed system setup.



*Peristaltic pump, controller, and SUB sold separately.

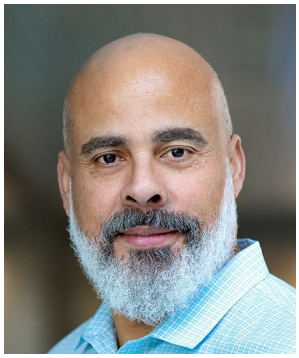
CORNING

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Critical success factors for tomorrow's cellular immunotherapies



PETER EMTAGE is a Venture Partner with Versant Ventures. Prior to joining Versant, Peter served as the Global Head of Cell Therapy Research at Kite Pharma, a Gilead company. Previous roles include Chief Scientific Officer at Cell Design Labs Inc., which was acquired by Gilead, and Vice President of Immune Mediated Therapy in the Oncology Innovative Medicines group at MedImmune. He has over 25 years of drug development experience in the fields of oncology, autoimmunity, infectious diseases, and inflammation.

Cell & Gene Therapy Insights 2021; 7(10), 1439–1445

DOI: 10.18609/cgti.2021.191

Q What are you working on right now?

PE: As a Venture Partner I am working within the Versant organization to deliver on a strategy of building innovative companies across different therapeutic areas and enabling platforms. The strategy is very diversified across the biotech space but essentially, it's where we see an unmet need and where we believe we can bring substantial benefit to patients.

With respect to platforms, we are always looking for and helping to develop platforms that can be used to create differentiated and novel therapeutic approaches.

Q It's around a year since you entered the venture capital world from the industry - what do and don't you miss about your previous roles in immuno-oncology and cell therapy research?

PE: I don't miss the levels of bureaucracy and slow decision-making in some areas of industry R&D. However, I do miss the bench to bedside development paradigm and the correlative data as it rolls off patient studies - looking at patient benefit and using correlative data to really refine an approach in a specific therapeutic area.

I find that what I have gravitated towards, which is a strong feature of Versant, is a science-heavy but very dynamic environment, which allows us to leverage the strengths of each other as well as our KOLs and partners within the space. This provides the team with the ability to come to a decision very quickly, or to formulate an idea or opinion on where we could take a specific technology platform or what areas we believe are important to drive innovation.

Q Tell us more about the Versant Ventures' approach to fostering innovation, particularly in the advanced therapies field

PE: I think what differentiates Versant from what I've been able to observe and understand in the space over the past twelve months is the scientific rigor– we have a very science-forward decision-making process.

It really stems from an understanding of the science, the ratification of an opinion across the group, and the seeking of guidance and insights where we don't have strengths from an incredible key opinion leader network that's accessible through the Versant experience. We are able to bring all of these elements together to evaluate or to innovate around building platforms and leveraging disruptive technologies across multiple areas, as we've done in the past with the likes of CRISPR Therapeutics, Bluerock Therapeutics, Century Therapeutics, and Graphite Bio. At Versant we aren't proscriptive about how we build companies. We can do it through our discovery engines, working directly with an entrepreneur/academic, or syndicating with other VCs. We let the opportunity at hand dictate the strategy.

Q You were at Kite Pharma during a very exciting period in both the company's and the overall industry's development - what are the key learnings from that experience that you apply in your current role with Versant's portfolio companies?

PE: I think that as you gain experience of a holistic drug development paradigm, be it in biologics, small molecules or cell therapies, you develop a keen sense of

DOI: 10.18609/cgti.2021.191

where some of the pressure points are when you start to think about partnering, clinical development and potential commercialization strategies. That's not only relating to the competitive landscape for which your therapy is targeted, but also understanding what the payers will do, and what the physicians in the community think and are likely to do. Leveraging this along with an understanding of the operational requirements of a start-up, mid-stage or late-stage biotech helps to build companies or outline a strategic direction to facilitate partnering or company goals.

"In the later stages of drug development, identifying key considerations for a successful partnership or the successful deployment of a drug in the commercial setting is critical to success."

Throughout my past in drug development, it's been a case of driving the science to the best outcome in Phase 1 and then, if it's deemed appropriate because of the response rate and durability, you move into late-stage development. Being able to think through that and bring some experience to bear on what those processes are - whether they are likely to enable a partnership or to enable a successful trial for one of our portfolio organizations - are some of the learnings I hope to bring to the organization. If we factor in the community physician early on in our thinking, it makes for a much better clinical development plan and opportunity for success.

So, I would say on the research end, it's about building teams, the right culture, and delivering on the pipeline. In the later stages of drug development, identifying key considerations for a successful partnership or the successful deployment of a drug in the commercial setting is critical to success.

Q What's your view of the current picture for cellular immunotherapy and how do you expect it to evolve over the near-mid-terms?

PE: If we look specifically at immuno-oncology, in the hematologic malignancies arena, there are commercial cell therapies at play in the relapsed refractory setting which are making their way into earlier lines of therapies or expanding indications. There are naked antibodies that facilitate antibody-dependent cellular cytotoxicity or ADCs against tumor cells that are also on the market, currently in relapsed refractory and heading into second line setting. And then we have bispecific antibodies that are now coming into the space. When you consider all of these approaches, patients with these malignancies have multiple options because there are such differentiated opportunities to bring about clear therapeutic benefit in these populations.

However, I do think that the ease of administration and associated toxicity will determine a winner as these various therapeutic modalities compete in the hematologic malignancies marketplace over the next 3-5 years. By that stage, I believe we will start to see the emergence of a preferred application or approach being deployed by the community physician.

When it comes to solid tumors, it's no-holds-barred. It's everyone for themselves, because we have yet to define a response and durability rate that rivals, let's say, CD19 CAR Ts in hematological malignancies. When I think about how the space needs to evolve, I separate those two oncology settings: one is driven by clear benefit being delivered to patients 4-5 years out from the original BLAs, while the other is yet to demonstrate the right targeting and the right biology to meaningful impact patient treatment.

In the US alone, approximately 580,000 patients a year die from solid tumor malignancies, and of those, the vast majority are epithelial derived. This group represents a dire unmet need. We really need to bring something that offers substantial benefit into those population, and over the last 10-15 years, we have seen the emergence of a fourth pillar of solid tumor treatment – immuno-oncology. And within I-O, I think that cell therapy has the greatest chance of really delivering the deep, durable responses that are needed in this space.

As I look towards the future of innovation within large and small biopharma and the academic realm, the greatest hurdle to cell therapy isn't another company, it's the tumor cell. It is evident that each indication, each line of each indication, and each stage of each line of each indication, can all carry differences that either attempt to block, prevent, or suppress cell therapies or IO approaches. We need innovation around targeting, but also innovation around the biology of these therapeutic cells that will allow them to compete in a hostile microenvironment - to outcompete the tumor and eradicate it.

I think that command and control technologies will be key, as will technologies that seek to unleash a better cell therapy product with respect to stemness and biology, and cell therapies that engender a holistic anti-tumor response rather than one that is focused solely on a single cellular approach like a T cell or a NK cell.

Finally, I think that once we have figured out how to do all of this in the autologous setting, it will be about bringing it into an allogeneic universal donor setting – approaches that will allow us to bring the cost point of cell therapies down sufficiently so that they can be accessible to all.

Q Can you go deeper on the specific technology platforms or modalities just coming over the horizon that catch your eye?

"...command and control technologies will be key, as will technologies that seek to unleash a better cell therapy product with respect to stemness and biology..."

PE: Companies like Century Therapeutics, who are creating and developing allogeneic products based on iPSC technologies, are not just trying to differentiate a T cell or NK cell - they are pushing the boundaries of acceptance in the recipient by mitigating host-versus-graft responses. I think that especially in this field of cellular immunotherapy, where the products can be pretty toxic, approaches that seek to balance efficacy and safety are going to be important. You can see that this sort of application, if

DOI: 10.18609/cgti.2021.191

successful, would really facilitate the efficient delivery of these programs out into the community and could potentially enable application in the outpatient setting, which would be game-changing.

Companies that are focused on this realm are very impressive. You have companies like Matterhorn, for instance, who are exploring novel T cell biology, employing MR1-restricted TCRs rather than the quintessential alpha-beta TCRs that most of today's cell therapies are based upon. That novel biology - the 'adjuvanticity' of that cell type and its ability to orchestrate a holistic response using non-classical MHC-mediated restriction – is the sort of thing that we need to investigate further. And then when you think about going beyond that, it's the NK space, it's the myeloid space - it's the ability to ask the question of whether another cell type can do a job, or are we relying solely on a classical T cell approach?

There is ample rationale, from migration to activity, for T cells being the base vehicular platform used today. But the question is, can a T cell do everything? I would argue that it can't. It can do a lot, especially in hematologic malignancies. But even there with the CAR T cell therapies you see that very quickly, 30-35% of patients stop responding because they have lost the target. So, I do think that technologies that focus on other platforms or other mechanisms of killing will start to push the boundaries of cell therapy in oncology.

Changing tack for a moment to think about where else the immune system can come in, we can also start thinking about harnessing T regulatory cells and modifying them to target autoimmune disease lesions with the goal of immune suppression. There are probably 8-10 companies in that space right now who are using that vehicle and platform to ask questions about mitigating autoimmune disease or potentiating solid organ transplant acceptance. I think it's an exciting field given the size of the patient populations and the potential to deliver something revolutionary to those patients.

There are many different companies in the space each with a distinct approach to addressing some of the longstanding and emerging questions critical for success. But what it boils down to is not only the target or the cellular vehicle, it's also about getting the immunology and the cell biology right, productive anti-tumor activity in a hostile environment.

I don't think that any one company or academic institution has all these pieces at its disposal at the moment, which really makes the race to cure somewhat based upon an organization's ability to focus. Again, when I look at companies like Century or Matterhorn and others, there is a discipline in building towards pipelines that can be strongly differentiated based on novel approaches.

Q Can you expand on the challenges these emerging technologies will need to address if they are to translate preclinical promise into clinical success?

PE: If you close your eyes and think about any disease setting in which you think a cell therapy could have an application, there are a couple of general aspects to consider if you are going to help these therapies to flourish and move the needle. One is the patient journey.

If you are developing a cell therapy in solid tumors, you first go into relapsed refractory late-stage patients. These are patients with an ECOG status of 1 or higher, which means

their ability to provide a blood sample to modify to make a cell therapy dose is rapidly diminishing with time. Therefore, approaches that allow you to rapidly turn around a cell therapy dose and get it back into patients are going to be of great interest; for example, in vivo transduction, point of care or allogeneic. There are organizations focused on *in vivo* transduction or transfection of T cells to provide a CAR or TCR that can then have a therapeutic benefit. It's being done with viruses and it's being tried with mRNA. I think that type of approach has a potential opportunity to revolutionize the space, if successful, because it would mean that your cell therapy is no longer cells in a bag - it could be DNA, or a virus or RNA in a tube.

Secondly, I think that mitigating toxicity will be absolutely crucial for the field. If you can't mitigate it, or at least get it to a point where a doctor in a community can administer the cell therapy, you are always going to have to rely on these specialized, authorized treatment centers to which patients must be referred. But if we are to ever get these therapies disseminated widely, we have to move away from the authorized treatment center model and out into the community. A doctor in the middle of Alaska needs to be able to receive the therapeutic and give it to the patient in the middle of Alaska, and not have to worry about sending that patient to Texas or Boston. So, technologies and approaches that seek to refine what that biology is and really bring the toxicity down to allow the therapy to disseminate out into the community are really interesting.

And thirdly, beyond the benefit shown in melanoma by Dr Steve Rosenberg, we have yet to see a substantial indication wide benefit from cell therapy in solid tumors. Going beyond melanoma to other solid tumors is a major challenge, especially in the metastatic setting. But I think technologies that are or induce anti-tumor polyclonality - enabling a multitargeted polyclonal approach -would represent a really interesting platform that will move the needle for cell therapy in solid tumors. The issue for cell therapies in solid tumors is one of response rate and durability. You have got to get them deep enough in their response to treatment that your durability has to be between 1-2 years rather than 6 months, because otherwise the cost just becomes prohibitive, at least within our current system.

To sum up, I would say there are a number of areas we need to focus on to really move the needle, but first and foremost is targeting and the right biology. If you don't have those, you will not give the T cell or the NK cell or the macrophage or whatever cell type you are using the ability to effectively compete in a tumor microenvironment and drive the type of response that you need. As long as you have got that differentiated response rate, it becomes a manufacturing issue of how you deploy the product to meet the average patient journey in the community. That to me is the success paradigm for wide adoption of cell therapies that are being developed at this point.

Q Finally, can you sum up your major goals and priorities in your work over the coming 12-24 months?

PE: My goals are to support the Versant Ventures' vision, to work within the team, and to enable innovation in areas where the promise for patients is highest.

DOI: 10.18609/cgti.2021.191

We are cultivating key concepts and platforms that we believe will be instrumental in enabling new treatment modalities for patients and their families.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

Article source: *Invited;.*

Submitted for peer review: *Jul 22 2021;* ***Revised manuscript received:*** *Oct 27 2021;* ***Publication date:*** *Nov 16 2021.*

CELL & GENE THERAPY INSIGHTS

NEW HORIZONS FOR CELL THERAPY:
EMERGING PLATFORMS

SPOTLIGHT

EXPERT INSIGHT

Next-generation DNA vectors: is the nS/MARt platform a viable alternative to viruses for autologous T-cell immunotherapy?

Matthias Bozza & Richard Harbottle

Cancer has a major impact on society and healthcare systems across the world, and by 2040 the number of new cancer cases per year is estimated to rise to almost 30 million. T-cell based adoptive immunotherapies rely on the delivery of genetically engineered T cells to patients. This methodology has developed enormously over the last decade, becoming one of the most promising therapeutic strategies for treating a range of malignancies. In recent years, several T-cell therapies have been granted Food and Drug Administration (FDA) approval including tisagenlecleucel (Kymriah®), axicabtagene ciloleucel (Yescarta®), brexucabtagene autoleucel (Tecartus®), and idecabtagene vicleucel (Abecma®). With increasing numbers of adoptive cellular therapies (ACT) in advanced clinical stages and the corresponding increased burden to create personalized pipelines, it is essential to explore alternatives. Ideally these manufacturing protocols need to be quick, reliable, safe, and financially sustainable. Currently, most protocols rely on viral vectors that have long and expensive manufacturing times that accordingly reduce the number of patients eligible for ACT therapies. Next-generation non-viral RNA and DNA vectors have emerged as an attractive alternative for introducing CARs or TCRs into immune cells; while maintaining a high efficiency of delivery, they are more versatile and are simpler and quicker to manufacture at scale, thus increasing the number of patients who can be treated while significantly reducing the vein-to-vein time of the treatment process. In this article we highlight the advantages offered by these alternative next-generation vector platforms with a particular emphasis on the nS/

MARt DNA vector system recently described by the authors at the German Cancer Research Centre, in Heidelberg.

Cell & Gene Therapy Insights 2021; 7(10), 1487–1493

DOI: 10.18609/cgti.2021.196

The Food and Drug Administration (FDA) has projected that by 2025, there will be up to 20 new cell and gene therapy products approved each year with more than 40 of these therapies potentially available on the market over the next 5 years. The incorporation of cellular therapeutics for cancer treatment [1] alongside surgery, chemotherapy, and radiotherapy comes with new challenges; two immediate barriers to the widespread use of ACT is the lack of affordable, rapid manufacturing and additionally, the challenging process of administering the cell-product to increasing numbers of patients. Therefore, beyond the success of current clinical trials [2–5], commercial-scale therapeutics in cancer might not be readily available for every patient, due to the simple lack of capacity to manufacture and deliver cell therapies [6].

The complexity of these treatments is not only limited by manufacturing issues, but also by a new shift in paradigm where the patient and hospital personal are part of the supply chain, with all the connected training, ethical, and legislative issues. Several University Hospitals have started to build their own cell therapy programs along with in-house manufacturing facilities [7], with the aim of scaling-down the batch size to deliver a large number of individual batches to local patients.

To generate genetically engineered CAR/TCR T cells, the transgenic material is currently typically introduced to the T cells using viral vectors (both g-retro and lentivirus) [8], whose manufacturing is costly with increasingly long lead times. The high diversity and complexity of viruses, the strict regulations required for good manufacturing practice (GMP), and an exponential demand for product has resulted in a manufacturing bottleneck with a limited number of companies capable of producing the vectors. Virus manufacturing for cellular therapies is probably the most complex and resource-intensive process in biological manufacturing and requires individually tailored processes.

In most clinical trials, peripheral blood T cells used for genetic modification are obtained via leukapheresis, isolated, activated and transduced with viral vectors that incorporate the transgene into the T cell genome leading to its expression on the cell surface [9]. Most of the time, the manufacturing process takes place in a different part of the globe from the patient, thus extending the vein-to-vein time and creating a complex supply chain.

Non-viral vectors such as messenger RNA (mRNA), plasmid DNA, and the CRISPR/Cas9 and transposon systems offer a viable alternative to viruses, offering increased versatility and a shorter manufacturing time; both crucial for generating personalized therapies where every cellular product needs to be uniquely created.

However, despite these advantages, most non-viral vectors also present some limitations and challenges mainly associated with immunogenic responses, persistence of expression, and unforeseen genetic integrations and rearrangements. Moreover, the manufacturing protocols developed and optimized for the manufacturing of T cells with viral vectors are rarely directly transferrable to alternative technologies and typically, large-scale delivery systems need to be employed, complicating the production in fully automated and closed systems. Commonly used plasmid DNA or mRNA benefit from a relatively

high safety profile being both non-viral and non-integrating systems but due to their nature, the expression of the CAR/TCR transgene will most likely gradually decline over time.

Clustered regulatory interspaced short palindromic repeat (CRISP)-associated 9 (Cas9) nuclease is a robust gene editing platform derived from a bacterial adaptive immune defense system [10] that has shown remarkable results and an improved efficiency of targeted transgene knock-in due to the optimization of more compact and precise Cas9 proteins. Because many patients are unable to receive engineered autologous T cells due to an inadequate numbers of lymphocytes available for manufacturing, with CRISPR/Cas9 it is possible to develop 'universal' healthy-donor-derived infusion products [11]. However, safety concerns have arisen when unexpected genomic aberrations and rearrangements have been demonstrated in cells modified with such systems [12].

Similarly, transposon technologies have undergone significant improvements in efficacy with the use of mixtures of DNA-RNA templates. The Sleeping Beauty (SB) transposase successfully entered the clinical stage as the first non-viral vector being used to generate CD19-specific-CAR T-cells [13,14], followed by the CARAMBA trial that aimed to test the feasibility and safety of autologous SLAMF7 CAR T cells [15]. There are currently a total of 14 active clinical trials in gene therapy making use of SB gene delivery. However, a recently described adverse event in a Phase 1 clinical trial highlighted the fact that T cells manufactured with PiggyBac transposons can be transformed by the vector leading to cancers in the treated patients, which raises concerns about the safety profile of the engineered T cells [16].

All of these technologies present several advantages over first-generation viral vector systems currently used. Only time and clinical trials will reveal which of these exciting platforms will demonstrate the most successful translation into the clinics and how they will compare to the established and robust viral approach that led the FDA approval of several CAR-T cells.

One of the more recently described classes of next-generation vectors which have been used for the genetic engineering of T Cells is the S/MAR nanovector, which has shown great promise in proof-of-concept studies [17].

S/MAR NANOVECTORS

Scaffold/matrix attachment regions (S/MARs) are short, AT-rich genetic elements enriched in DNA topoisomerase II binding sites, whose function is to anchor chromatin to nuclear matrix [18]. The ability of S/MARs to stably attach to nuclear matrix proteins led to an exploration of their use as episomal gene therapy vectors [19]. Earlier prototypes showed promise but had significant limitations [20–22] – specifically, their manufacture relied on antibiotic selection, and thus contained immunogenic bacterial sequences that causes vector-mediated toxicity and transgene silencing in eukaryotic cells, which leads to inefficiency and a reduction in long-term transgene expression. Next-generation nS/MARt DNA vectors do not share any homology with the original vectors and they were optimized and refined in all components to reduce their impact on target cells and to improve their rate of transcription and transcript stability [23]. They are much smaller (and consequently easier to deliver) and based on an a minimally-sized, antibiotic-free selection system of ~450bp [24] that make these types of vectors suitable for clinical application. This class of vector has no theoretical limits of size – the actual limit in capacity of this vector system is driven by the limited capability to effectively handle large genetic constructs and to efficiently administer them to cells via electroporation or other means. S/MAR DNA vectors have been described which are over 100kb in size and comprise entire genomic loci [25]. This size is, of course, unnecessary for cellular therapies, but it does illustrate that without viral

packaging constraints there is the potential for increased genetic capacity in the S/MAR DNA vector system.

NS/MART DNA VECTORS AS A VALUABLE ALTERNATIVE TO LENTI- & γ-RETROVIRUS

The aforementioned refinements to the nS/MARt DNA vector system resulted in a range of improvements in performance – there is a marked increase in gene transfer efficiency and persistence as well as a reduction in vector-mediated toxicity, which is described in a recent Science Advances paper [17]. For example, they provide a ten-fold improvement in transfection efficiency compared to the original prototype S/MAR based pEPI vector [19]. nS/MARt vectors were also evaluated for their efficiency to transfect and persist in primary human $CD3^+$ cells using clinically approved large-scale electroporation devices. In these experiments the new vectors achieved 60–80% overall transfection efficiency in 3 x 10^8 cells, and the expression of the chimeric antigen receptor (CAR) was detected for at least 1 month upon cell administration into animal models. To investigate the efficiency and effect of transgene delivery with nS/MARt vectors on primary human T cells relative to lentivirus, isolated $CD3^+$ cells were engineered with nS/MARt vectors or lentiviruses carrying the same expression cassette with the carcinoembryonic (CEA)-CAR transgene. Both vectors led to similar percentages of successfully engineered CAR-T cells, but cells transfected with nS/MARt showed a significantly higher CAR expression that resulted in an improved capacity of targeted tumor killing (Figure 1). By means of single-cell RNA sequencing, we revealed that lentivirus transduction led to a greater disturbance of the T cells' homeostasis relative to those engineered with nS/MARt vectors. The potency of the nS/MARt CAR-T engineered cells were found to be functionally superior relative to the lentivirus engineered cell at lower effector-to-target ratios *in vitro*, most likely due to the less disruptive effect of S/MAR DNA vector-mediated transfection relative to lentivirus-mediated transduction. This is a result also supported by data obtained with cells engineered with the non-viral transposon vectors, which have shown improved potency relative to lentivirus engineered cells at equal concentrations [26].

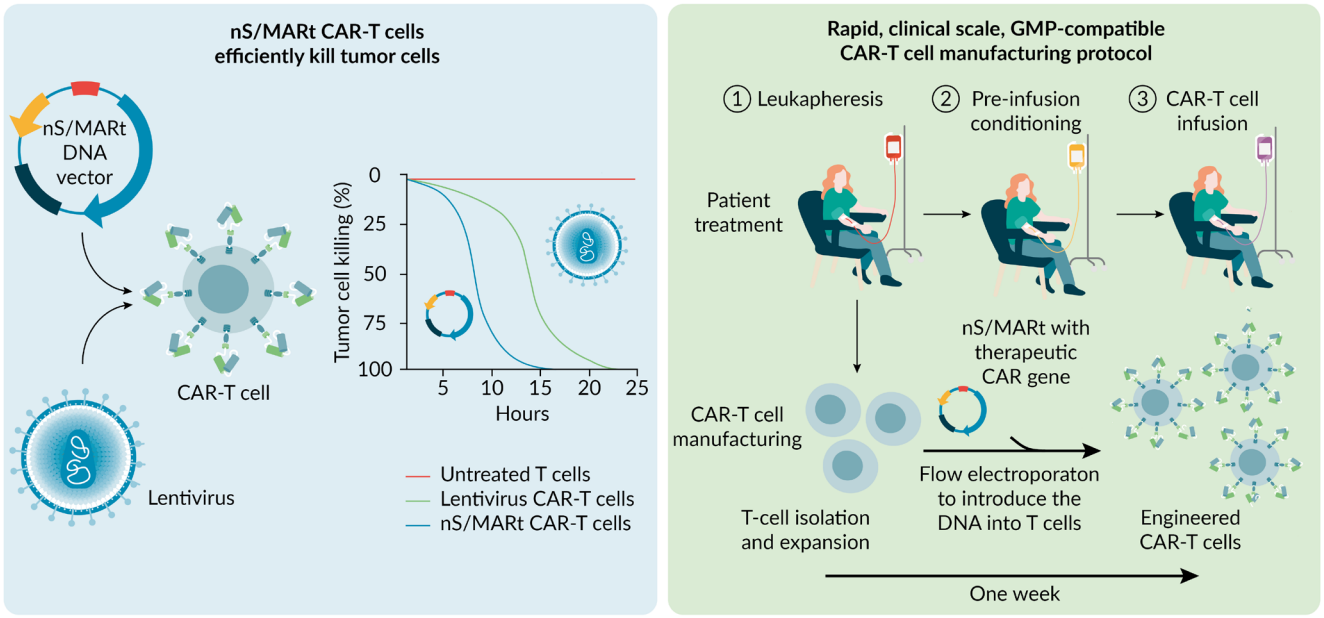
Time is of the essence: speed & cost of manufacture

With an increasing demand for ACT products and exponential growth of novel personalized pipelines that identify patient-specific reactive T cell receptors, the time necessary to manufacture the starting material and the final product represents the most critical limiting factor. The production of viral vectors requires dedicated infrastructure, but it is also a laborious and cost intense process that requires highly experienced personnel. Large-scale viral vector manufacturing relies on a multistep process, which begins with the culture of a packaging cell line in a facility that uses Good Manufacturing Practice (GMP). The cells are transfected with the plasmids that make up the lentiviral vector (that requires a previous production to be readily available off-the-shelf), and the vector-producing cells are expanded in culture. The vector is then purified from the cells and the culture debris and filtered to ensure sterility before individual aliquots are cryopreserved. Ran *et al.* [27] estimated the cost for generating GMP grade viral vectors for 30 patients at approximately US$900,000 with an expected delivery time of 1 year. Developing more efficient, more accessible, and economically feasible expression vectors is therefore a principle strategic task, and is the crucial prerequisite for the extended implementation and successful clinical application of ACT. A non-viral nS/MARt DNA vector can offer a valuable alternative as it can be produced at a high yield (2.6 g/L) with a single-step purification process in only a few days. In addition to a significant reduction in the time and

DOI: 10.18609/cgti.2021.196

FIGURE 1

The efficacy of CAR-T cells manufactured with nS/MARt DNA vectors compared to lentiviruses (left panel) and a flow diagram illustrating the process and the relative speed with which CAR-T cells can be engineered using this next-generation DNA vector technology (right panel).



costs of manufacturing (estimated as approximately US$9,000 per patient), it also offers a more prolonged shelf-live compared to viral systems. Once the vector is produced, the final ACT products can be generated in only 5 days, in comparison to the minimum of 15 days necessary when integrating viral systems are employed. Thus, the most significant benefit will be for the patient who can, in principle, gain access to the therapy in only 1 week when the platform may be used 'off the shelf'. For aggressive cancers especially, this might be a crucial point.

SUMMARY

The key advantages of these next-generation DNA vectors are their unlimited capacity, low risk of integrative mutagenesis, robust transgene expression, persistence in dividing and non-dividing cells, low immunogenicity, relatively low cost of production, and ability to enable rapid T-cell manufacturing. The speed of manufacturing is particularly important given the continuous mutation of cancer cells and can be critical for tumors that are more aggressive. For example, the most common neoepitope mutation in glioblastoma, the EGFRvIII fusion, is lost in half of tumors by the time of recurrence 9 months later, even in the absence of selective pressure. The potential to rapidly identify novel targets, to generate patient- and tumor-specific DNA vectors within days, and to then use them to effectively engineer T cells within weeks, will be a crucial advantage in the fight against cancer. A vector platform that can provide this speed of design, vector production, and efficient and effective T-cell manufacture will prove to be particularly valuable as demand for ACT increases. It is likely that as our vector technologies improve, new non-viral vectors such as those described here will be increasingly used in clinical application in place of viral vectors.

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DOI: 10.18609/cgti.2021.196

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Contributions: *All named authors contributed equally, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *We would like to thank the authors of the articles that we used as the basis of this review. We would also like to thank Patrick Schmidt who jointly led our project and the other co-authors, colleagues and collaborators at the DKFZ and NCT Heidelberg for their support.*

Disclosure and potential conflicts of interest: *DKFZ Heidelberg and the Nature Technology Corporation hold two patents: WO2019057774A1 and WO2019060253A1.*

Funding declaration: *This work was supported by the German Cancer Research Centre in Heidelberg and the NCT 3.0 Proof of Concept Program.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 20 2021;* ***Revised manuscript received:*** *Nov 9 2021;* ***Publication date:*** *Nov 19 2021.*

CELL & GENE THERAPY INSIGHTS

NEW HORIZONS FOR CELL THERAPY: EMERGING PLATFORMS

SPOTLIGHT

INTERVIEW

Enhancing the depth and durability of patient responses to CAR T cell therapy

JENNIFER BROGDON, PhD, is Executive Director, Head of Cell Therapy Research, in the Department of Exploratory Immuno-Oncology of the Novartis Institutes for BioMedical Research (NIBR) in Cambridge, MA. Jennifer leads development of next generation CAR-T cell therapies for oncology and is responsible for driving innovation, strategy and building a therapeutic pipeline from target discovery to first in human (FIH) clinical studies. Jennifer has broad expertise in antibody, small molecule and cell & gene therapy programs. She helped drive the development of Kymriah – the first CAR-T cell therapy approved by the FDA – and received the Novartis Distinguished Scientist Award for that work and other contributions to novel therapies for multiple hematological and solid malignancies. Her drug discovery experience includes >14 distinct therapeutic immuno-oncology programs that have reached Phase I/II clinical trials. She holds a PhD in Immunology from Duke University and a postdoctoral fellowship in Immunobiology from Yale University.

Cell & Gene Therapy Insights 2021; 7(10), 1423–1428

DOI: 10.18609/cgti.2021.187

Q What are you working on right now?

JB: We have now seen the proven effectiveness of CAR T cell therapy in B-cell malignancies with Kymriah®. As the field continues to evolve in that space, we are looking

at how we can expand further into hematological malignancies. We are also seeking to revolutionize the manufacturing so that we can improve accessibility to patients.

So we are expanding with new targets and new indications, but also bringing forward a next-gen pipeline based on a novel manufacturing platform.

Q Tell us about your journey in I-O - and in cellular immunotherapy in particular - at Novartis. How have your specific activities evolved as the field has flourished over the past decade?

JB: We started back in 2012 with the collaboration with the University of Pennsylvania, which was aimed at developing CTL019 for B-cell malignancies. At the same time, we initiated a broader research collaboration with UPenn - a collaborative effort that spanned preclinical, translational, and clinical R&D, manufacturing, and ultimately, on our side, commercialization.

We very quickly realized the value that the R&D engine of the Novartis Institutes for BioMedical Research (NIBR) could bring to this new modality, and we embarked upon a number of efforts to raise the bar in terms of how to design, optimize, customize, and select new therapeutic candidates.

As we have built out the most comprehensive CAR-T manufacturing footprint in the world – we now have seven facilities across four continents - we've also developed our own internal Phase 1 capabilities. In the early days, UPenn ran a lot of those initial Phase 1 studies as it represented a faster route into clinical testing and evaluation. But the more we started to learn from those studies, the more we realized the value of bringing those capabilities in-house as well.

Our focus remained largely on hematological malignancies through this period, but we did dip our toes into solid tumors with a couple of programs with UPenn. We learned a tremendous amount from those studies. Even though you don't see efficacy, there is a lot of interesting activity within the tumor, and we started to glean understanding around the challenges of solid tumors. We also recognized the need for appropriate targets as we think about these therapies moving forward. That has led to our recent collaboration with TScan on looking for novel targets in solid tumors that have a better safety profile than traditional CAR targets.

Q What would you pick out as the key learnings you take from the development of the likes of Kymriah, and apply to the next generation of cellular immunotherapies coming into the Novartis R&D pipeline?

JB: Overall, the most powerful thing we took away from Kymriah is how you can engineer the immune system to fight cancer in a very definitive and durable way. I think the real-world evidence now coming out really bears that out in terms of clinical benefit, safety, and durability of response.

DOI: 10.18609/cgti.2021.187

Of course, the one key challenge that everybody is facing in this new space is how to make an autologous, personalized therapy widely available to all the patients who need it. Our approach has been to examine the manufacturing process and how it affects the final product characteristics, the biology behind the cell therapy itself, as well as gain understanding from the patients themselves how these products are engendering the *in vivo* activity and efficacy we see. We look at how we can apply our understanding of T cell biology to enhance that potential response and maximize the potential benefit that patients will gain from it.

"...the one key challenge that everybody is facing ... is how to make an autologous, personalized therapy widely available to all the patients who need it."

We are now focusing on maximizing optimal T cell function while simplifying the manufacturing in a way that ensures both the best product quality and the scalability to benefit more patients in the long run.

Q Stepping back for a moment, how do you view the key trends and current state of the art in cancer drug development, and how do they impact Novartis's early-stage R&D pipeline decision-making?

JB: I think it's safe to say that there is no one way to cure cancer! It's a tough disease, it's smart, and it likes to try to find ways to evade the immune system as well as targeted therapies. We have taken a holistic approach to this challenge and we now have four different therapeutic modalities in our Oncology portfolio, which I believe is a degree of diversity unique to Novartis. The different approaches we are pursuing include targeted therapy, radioligand therapy, immunotherapy, and of course, cell and gene therapy.

This approach affords us the opportunity to maximize the different combinations of approaches we can use to try to meet the needs of the patient populations we are trying to treat.

Q Can you go deeper on how you see the combination therapy development picture involving cellular immunotherapies continuing to evolve?

JB: The field is moving more and more into solid tumors. =As we build upon the proven benefit in B-cell malignancies, solid tumors represent a space where combination therapies are going to be required in order to achieve transformative benefit for patients. I think it's highly unlikely that we are going to be able to use a single infusion of a cell therapy and find a definitive response in solid tumor patients. It's therefore going to be very important to understand what are the underlying mechanisms involved in the specific indications, and how

one can go about applying different modalities in combination to try to overcome the various challenges solid tumors present.

These challenges run the gamut from target heterogeneity and how you select your target, to trafficking of the immune cells, such as T cells, so they reach the required location. The immune cells also need to infiltrate and proliferate so they can overcome the tumor microenvironment (TME) immunosuppression and mediate the appropriate tumor killing. The fact there are several different components that need to be tackled suggests that combination therapy is going to bear out as something that is important.

Of course, how we are going to find the right combinations remains a major ongoing challenge for the field, even in the spaces of immunotherapy and targeted therapy. We will certainly need to spend some time and effort on advanced technology platforms that can interrogate the TME and help us to understand patient immune responses, so that we can employ a science-driven approach to identifying the best combinations.

Q What specific enabling technology innovations will play a key role in unlocking solid tumors for the cellular immunotherapy field?

JB: We have got to find ways of doing a better job of looking at on-treatment biopsies or on-treatment immune responses. If we can find technologies that will advance the field in that regard, it will be enormously valuable.

We are learning a lot from things like spatial transcriptomics and single-cell RNASeq profiling. We have also partnered with SOMAscan to look at SOMA (Slow Offrate Modified Aptamer) technology platforms. All of this is an attempt to look at multiplexed and multifunctional immune responses that are happening both at the time of treatment and during the course of treatment. We are looking for specific pharmacodynamic effects and other readouts we can use to track that activity and understand where it needs to pivot in order for us to see the best outcome.

It's a challenging area and one that is still evolving – thankfully, quite rapidly.

Q Looking to the future, how will Novartis continue to evolve – firstly, in relation to off-the-shelf allogeneic cellular cancer immunotherapy R&D?

"We are learning a lot from things like spatial transcriptomics and single-cell RNASeq profiling."

JB: The field has exploded recently in the number of opportunities on the allogeneic, off-the-shelf front. We know right now that autologous T cell therapy works in patients and we can get durable and persistent responses. I think that if you look at the allogeneic platforms, it's still very early days in terms of seeing if they can deliver the same

DOI: 10.18609/cgti.2021.187

level of clinical benefit and the same durability of response that we are getting with autologous therapies. We will continue to watch the field and see where it goes.

We have learned what is required to provide global access to this type of medicine. As we channel these learnings back into our research labs at NIBR and interrogate them, we try to imagine what the next generation of cell therapy can look like. I think we are cognizant of the key challenges in this space and we try to balance those out with where we look to invest in our innovations.

Q How about in terms of the growing influence of gene editing and non-viral gene delivery platforms in the space?

JB: While most efforts to date have focused on lentiviral or retroviral vectors, we are now seeing several different approaches coming through. For example, there are various transposons that are being explored for their potential to improve delivery and cater for larger payloads, AAV technology platforms are also being used in tandem with CRISPR gene editing to improve targeting. I think we'll continue to see those approaches evolve. There is a lot of work yet to be done to understand their specificity and safety, though – so far, we have only seen data from a small number of patients.

Q Finally, can you sum up your chief goals and priorities for your work over the coming 12–24 months?

JB: We will continue to advance our next-gen pipeline. We're focusing on science to drive our next innovations, and we have a couple of programs in the clinic now that utilize our new manufacturing platform.

This platform will take up a lot of our focus as we try to understand its full potential based on the science behind it. Essentially, we have developed a novel manufacturing process that preserves T cell stemness, which is an important T cell characteristic that is closely tied to the therapeutic potential of T cell therapies. As we develop these programs, we are looking to not only make something that is scalable, but to also provide patients with a product that has greater proliferative potential and fewer exhausted T cells, which will hopefully lead to deep, durable responses and improved long-term outcomes.

We have a heavy focus on a couple of programs that are based on this platform, as well as developing new products to come along behind.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Brogdon is a Novartis Insititutes for Biomedical Research (NIBR) employee and stock holder. She is the inventor on a number of content related patents as part of her employee responsibilities with NIBR; although no royalties are received.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Submitted for peer review: *Sep 28 2021;* ***Revised manuscript received:*** *Oct 22 2021;* ***Publication date:*** *Nov 17 2021.*

DOI: 10.18609/cgti.2021.187

CELL & GENE THERAPY INSIGHTS

NEW HORIZONS FOR CELL THERAPY: EMERGING PLATFORMS

SPOTLIGHT

INTERVIEW

A question of control? Next steps for novel TIL therapeutics

JAN TER MEULEN is a biotech executive (MD, PhD Virology, Board Certified Clin Microbiologist, Diploma Trop Med) with broad experience in immuno-oncology, vaccines, recombinant antibodies and infectious disease R&D, from discovery through phase II clinical trials. He has 16 years of industry experience in leadership positions, including 11 years at biotech companies (CSO at Immune Design Corp. and Executive Director at Crucell Holland NV) and 5 years in pharma (Executive Director & Head of Vaccine Basic Research Department at Merck & Co.). He has conducted extensive cross-functional work in the field of antibodies and vaccines, including discovery research, preclinical development, process development, clinical development, regulatory affairs, and business development. Jan was recipient of a 5-year Howard Hughes International Research Scholarship, and has been a reviewer/consultant for the NIH, European Commission, German Ministry of Science and Education, French Research Agency, Medical Research Council UK, and South Africa.

Cell & Gene Therapy Insights 2021; 7(10), 1447–1454

DOI: 10.18609/cgti.2021.192

Q What are you working on right now?

JTM: When I joined Obsidian Therapeutics roughly a year ago, we had just started to really explore regulating cytokines in tumor-infiltrating lymphocytes (TILs). We strategically selected IL15 as our first cytokine target, and most of the company is

now working on bringing that product – TILs that express membrane-bound IL-15 that can be regulated (cytoTIL15™) – to the clinic.

We anticipate entering the clinic in less than a year, so we are currently doing all the necessary work in preparation to file an IND, which of course, for a small company, requires that everybody holds hands.

In addition, we have a partnership with Vertex around exploring regulation of gene editing in an indication that is of interest to them - that's something else my group is working on. And beyond that, we are building a pipeline of next generation TILs, and looking at other applications of our regulatory technology. This platform is a molecular on-switch. It can regulate multiple proteins in a cell - intracellular proteins, membrane-bound proteins, secreted proteins - so it has potentially wide applicability for both *ex vivo* and *in vivo* expression and regulation of proteins. Our current target indications are in oncology, but there are potentially other therapeutic areas where it could be applied.

Q You have enjoyed an extensive career in the vaccines and biopharma industries - tell us about your journey towards and into cell and gene therapy, and what attracted you to the field in the first place?

JTM: It's interesting because I started out doing something very different. I am an MD by training - I'm board certified in clinical microbiology - and have a PhD in virology. I was very interested early on in viral immunology so I studied that for a while, including in some interesting tropical viruses. In fact, I spent quite some time in tropical virology.

That work got me into studying immune responses in patients on a molecular level, both antibody responses and T cell responses, and that in a way brought me naturally to generating recombinant antibodies. That was mainly phage display repertoire cloning from patients - for example, yellow fever patients in Africa. We cloned antibody repertoires in that indication - neutralizing antibodies on the wild type and vaccine viruses - and studied T cell responses and cloned T cells.

This led me directly to the question of how to make better vaccines, which I pursued for a while in academia. However, it got more and more difficult to find the funding (not least because this was rather applied research, and such research in the field of tropical medicine was also not exactly something that was easily fundable) so I then went to industry.

I joined a small and at that time unknown company in The Netherlands called Crucell, which became a powerhouse for generating human recombinant antibodies. We also had adenoviral platforms to make vaccines and cell lines. The hook that first brought me to Crucell was that they were working on the first Ebola vaccine, which was an adeno-based vaccine that came out of the NIH's Vaccine Research Center. But after working on that for a while, I switched to generating

"...we are building a pipeline of next generation TILs, and looking at other applications of our regulatory technology."

 DOI: 10.18609/cgti.2021.192

monoclonal antibodies and became head of the antibody group. We went on to make the first human monoclonals against SARS-CoV1. We showed synergy of certain combinations of monoclonals targeting different epitopes in the receptor binding domain, delineating some general principles for how you can neutralize SARS with a cocktail of antibodies preventing its escape. That work was revisited last year and created much greater interest than it did a decade and more ago when the first SARS outbreak occurred. We continued in a similar vein by identifying the first human monoclonals that could broadly neutralize influenza by targeting the stem of the hemagglutinin – an approach that is still being used to try to generate universal flu vaccines.

That work must have caught the eye of Merck Sharp & Dohme because I was recruited to run the early vaccine discovery research work for them on the East Coast. I was there for 5 years, mainly developing vaccines for infectious disease indications. From there I moved on to an immuno-oncology company in Seattle - Immune Design - where we developed therapeutic cancer vaccines based on lentiviral vectors targeting dendritic cells, the prototype for which came out of the lab of David Baltimore at Caltech. We also developed toll-like receptor agonists for intratumoral treatment. We took those assets through Phase 1 and Phase 2, into Phase 3, but then hit something of a roadblock in terms of enrollment of patients and we got into choppy waters. We were bought out by Merck.

At that point, I wanted to see where I could apply my learnings in vaccines and immunology, especially in T cells, and I found Obsidian who were just about to launch their TIL program.

Q Can you tell us more about Obsidian's technology platform/approach and what differentiates it in the busy cellular immunotherapy space?

JTM: I would say Obsidian is more than just a cell therapy company. We have a technology that allows controlled expression of any protein in genetically manipulated cells with the help of small molecules.

We leverage drug responsive domains - destabilizing domains (polypeptides), which when fused to a protein of interest and in the absence of a ligand, assume an unfolded state. They get recognized by the quality control mechanism of the cell and are degraded in the proteasome. Then, when you add the small molecule ligand that binds to this destabilizing domain, you stabilize it – hence, you stabilize the entire fusion protein, and your protein of interest can be active.

In other words, it's effectively a molecular on-switch for the activity of the protein. We use that to express IL-15 in TILs, but we have also used it to express and regulate other molecules such as CD40 ligand and IL-12, which are programs currently partnered with Bristol-Myers Squibb. And most recently, we have used it to control the expression of gene editing in our collaboration with Vertex.

Going back to my career journey, what drew me to Obsidian was firstly the broad applicability of the platform, but secondly it was the engineering of TILs, because what I learned through my time in vaccines and then in immuno-oncology is that you need a certain number of T cells in order to get a meaningful clinical response. However, those cells are very hard to generate with vaccines, in the face of the chronic antigenic exposure that occurs in a tumor setting.

What you need to do in that case is to give the patient a large number of T cells - in this case, autologous T cells – but you have to armor them with something so that they become more active. And we believe engineering TILs with IL15 makes TILs not only more active but much more persistent. We now have a treatment that takes these TILs from patients and makes them persist much longer as well as more active and potent – certainly, our preclinical work shows them to be more polyfunctional than IL-2-derived cells.

So my career path brought me to a point where I realized that the key to successfully treating cancer probably lies in adoptive T cell therapy, and that these cells need to be equipped with factors that make them more active and help them persist. And ideally, you would want to control the expression of these factors because they can either be toxic or, if you stimulate cells chronically with certain cytokines, for example, they will lead to T cell exhaustion. I believe that moving forward, molecular switches will play a very important role in preventing those eventualities.

In terms of what differentiates the Obsidian platform, there are other switches out there that are currently used in CAR T cells, for example, but the vast majority of them rely on inbuilt mechanisms by which the cell switches certain things on and off when it encounters certain antigens, or there is a logic gating of signals. While that is clearly clever and interesting, the problem is you don't really have any handle on it once the cells are in the patient. You engineer something into the cells, you give the cells to the patient, but you cannot then control them or call them back unless you have a kill switch. I was never that convinced by the idea that killing a cell product that cost you a lot of money to generate is the smartest thing to do. I would rather favor a scenario where if the cells don't do what you want them to do - if you get cytokine release, for instance – then you dampen the activity or switch that activity off instead of killing them outright.

We can titrate the biological activity of our molecules that armor these cells with the help of the FDA-approved small molecule drug we use. This gives the physician the opportunity to withdraw the drug if the activity becomes too strong, or if there are signs of T cell exhaustion. We are currently exploring pulse-dosing regimens preclinically to see if that is beneficial for treatment. In general, we think that having the opportunity to steer the cells towards a certain desired effect, or to withdraw them, by using something else that you can give the patient makes much more sense than hoping the cells will do the same thing on their own.

Q Can you go into more depth on the synergy between TIL therapy and Obsidian's platform across both liquid and solid tumors?

JTM: As we all know, CAR T cells have been extremely successful in treating hematologic malignancies, but they have their limitations. Firstly, you can only use them to target surface-expressed antigens, and secondly, you have to make sure you don't have off-tumor on-target activity, because once you direct these cells towards antigens that are not exclusively expressed on a cell type that you can eliminate, you run into all sorts of toxicity problems. Even with the current CAR T cells in hematologic malignancies, there can be strong toxicity in the form of cytokine release syndrome or neurotoxicity, for example, which

DOI: 10.18609/cgti.2021.192

are manageable to a certain extent but still a problem. The bigger problem, however, is that there are very few antigens that are expressed exclusively on the surface of tumor cells. And there is also a huge problem in terms of getting the cells to the tumors, into the tumors, and overcoming the immunosuppressive tumor microenvironment (TME).

"More recently ... companies have revisited the fairly old idea that you can use TILs extracted from the tumor biopsy of a patient, expand them in cell culture, and then give them back."

In response, scientists began looking to engineer T cells with something else. They started to pop recombinant T cell receptors (TCR) into peripheral T cells, thus directing them to tumors. The problem there, though, is that typically those are directed against a single antigen that's intracellularly expressed. Scientists then looked to target a tumor-specific antigen, but targeting a single antigen proved problematic because firstly, every single tumor cell needs to express it for the treatment to be effective, and secondly, you immediately get immune escape if your treatment is effective because the tumor will stop expressing that antigen or just delete it. So these obstacles remained.

More recently, in the past five years, companies have revisited the fairly old idea that you can use TILs extracted from the tumor biopsy of a patient, expand them in cell culture, and then give them back. This is a form of adoptive cell therapy that was pioneered by the lab of Steve Rosenberg at the NIH. It can be very successful on its own in melanoma - some clinical centers have had complete response rates of up to 30% in clinical trials. There has also been promising data in other immunogenic tumors such as cervical cancer, head and neck cancer, and lung cancer. In general, it is believed these treatments can work very well because you have a naturally selected repertoire of T cells that recognize tumor antigens. Currently they need to be expanded *in vivo* and in the patient with recombinant IL-2, which often results in severe systemic toxicities that limits the use of conventional TIL treatment. We are engineering TILs with membrane-bound interleukin 15 (mbIL15), to make them completely independent of IL2 and enhance their potency and persistence. This year we have presented preclinical data at multiple conferences (ASGCT, ESMO, SITC) that IL15 can do just that, both *in vitro* and in mouse models, showing that our "cytoTIL15™" are both *in vitro* and *in vivo* more persistent and potent than conventional IL2-derived TIL. We are also working on a pipeline of follow-on modifications building on cytoTIL-15 to increase their activity in cold tumors.

Iovance, who has been the trailblazer in the TIL area for a couple of years, may get an approval for their conventional TIL treatment next year - that will be very good for the field and it's reflected in a lot of recent business activity in the space. We now have public TIL companies, including Iovance, Instil Bio, and Achilles Therapeutics, and we just raised a big Series B. Considering the current interest both in academia and industry, I think we will see more TIL treatments entering the clinic. And an increasing percentage of these will be genetically engineered TIL. I think that of all the cell therapy treatments currently being pursued, they probably hold one of the strongest promises in terms of solving some of the problems around

recognizing multiple antigens and delivering active T cells that can overcome immunosuppression in the tumor.

Q What more can you tell us about Obsidian's early-stage foray into the gene editing realm with Vertex, and what is your mid-to-long-term vision for the impact of gene editing on the cellular immunotherapy space?

JTM: The Vertex deal was driven by the desire to regulate gene editing, especially in indications where you don't want the editor to be active for a very long time.

If you think of certain genetic diseases, including those in children, which you may be able to successfully treat with editing in the near future - with technologies that have to rely on permanent expression of the editor, like lentiviral integration, AAV to a certain extent, and others - and you don't have the means to switch off that editor, you may be looking at 10-20 years of continuous activity of a gene editor. And while they are very specific, it's clear that there is a low but real risk of off-target genetic toxicity, and that will just increase over time.

There is also the fact that you don't need to have an editor active for years and years. The editing process itself is not going to take very long - if you were able to reach your target cells of interest with your delivery system to the extent that you could get clinical improvement or cure your disease, you should be able to switch off the editing after perhaps just a few weeks of treatment, and maybe less than that.

I think that in biological treatments in general right now, one important trend is trying to be able to regulate activity, especially of very active treatments.

The field has been very good at coming up with active treatments such as the various gene editing platforms and the cell therapies we talked about earlier, but where it is somewhat lagging is in the control of these treatments. And that may have implications for both safety and efficacy. It's always better to be able to control a treatment than not be able to.

I believe that regulated cell therapy is going to be extremely important moving forward in cancer, but also in the treatment of other chronic diseases. Think of Parkinson's disease, for example, where we could regulate expression of the enzymes that are needed to produce dopamine in the brain. There are many such instances where regulation of genetically modified cells that you implant into the patient becomes really important.

"...regulated cell therapy is going to be extremely important moving forward in cancer, but also in the treatment of other chronic diseases."

Specifically in cell therapy, as I discussed before, there is and will be a huge push to armor cells, to engineer them with properties that make them more active. But the more active they become, the more important it becomes to be able to regulate that activity. Because at the end of the day, the biology is incompletely understood and again, you

DOI: 10.18609/cgti.2021.192

cannot call a cell therapy back - you give it to the patient and that's it. Since you cannot call it back you only have two options – the kill switch to simply get rid of the cells, or some other mechanism to control the activity. And the risk that these cells might do something that you don't want and you cannot control is only going to increase with every round of innovation where we make these treatments more effective. Several approaches rely on hard-wiring control mechanisms into cells which get triggered by receptor-ligand interactions *in vivo* that cannot be controlled further externally. Giving the physician control over the expression of a cellular factor in a genetically modified cell via reversible dosing the patient with a small molecule drug, the Obsidian approach, takes control to the next level.

Q Are there any further 'on the horizon' technologies or platforms that you expect to see coming to the fore for the cellular immunotherapy field over the foreseeable future?

JTM: Precision medicine is obviously the ultimate goal. You want to have a biological treatment tailored to the individual patient, whether that involves targeting certain mutations that a patient's cancer or adapting the treatment to the make-up of the immunosuppressive microenvironment of the tumor, or correcting a specific genetic defect. I think there are a lot of very interesting technologies now being developed to potentially achieve this - the problem is there is a big gap between the proof of principle of these approaches *in vitro* and in mice, and actually being able to manufacture them and take them into the clinic. And that gap may even grow larger as these therapies become more and more experimental. For example, we are going to continue to see a lot of interesting gene editing technologies being developed that go beyond just CRISPR - base editing, editing RNA, etc. But the question will remain: how do you take them into the clinic? It will require not only money - the money seems to be there anyway, at least for now - but conceptually, what is a fast way to generate the data that will allow the regulators to let you perform these clinical trials, especially from a safety perspective?

The models are always a problem, too. The more personalized these treatments get, the less relevant some of the crude mouse models become, and there are no really good models using human tissues to test them *ex vivo*. This is being worked on - for instance, it would be great to have a three-dimensional tumor model, and organoids are being developed that have a reconstituted immune system in some shape or form, which allows *in vitro* testing of treatments using a chunk of the patient's tumor and maybe some of the patient's immune system. But we are not there yet.

Once that is in place, and especially if it's amenable to high throughput, then I think we will start to see real breakthroughs happening much faster. But for now, bridging the gap from mice to a Phase 1 with the ever-more complex and personalized treatments that are coming through is going to require a lot more effort.

Q Finally, can you sum up some major goals and priorities, both for yourself in your own work and for Obsidian as a whole, over the coming 12–24 months?

JTM: Our main goal is our first Phase 1 clinical trial next year with cytoTIL15, which we hope will show clearly that by replacing IL-2 with IL15, we can open up TIL treatment initially for a much larger group of melanoma patients. We hope it will then show efficacy in solid tumor malignancies beyond melanoma, such as lung cancer.

I will continue to be very active building a pipeline and addressing questions of the TME of colder tumors and am are very interested in going beyond the more immunogenic tumors and into the likes of sarcoma and potentially some GI malignancies, for example.

Overall, the goal is to grow Obsidian into a powerhouse of cell therapy and potentially, of gene therapy. We started to venture into gene therapy with Vertex and we're going to see how that develops - I would not rule out that we also grow that branch of the business. But I guess that really depends on various factors: how the field is developing, where you see breakthroughs, etc. I believe you have to do cutting-edge science these days to be competitive at all, and of course, as a small company, you cannot do cutting-edge science in lots of different areas - you have to focus.

So in a way, we will see where the journey takes us. But I'm quite optimistic for our first product here to make an important impact. And then I think things will flow from there.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr ter Meulen is an Obsidian Therapeutics full-time employee and shareholder. He is a non-executive director at Virometix AG, Switzerland.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

Article source: *Invited.*

Submitted for peer review: *Sep 16 2021;* ***Revised manuscript received:*** *Oct 26 2021;* ***Publication date:*** *Nov 16 2021.*

DOI: 10.18609/cgti.2021.192

SPOTLIGHT

INTERVIEW

New-generation T cell immunotherapy at the intersection of adaptive and innate immunity

DANIEL COREY is founder and chief executive officer of CERo Therapeutics. A board-certified hematologist, Dr. Corey led the foundational discovery research to identify and define the therapeutic utility of chimeric engulfment receptors and advance understanding of the basic biology of innate immune responses. Dr. Corey received his medical degree from the University of Washington Medical School and completed his residency in internal medicine at Duke. Dr. Corey completed a series of fellowships in cell biology, immunology and hematology at Duke University and Stanford University.

JOHN ROSSI leads the Translational Medicine team at CERo. Prior to joining, Mr. Rossi was Senior Director of Translational Medicine and Head of Cell Therapy Clinical Pharmacology at Kite, a Gilead Company. While at Kite, Mr. Rossi led translational activities supporting the clinical development and global approvals of YESCARTA® and TECARTUS® CAR T cell therapies. Mr. Rossi's team also supported work focusing on the mechanistic understanding of engineered cell therapy products under strategic collaborations with the NCI and numerous leading academic institutions. Before Kite, Mr. Rossi spent 13 years in oncology drug development at Amgen working with teams to identify novel biomarkers for hematologic and solid tumor drug development programs.

REMUS VEZAN MD PhD, joined CERo in April 2021 from Kite, a Gilead Company, where he spent the last four years in cell therapy clinical development, most recently as Executive Director of Clinical Development. At Kite, Dr Vezan oversaw the clinical development of more than 20 CAR T-cell trials, as a single agent or in various combinations for the treatment of B-cell malignancies. Under his leadership, YESCARTA® and TECARTUS® were granted various global approvals. Before Kite, Dr Vezan served as Medical Director at Pharmacyclis, an Abbvie Company. Dr Vezan completed his medical training (MD and PhD) at the University of Medicine and Pharmacy Cluj, Romania and University of Bern, Switzerland.

Cell & Gene Therapy Insights 2021; 7(10), 1455–1464

DOI: 10.18609/cgti.2021.193

Q What was the vision for founding CERo? What is the company mission?

DC: The vision for CERo is to develop a multifunctional T cell platform at the intersection of innate and adaptive immunity with enhanced capabilities that can be combined and applied across multiple malignancies, including solid tumors.

There has been emerging interest in strategies to effectively engage the immune system. These approaches have utilized various immunotherapeutics to enhance tumor cell phagocytosis, promote antigen processing and presentation, or remodel the tumor microenvironment, for example. We have taken a synthetic biology approach to introduce some of these learnings into a new-generation T cell platform. In this way, we rewrite T cell elimination programs and take advantage of plasticity within the lymphocyte lineage, thereby reprogramming T cells to enhance their adaptive and innate functionality.

CERo's platform allows for combination with various targeted small molecules and to also integrate with various T cell therapies, such as CAR T cells or engineered TCRs. There is broad utility in this approach, which we think will be important for solid tumors in particular.

Q Tell us more about your backgrounds and the main reasons why you joined CERo

RV: I've spent the last decade doing drug development in hematological and solid malignancies, and have had the chance to contribute to bringing small molecules and more recently, cell therapy product into clinical use, helping many cancer patients to get back to normal life.

Here at CERo, we have a good understanding of the great unmet medical need in many of the disease areas, but also the potential that cell therapy can have in addressing at least part of that need. And for me personally, there is a commitment to advance the field of cell therapy for the betterment of many patients with cancer. With my experience in designing and implementing clinical trials and building teams, I consider that by bringing my clinical expertise in cell therapy, and also my energy and commitment, I can help CERo to achieve the goal of developing a novel therapeutic modality.

Beside the scientific merit of the approach, I think we have a very talented and ambitious team with both academic and industry experience in synthetic biology, immunology, and T cell engineering. This is also a group with a great culture based on science, as well as a

great vision and desire to help patients. We have many notable physicians and scientists affiliated with the Scientific Advisory Board, all with a track record of success in cell therapy, immunology, and oncology: like Rick Klasuner, our Founder Larry Corey, Rafi Ahmed, Adrian Bot, and more recently, Antonio Lanzavecchia. And of course, as with any such endeavor, the financial support is also very important. CERo has some solid and preeminent investor partners in Arch Venture, MilkyWay and Sequoia.

"The approach combines both innate and adaptive function alongside novel induced targets to expand T cell functionality. Our core technology is a class of innate immune receptors called CERs (Chimeric Engulfment Receptors) - the genesis of our name, CERo"

- Daniel Corey

JR: I have been working in pharma for the past 20 years, about 15 of which have been spent in oncology. In that time, a couple of things stand-out: 1). the huge unmet need that exists for the vast majority of patients with cancer, and 2). the challenges that we currently face in the drug development arena to meet those unmet needs.

I had the fortune of working at Kite Pharma for a period of time during the development of two CAR T cell products that made it all the way from first in human clinical trials through to commercialization. At Kite, I saw the promise of cell therapy first-hand as well as the many challenges, definitely something that attracted me to CERo's novel platform. We are building on the second-generation CAR technologies with something that is very new, and which we feel is going to potentially help to address some of the unmet needs through delivery of cell therapy products that may overcome metastatic disease in the solid tumor setting. CERo's long-term goal is to develop a suite of products that can be used across multiple indications - leukemia all the way through to solid tumors. We believe this can be achieved through synthetic biology and the plasticity and polyfunctionality of T cells, which Dan mentioned.

Q What's unique to Cero's platform and approach?

DC: The approach combines both innate and adaptive function alongside novel induced targets to expand T cell functionality. Our core technology is a class of innate immune receptors called CERs (Chimeric Engulfment Receptors) - the genesis of our name, CERo.

Interestingly, introduction of a CER into a T cell, leads to transcriptional and signaling cascades, which enable these engineered cells to capture tumor cell fragments. This novel biology introduces innate immune features designed to promote tumor antigen uptake and antigen presentation.

We also believe the mechanism of action will promote a favorable tumor microenvironment, and integrate functions analogous to CAR T cells, such as target antigen-specific T cell activation, cytotoxicity, proliferation, and cytokine production. Our hope is that this integrated approach leads to an initial primary effective CER-T mediated tumor cell clearance, as well as activating a secondary immune response that amplifies and sustains a bystander anti-tumor effect. In this way, the platform offers the potential to address a broad range of tumor types with a single T cell therapy product. However, the approach also lends itself well to synergies with various cytotoxic and immune-based therapies, including combinations with small molecule agents or other chemotherapeutics, and other engineered cell therapies such as CAR-T or TCR-T cell therapies, for which we have a current collaboration with Lyell Immunopharma.

JR: We are leveraging existing engineered autologous T cell manufacturing technology, so that's not novel, but there are a couple of aspects that are very new. Firstly, there's the CER T cell drug target: inducible stress antigens that are found across cancer indications. We feel we can overcome antigen escape, which has been seen with the current class of CD19 and BCMA directed CAR T cells, for example. Additionally, these inducible stress antigens can leveraged to make a single cell therapy product with utility across numerous indications, including the most common types of solid cancers (lung, ovarian, colorectal, etc.).

Another element that is very unique is the concept of bringing in innate immune function alongside adaptive immune function into a single T cell product. We are hypothesizing this will have ramifications in terms of the activity of the product, especially in the solid tumor arena where there are multiple barriers that an engineered cell therapy product has to overcome – the hostile tumor microenvironment (TME) and target heterogeneity, for example.

It's a complex area and of course, something that we will need to demonstrate both preclinically and clinically: that through enhanced phagocytosis and antigen uptake, we are promoting HLA class I and class II presentation of antigen, which could lead to additional recruitment of immune machinery to tumor sites to aid in full tumor clearance. We hypothesize that a secondary anti-tumor immune response could potentially lead to longer and more durable response across some of the indications we are hoping to go into.

To summarize, the concepts we are really hoping to get across are novel and inducible stress antigens as targets, as well as bringing in innate and adaptive immune function into single T cells.

Q In what other ways are CERo's product candidates differentiated from other cell therapy modalities, especially CAR T cells?

JR: Inducible tumor stress antigens that are not fully under genetic control allow us to use approved small molecules across various indications to drive up our target. We can also induce the target through synthetic biology. One could think of a bisistronic configuration with the CAR targeting an antigen alongside a CER, targeting an inducible stress

DOI: 10.18609/cgti.2021.193

antigen, and then again, bringing in this novel secondary innate function. Additionally, at key points, CER T cells retain many of the key features of CAR T cells – cytolytic activity, proliferative capability, production of cytokines and chemokines that aid in immune function, and recruitment of myeloid-related cells to the site of tumors. When you take all these concepts together (targets, functionality) we believe it gives us access to a broad range of tumor types.

> "the concepts we are really hoping to get across are novel and inducible stress antigens as targets, as well as bringing in innate and adaptive immune function into single T cells."
>
> - John Rossi

Q What key learnings and experience do you bring with you to your current roles - for example, from the likes of Kite Pharma?

RV: Between us, we have worked on many clinical programs covering small molecules like BTKi, biologics, and more relevant to CERo, autologous CAR T cell therapy. We were part of the success of these approaches in various malignancies, and we saw how cell therapies changed the treatment paradigm in lymphomas. But we also learned first-hand about the limitations and challenges patients are facing with receiving these types of therapies.

As you know, the current approved anti-CD19 CAR T cell therapies have notable toxicities, including cytokine release syndrome, neurological toxicities, and in some cases, prolonged cytopenias. Whilst these are toxicities that the field much better understands and manages these days compared to even just a few years ago, the problems are still not solved. There is room for improvement – for more learnings and better understanding of mitigation solutions that would lead to products with a better safety profile, and hence, broader access for patients to these treatment modalities with a curative potential.

In terms of disease control, approximately 60% of non-Hodgkin lymphoma patients, for example, do not respond or will later relapse after CAR T cell therapy. For these groups of patients that have failed CAR T therapy, unfortunately, there are limited further viable option as there are no other curative treatments left. And there are other important factors which contribute to the success of a cell-based therapy for a patient, such as access to or availability of a specialized highly-trained medical center, eligibility to receive the therapy, disease control during the manufacturing turnaround time, and reimbursement. We believe that novel cell therapy approaches or novel treatment modalities are needed to improve and optimize both the toxicity profile and the durability of responses of current approved CAR T cell products. We now have a much better understanding of the mechanism of relapse and tumor resistance - learnings that we gathered mostly from translational studies and analysis implemented in our previous cell therapy clinical trials.

JR: Having been at Kite Pharma very early on, when the company was just slightly larger than CERo is now, I was able to learn important organizational strategies to build an organization to support the advancement of pivotal cell therapy clinical trials that will position products for successful commercialization. Secondly, I was able to work on the technology early, in collaboration with Jim Kochenderfer and Steve Rosenberg at the National Cancer Institute, which laid the groundwork for how to look from a translational perspective at patient samples in order to understand basic things - from clinical pharmacology all the way through to product attributes and their association with clinical outcomes. There are certainly lessons learned from that experience to bring to CERo and in addition, all of the experience gained with regards to regulatory processes related to development of cell therapy products. Having worked on several INDs, BLAs and SPLAs in global filings for CD19 CAR T cell products, is invaluable experience that I hope to bring to CERo.

Scientifically, I'm hoping to integrate prior learnings to help CERo develop sound strategies that test various aspects of CER function in the human patient, so that we can quickly drill down on the mechanism of action in real-time. If there are toxicities, which there likely will be, we'll want to understand the root cause as well and as quickly as we can.

I'm really excited to get to that point where we can start to bring in a very sound translational strategy to help define and solidify this new mechanism of action that a CER T cell affords us in human clinical trials.

Q What are the chief considerations in terms of the integration of engineered cells with targeted therapies?

> "We are aiming to initially produce a cell therapy product that can be combined with an approved targeted small molecule to induce the tumor target, leading to an improved therapeutic index that will hopefully lead to complete tumor clearance."
>
> - Remus Vezan

RV: We are aiming to initially produce a cell therapy product that can be combined with an approved targeted small molecule to induce the tumor target, leading to an improved therapeutic index that will hopefully lead to complete tumor clearance. There are some key considerations, though.

The small molecule needs to concretely not interfere with the engineered T cell functions, or the manufacturing process. We know that certain classes of small molecules, like ibrutinib, may enhance T cell function, but others such as bendamustine may have a negative impact on the T cell function or manufacturing outcome.

There are other very important considerations with regards to safety. We want to

DOI: 10.18609/cgti.2021.193

make sure we have minimal safety concerns with this treatment modality, and that benefit-risk ratio and therapeutic index are improved overall.

Our next steps will be to combine CER-T products with other therapies, including CAR-Ts, with the ultimate goal of creating this unique hybrid T cell that leverages CAR T cell functions as well as the CER-T function.

JR: Going back to what I was discussing earlier around translational findings, the hope is that through the early proof of concept studies in the hemalignancy space, we will be able to adapt and extrapolate those learnings to help inform on how we can best approach solid tumor indications - for example, whether it's with a bisistronic CAR/CER-small molecule pairing, or if we have to modify our intracellular signaling domains. I think a lot of that is going to be borne out from the early clinical trials as we strive to hit the much higher bar set by solid tumors. The clinical development path that Remus is laying out will help us to get there, but of course, it's going to have to be backed with both the clinical and translational science that each of us is hoping to bring in.

Q Can you tell us about the manufacturing model and your plans in that regard?

JR: We are able to leverage current manufacturing processes that are used to make commercial second-generation CAR products, including viral vector delivery systems with transgenes that carry the CER gene into autologous T cells. Much of that has been worked out for us. Our initial approach is going to be lentiviral-based T cell manufacturing, which is unlikely to involve selection of specialized T cell subsets to make the product - that could change over time, as we learn more about the functionality of the product and which T cell subsets are playing a major role in tumor control or lead to toxicity.

The overall goal is to have a relatively simple, streamlined, and scalable process that will enable us to quickly and reliably receive apheresis material, turn around the product manufacture, and get the final product back to the patient. So the model will be very similar to that of current autologous CAR T cell therapy products, likely with a centralized manufacturing facility making the product after receiving cells from various clinical centers.

For the early days in the clinical trial setting, we will likely partner with a contract organization to tech transfer our manufacturing know-how as we head into proof of concept studies, and then we'll go from there. Obviously, we know that manufacturability provides many challenges. Our goal of potentially pairing with small molecules also needs to be taken into consideration: we know from our previous experience that certain prior treatments can have a deleterious effect on T cell function. Again, bendamustine is an example – it's more and more commonly used in the B-cell malignancy space, but I think the field is learning that there are implications to the timing and degree of its use. So we will need to understand challenges in that area that we face with our patient populations - prior lines of treatment and so forth have to be factored in.

If we are successful and the products work in the way we hope they will, our manufacturing model should lead to products that are accessible more broadly to patients. It's a challenge in the autologous setting, of course, and if we have something that really works well, we will have to start considering allogeneic approaches. Although I would add my belief that gene editing and a lot of the rules for allogeneic T cell products still need to be worked out, and that still feels a long way off.

Q Can you go into more depth on CERo's harnessing of synthetic biology and bioinformatics?

DC: The synthetic biology approach draws on some of the early success in architecture design from second-generation CAR T cell therapy products and applies them to innate immune synapses.

These parallel systems are amenable to engineering approaches, just as the T cell/antigen-presenting cell interface has been used to develop chimeric proteins to incorporate multiple signaling pathways in the single molecules for optimal T cell activation programs. We have applied similar design principles, but with distinct signal recognition domains to capture innate function alongside the clinically validated adaptive capabilities of CAR T cells.

RV: With more and more cell therapies approved or in clinical development - including autologous and allogeneic products, NK cells, etc. - the role of bioinformatics will increase and play a very important role in the future in patient selection. It will help us identify the patient population that would benefit most from a given therapy, either alone or in combination, based upon various elements like tumor-specific markers, clinical criteria, and number and type of prior therapies received. We are focusing on implementing all of these tools that would increase the clinical success.

JR: Just to add to the synthetic biology piece, we have learned a lot regarding CAR function and how the co-stimulatory domains will impart different functionality into T cells. What we are hoping to do now is to build on that by developing, through novel transgene design, a CER T cell that has anti-tumor function and brings in this innate capability, whilst also having a gentler cytokine secretion profile, for example. We want to be able to dampen down certain inflammatory cytokines that have been very strongly associated with cytokine release syndrome and neurological toxicity/ ICANS, which seems to be a class effect with CAR T cell therapies, at least in the B-cell malignancy indications.

This plays into all the tools that are available to us now to quickly test different intracellular signaling domains and different combinations - to try to tease out those beneficial attributes of a CER T cell product that we want to ultimately move forward to our first IND. So we are leveraging a lot of the work that has been done and a lot of the tools that are already available to us to bring in this new biology. But it's all done with a synthetic approach, thanks to some really talented immunologists and molecular biologists that we have at CERo.

DOI: 10.18609/cgti.2021.193

Q You've touched on the CER-T cell products in the combination setting – can you tell us more about the types of molecules or treatments that could be combined with CER-Ts to increase the therapeutic index?

RV: Due to a novel mechanism of action and this concept of tumor agnostic inducible target, the CER-Ts can be combined with a broad range of different classes of small molecules. We feel that our platform and products will be amenable to various combinations across both hematologic malignancies and solid tumors. But again, the other important goal is to have this single cell therapeutic product, autologous or even allogeneic, that integrates a CER and a CAR in a bisistronic or bispecific construct, which could be used as a single treatment modality for multiple cancer indications.

Q Finally, what more can you share in terms of ongoing preclinical development plans and the eventual translation of your therapeutic candidates into the clinic?

RV: In terms of clinical and regulatory timelines, we are planning for FDA interaction in the first half of 2022, with an IND and initial proof of concept study in hematologic malignancy later in 2022 and we aim to quickly expand our clinical development into solid tumors after that.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *D Corey holds patents related to Chimeric Engulfment Receptors and their uses. D Corey holds stock options in CERo Therapeutics Inc. J Rossi is an equity holder at Gilead. The authors have no other conflicts of interest to disclose*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Jun 8 2021;* ***Revised manuscript received:*** *Nov 3 2021;* ***Publication date:*** *Nov 24 2021.*

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DOI: 10.18609/cgti.2021.193

CELL & GENE THERAPY INSIGHTS

NEW HORIZONS FOR CELL THERAPY: EMERGING PLATFORMS

SPOTLIGHT

EXPERT INSIGHT

Artificial intelligence: accelerating translational cell therapy development through computational methods for neoepitope discovery

Maurizio Chiriva-Internati, Lucia Piccotti, Luca Zammataro, Michael C Ryan & Leonardo Mirandola

A surge in the efforts to identify cancer-specific antigens as targets for immunotherapeutic approaches has characterized the past few decades. However, the clinical use of tumor-associated antigens has been mostly limited to cancer/testis antigens, restricting its application to certain cancers and limiting the weak immune response elicited by self-antigens. Neoantigens resulting from somatic mutations in cancer cells represent an alternative immunotherapy target voided of such limitations. Similarly, cancer-specific protein isoforms or mutational hotspot antigens provide the advantage of tumor tissue specificity coupled with low thymic selection and central tolerance. Choosing the best antigenic determinants is critical for successful adoptive cell therapy; the availability of algorithms for clinical data mining and to predict the most immunogenic epitopes is thus a powerful promise for the rapid advancement of cancer medicine. The state of the art of computational neo-antigen research is discussed here.

Cell & Gene Therapy Insights 2021; 7(10), 1471–1485

DOI: 10.18609/cgti.2021.195

IMMUNOTHERAPY AS CONVERGENCE OF MEDICINE, BIOTECHNOLOGY & ARTIFICIAL INTELLIGENCE

The last decades have seen a shift in therapeutical modalities and the efficacy of cancer treatments. The modern therapeutic vision introduces the immune system into the scenario, flanking the surgery, chemo, radio, and target therapy: this new vision is known as cancer immunotherapy.

Historically, cancer immunotherapy dates back to 1868, when Busch and Fehleisen reported an intentional infection of a cancer patient with erysipelas [1]. Indeed, the two German physicians had already noticed several cases of cancer regression in patients after accidental infections in the past. These observations led them to enterprise a new road in cancer therapy based on intentional infection with bacteria.

Ten years later, William Coley at Memorial Hospital in New York replicated the approach of his predecessors, treating a 43-years-old patient with sarcoma by injection of heat-inactivated bacteria (Coley's toxin) which resulted in a long-term regression of the tumor. After these intriguing episodes, for almost a century and a half, several discoveries in immunotherapy followed, characterized by clinical findings and pieces of evidence supporting the implication of the immune system in cancer. But, the crucial role of immunotherapy as a powerful approach in the fight against cancer was predicted in 1977 by Professor Lloyd J Old [2].

In the following 50 years, the field of immune-oncology has progressed at a breakneck pace resulting in impressive outcomes in treating hematologic and solid tumors [3]. In the 1990s the identification in melanoma of the first human cancer antigen recognized by cytolytic T lymphocytes [4] paved the way for new strategies for targeted immunotherapy.

As the therapies progressed, the belief that tumor genetic alterations played a fundamental role in the success of immuno-oncology became more evident. Indeed, thanks to the rise of new biotechnologies, and genome-sequencing techniques, the face of cancer therapy was dramatically changed by the discovery of alterations harbored by tumor cells, such as gene fusions, mutational frameshifts, splice variants, and endogenous retroelements, and other tumor-specific abnormalities. All these genetic and transcriptional alterations can impact protein sequence, structure, and composition; consequently, new immunogenic epitopes become available for presentation by the automatic mechanism of the cancer cell proteasome cleavage [5–7].

As shown in Figure 1, the discovery of CTLA-4 blocking antibodies in cancer treatment, dating back to 2011, opened new roads in developing autologous cell-based cancer vaccines and was pivotal to the exponential acceleration of cancer immunotherapy. In 2010, Sipuleucel-T was the first autologous cell-based cancer vaccine FDA approved in prostatic cancer. In 2011, Ipilimumab was the first immune-checkpoint inhibitor (IC) FDA approved in melanoma treatment. Finally, it dates back to 2012, the discovery of the CRISPR/Cas9 system by JA Doudna and E Charpentier.

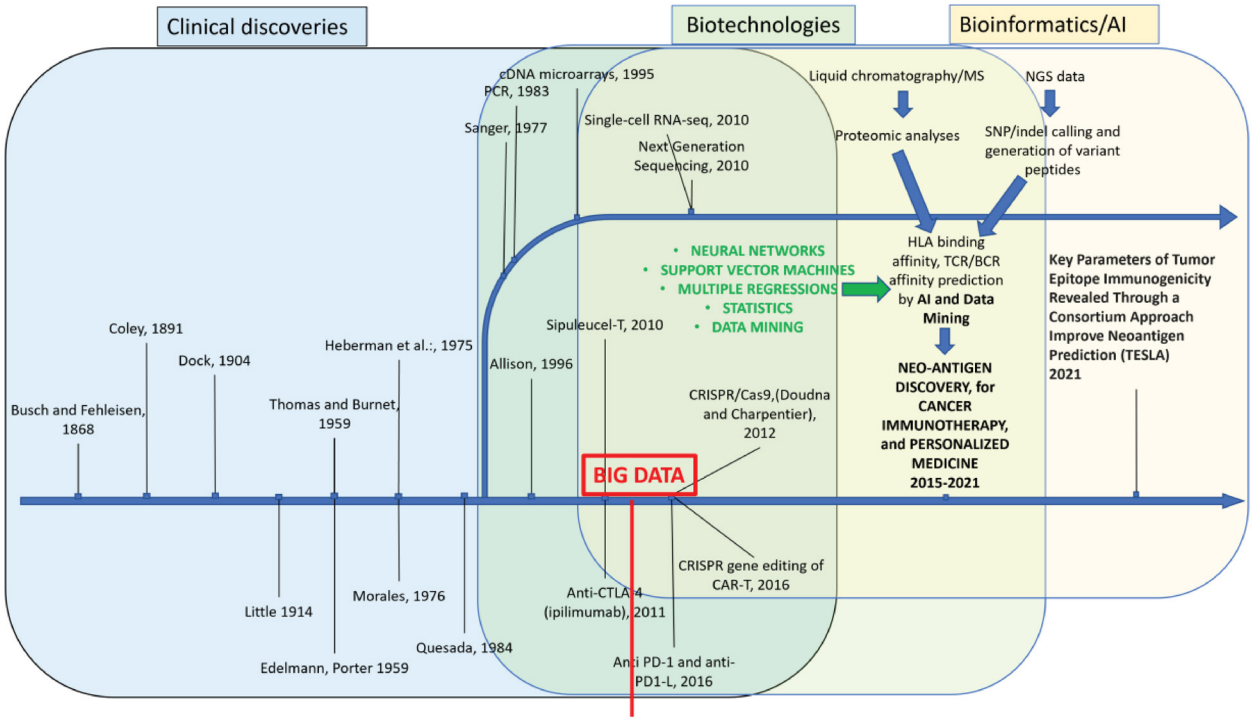
However, despite the promising clinical results obtained with neoantigen-based cancer vaccines and adoptive cell therapies to prime host immunity, the identification of novel neoepitopes suitable for these clinical applications was (and remains) cumbersome and labor-intensive. The lack of predictive methods to determine the somatic mutations in tumors and predict which neoepitopes will elicit a specific anti-tumor response represents a significant hurdle to obtaining effective cancer treatments [8,9]. Thus, the application of computational tools has been revealed to be crucial to expedite the research of neoantigens and neoepitopes suitable for immunotherapy [10].

Therefore, the last decades have been characterized by an incredible effort toward neoantigen and neopitope discovery whose development has been crucially interdependent with the growing production of Big

DOI: 10.18609/cgti.2021.195

FIGURE 1

The recent progress of cancer immunotherapy has resulted from an accelerated convergence of medicine, biotechnology, and information technology, respectively represented by three colored squares: blue square clinical discoveries, green square biotechnology, and yellow square bioinformatics/Artificial Intelligence.



Initially, the three disciplines appears to be well distinct. After 2011 (marked by a red line), immunotherapy has been subjected to exponential increase, triggered by the explosion of Big Data due to discoveries in the field of medical immunology. Clinical Discoveries Era: Busch and Fehleisen, 1868: first intentional infection of a cancer patient with erysipelas for the first time. Coley, 1891: first injection of heat-inactivated bacteria (Coley's toxin). Dock, 1904: first observation of a 42-years old woman in remission of leukemia after an episode of influenza infection. Little, 1914: discovering genetic basis of rejection in transplantable tumors. Thomas and Burnet, 1959: formulation of a Theory for Immune Surveillance in cancer. Edelmann a and Porter, 1959: chemical structure of antibodies (Nobel Prize). Heberman, 1975: first description of NK cells lysing tumor cells. Quesada, 1984: first report of interferon response in leukemia patients. Allison, 1996: discovery of CTLA-4 blocking antibodies in cancer treatment. Biotechnology Era: This era is characterized by a list of successes in developing autologous cell-based cancer vaccines. Sipuleucel-T, 2010: first autologous cell-based cancer vaccine FDA approved, in prostatic cancer. Ipilimumab, 2011: first immune-checkpoint inhibitor (IC) FDA approved in melanoma treatment. CRISPR/Cas9, 2012: Discovery of the CRISPR/Cas9 system, reported by JA Doudna and E Charpentier. Anti-PD-1, 2016: a third class of ICIs, PD-L1 (atezolizumab), is approved for the bladder cancer treatment. CRISPR gene editing of CAR-T, 2016: First test in humans of CRISPR gene-editing for CAR T-cell therapy. Bioinformatics/AI era: Artificial Intelligence (machine learning algorithms like Neural Network, Support Vector Machines, Multiple Regression, become pivotal in analyzing and integrating Big Data from biology and medicine. The peak of this integration happened recently, when genomics, proteomics (liquid chromatography/Mass Spec) of HLA-peptide binding characterization reached a high level. The NGS technology has produced a lot of data around probable peptides presented by the MHC-I/II system, based on tumor patient SNPs. The support of AI in screening patient-specific neo-antigens has accelerated innovation in Personalized Medicine. In 2020, a new Consortium based on the TESLA system had focused on defining critical parameters for Tumor Epitope Immunogenicity to improve Neoantigen Prediction (TESLA) [11,12].

Data through Next-Generation Sequencing. Indeed, quick and efficient identification of non-synonymous somatic mutations in cancer has been made possible by new bioinformatics tools to analyze large datasets [10]. Moreover, biotechnology advancements have converged with and generated the necessary information for the simultaneous rise of artificial intelligence (AI) applications for medical sciences.

AI (machine learning algorithms like Neural Network, Support Vector Machines, Multiple Regression) has thus become pivotal in analyzing and integrating Big Data from biology and medicine. The peak of this integration has been reached with the advancement of high accuracy proteogenomic methods aiming to directly measure the pool of peptides binding specific MHC complexes. The NGS technology has produced a lot of data

around probable peptides presented by the MHC-I/II system, based on tumor patient SNPs. The use of AIs to perform patient-specific neoantigen identification is critical component of the modern concept of Personalized Medicine in cancer treatment. A clear example of successful integration of all these findings is the first test in humans of CRISPR gene-editing for CAR T-cell therapy in 2016 following the game-changing discovery of the CRISPR/Cas9 system in 2012 by JA Doudna and E Charpentier (Figure 1) [11,12].

From this point in the timeline, the areas of Medicine, Biotechnology, and Bioinformatics with AI, dramatically started converging, producing the current vision of immunotherapy based on neoantigens characterization. This vision led, in 2020, to the establishment of a new Consortium based on the TESLA system that had focused on defining critical parameters for Tumor Epitope Immunogenicity to improve Neoantigen Prediction (TESLA) [11]. Today, the Consortium represents a vital reference point for computational scientists developing novel algorithms for Tumor Epitope Immunogenicity prediction (Figure 1).

THE ADVENT OF AI IN NEOEPITOPE IDENTIFICATION

As mentioned in the previous paragraph, the discovery of neoepitopes dates back to the advent of genome sequencing technology. The appropriate selection of neoepitopes for immunotherapy depends on the pattern of proteolysis of the antigenic proteins, the transport of peptides by the transporter associated with antigen processing (TAP), their binding to the histocompatibility complex (MHC), and their ability to trigger T-cell response.

Tumor antigens can be divided into self-antigens, expressed in healthy and cancer tissues and subjected to host tolerance in the thymus thymic regulation when utilized as immunotherapy targets, and non-self-antigens, or neoantigens. Cancer testis antigens [13] differentiate themselves for being self-antigens that present the advantage of low or null expression in healthy female tissues and protection of testicular tissues due to presence of the blood–testis barrier, a physical barrier between blood vessels and seminiferous tubules, which can hinder the passage of engineered immune cells. Testis antigens have thus been extensively investigated as targets for cancer immunotherapy. Unfortunately, not every tumor expresses such antigens, thus limiting the clinical application to only a selected pool of pathologies.

Compared with self-antigens, neoantigens have the advantage of escaping thymic regulation and being more tumor-specific, thus reducing the risk of on-target off-tumor toxicity. The major histocompatibility complexes' presentation of these tumor-specific neoantigens can trigger T-cell response [14], driving an immune response directed against cancer cells [5–7].

The immune system does not recognize cancer antigens as self; that's why they are defined as neoepitopes [15]. They can be presented by antigen-presenting cells, such as dendritic cells, or directly by the cancer cells and can drive the adaptive immune system to target cancer cells [13] selectively. Therefore, neoepitopes represent strong candidates for personalized cancer immunotherapy [8].

The last decade has seen intense efforts to generate novel and effective computational models to predict neoantigens and neoepitopes that could be effectively targeted in immunotherapy. Various software and *in silico* tools to predict immunogenic neoantigens have thus been generated, each with different strengths and weaknesses [16–18]. The majority of them are based on Machine Learning technologies, more broadly defined as artificial intelligence (AI).

With the term AI, we refer to a broader concept of machines (or algorithms) that can do tasks in a way that we would consider 'smart'. With the term 'Machine Learning' (ML), we mean a specific AI-based application can give algorithms access to learn for themselves. Thus, AI and ML are two definitions that wrap the same concept: they are often erroneously used interchangeably. But

DOI: 10.18609/cgti.2021.195

their distinction is around the idea of 'data accessibility'. Therefore, ML is a form of AI that enables a system to learn from data and not by explicit programming. Neural networks (NNs) are ML algorithms inspired by the biological neural networks of animal brains. At the same time, Deep learning (DL) represents a special implementation of NNs that embodies NNs in successive layers. DL is thought to emulate how the human brain works, so computers can be trained to deal with poorly defined abstractions and problems.

Most of the currently available algorithms predict the binding affinity to MHC [19–21], with more recent platforms providing data on gene expression, variant alleles, and clonal mutations. Some computational tools such as NetChop [22] can predict antigen processing patterns; others, such as NetCTL [23], can predict peptide transport. However, the modeling of peptides binding to MHC-I remains the main feature of most platforms.

Neural network algorithms (e.g., NetMHC [24]) can predict antigens that can form epitopes with the molecular fitting with MHC-I and include tools for molecular modeling simulation to predict amino acid orientation regarding the MHC binding site and strength of molecular interaction force. The Biomolecular Modeling algorithms are complicated because ~5,000 alleles encode MHC-I in humans, and each subject can express six of them. Experimental data from 50–100 measurements of peptide affinity for a specific MHC allele are initially required to achieve sufficient predictive accuracy in these models. Algorithms that consider allele structural similarities are being pursued to fill the experimental data gap for some alleles [25,26]. A universally accepted and well-validated computational platform for predicting neoantigens suitable for efficacious cancer therapies is thus paramount [27].

ADOPTIVE CELL THERAPY

Adoptive cell therapy (ACT) has been at the forefront of these efforts, with immune checkpoint inhibitors (ICI) and chimeric antigen receptor (CAR) immune cells being the most successful strategies in preclinical and clinical studies. By combining antibody-binding domains with the constant regions of the T-cell receptor (TCR), CAR can confer antibody-type specificity to lymphocytes [28]. The excellent results obtained with this approach led in 2017 to the FDA approval of the therapeutic use of CAR T cells for pediatric patients with relapsed B-cell acute lymphoblastic leukemia (Kymriah®) and then for the treatment of adult patients with relapsed or refractory large B-cell lymphoma (Yescarta®) [29]. Unfortunately, CAR technology has some critical limitations linked to the expression of target antigens on normal tissues, which can cause on-target off-tumor toxicity leading in turn to cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [30].

ANALYSIS PLATFORMS & AI: STATE OF THE ART

RNA-Seq has proven to be crucial to advance the identification of antigens for immunotherapy. Furthermore, the availability of publicly perusable proteogenomic repositories has allowed immunologists and cancer biologists to access large sets of data. In particular, the Cancer Genome Atlas (TCGA), a public program for the genomic profile of cancer that includes transcriptomics data from 33 human cancer types, and from the Genotype-Tissue Expression (GTEx), a public program to study tissue-specific gene expression that includes data for 54 healthy tissue sites provide unprecedented richness of resources. TCGA contains genomic and transcriptomic data for approximately 10,000 tumor samples and 726 tumor-adjacent tissues, while GTEx contains data for over 8,000 non-tumoral tissues. The predominance in the number of tumor samples could introduce a computational bias in differential analyses if data from the TCGA repository are only analyzed. Computational algorithms to compare data from TCGA with

data from GTEx can mitigate such bias risk as achieved by the Xena project of The University of California Santa Cruz (UCSC), where data from TCGA and GTEx were reprocessed based on a common pipeline. The most used visualization and analysis platforms available online for analyzing gene expression from TCGA and GTEx data are cBioPortal, Xena, Expression Atlas, and HPA.

Most of the *in silico* predictors available today, like NetCHOP [22] and NetMHCpan [31], are based on Machine Learning, a branch of AI relying on learning from experimental data [32]. Sometimes predictors present integrated frameworks, like FRED-2 [33] and pVAC-seq [33,34], that also offers the possibility to embed high-throughput DNA/RNA-seq data combined with functionalities of HLA genotyping [35], and like TIminer [36] that integrates RNA-seq data and somatic DNA alterations to characterize immune infiltrates and quantify tumor immunogenicity. Most of these tools can identify cancer-selective neoantigens and detect patterns of peptides with high MHC binding and can calculate the suitability, for a short list of peptides, to be antigenic [32,37].

The Immune Epitope Database (IEDB), a manually curated database of experimentally characterized immune epitopes, is complemented by the Immune Epitope Database Analysis Resource [38]. This site provides a collection of tools for the prediction of IC50 values for peptides binding to specific MHC molecules, prediction of epitope candidates based upon the processing of peptides in the cell, prediction of B-cell epitopes, and the detailed analysis of a known epitope sequence or group of sequences including the HLA coverage in the human population.

The Waikato Environment for Knowledge Analysis (WEKA) is an open-source machine learning project with a wide range of algorithms for data mining, comprehensive packages for pre-processing, classification, regression, clustering, association rules, and visualization of epitopes [39]. For example, the tool WEKA Explorer can analyze epitope datasets to build prediction models for B cells [39].

Few quantitative modeling methods exist to predict with accuracy the binding affinities between peptides and MHC molecules: SVRMHC method is based on support vector machine regression (SVR) [40]. DeepHLApan [41] is a deep-learning-based approach to predicting neoantigens, considering binding peptides to MHC and immunogenicity.

The immune-dominant epitopes for specific HLA are identified with online platforms such as NetCTL-Pan, NetCTL-Chop, NetCTL, Antigen, NetCTLIIPan, and EPIMHC50.

As previously discussed, these are methods that utilize models driven by experimental peptide-MHC binding data. Machine learning is necessary to generate accurate predictive models. The system is trained with a list of binding peptides and non-binding peptides to a specific HLA allele. Artificial neural networks (ANNs) are the most significant example of such machine learning prediction models.

Kiromic Biopharma, Inc has developed DIAMOND, an artificial intelligence and cognitive machine, and deep learning platform that can mine large genomic and proteomic datasets contained both in Kiromic proprietary clinical libraries and in public data repositories such as the Human Protein ATLAS, TCGA, and GEO [42]. DIAMOND is an Immunotherapy Builder System Platform (IBSP). IBSP is a specifically designed suite of programs that can facilitate '*in silico*' discovery of novel disease-associated targets and numerous narrow targets to a significantly smaller subset with high immunogenic potential. Thereby, DIAMOND was uniquely designed to facilitate the resource-efficient development of novel immunotherapies.

DIAMOND presents as an integrated system designed to tackle whether a predicted epitope can be used for specific therapeutic aims. Whereas the other platforms are often limited to specific research tasks, DIAMOND comprehensively integrates databases and prediction scores for clinical purposes, indicating straightforward solutions and quick strategies for immunotherapy.

DOI: 10.18609/cgti.2021.195

Disease-associated targets (e.g., disease-associated amino acid sequence variants) can be considered disease-associated antigens that have immunogenic potential, according to the assessment of major histocompatibility complex (MHC) interaction or T-cell receptor (TCR) interaction and B-cell receptor (BCR) interaction. Tumor somatic driver mutations are directly linked to or cause the transformation process in cancer. Also, sporadic mutations associated with the genetic instability of rapidly expanding tumor cells are associated with 'passenger' neoantigens. Such antigens have great potential for immunotherapy because these neo-antigens and neo-epitopes are not 'protected' by thymic selection and central tolerance [42].

For this reason, the DIAMOND platform consists of multiple modules. These modules can narrow many amino acid sequences in an input amino acid sequence to a subset identified with immunogenic potential.

In detail, the DIAMOND system encompasses four primary modules:

1. A Differential Expression Module (DEM);
2. An MHC Allele Affinity Determination Module (MAAM);
3. A T-Cell Receptor Immunogenicity Determination Module (TIM); and
4. A B-Cell Receptor Epitope Determination Module (BEM).

With its DEM module, DIAMOND can identify the tumor-selective overexpression of specific genes and analyze their distribution and methylation across entire datasets from clinical studies. A novel capsule artificial neural network provides the additional feature of mapping the exact gene portion able to elicit an immune response, making DIAMOND a flexible tool able to expedite neoepitope discovery for immunotherapy.

While the DEM works on the characterization of the epitope expression at the gene level, MAAM, TIM, and BEM produce evidence of MHC-1/II presentation and T/B cell affinity of the selected epitopes. Moreover, DIAMOND includes a fifth dedicated module, a Sequence Acquisition Interface (SAI), which acquires an amino acid sequence from a gene of interest. MAAM, TIM, and BEM receive the amino acid sequence from SAI for processing.

For the B-Cell Receptor Epitope Determination Module (BEM), DIAMOND embodies BepiPred-2.0, a random forest algorithm trained on epitopes from antibody-antigen 3D structures and linear epitopes available in the IEDB data repository.

For the T-Cell Receptor Immunogenicity Determination Module (TIM), DIAMOND is based on NetCTL, a computational approach to CTL epitope prediction, integrating MHC-I binding, TAP transport efficiency, and proteasomal cleavage predictions.

Finally, for the MHC Allele Affinity Determination Module (MAAM), DIAMOND uses MHCflurry 2.0, an OpenSource Python 3.4+ library that uses the Keras neural network library via the Tensorflow or Theano backends.

Although the various algorithms used in DIAMOND can result in redundancies, the reference associated with MHCflurry reports evidence that this Python library was tested in benchmarks reported by O'Donnell *et al.* in their paper, the MHCflurry performances were analyzed in MONOALLELIC and MULTIALLEIC benchmarks together with NetMHCpan and other AP models. The algorithm, therefore, follows the STAR methods, as reported by the authors. Moreover, MHCflurry has been reported as one of the approaches encompassed by the TESLA Consortium.

The Kiromic Genomic Research Application (KGRA) utilizes a database of over 1.5 billion records from clinical samples of diseases such as cancer, metabolic syndromes, infectious diseases, and autoimmune disorders. By using the reprocessed data produced by the UCSC Zena group's TOIL pipeline, DIAMOND is able to perform comparative analysis of the expression of gene isoforms across the heterogeneous data sources: TCGA, TARGET [43], and GTEx [44]. More in detail,

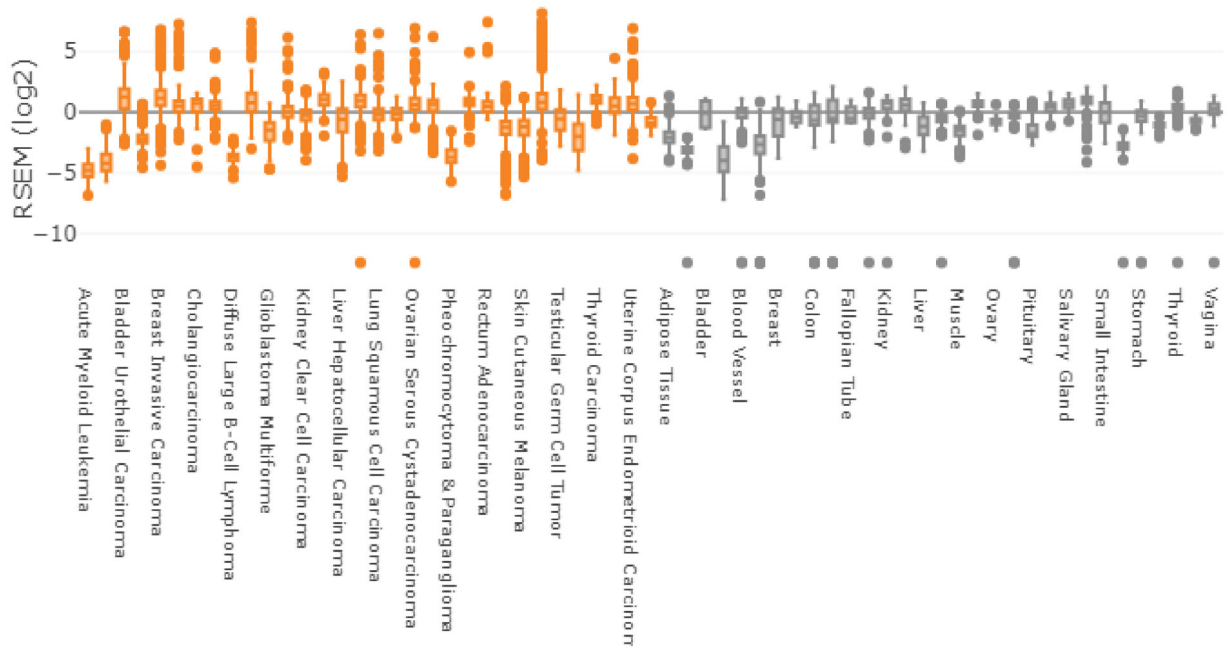
the RNA seq CB pipeline from Memorial Sloan Kettering data is utilized to reprocess raw RNA-Seq data. STAR [45] is then used to align them, RSEM and FeatureCounts [46] to quantify gene expression, mRIN [47] to evaluate sample degradation, RSeQC [48] to measure the strandness and quality of samples, and SVAseq [49] to correct batch biases. The results of the meta- and convolution analyses and of the standardization and normalization of multiple experimental datasets, including RNA-seq and microarrays of healthy and disease tissues, are summarized in clear, user-friendly graphic outputs (Figure 2).

Comparing prediction output from computational algorithms to experimental biology

FIGURE 2

DIAMOND analysis of *ERBB2* gene expression in tumor and normal tissues.

A



B

Category	Expression Mean	Expression Var	▼Difference from Referer	Corrected P Value	Cohen's D	Effect Size
Breast Invasive Carcinom	13.76184	2.21118	1.37043	** < 0.00001	0.83982	Large
Bladder Urothelial Carcinc	13.58318	1.99628	1.19177	** < 0.00001	0.74553	Medium
Stomach Adenocarcinoma	13.4659	2.68761	1.07449	** < 0.00001	0.63085	Medium
Esophageal Carcinoma	13.40473	2.58588	1.01332	** < 0.00001	0.60022	Medium
Thyroid Carcinoma	13.40298	0.29466	1.01157	** < 0.00001	0.7748	Medium
Kidney Papillary Cell Carc	13.39561	0.46123	1.0042	** < 0.00001	0.75103	Medium
Skin	13.32326	0.30168	0.93185	** < 0.00001	0.713	Medium
Lung Adenocarcinoma	13.27258	1.22255	0.88117	** < 0.00001	0.59838	Medium
Uterine Corpus Endometri	13.21198	1.6308	0.82057	** < 0.00001	0.53272	Medium

Data were extracted and analyzed with DIAMOND from datasets of accurate transcript quantification from RNA-Seq data. Data from TCGA and GTEx were integrated for visualization of gene expression across multiple diseases and cancers. To do so, RNA-Seq data were reprocessed from raw sequencing reads using the RNAseqCB pipeline from Memorial Sloan Kettering. (A) DIAMOND visualization of gene expression data with tumors represented in yellow and normal tissues in grey. As reported in the literature, an upregulation in various cancer tissues, including melanoma and urothelial carcinoma, is visible here. (B) Screenshot of the table generated by DIAMOND showing that the highest significant differential expression for ERBB2 is observed in invasive breast carcinoma.

DOI: 10.18609/cgti.2021.195

TABLE 1

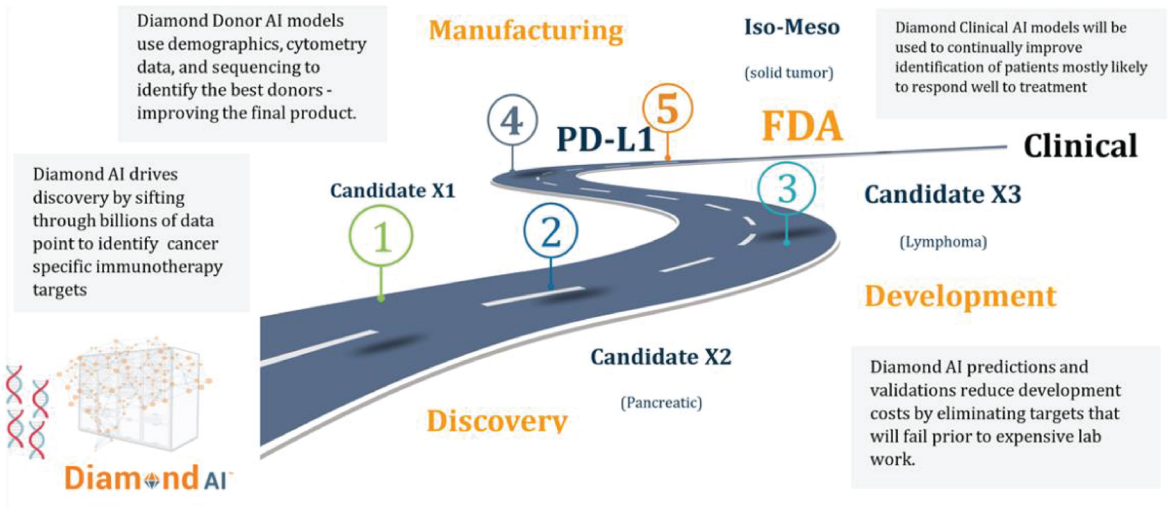
Comparison of IC50 and affinity, presentation, and processing predicted scores.

Protein	Peptide	HLA	Measured IC50 (nM)	DIAMOND IC50 (nM)*	DIAMOND-Affinity	Presentation Score	Processing Score	NetMHCpan4.1 IC50 (nM)	NetMHCcons IC50 (nM)	SMM/IEDB IC50 (nM)
HER-2 (Homo sapiens)	KIFGSLAFL	HLA-A*02.01	33.3	14.062	Strong	0.859	0.055	10.76 <= SB	15.2	64.14
HER-2 (Homo sapiens)	KIFGSLAFL	HLA-A*68.02	3333	293.263	Strong	0.241	0.055	Missing allele	1384.4	1001.36
Influenza matrix protein M1 peptide	GILGFVFTL	HLA-A*02.01	1200	19.658	Strong	0.849	0.12	7.27 <= SB	13.49	48.65
OVA (Gallus gallus)	SIINFEKL	HLA-A*02.01	No binding	5106.719	Weak	0.113	0.547	15140.42	2483.17	76950.24
NY-ESO-1 (Homo sapiens)	SLLMWITQC	HLA-A*02.01	13487	247.331	Strong	0.258	0.036	422.15 <= WB	608.33	270.46

[51–55].

FIGURE 3

Diamond AI drives discovery by sifting through billions of data points to identify cancer-specific immunotherapy targets.



Diamond AI predictions and validations reduce development costs by eliminating targets that fail before expensive lab work. Diamond Clinical AI models will continually improve the identification of patients most likely to respond well to treatment.

data is paramount for validating predictive informatics tools. The parallelism observed by comparing DIAMOND predictions with experimental data of expression and immunogenicity of well-characterized immunotherapy antigens thus supports the platform's reliability.

In Table 1 [50–54] algorithm predictions of IC50 are compared to wet lab data for a set of well characterized peptides already utilized in clinical settings. Receptor tyrosine-protein kinase HER2 is a growth-promoting 185 kDa transmembrane glycoprotein encoded by the *ERBB2* gene and is a well-described target for cancer immunotherapy. Cytotoxic T lymphocytes specific for HER-2 HLA-A2 binding peptide p369–377 (KIFGSLAFL) can target HER-2 overexpressing breast cancer cells; this epitope has thus been used in many vaccines [55]. As shown in Figure 2, DIAMOND meta-analyses of expression data displayed a significant ($p < 0.00001$) upregulation in various cancer tissues compare to normal is computed with the highest differential expression observed in invasive breast carcinoma and bladder urothelial carcinoma. Moreover, a strong binding of the peptide HER-2 p369–377 (KIFGSLAFL) to HLA-A2 is predicted, in accordance to experimental data [55].

Similarly, data reported in the literature correlate with the computational analysis of gene expression and immunogenicity predictions for New York Esophageal Squamous Cell Carcinoma 1 (NY-ESO-1), a cancer-testis antigen encoded by gene *CTAG1B*. DIAMOND was able to calculate *CTAG1B* gene overexpression in cutaneous skin melanoma, lung adenocarcinoma, and sarcoma. Moreover, the epitope 157-165, successfully targeted in clinical trials for patients with HLA-A*02:01, was predicted to have an MHC binding affinity of 0.289 and a prediction score of 0.4456 for Supertype A2.

In conclusion, our system effectively combined predictions of peptide processing, HLA binding, and T-cell activation. This is crucial to avoid high false-positive rates, which are often a problem of current prediction methods, which often cannot discriminate peptides

DOI: 10.18609/cgti.2021.195

presented by target cells but are not efficient in triggering a T-cell response.

Predictive AI algorithms accelerate the development of new clinical translational therapies (Figure 3). The ability to single out tumor specific targets is pivotal to direct the innate and adaptive immune system against tumor tissues while dramatically reducing side effects for the host. This discovery process is complex and the quantity of quantity of suitable targets is limited. The state of the art of target discovery and prioritization for anti-tumoral cell-mediated responses is negligible when compared with the field of infectious diseases. Hence, the demand for novel methods to uncover novel neoantigens and neoepitopes.

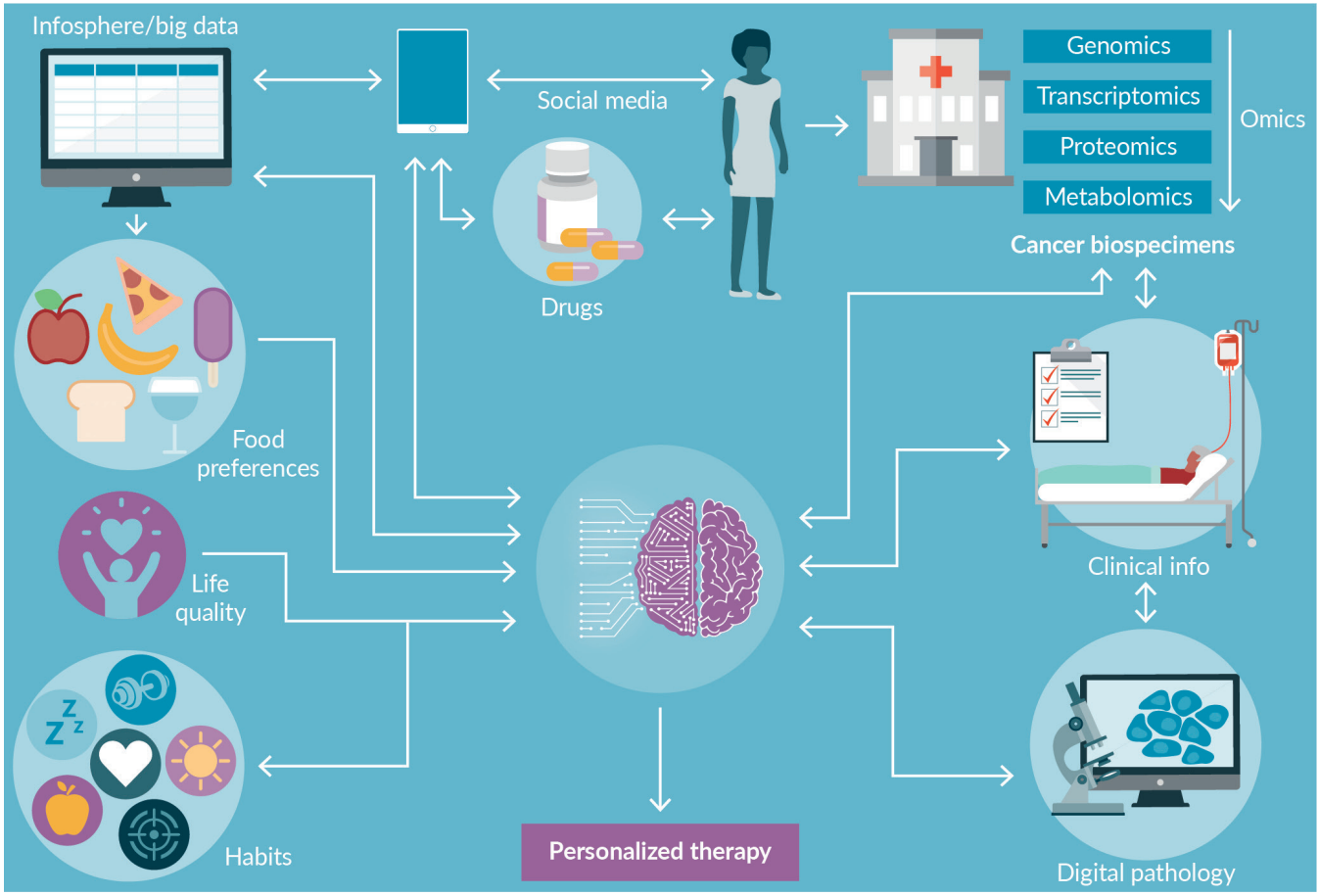
The capacity to further expand these methods to a wide range of different targets for cancers of different origins, mainly solid tumors, is crucial for developing novel cancer cell therapies. Consequently, employing innovative systems to recognize and authenticate cancer-specific antigens will lead to faster and more dynamic clinical development for cancer patients' treatment.

CONCLUSIONS

In the future, AI will optimize and personalize immunotherapy by integrating the patient biospecimen's OMICs. Genomics/

FIGURE 4

The future of AI in immunotherapy.



AI in immunotherapy will permit integration of the cancer biospecimen results (encompassing Genomics, Transcriptomics, and Metabolomics data) with BigData, encompassing different levels of information, such as drugs, food preferences, patient's life quality, and habits. This kind of 'super' integration, will require specific algorithm features, like the possibility of facing an internal AI dataset with web-oriented technology, (computers, phones, and any kind of personal devices) This information will be integrated with clinical data from the Hospital, or other external sources of information,(i.e.: Digital Pathology).

transcriptomics will characterize the patient biospecimen through the production of high throughput data.

Thanks to Machine Learning, it will be possible to integrate heterogeneous cancer multi-omics data such as somatic copy number aberrations (CNA), messenger RNA (mRNA) expressions, and clinical data of patients diagnosed with cancer [56]. Merging different information levels into different architectures will lead to deep analyses and comparisons concerning specific data features.

Immunotherapy is based on neoantigen prediction, which means that it will be possible to analyze the major histocompatibility complex (MHC)-eluted peptides by mass-spectrometric (MS) through the patient biospecimen lysis. The pMHC complexes will be captured, and peptides will be purified using MHC-specific immobilized antibodies through immunoaffinity purification.

By the Single-cell RNA sequencing (SC-RNA), it will be possible to characterize, for each patient, the Tumor Micro Environment (TME), which has a pivotal role in cancer. Indeed, its determination will enrich the AI's input datasets, permitting a better classification of the tumor environment and significant success in personalizing the therapy.

In particular, the TME's stroma promotes tumor formation and progression, which are influenced by the surrounding signals. Cell–cell and paracrine interactions between cancer-associated fibroblasts (CAFs) and cancer cells are involved in programming the stroma. The SC-RNA sequencing will also be fundamental in defining the major histocompatibility complex (MHC) class I antigen presentation pathway for peptides recognized by $CD8^+$ cytotoxic T cells.

Data mining will permit integration of the cancer biospecimen with the BigData and social media, encompassing different levels of information, such as drugs, food preferences, patient's life quality, and habits. This information will be integrated with clinical data from various sources of information (i.e. Digital Pathology). This kind of 'super' integration will require specific algorithm features, like the possibility of facing an internal AI dataset with web-oriented technology (computers, phones, and any personal devices) (Figure 4).

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DOI: 10.18609/cgti.2021.195

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *The authors thank Teri Fields for her grammatical and orthographical evaluation of the manuscript.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *This research received internal funding from Kiromic R&D.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 21 2021;* ***Revised manuscript received:*** *Oct 27 2021;* ***Publication date:*** *Nov 25 2021.*

OCTOBER 2021 Volume 7, Issue 10

CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

FASTFACTS

PATfix™ pDNA platform for monitoring pDNA purification process

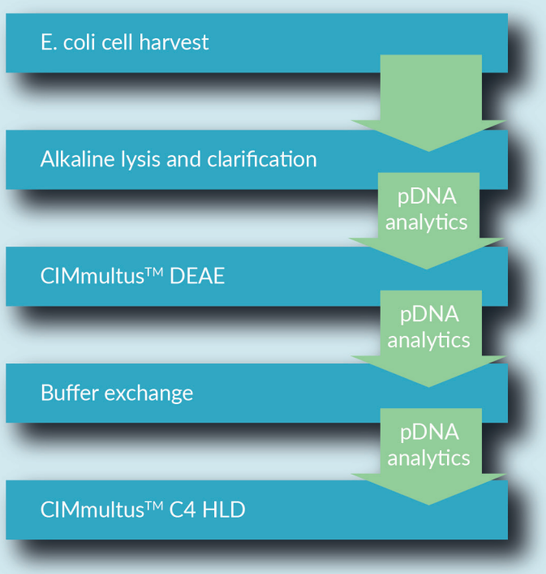
Nejc Pavlin, Project Manager in Process Analytics Development, BIA Separations, now a Sartorius company

PATfix™ HPLC system is a new platform with a variety of upstream and downstream applications.
This poster focuses on the use of a specialized PATfix™ platform for rapid, at-line analysis of plasmid (p)DNA during purification.

Cell & Gene Therapy Insights 2021; 7(10), 1437; DOI: 10.18609/cgti.2021.190

pDNA, as an enabling product, is critical in the production of mRNA, AAV, and other therapeutic vectors. Increasing yield and purity in the production of pDNA is a vital step in meeting demand for therapeutic vectors. Supporting rapid process development and optimization, PATfix™ pDNA analytical platform provides reliable in-process control for pDNA process development and production.

Figure 1. pDNA purification scheme.



PATfix™ pDNA platform is optimized for pDNA analytics and allows users to perform analytics in a rapid at-line fashion, while minimizing the requirement for skilled operators. This is made possible by built-in analytical methods and customized software with easy-to-follow user guides. A complementary, certified pDNA standard streamlines the identification and quantification of bioprocess species.

pDNA PURIFICATION CONTROL USING PATfix™ pDNA PLATFORM

Typical purification of pDNA using monolithic columns from CIMmultus™ line, consists of alkaline lysis and clarification, capturing on CIMmultus™ DEAE column, buffer exchange, and polishing of pDNA on CIMmultus C4 HLD column **(Figure 1)**.

Each step of the purification process can be qualitatively and quantitatively monitored by employing a fast and high-throughput PATfix™ HPLC system, equipped with CIMac™ pDNA column, and certified pFix5 plasmid standard produced by BIA Separations.

CASE STUDY

The built-in pDNA analytical method performed on the CIMac™ pDNA column allows us to efficiently monitor the pDNA content in the harvest, optimize the conditions of alkaline lysis, and ensure the best possible extraction of plasmid pDNA from the cellular harvest **(Figure 2A)**.

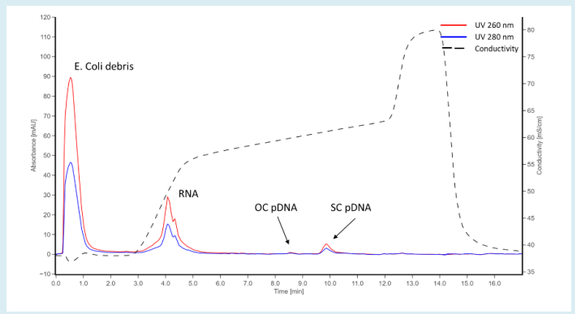
With the ability to separate pDNA from impurities (RNA, E. coli debris, etc.) in the sample, the CIMac™ pDNA column enables us to control the production of pDNA and follow the reduction of impurities through the purification process **(Figure 2B & 2C)**. In addition, its high selectivity clearly distinguishes between different pDNA isoforms, such as open circular plasmid (OC) and supercoiled plasmid (SC) **(Figure 2B & 2C)**.

CONCLUSION

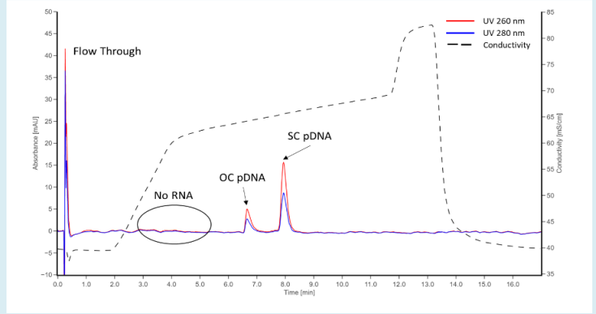
The PATfix™ pDNA platform offers a fast and efficient solution for problems related to pDNA upstream and downstream processes. Using the platform, clients can optimize pDNA production and purification procedures according to their needs, increasing its yield and purity, and reducing cost of production.

Figure 2. Analytical chromatograms using PATfix™ pDNA platform showing the composition of samples in different stages of pDNA purification: A) neutralized alkaline lysate, B) DEAE plasmid elution, and C) C4 HLD plasmid elution.

A



B



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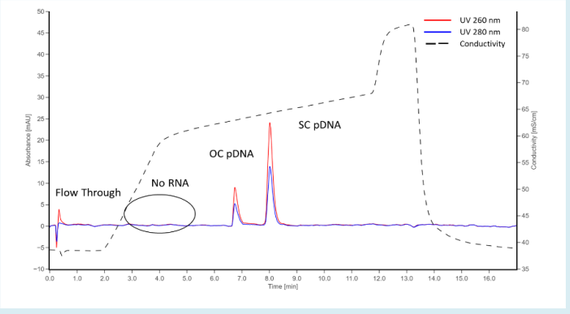


FASTFACTS

Improving the enrichment of mononuclear cells for CAR T cell therapies: the importance of platelet depletion

Isabelle Dalle Fusine, Product Marketing Manager for Digital and Automation, Cytiva

Enriching mononuclear cells by removing other cell types and contaminants is a critical step in CAR T cell therapy production. This poster explains why, how, and when you should carry out platelet depletion to achieve successful T cell enrichment.

To produce CAR T cell therapies, scientists start by collecting leukapheresis products that contain peripheral blood mononuclear cells (MNCs). They then remove all other cell types to enrich the T cells. In autologous therapies, variability in the source material can affect performance.

Removing extraneous cells at the beginning of the process helps scientists to reduce the variability of leukapheresis products between individual patients, and standardize the development of very complex therapies.

WHY REMOVE RED BLOOD CELLS AND PLATELETS?

Leukapheresis product contains two cell groups: MNCs and non-MNCs. Scientists remove all non-MNCs, including red blood cells (RBCs), granulocytes, and platelets.

Regulators consider RBCs an impurity — it's essential to deplete enough RBCs to avoid contamination and produce CAR T cells successfully **(Figure 1)**.

Additionally, scientists must remove platelets from the product to avoid the risk of activating platelets. Activation can be triggered by mechanical force, changes in temperature and cell density, and engagement of platelet receptors — all events that can occur later during processing or storage. Activated platelets cause cell aggregation, clump formation, and changes to cell density, all of which can affect the efficiency of your production **(Figure 2)**.

HOW AND WHEN?

Today, the most common way to enrich MNCs is to remove RBCs using density gradient medium, and to follow up with washing steps to deplete any remaining platelets. However, washing steps can activate remaining platelets, reducing monocyte viability and compromising RBC removal **(Figure 3A)**.

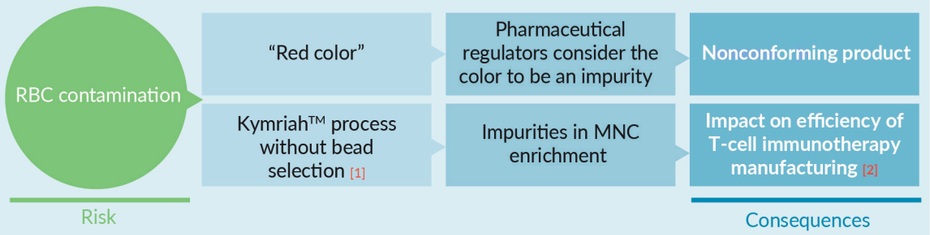
We developed an alternate process to address this challenge. We begin with a washing step to remove platelets, followed by density gradient medium separation. This configuration reduces the risk of platelet activation and RBC contamination **(Figure 3B)**.

To further reduce the risks associated with MNC enrichment, scientists can use Sepax™ C-Pro or Sefia™ systems to automate their process, and make production safer, faster, and less labor-intensive.

CONCLUSION

When performed successfully, the MNC enrichment step helps to reduce donor cell content variability and standardize autologous CAR T processing. We recommend removing platelets first and then RBCs — this sequence reduces RBC contamination and protects product performance. In addition, working with a closed and automated system can minimize other potential risks including contamination, operator variability, and errors, while reducing procedure time and improving efficiency.

Figure 1. Consequences of RBC contamination.



MNC: Mononuclear cells. RBC: Red blood cells.

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Figure 2. Reasons to remove platelets.

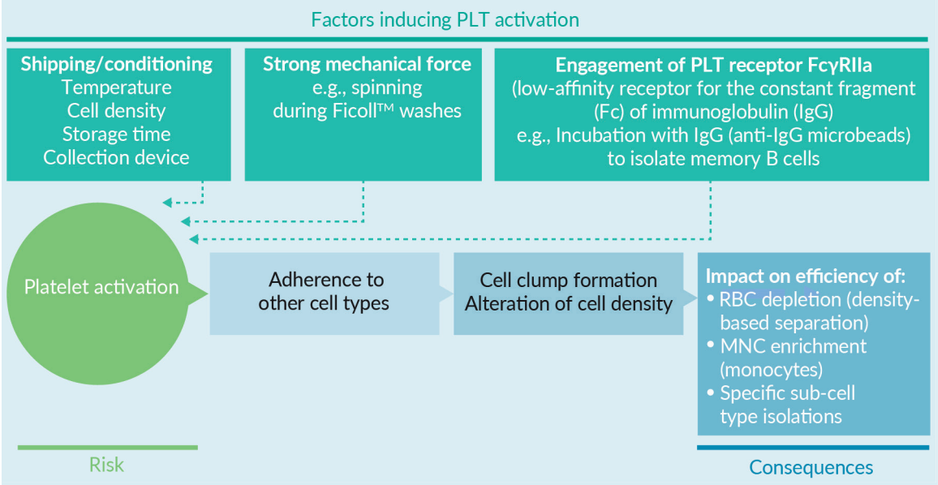

Figure 3. MNC after density gradient medium separation before (A) or after (B) platelet depletion.

A

B

CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2021; 7(10), 1275; DOI: 10.18609/cgti.2021.170

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Midstream unit operations: unsung heroes in AAV process development

Ratish Krishnan & Matthew Roach

Although the quest for a templated process to accelerate the race to commercialization for cell and gene therapies has largely remained elusive, we are starting to see scientific data from process development experts. One underappreciated area of process development is midstream unit operations, which consist of steps such as cell lysis, DNA digestion, and clarification. Here, an example of a fruitful collaboration between Merck and Precision BioSciences to develop an integrated approach to an adeno-associated virus (AAV) harvest step will be presented, with key takeaways that can be implemented in everyday process development.

Cell & Gene Therapy Insights 2021; 7(10), 1309–1321

DOI: 10.18609/cgti.2021.173

This article will focus on cell lysis, nuclease digestion, and clarification steps. Lysis is commonly practiced in two main ways – physical or chemical – with each having its unique advantages and disadvantages. The main drawback of physical lysis is the high capital expenditure and possible thermal degradation of the virus. Chemical lysis using detergents such as TWEEN® and Triton® is the most common method. Despite the need to demonstrate the removal of these added detergents in the process, they remain popular since they are convenient, scalable, and can be cost-effective.

The lysis step is typically followed by nuclease digestion to eliminate residual DNA. Benzonase® endonuclease is often employed in the viral vector space and is cited in many journals and biological license applications. The enzyme breaks down virus-nucleic acid complexes, reduces the viscosity of process intermediates, and prevents fouling of downstream equipment. It is easily removed in subsequent

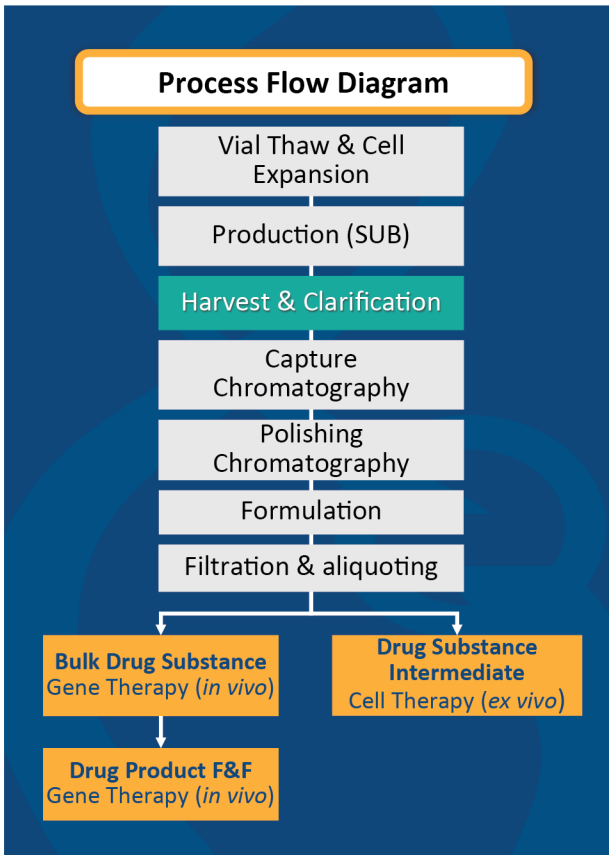
steps and has been used in several clinical trials and commercialized products, thereby assuring patient safety. One unit of the enzyme degrades approximately 37 µg of DNA in 30 minutes to as low as 3–8 base pairs, or less than 6 kDa. With the launch of Benzonase® endonuclease Safety Plus, the enzyme is completely animal-origin free, tracking back to upstream raw materials used in the fermentation process.

The workhorse of midstream is undoubtedly the clarification filters used in depth filtration. Filtration is typically carried out in two steps; primary filtration to remove large particles and secondary filtration for the removal of colloids and other sub-micron particles.

In this article, we will highlight data from a collaboration between Merck and clinical-stage biotech company Precision BioSciences to optimize these midstream unit operations and improve yield.

FIGURE 1

Overview of AAV production process.



INTRODUCING PRECISION BIOSCIENCES

Precision BioSciences is a clinical-stage biotech with pipelines for both allogeneic CAR T and *in vivo* gene editing, based in Durham, North Carolina. Their gene-editing technology, ARCUS®, is based on a naturally occurring genome-editing enzyme called a homing endonuclease. Having observed some of the limitations of other gene-editing technologies, Precision BioSciences looked to nature for better specificity, accuracy, and versatility, starting with the natural enzyme I-CreI from algae and reprogramming it to target new genetic sites.

With the ARCUS® technology, Precision BioSciences set out to target two main areas, the first being permanent correction of genetic disease with *in vivo* editing, and the second being the optimization of allogeneic CAR T cells for deep and durable responses to cancer. The company is currently in clinical trials for multiple cell therapy programs targeted towards a number of lymphomas. It also has a robust *in vivo* gene editing pipeline.

Both *in vivo* gene editing and CAR T cell applications utilize AAV, so Precision BioSciences has developed internal capabilities and knowledge around AAV production and the eventual manufacturing process.

DEVELOPING A CLARIFICATION PROCESS

AAV is unique when compared to traditional biologics. Production processes typically require the addition of plasmid or virus to deliver the genes required for AAV production, and the removal of process-related impurities is even more critical as the ratio of impurities to product is higher than traditional biologics such as monoclonal antibodies.

With this in mind, Precision BioSciences set out to develop its harvesting clarification process with three primary goals. First, to ensure a high viral genome recovery throughout the harvest process by reducing host cell debris and contaminants. Second, to ensure it is a

DOI: 10.18609/cgti.2021.173

platform block that can be used for many different AAV serotypes, to target different tissues. Lastly, to lower the cost of goods and complexity of the process, specifically by reducing the total filtration area required for a given batch and the number of types of filters needed for the filtration. Figure 1 gives an overview of the harvesting process, including lysis with endonuclease treatment and depth filtration.

Here, we will focus on the results from the development of Precision's platform depth filtration step for AAV clarification. TWEEN® 20 was used to lyse cells and release intracellular AAV, followed by endonuclease treatment to reduce contaminating host cell DNA, depth filtration, and 0.2-µm filtration to remove additional host cell debris.

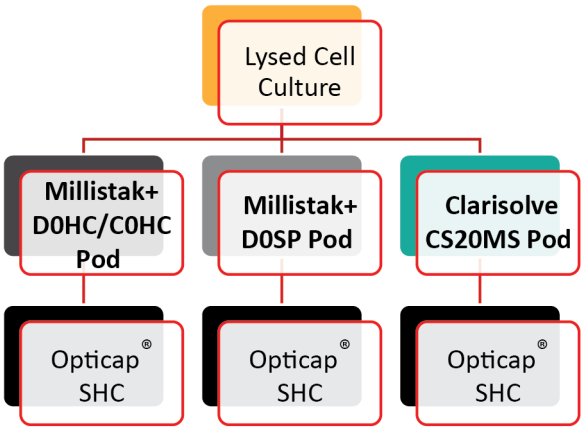
DEPTH FILTRATION SCREENING STUDY

Based on the data mining summary provided by Merck, and with the support of Technical and Scientific Solutions team, a limited number of depth filters for filtration of lysed cell culture were screened with Precision BioSciences. One of the benefits for Precision BioSciences in their collaboration with Merck was the ability to rely on Merck's expertise about their products. For example, Merck scientists were able to rule out filters that were unlikely to work for the process based on their prior experience with AAV production.

We took lysed cell culture through three different depth filtration trains, one D0HC and C0HC, one with D0SP, and one with CS20MS – all followed by the Opticap® SHC filter from Merck (Figure 2).

FIGURE 2

Screening of Millistak+® HC Pod Depth Filters, Millistak+® HC Pro Pod Depth Filter, and Clarisolve® Depth Filter.



D0HC *et al.* level is "depth filtration".

The Millistak+® HC Pro Depth Filter utilizing the D0SP media series was selected for further testing and scale-up as it achieved the highest throughput in the depth filtration screening study (Table 1). Additionally, it provided high viral genome and viral particle recovery and was capable of producing a stable clarified lysate, monitored via turbidity measurements. We saw viral genome recovery and viral particle recovery above 100% in this experiment. This level of variance was within the normal observed range for both assays at this time of analytical development. Interestingly, the Millistak+® D0SP pod depth filter was able to provide throughput much greater than the Clarisolve® 20 and Millistak+® D0HC/C0HC filtration trains, although all performed well enough to scale. Due to time constraints, further process development was performed with just the Millistak+® D0SP filtration train.

TABLE 1

Key results from the depth filtration screening study.

Filter	Throughput (L/m²)	Viral genome recovery (%)	Viral particle recovery (%)	Stability of the clarified lysate
Millistak+® D0HC/C0HC Pod depth filter	179	111%	120%	3 days
Millistak+® D0SP Pod depth filter	444	100%	109%	7 days
Clarisolve® CS20MS Pod depth filter	218	101%	124%	3 days

FIGURE 3

Throughput with increasing pressure for three pods from Merck.

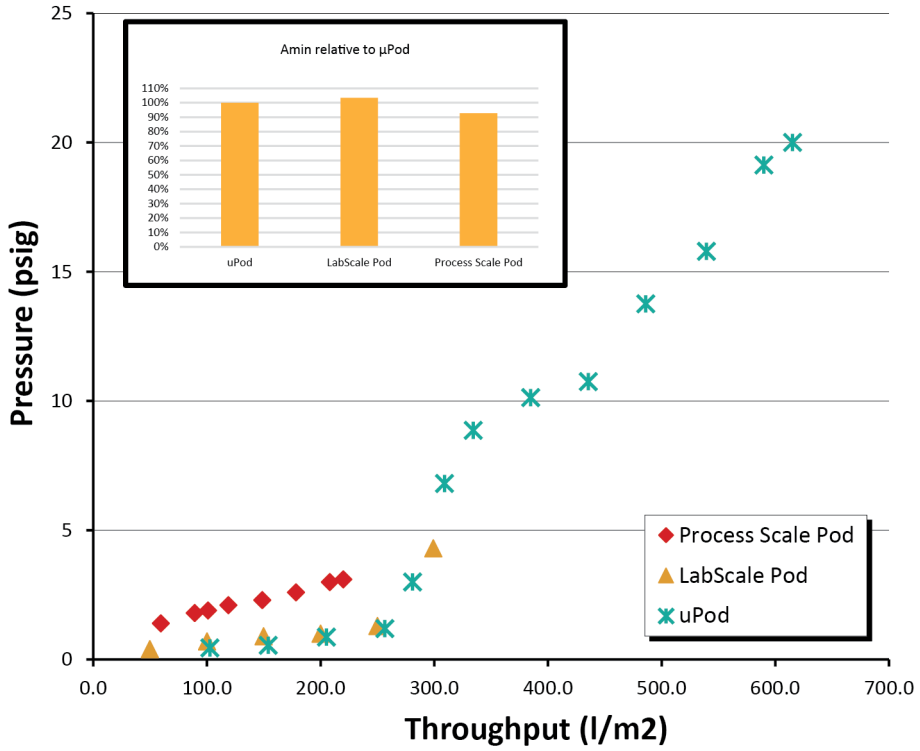
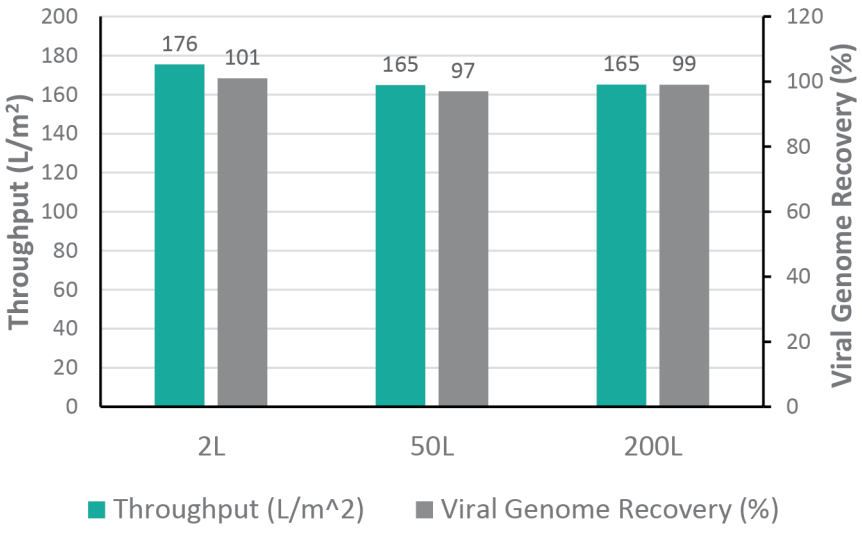


FIGURE 4

Throughput and recovery results across scale.



DOI: 10.18609/cgti.2021.173

SCALE-UP BASED ON SELECTION FROM DEPTH FILTER SCREENING

Merck has a range of depth filtration offerings across various process scales. Figure 3 shows pressure as throughput increases across each of the pod depth filter offerings. This case study with AAV gave Precision BioSciences confidence that the process could be effectively scaled up. Additionally, the pilot and process pod depth filtration system minimize bioburden risk through a single-use flow path and the ability to sterilize the pod with an autoclave.

After a lead depth filter was chosen, the Millistak+® D0SP filtration train was scaled up to 2L, 50L, and 200L, while monitoring the throughput and viral genome recovery. At a near-constant throughput, very similar viral genome recoveries were achieved across scale-up, indicating that the case study conducted earlier by Merck was accurate (Figure 4).

One of the original goals was to ensure that the selected depth filtration train was capable of clarifying feed streams effectively, even when challenged with a new AAV serotype. After selecting a lead depth filter, the Millistak+® D0SP and OptiCap® SHC (sterile, high capacity) filtration train was advanced with 2-liter bench-scale runs of four different AAV serotypes carrying the same transgene, to confirm that the clarification train worked as a platform. Figure 5 details the throughput and viral genome recovery for each of these filtrations. Notably, even when the filtration train was challenged with a higher throughput, all serotypes achieved high viral genome recovery utilizing the Millistak+® D0SP and Opticap® SHC filtration train, with viral genome recovery at 84–96%.

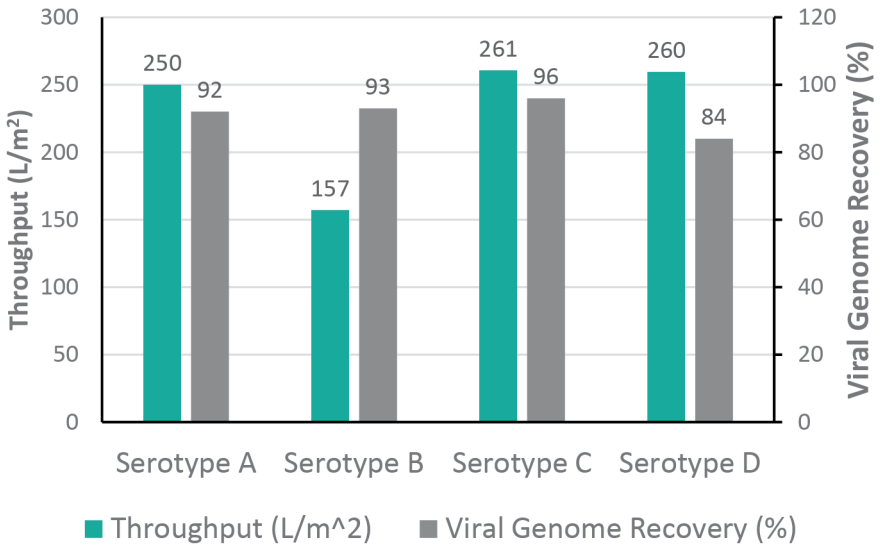
CONCLUSIONS

These data show how collaboration between process and product experts can help solve problems in bioprocessing. Key takeaway messages from the study include:

- Focus on DOE approaches for optimization: it is very important to screen a variety of filters to data-mine the best fit for your process;
- All decisions should be based on the foundation of data;

FIGURE 5

Bench-scale throughput and recovery results across serotypes.



- Remember that adjacent unit operations impact clarification;
- Begin with the end in mind: think about scale-up from the start;
- A platform approach is possible: unit operations could be templated for other programs with additional optimization work;
- Moving from two-stage to single-stage filtration lowers the cost of goods and simplifies the process.

ASK THE AUTHORS

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Senior Strategy Consultant, Novel Modalities BioProcessing Group, Merck

Matthew Roach
AAV Process Development Team Leader, Precision BioSciences

Authors Ratish Krishnan (Merck) and Matthew Roach (Precision BioSciences) answer your questions about midstream unit operations.

Q When screening depth filters, which is better: high yield or high throughput?

RK: I would say the selection of a depth filter is based on balancing a lot of factors. Oftentimes we look at yield first, but if yields of the filter you've evaluated are comparable, sometimes the decision is based on the throughput. Other considerations may also include impurities, if applicable, like host cell protein and DNA. So I would say it's not a straightforward answer, but completely dependent on your feed, scaling requirements, and process expectations after you analyze the data.

DOI: 10.18609/cgti.2021.173

Q And what is the difference between Millistak+® D0SP and Clarisolve® C0SP filters? How are they different from Clarisolve® filters?

RK: The Millistak+® HC Pro high-capacity synthetic media are a family of synthetic depth filters, providing cleaner and more consistent depth filtration media compared with usually used diatomaceous earth and cellulose esters. Multiple media grades are available and are used in primary and secondary clarification as well as downstream filtration. The Millistak+® D0SP is a four-layered depth filter media, which also includes an upstream non-woven layer to improve the filtration capacity, primarily used in direct harvest applications. Clarisolve® C0SP is also a four-layered depth filter media and it may be used either for direct harvest or secondary clarification purposes. Both have the benefit of synthetic materials, formulation design, and disposable parts, and can clarify anywhere from 5 liters to 22,000 liters.

Clarisolve® technology was developed to address the challenge of high cell density feed streams, where pre-treatment methodologies like PDADMAC or similar flocculating agents are typically used. It has a gradient density structure and is designed for the particle size distribution of pre-treated feed streams. Clarisolve® filters with the designation -20, -40, and -60 refer to the particle size distribution after treatment with flocculants or acid precipitation. This enables a single-stage clarification of the pre-treated feeds, and thereby reduces your footprint and eliminates the need for a secondary stage of clarification.

Q What is the advantage of using the step filters as opposed to using other depth filters, such as glass fiber filters?

RK: Glass fiber filters have very fast flow rates, high loading capacity, thermal tolerance, and particulate reduction. What we've seen, when you look back at monoclonal antibody bioprocessing, is that depth filters have become the staple in clarification technologies. They have better scalability, and better removal of residual impurities and sediments. In theory, you could use glass fiber filters, but we've seen depth filters being preferred in clarification operations.

Q Do you have any further data that confirmed the selected filtration train?

MR: Absolutely, yes. We have confirmed this with larger scales and across many different serotypes and transgene combinations, and even in just routine research production, we've seen this be a success. So it's definitely become a platform step for us.

Q How do you explain a greater than 100% recovery for every filter used in the filter study?

MR: With any assay, you're going to have inherent variability, even on the same plate. For an assay like ddPCR, we might see a 1–8% CV value. The viral particle titer is measured by ELISA, which has a smaller dynamic range to work with and so is a lot more variable.

Q How did you measure the stability of the clarified lysate?

MR: The data we showed here was gained through turbidity measurement of the clarified lysate. Since then, we have taken that through to capture chromatography as well, and soon we plan to take this to a final indication of stability through chromatography, and then testing out infectivity and potency.

Q Did Precision BioSciences evaluate alternative filtration options?

MR: We did, although we haven't included that data here. We're confident that this train was the best that we tested out. I think the recovery data speaks for itself and our host cell protein clearance and host cell DNA was more than sufficient as well.

Q You mentioned that your lysis is based on TWEEN® 20. Can you share the concentration, temperature, and time of incubation? Were the experiments performed on fresh or thawed harvest?

MR: There was a final concentration of 1%. The temperature was 36–37°C, and the time of incubation was 30 minutes for lysis. The experiments were performed on fresh harvest material on the day of harvest.

Q Were there any impacts of Millistak+® D0HC or Clarisolve® C0SP filters on AAV potency or host cell proteins?

MR: No. When we've compared the final material across the train (not at the step for stability) we have seen no difference in potency or final host cell protein from the processes.

Q What detergents were used for cellular lysis? And were the matrix effects on the analytics taken into account?

MR: We used TWEEN® 20 and the matrix effects were something we put a lot of effort into investigating. Our ddPCR team here at Precision BioSciences have worked

DOI: 10.18609/cgti.2021.173

tirelessly and tested a ton of conditions to ensure our assay is telling us exactly what we think it is – which is critical to making sure that the data we're showing here are accurate.

Q Have you been able to optimize the use of Benzonase® endonuclease Safety Plus in your process?

MR: Yes, absolutely. Ratish and his colleagues at Merck were able to help out with that and have pre-set design of experiments that you can go through for it. Making use of those resources has saved us a lot on cost of goods and also made us confident that we are reducing host cell DNA at that step as effectively as possible.

RK: It's also very important to look at the unit operations connected to your depth filtration. Whether you're using Benzonase® endonuclease before or after, it's important to analyze the impact of each unit operation. The need for optimization is there, but it also depends on the analytics you trust at that point, so it often gets overlooked. If you optimize the Benzonase® endonuclease and then you do your clarification then you could get better surface area from your depth filters – it definitely has a knock-on effect.

Q Could you talk about the need for salt addition after cell lysis or Benzonase® endonuclease treatment and its impact on the filter performance?

MR: It's typically recommended to add salt for activation of Benzonase® endonuclease to stop that reaction. But we've actually seen the addition of salt helps us with recovery, and getting through that filtration process. So we've kept it in our process for both inactivation of Benzonase® endonuclease and ease of clarification.

Q For the depth filter trains, are you priming before loading the crude harvest?

MR: We're just doing a water flush – we're not doing a buffer flush. We have experimented a little bit with that but we haven't seen a large difference in recovery or stability.

Q Have you investigated implementing the addition of Benzonase endonuclease after clarification?

MR: That's something that we haven't studied but I think it's an interesting concept and maybe something that would pair really well with a TFF step after clarification.

RK: It really depends on how you want the train to be designed – there are lots of options. We have customers who use Benzonase® endonuclease before or after clarification, or even both.

Q How much clearance of host cell DNA does the Benzonase® endonuclease provide? And do the subsequent chromatography steps help to reduce any residual hcDNA?

MR: I can't speak to exact values, but we do see a large host-cell DNA reduction there. We also see a large reduction across our chromatography steps as well. I think this will be really dependent on your production processes – whether you're using a triple transfection system versus the baculovirus system, or other viral means of introducing the genes to produce AAV. Plus, what cell densities you're operating with.

Q Were there differences in the Opticap® SHC filter throughput based on the depth filter used?

MR: We have seen a difference in Opticap® SHC filter throughput depending on the filter, and it really seemed to correlate with where the cut-off is for the lower filter. For example, the Millistak+® D0SP filter has a higher final cut-off than Clarisolve® C0SP filter. The closer the cut-off of the depth filter is to 0.2 microns, the higher the throughput for the Opticap® filtration. We've been okay with our current train, but if you're trying to minimize the filtration area of the Opticap® and want to increase the number of depth filters on the other side prior to that, you can do that. It just depends on the facility design and process design.

Q What are your thoughts on investigating osmolarity, especially during the lysis step?

MR: It's a really interesting concept and there have been a couple of recent presentations by groups looking at that. It's something that we've measured, but it's not something that we've delved into too deeply yet.

Q How important is precise control of temperature and pH during the Benzonase® endonuclease step?

RK: Very important! I'd recommend taking a close look at the datasheet for Benzonase® endonuclease, because it's got a specific window of operation in terms of pH, temperature, and salt concentration. So it's important to make sure that your process is compatible with that. For a lot of customers adding Benzonase® endonuclease to a

DOI: 10.18609/cgti.2021.173

bioreactor is much easier because you've got the temperature control right, versus using it as a standalone operation, where you might have to do your Benzonase® endonuclease step at room temperature instead.

Q How would different types of depth filters affect the overall lysate stability?

RK: I don't think it's a major concern. Most of the depth filters we've used don't appear to affect the lysate stability. Obviously, it's feed-dependent, but given AAV is produced in HEK cells or SF9, there are probably much lower cell densities than is typical and I don't see them affecting the stability overall. Common practice is an overnight hold at around 4°C or no hold in continuous processing.

MR: We haven't seen any effect on lysate stability either. But regardless, you definitely want to measure that and be sure that you're not missing out on something that may surprise you later.

BIOGRAPHIES

Ratish Krishnan
Senior Strategy Consultant, Novel Modalities BioProcessing Group, Merck

Ratish Krishnan is a Senior Strategy Consultant in the Novel Modalities BioProcessing group for the Americas at Merck. He is passionate about providing solutions to bring treatments to market. A Process Development Scientist by background, he has over 13 years of experience in vaccine, monoclonal antibodies and viral vector modalities from pre-clinical to late-stage process characterization, validation and commercialization activities such as BLA authoring. As a Biochemical engineer, he holds a Master's degree in biotechnology from the Pennsylvania State University. Ratish has managed process development teams at Novartis and Pfizer prior to his current role where he serves as global subject matter expert for viral vector manufacturing and provides strategic guidance to internal stakeholders and key customers. He is active in his thought leadership activities at scientific conferences, technical webinars and key authorship contributions in peer-reviewed articles and white papers.

Matthew Roach
AAV Process Development Team Leader, Precision BioSciences

Matt leads the AAV Process Development group at Precision BioSciences, which is focused on designing and implementing new strategies for the production and purification of adeno-associated virus. Matt completed his Bachelor's degree in Biological Sciences at North Carolina State University and his Master's degree in Microbiology and Cell Science at the University of Florida. Prior to Precision, Matt spent time at Pfizer working on the purification of AAV and the Biomanufacturing Training and Education Center training industry professionals on downstream bioprocessing operations.

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Roach is an employee of and stock holder in Precision BioSciences.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Article based on a webinar which can be found here.*

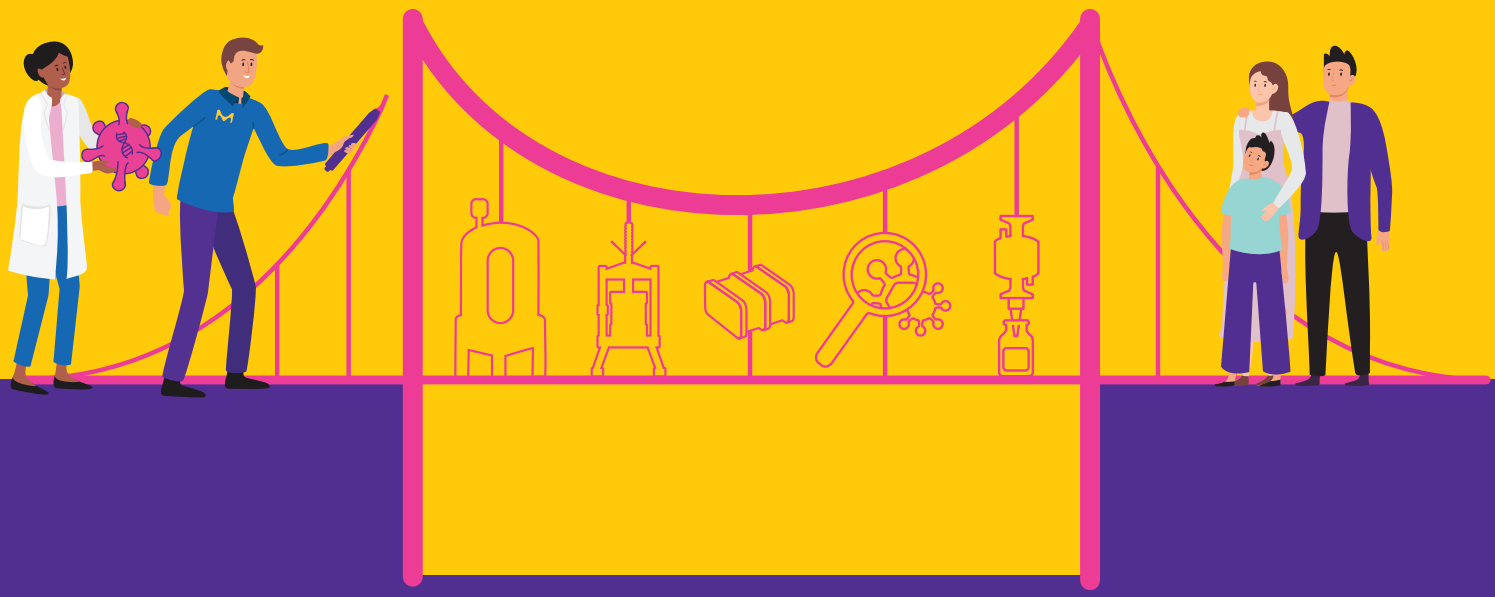
Webinar recorded: *Aug 18 2021;* ***Publication date:*** *Nov 16 2021.*

DOI: 10.18609/cgti.2021.173

Draw on our experience

To bring your gene therapy to life

Products – Testing – Manufacturing



Developing a new gene therapy is rewarding but there are unique challenges ahead. Lack of process templates, scale-up challenges and evolving regulatory guidelines can slow your progress.

We're here to help. With 30+ years of gene therapy experience we bring expertise in viral vector manufacturing and testing as well as a global organization to integrate regulatory, safety, and process development to fit your needs.

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Please visit **MerckMillipore.com/genetherapy**

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Mass spectrometry-based AAV protein characterization

Andrew Hanneman, PhD, Scientific Advisor, Biologics Testing, Charles River Massachusetts

The FDA Advisory Committee on Cellular Tissue and Gene Therapies recently convened a meeting to discuss the toxicity risks of AAV vector-based gene therapy products and called for increased research in this area. At Charles River, we conduct research to characterize AAVs, including capsid structure and a wide variety of process- or product-related impurities. A key technique we employ is high-resolution mass spectrometry, and this poster will describe how Charles River uses mass spectrometry for a range of AAV protein characterization activities.

Cell & Gene Therapy Insights 2021; 7(10), 1435; DOI: 10.18609/cgti.2021.189

Charles River provides analytical testing from initial R&D through to commercial lot release. Mass spectrometry (MS) is used to support a wide variety of analytical tests and is especially critical early in the development program, providing key proof-of-concept characterization data supporting IND-enabling and process development studies. Even fully validated analytical methods will occasionally need to be supported by investigative studies. For example, if a new peak is observed in a chromatographic method, it will need to be characterized. Typically, MS is used to carry out these investigations.

Throughout the drug development journey, it is important to keep in mind the need to have fully validated methods in place by clinical Phase III. ICHQ2 guidance is generally referenced to guide validation according to the intended use of a specific test method, including tests of identification, impurity tests, and content or potency assays. MS may be used to support an identity test, such as the development of a peptide map that provides a unique fingerprint of a specific AAV product. Similarly, MS is typically used to characterize components observed in various impurity tests, and to support content assays such as high-performance liquid chromatography (HPLC) tests for determining the ratio of viral proteins in an AAV capsid.

The intended uses of MS test methods and the information they provide are summarized in **Table 1**.

Table 1. AAV mass spectrometry-based protein analytics.

Intended uses	Information obtained
▶ AAV protein characterization for proof of concept and IND-enabling studies	▶ VP-1/2/3 protein content ratio (1:1:10)
▶ Support of lot-to-lot comparability studies	▶ Full-length VP sequency confirmation
▶ Support around method validation activities:	▶ Detailed amino acid sequence analysis, including novel chimeric sequences
▶ Serotype identity tests, e.g., a peptide map	▶ Post-translational modifications:
▶ Stability-indicating impurities tests	▶ Acetylation
	▶ Phosphorylation
	▶ Glycosylation
	▶ Disulfide linkages
	▶ Degradation events:
	▶ Deamination
	▶ Oxidation
	▶ Presence of host cell proteins

MASS SPECTROMETRY-BASED AAV CHARACTERIZATION IN ACTION

One example of how MS is used to characterize AAV proteins is presented here. An important initial step in an AAV characterization program is the AAV subunit reverse phase LC-MS method used to characterize VP 1, 2, and 3. Charles River provides platform methods for initial characterization (proof of concept to IND), and customized methods for lot-to-lot characterization, stability-indicating methods, and lot release **(Figure 1)**.

Incorporating mass spectrometry detection early in a development program offers a number of advantages. These include obtaining precise characterization of each AAV viral protein HPLC peak, including post-translational modifications, end terminal variants, alternate VP truncation variants, and phosphorylation events **(Figure 2)**.

Figure 1. AAV subunit reverse-phase HPLC.

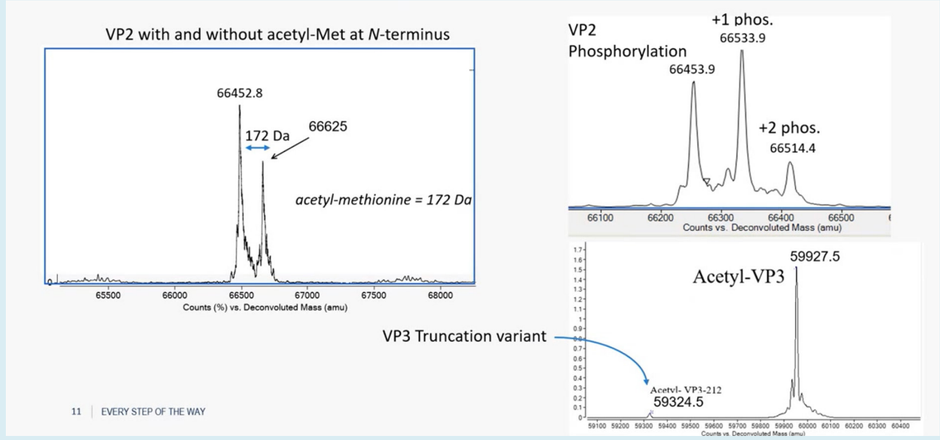
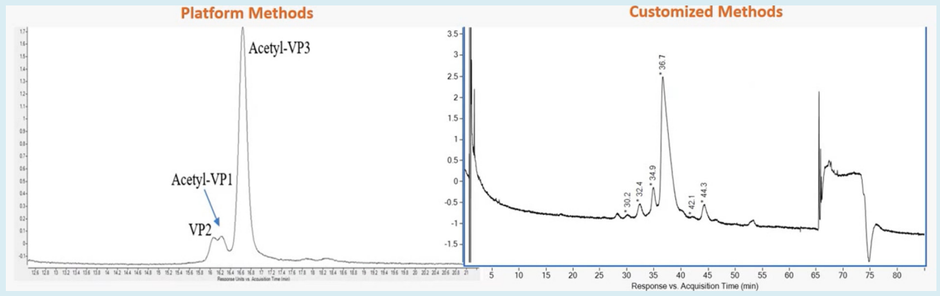


Figure 2. AAV subunit LC-UV-MS analysis to characterize post-translational modifications.



CELL & GENE THERAPY INSIGHTS

PODCAST INTERVIEW with:

Jeffrey Hung, General Manager, Vigene Biosciences, a Charles River Company; **Daniel Smith,** Executive Director, Global Cell and Gene Therapy Portfolio, Cobra Biologics, a Charles River Company; and **Horst Ruppach,** Executive Director, Scientific and Portfolio, Global Biologics, Charles River



Key topics in advanced therapy manufacturing: quality, safety, and supply

Cell & Gene Therapy Insights 2021; 7(10), 1411–1422

DOI: 10.18609/cgti.2021.185

Three experts in advanced therapy manufacturing discuss the challenges – and opportunities – facing cell and gene therapy today, including intensifying viral vector processing, strengthening supply chains, and navigating the ever-changing regulatory landscape.

Q As evidenced by recent meetings such as that conducted by the FDA's Cellular, Tissue, and Gene Therapies Advisory Committee, the safety of viral vector-based gene therapies is firmly in the regulatory spotlight at present – how are specialist CDMOs such as Vigene helping to address this key issue?

JH: On the organizational level, there are three lines of work we are following right now. The first is molecular gene therapy design. As you know, a gene therapy is only as good as the gene therapy on the plasmid, and later packaged into the viral vectors. As a development company for viral vectors, we have seen a lot of gene therapies that are not stable, causing the batch-to-batch and lot-to-lot variability, because the design was not right. I would like to see the industry standardize and be better at making stable and consistent gene therapy molecular design.

The second line of work is process development. We have often seen cases where the development process design was not optimal for gene therapy production. We have to rework a lot of processes we receive to make the process more robust and reduce impurities to a level that is safe for patients. That is critical, and we have done a lot of work on the process optimization and process development on our side.

The third line of work we have been doing is implementing best practices in the operation of gene therapy manufacturing with quality and safety. That is the last mile to the patient, so we need to implement good design and execute it flawlessly.

Q What is the latest progress in enabling viral vector process intensification, and where is further work required?

JH: Vigene was founded with the vision to make gene therapy affordable, so process intensification (scale-up) is core to our mission. I would like to highlight three aspects of how we achieve that goal.

The first is upstream process intensification. For example, we have been working on cell line development. The viral vector cannot amplify cells by itself for safety reasons, so all recombinant viral vectors have to be packaged artificially in cell lines. These cell lines differ dramatically from one another in signs of productivity and stability, so optimizing the cell line is important.

Second is bioprocessing intensification, including perfusion, is critical. If we can increase the yield of cells by a factor of two or four, the yield of viral vector will increase accordingly.

Third is downstream optimization, relying on advances in material science for downstream columns and membranes. Right now, we are partnering with several suppliers and partners to develop and verify those new downstream technologies.

Q What would be your advice to gene therapy developers struggling with the requirement for earlier process-related decision-making brought about by reducing development timeframes?

DOI: 10.18609/cgti.2021.185

JH: I have three pieces of advice, all centered around quality by design. The first is quality by molecular design – how to structure the promoter, how to structure the stuffer, and how to design the plasmid such that undesired packaging will be minimized. All of these are determined by the molecular design of the plasmid and gene therapy itself.

The second is the quality by process design. Manufacturing can only be as good as the process development and process itself. In other words, the safety, purity, and potency can only be as good as the process we develop. That means a lot of things need to be built in to consider maximizing potency and minimizing impurity.

The third is quality by material design. A lot of academics and gene therapy designers are working with materials, especially critical supplies, that are not GMP ready. When it comes to gene therapy manufacturing, we then have to go back to the drawing board and re-do all the materials supply and design. That takes a lot of time and brings a lot of risk to the gene therapy program.

DS: I completely agree with Jeff on all of that. From a development point of view, it's also very useful to help developers to think about the ultimate quality attribute they require from their viral vector, early on in that development lifecycle and how best to achieve that. What dosages are they looking for? What's their population size of indication they need to go after? We try to help them map out early on not just how much to make to support the patient population but how much is required for analytical development, qualification of assays, stability-indicating assays, so they have a clear roadmap of how much material is required for the development phases, early clinical phases, and late clinical phases before they get to commercial realization.

Given the speed that some of these products move through the clinical phases (for example, after being granted orphan indication and breakthrough status with the FDA) you may not have a lot of time as a manufacturer to change the processes between phases. Therefore, the process they start with at early phase must be the process they end up with at commercial phase. We want to help people make the right decisions at the front end because it's a lot of time and cost if you get it wrong as you move forward through the different phases.

HR: I would add that GMP aspects should be considered very early – in the preclinical phase, maybe even earlier. Even if you set up assays, those assays may be fit for purpose but not fit for GMP, and switching the assay, method, or equipment can cause major delays when moving through clinical phases.

Here at Charles River, we have the GMP background and the preclinical background to know what needs to be covered at a very early stage and smooth the path from the clinic to commercial. That's one of the strengths we can offer to our clients.

Q Ensuring a sufficient supply of high-quality plasmid has been identified as a key potential bottleneck for the cell and gene therapy field moving forward. How is Charles River positioning to address that?

DS: To put it bluntly, everything starts with plasmid. Currently, most viral vectors are made through transient transfection using a combination of 3–5 different plasmids to make

the viral vector. Therefore, it's really important to make sure the supply chain for plasmid is robust.

So how are we doing that at Charles River? Different developers can access plasmid at different grades through their development lifecycle. First. There is research use-only grade plasmid, and acquisition of Vigene and Cobra Biologics allows Charles River to make research-grade plasmid very quickly.

The next grade of plasmid is what we would call high-quality grade plasmid or GMP-ready plasmid. Again, Cobra and Vigene can supply plasmids of sufficient quality from both in terms of absolute functionality of those plasmids but also, within a regulatory environment, to allow traceability and confidence that the analytical support around it is in place. Documentation allows customers and potential developers to use that type of plasmid to file the right regulatory framework to support their clinical trial applications. As we move on in the development phase from Phase 1 through to Phase 2 and commercial, again GMP grade plasmid is important to be able to again ensure a level of quality and compliance for the product.

Charles River, through the acquisitions of Cobra and Vigene, now has a strong network of service offerings, in the US and Europe, to allow customers and clients to go from research-grade through to high-quality grade and GMP-grade plasmid.

There is a bottleneck in the industry for plasmid supply; however, by streamlining and harmonizing some of the service offerings across the CDMO network within Charles River, we're able to allow customers to access whatever plasmids they want, whenever they want, at whatever grade they want. We are ensuring that we build the appropriate capacity for either larger scale-up or larger scale-out at those different grades as the industry demands it.

Q How do regulatory and scientific requirements for plasmid differ for different products, for example, lipid nanoparticles, adeno-associated virus, or lentivirus?

DS: Again, I think it's interesting to dive into the different types of regulation around the use of plasmids and what plasmids can be used for. We've talked about viral vectors and how plasmids can be used transiently to support the production of viral vectors.

When you think about what the regulations are there for, it's to ensure patient safety from the point of view of clinical trials. The plasmids themselves are never going to be the product. They are there as critical starting materials to feed into vector production and, in the case of lentivirus, to make lentivirus that then goes on to potentially transduce a

> "...the industry is now starting to ... assess what is really important from a regulatory and a specification point of view to make plasmid for critical starting material."
>
> - Daniel Smith

DOI: 10.18609/cgti.2021.185

human cell for a cell therapy-based product – there are degrees of separation between the plasmid and the patient.

The regulatory environment is evolving. Traditionally, people have adopted the same approach for plasmids as starting material as we would apply for plasmids for direct clinical use. That means a lot of platforms, specifications, and analytical methodology have been built around the need to ensure patient safety from a clinical point of view. However, I think the industry is now starting to challenge that paradigm and assess what is really important from a regulatory and a specification point of view to make plasmid for critical starting material.

A case in point here is an mRNA sequence within a lipid nanoparticle. When it comes to the plasmid that was used to make the mRNA, is it more important that we remove all the residuals from it from host–cell protein, host–cell DNA, and host–cell RNA, or is it more important that the sequence is absolutely correct? It's an ongoing discussion.

The FDA and EMA have both recently announced new guidelines for the production of plasmids as critical starting materials – I would like to see more harmonization between those two sets of guidelines. As part of the CDMO network within Charles River, we need to understand how to apply the guidelines appositely across our network to give customers confidence that the plasmids we make for them are fit for purpose, both from a safety perspective and a utility perspective.

HR: I have a question for Daniel. Lentiviral vectors are also a critical ancillary or raw material because they are not typically given directly to patients but used as a material to transduce cells. However, the FDA advises that retroviral vectors should be considered like drug substances. Is that the same or similar with plasmid?

DS: To a certain extent it is. The recent guidelines have three main areas of compliance. Full GMP, non-GMP, and within the 'principles of GMP', which I would call a gray area in the middle.

Plasmid that is made for transient transfection for viral vector falls under principles of GMP, as does a lentiviral vector used for modification of cells for cell therapy. Plasmid that is used to make mRNA also falls under principles of GMP but mRNA itself falls under full GMP because it is the clinical product.

However, I don't think people have really adopted this approach yet for lentiviral and retroviral vectors. There's still a lot of discussion around what is the absolute regulation around this. And I think the other thing to consider here is that the regulations are also linked to the phase that you're at.

Principles of GMP are very easy to apply for early-stage material. As you move through to late-stage, Phase 3, and ultimately commercial material, most quality systems from large pharmaceutical or biotech companies will insist you go to full GMP. I think it's a sliding scale between early-phase to late-phase, principles to full GMP. Horst and Jeff – it would be great to get your thoughts on this.

JH: I totally agree with you, Dan. We need to design the process such that it can be easily scaled to be GMP compliant. For instance, if we are talking about the master cell bank for *E. coli*, it's better to structure and make a full GMP compliant master cell bank to start with instead of a research-grade master cell bank to make the early-phase clinical trial plasmid and

later on the viral vector raw material. That's just one example of how we can be compliant and structure the program to be fully integrated with commercial readiness.

DS: We've talked about process, and we've talked about the different regulations and how people are applying those, but I think the analytical and characterization side is also important here and there is less flexibility in that space. To be able to release products under the principles of GMP, the analytical assays and methodologies need to be fully qualified, if not validated, for certain points. With a very strong baseline for analytics, it's difficult to characterize your product as well.

There has always been a phase-appropriate approach to analytical characterization and regulation – whereas for early-phase you might use fit-for-purpose assays, you might go on to use qualified assays, and only at late stage go for full validation of those assays. It's the same principle we're trying to apply here. I think it's really important to make sure your analytical characterization packages are, if not fit for purpose, at least properly qualified for principles of GMP work.

HR: These aspects are very important – we could fill an entire podcast talking about it so I'll just add that often our clients have a different understanding of qualification of an assay and phase-appropriate qualification. It's a challenge because you have compendial methods that are more or less easy to use, and various other assays, so we have a lot of discussion with clients about what is needed. It's a topic where clients have a lot of confusion in terms of what the regulations mean for their specific case. And the answer frequently, unfortunately, differs case by case.

Q What lessons have you picked up during the COVID-19 pandemic and response to it?

DS: It's been an interesting 18 months. I've been privileged to work in an organization, Cobra Biologics, that has been at the forefront of the production of viral vector vaccines for the pandemic, DNA-based vaccines for the pandemic, and DNA raw materials to support mRNA vaccines for the pandemic.

What have I learned through that? That if you all work cooperatively and collaboratively, you can achieve a lot very quickly. It takes a common purpose to be able to move things rapidly through development, scale-up, and into GMP environments, with the right level of qualification, validation, and compliance, and with a real foresight on how to move this as quickly as possible without compromising on patient safety or cutting any corners. Working with a common purpose and in collaboration, we have been creative and challenged our normal paradigms of working.

We have also had to learn to ensure robustness and resilience in some of our workflows, and I think the most important thing is planning around this. The last 18 months have been a rollercoaster and what we're seeing now as a result of all of that is extended lead times and a lack of robustness in supply chains. Business continuity, good supply chain planning, and the ability to move things around quickly within the appropriate regulatory framework are

DOI: 10.18609/cgti.2021.185

essential to get things done. That has been a phenomenal challenge, and it's been a huge privilege to work with some really dedicated people to get things moving.

Q Can you give an overview of current regulatory standards or guidance for viral clearance in gene therapy manufacture?

> "The question most clients have is … Do I need to analyze the viral clearance capacity of the downstream process? Of course, if you go commercial you have to analyze."
>
> - Horst Ruppach

HR: First, let's clarify the terminology. Viral clearance means the capacity of the product purification process to remove or inactivate adventitious viruses. As you can imagine, for vectors like AAV or lentivirus, the capacity to remove or inactivate viruses is limited, because the product itself is a virus particle. By contrast, for recombinant products, there are usually strong viral clearance capabilities. For the cell therapy area, you don't have any capabilities to remove or inactivate viruses in the production process.

When we talk about viral clearance, the ICHQ 5A guidance is most often referenced, even though the scope of this guideline does not address viral vectors. However, the ICHQ 5A is currently under revision and the scope will be expanded to include gene vector products.

The question most clients have is: do I need to apply it? Do I need to analyze the viral clearance capacity of the downstream process? Of course, if you go commercial you have to analyze. It's part of the validation of the manufacturing process before going commercial: the demonstration of the capacity of the downstream process to remove or inactivate viruses.

However, many questions come to us about early-stage. For example, a client may ask: what about if we want to step into clinical Phase 1, do we need to analyze viral clearance capacity at that stage?

This is a little bit confusing if you look at the regulation. For instance, the most recent FDA guidance, *"CMC Information for Human Gene Therapy IND Applications"*, does not request general viral clearance validation when you step into clinical Phase 1. However, there is one exception. If there is a viral contaminant, you should demonstrate, even at that early stage, the capacity of your downstream process to remove those viral contaminants. A known viral contaminant could be, for instance, a helper virus. If you use a helper virus in the manufacturing process, you need to demonstrate that this helper virus is inactivated or removed in the downstream process.

The same principle applies if you use a baculovirus system – you should demonstrate the removal of baculovirus or HSV if that modality is used. And for some production cell lines like the Sf9 insect cell line, there are reports that this cell line is contaminated with rhabdovirus, so it's a known contaminant. If this is confirmed, you must demonstrate the clearance of this virus as well at early phases.

There is also a European draft guidance for investigational ATMPs, and these are much clearer, saying the process and the viral removal inactivation steps are expected to be validated

prior to the first-in-human clinical trials. It may change, but right now the expectation in Europe is that you analyze the viral clearance capacity in general, independent of whether you have a relevant viral contaminant like adenovirus or not.

Q What would you pick out as the key recent advances in terms of the available assays and analytical tools, and what are some of the important considerations in employing them?

HR: Very important, especially in the cell therapy area, are rapid testing capabilities. There are already solutions available like for mycoplasma and sterility testing. Some assays can reduce the turnaround time for sterility testing down to 7 or even 3 days, whereas compendial methods take at least 14 days.

Another development I see is performing on-site testing instead of shipping materials to a CRO, especially for in-process testing and release testing. The above-mentioned sterility testing technologies are set up for ease and robust use – ideal for on-site testing. Rapid mycoplasma testing still requires PCR logistics and expertise. The next step is from on-site testing to online monitoring. I have seen online monitoring systems that are connected to the bioreactor and do deep analytics of the phenotype of cells. They are so sensitive that they can differentiate infected cells from non-infected cells. Those tools are also used to analyze the transfection process in the bioreactor because they can even differentiate transfected and non-transfected cells.

Another technology that I regard as highly important for the characterization of starting material, especially cell banks, is high-throughput sequencing technology (next-generation sequencing). This is a comprehensive tool that can be used for two aspects. One is to screen for pathogens that like mycoplasma or viruses. The potential of this technology is that it can find and identify any kind of contamination – even unknown contamination – because it sequences any nucleic acids that are in the sample. Another use is to genetically characterize cells. For instance, the copy number of vectors, off-target integrations, and identity of cell lines like iPSCs.

I regard next-generation sequencing technologies as the most important technology that we will see used in the future for the quality assurance of critical raw materials and products, whether it's gene vectors or cell therapy products.

The challenge is that next-generation sequencing is a complex technology. It requires processing of data like data filtering. There are many, many aspects that you must consider. And under GMP it's even more challenging. However, many groups are working on these challenges, including regulatory agencies like the FDA, who have built working groups to make it possible to use next-generation sequencing in a GMP environment.

Q CMC has certainly been in the spotlight of late with late-stage cell and gene therapy developers running into issues with the regulators – what would be your advice to early-stage developers seeking to prepare for an increasingly stringent regulatory environment?

DOI: 10.18609/cgti.2021.185

HR: In addition to the points already covered, I would add that taking care of data integrity is very important. Protection and traceability of data is an important demand and should be considered very early on in the development process.

As you develop your product, you should document what you do, and you should take care that the data you create is securely stored for use as justification for the next steps. If you have a clear and documented path of how you selected the assays, how you created data, that will help you later in moving forward and avoid delaying the process. Even though that data may not be under GMP, if it is well documented it will be appreciated by the regulators as supportive data to justify your approach when you are in clinical phases.

For example, there are specific guidelines for potency assays to demonstrate the functionality of the product, in an *in vivo* or *in vitro* assay. This can be a complex and time-consuming assay. If you use equipment for cell-based *in vitro* assays, make sure that this equipment is part 11 compliant, which means it fulfills GMP requirements.

If you use equipment that is not part 11 compliant and you step into clinical phase, this equipment will not be accepted, so you must switch to new equipment. That means you may start from the beginning because you need to create new data, and the data might look different than what you have created so far.

You don't need to follow GMP rules in the documentation from early on, but the more you document, and the better you consider the aspects at an earlier stage, the better and more smoothly you will move forward from Phase 1 to Phase 2, Phase 3, and into commercial.

JH: Horst is absolutely right; data integrity is critical and many developers miss that. In addition, there are other aspects that academic, or early-stage gene therapy developers often don't consider. Therefore, I would suggest that a gene therapy developer contact experts like Charles River lab as early as possible. We're happy to provide consultative suggestions and services so they are staged for success very early on. It pains me to see programs that are not staged or designed well, so that we have to rework a lot of design, which wastes a lot of time and money.

Q What are the specific benefits to the integrated solution that the combination of Cobra Bio, Vigene Biosciences, and CRL provides to the advanced therapies community?

JH: I think first and foremost is speed. Charles River now has an integrated end-to-end solution from plasmid cell supply to viral vector and testing capability.

I would like to actually start from the end – testing. The testing takes as much time as

"I would suggest that a gene therapy developer contact experts ... as early as possible. We're happy to provide consultative suggestions and services so they are staged for success very early on."

- Jeffrey Hung

the manufacturing of cells, so if we can integrate the manufacturing with testing, and build in a lot of preparation work, that can save a lot of time and money for a gene therapy development program.

DS: From my perspective, we are able to offer customers choice, with multiple entry points to their development and manufacturing approach. Some developers will want the full service – plasmid, viral vectors, and cell therapy manufacture, all tested through the biologics function. Others will want to dip in and out at different points of that. Our integrated approach allows us to offer that choice to customers, and ultimately help them reach their patients quicker than they otherwise could have done.

HR: Testing and characterization are critical to the quality and safety of the product, and Charles River has been doing that testing for more than 20 years. Integrating this expertise and experience into the CDMO space ensures best testing strategies and strong support if you run into trouble with testing results.

If the client gets everything from one place, they don't have to manage multiple master service agreements and leaves them free to focus on what matters – getting therapies to patients.

BIOGRAPHIES

Jeffrey Hung, PhD

General Manager, Vigene Biosciences, a Charles River Company

Dr. Hung has over 20 years of experience in the gene therapy, synthetic biology and drug development. He joined Vigene in 2016 and orchestrated the acquisition of Omnia Biologics, a GMP manufacturer of viral vectors of 15 years. He has also overseen Vigene's expansion into GMP manufacturing and new product areas such as biosensors. An experienced entrepreneur, Dr. Hung was instrumental in successfully growing GenScript and SABiosciences, two previous companies, to IPO and acquisition stage, respectively. He also previously held the position of Chief Marketing Officer at ATCC. Jeffrey is the author of multiple patents, publications, and book chapters. He holds a PhD in genetics from Cornell University, an MBA from UC Berkeley, and a B.S. in biology from Peking University.

Professor Daniel C Smith, PhD, FRSB

Executive Director, Global Cell and Gene Therapy Portfolio, Cobra Biologics, a Charles River Company

Following the acquisition of Cobra Biologics/Cognate BioServices by Charles River Laboratories (CRL) in April 2021, Professor Smith was appointed Executive Director, Global Cell and Gene Therapy Portfolio within the CRL Corporate Development and Strategy function. Prior to acquisition, Professor Smith was the Chief Scientific Officer across the Cognate/Cobra CGT CDMO portfolio (2020–2021), and Cobra Biologics (2014–2020), driving CDMO innovations and partnerships across plasmid DNA, viral vectors, and latterly, (gene-modified) Cell Therapies, with respect to development and production. Previously he was Knowledge Transfer Manager and Senior Technologist for BioProcessUK. Daniel spent five years (2005–2010) at Cobra in roles including Senior Scientist, QC Team Leader, Head of Process Technology Transfer and Commercial Scientific Development Manager. He has over 8 years academic research experience, 30+ research publications to his name and a PhD in Molecular Cell Biology, and a BSc (Hons) in Biochemistry. Daniel holds Honorary Industry Professor positions at both the University of Kent, UK and at the University of Warwick, UK.

DOI: 10.18609/cgti.2021.185

Horst Ruppach, PhD

Executive Director, Scientific and Portfolio, Global Biologics, Charles River

Horst Ruppach studied chemistry at the University of Cologne and the University of Marburg, Germany, and earned his PhD in virology (HIV) at the Georg Speyer House, Frankfurt. He has 25 years of experience in the field of virology. His expertise is in virus safety testing and virus/prion clearances studies requested for all biopharmaceuticals and medical devices using animal- or human-derived materials. Dr. Ruppach is currently responsible for the business development of Charles River's viral clearance and virology service worldwide.

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *This is a transcript of a recorded podcast, which can be found here.*

Interview conducted: *Nov 8 2021;* ***Publication date:*** *Nov 24 2021.*

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SUPPLY CHAIN CHANNEL: Trends & Opportunities in Raw Material Sourcing

October 2021

Volume 7, Issue 10

INNOVATOR INSIGHT

INTERVIEW

CELL & GENE THERAPY INSIGHTS

TRENDS & OPPORTUNITIES IN RAW MATERIAL SOURCING

INNOVATOR INSIGHT

Understanding raw material performance: quality and consistency of cytokines for translation to the clinic

Bernd Leistler

A deep understanding of origin, performance and quality of raw materials in cell and gene therapy development is crucial, but international standards for these raw materials are still missing in our industry. The challenge lies in the fact that raw biological materials are inherently variable, while batch-to-batch consistency is essential for successful and long-term commercialization of therapies. There is also the big debate of when to apply rigid standards, is commercial-scale manufacturing too late? One category of raw materials that require further standardization and characterization is cytokines, growth and differentiation factors (here named cytokines for simplicity). Cytokine quality and performance are directly linked to the clinical and commercial success of a therapy. However, there are important quality considerations to address during preclinical research to ensure your therapy is set up for regulatory and commercial success. Even cytokines that were originally developed for other uses, including those used as human therapeutics themselves, are not necessarily suited for use in cell and gene therapy manufacturing. Limited information on the potency and other critical attributes of the materials makes it difficult to define specifications for those reagents and to investigate the material. This article will explore ways of easing translation from preclinical development into the clinic, the importance of using animal-derived component-free (ADCF) cytokines, how to compare cytokines from different vendors and the value of international units of measurement.

Cell & Gene Therapy Insights 2021; 7(10), 1147–1152

DOI: 10.18609/cgti.2021.153

CYTOKINE ACTIVITY MEASUREMENTS & INTERNATIONAL STANDARDS

Defining and measuring the possible effects of a given cytokine is not a simple, black-and-white task; this is because of the inherent variable nature of these tiny but powerful messengers. Cytokines can impact a multitude of cells through the signaling pathways they initiate, in a wide variety of ways. Their effects can depend on various factors including the target cell, its environment, cell culture etc., adding another layer of variability to an already complex picture.

A cytokine's biological activity should therefore be measured by its effect on a particular cell type. However, there is not yet a recognized industry standard for these measurements or their units, also because different protocols may be based on different modes of action of the same cytokine. This can be challenging for developers when trying to demonstrate reproducibility and comparability to regulatory authorities.

What we do have currently are 'international units' (IU) developed by the World Health Organization (WHO). IU are calculated using a standardized assay in which the cytokine of interest is tested side by side with the defined WHO standard, which can be obtained from the National Institute for Biological Standards and Control (NIBSC). The activity of the cytokine is then normalized using this standard. Such a standardized assay should in addition be validated following the applicable ICH guidelines, according to each lab's specific conditions.

The NIBSC's definition [1] of international units is:

"International units (IU) are assigned to international standards or other reference materials to allow the assessment of 'biologicals' in a consistent internationally agreed manner.

Biological reference materials, with an assigned value in IU, may be used in situations where physico-chemical determination of international standard units, e.g. mass, is not possible or not appropriate. There may be no agreed validated reference methods of determination available, or a simple mass unit may not adequately define a clinically-relevant measure of activity e.g. glycoprotein hormones."

Examining the specifications for activity used by several leading cytokine providers indicates that IU are not broadly used by all suppliers. If these units were to be universally adopted as a global standard, it would allow the cell and gene therapy industry to:

a. Achieve comparability in the activity of cytokines in an internationally agreed manner and more easily evaluate cytokines from different raw material providers;

b. Produce comparable and reliable data, demonstrating batch-to-batch consistency for regulatory submission.

Batch-to-batch consistency is vital for regulatory approval and sustained commercial viability.

HOW TO PERFORM EFFECTIVE COMPARATIVE SIDE-BY-SIDE TESTING

There are many ways to measure the biological activity of cytokines, but quantifying all the potential activities of a cytokine in one single numeric value is not possible. We recommend measuring one defined effect – e.g. stimulation of cell proliferation – on one defined target cell under standardized conditions. The assays should be validated following ICH guidelines for each cytokine and should be performed according to SOPs under a GMP quality assurance system and using qualified equipment.

DOI: 10.18609/cgti.2021.153

It's important to be vigilant when comparing units of activity between different sources, as different suppliers may have their own methods for defining a unit. The method used must be the same between sources for a unit comparison to be valid. The only way to make a reliable comparison is via side-by-side testing. Unfortunately, this side-by-side testing can be arduous and time-consuming, here are some considerations to ensure effective testing.

Effective comparative side-by-side testing in three steps:

1. Determine the activity in IU/mg: use identical proliferation assays and calibrate against an international reference standard;
2. Determine the protein content in µg: use identical assays for all samples (the specific cytokine activity is dependent on the amount of cytokine tested). Note that different sources of international standard and test material may result in different molecular weights (e.g., caused by different amino acid sequences or different levels of glycosylation);
3. Determine the purity: preferably using a HPLC method.

WHY USE CYTOKINES FREE OF ANIMAL- & HUMAN DERIVED COMPONENTS?

Materials of biological origin, particularly of human or animal origin, can present risks, including transmission of adventitious agents or introduction of biological impurities.

Using cytokines free of any animal or human-derived components:

- Minimizes potential variables associated with animal or human-derived components
- Eliminates the risk of introducing biological contaminants in your cell and gene therapy process
- Speeds your time to market and saves money: no viral safety studies needed

To allow cell and gene therapy developers to perform a risk assessment of the raw materials used in their manufacturing process it is important that raw material suppliers offer a well-defined animal-derived component-free (ADCF) policy. ISO Technical Standard-20399 [2] defines two ADCF levels:

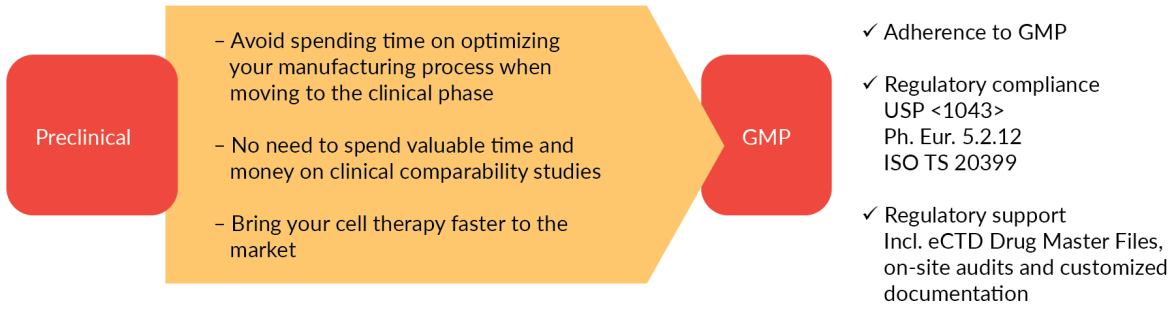
- Level 1 (product level): the raw material does not contain any materials from animal or human source as its ingredients.
- Level 2 (production level): in addition to ADCF level 1, raw material is produced without the use of any materials from an animal or human source. This includes excipients, equipment or containers that come into contact with the raw material during production.

SEAMLESS TRANSITION FROM PRECLINICAL TO CLINICAL DEVELOPMENT

Translation from lab to clinic and subsequent scale-up to commercial levels present our industry with many challenges, including reliability and reproducibility. GMP-grade raw materials that are required in later clinical phases may have different characteristics to those used in earlier research phases, as they are subject to different manufacturing protocols or quality standards. Changing raw materials during clinical development is time consuming and costly, requiring comparability studies that can have serious regulatory implications. Switching to GMP grade raw materials in the early clinical phase offers an economic benefit and saves time. A study from the Tufts Center for the Study of Drug Development (CSDD) estimated that the costs of an amendment in phase Ill are more than three times than those for a Phase 2 trial [3].

FIGURE 1

Seamless transition from preclinical to GMP.



To enable a safe and effective translation to the clinical stage we recommend using appropriately characterized cytokines of comparable performance in your preclinical and early development phase. These cytokines, we call them 'preclinical grade', should be produced under comparable conditions as the GMP equivalent, offering equal product performance. That way, you can switch directly to GMP-grade raw materials and avoid additional process optimization and laborious, expensive comparability studies (Figure 1).

CONCLUSION

Characterizing and measuring the biological activity of cytokines is a critical factor in the clinical and commercial success of cell and gene therapies. International standards are crucial for comparability of cytokine activity and, therefore, batch-to-batch consistency and the industry must work together to develop and adopt standards.

The earlier and easier the switch to GMP grade materials can be made, the more cost and time effective it is. Ideally this will be done prior to the clinical phase to avoid comparability issues and potential regulatory hurdles.

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DOI: 10.18609/cgti.2021.153

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 6 2021;* ***Revised manuscript received:*** *Sep 29 2021;* ***Publication date:*** *Oct 14 2021.*

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CELL & GENE THERAPY INSIGHTS

TRENDS & OPPORTUNITIES IN RAW MATERIAL SOURCING

CHANNEL CONTENT

INTERVIEW

Key considerations and risk management best practice for placenta-derived cell therapy raw materials

RUTH GOLDBERG joined Pluristem in 2009 and currently serves as Compliance and Methods Validation Manager. Ruth has over 14 years of experience in the biotech industry in several leadership positions. She is skilled in Biotechnology, Life Sciences, Quality Assurance, Quality System, CMC, and Pharmaceutical Industry; a strong education professional with PhD in Cell Biology and Immunology, Technion.

LIOR RAVIV joined Pluristem in 2011 and currently serves as Vice President of Operations & Development. Prior to that Mr Raviv served as Process development engineer and Projects manager and Product development Team leader at Pluristem. Prior to joining Pluristem and during the years 2010–2011, Mr Raviv held the position of R&D Analytical Researcher at Teva Pharmaceutical Industries. Mr. Raviv holds a M.Med.Sec in pharmacology from the Ben Gurion University and a BSc, in Biotechnology engineering from the Ben Gurion University.

Cell & Gene Therapy Insights 2021; 7(10), 1243–1252

DOI: 10.18609/cgti.2021.166

Q Can you introduce us to the specific raw materials-related considerations for Pluristem's product pipeline?

RG: According to EMA Draft Guideline on Quality, non-clinical and clinical requirements for investigational advanced therapy products in clinical trials (2019), starting materials are for example, 'donated cellular material (cells or tissue) from single or multiple donors, once processed' and 'additional substances (e.g. scaffolds, matrices, devices) when combined as an integral part with the manipulated cells'. The source material for the manufacture of Pluristem's investigational products is a placenta donated by a woman who has undergone elective caesarian section following a full-term pregnancy. Placentae are used for research purposes, for process development or for the manufacture of a clinical-grade product suitable for clinical trials. The manufacturing process of the clinical-grade product is detailed in a Biological, Chemical and Pharmaceutical Quality document that is submitted and approved by relevant regulatory authorities.

All Medical Centers donating placentae are required to have the approval of their local Ethic Committee (EC), and, if required by EC, approval from Israeli Ministry of Health (MoH). Prior to donating a placenta, the donor will sign an Informed Consent form before any procedure is performed.

The donor eligibility process includes screening of the donor for the risk of communicable diseases via a questionnaire, physical examination, review of medical records, and testing of the donor's blood sample for detection of infectious diseases.

Raw materials (RMs) used in manufacturing of clinical products should be purchased from the user's approved suppliers. RMs approval should be based on a qualification program. Qualification of RMs should be based on the risk assessment of each raw material, as assessed by R&D and QA departments. The goal of raw material risk assessment is to proactively identify risks that could contribute to an interruption of raw material sourcing, raw material performance, or the material qualification essential to the supply of safe and efficacious final cell therapy products. Risk assessment should employ a quantitative approach – for example, assigning a point value to each risk parameter for a RM, which results in cumulative scores that prioritize effort and resources for decreasing the risks associated with RMs. Based on the risk assessment, a qualification classification should be designed for each RM.

As biological raw materials are more difficult to characterize, because they have complex biological activities and high variability from lot to lot, specific characterization testing may be needed to assess a variety of quality attributes.

> "Risk assessment should employ a quantitative approach..."
> Ruth Goldberg

Performance variability of such materials may have an impact on the potency and stability of the final cell therapy product. Examples of complex functionality testing for RMs may include, for example, growth promotion testing of individual lots of Fetal Bovine Serum (FBS) on cells used in manufacturing,

DOI: 10.18609/cgti.2021.166

and *in vitro* tissue culture toxicity assays for individual lots of Dulbecco's Modified Eagle Medium (DMEM).

Q Are there any particular materials that carry additional risk – for example, that are single-sourced – and what is your approach to mitigating this risk?

LR: In the cell therapy field almost every RM or disposable is single source. One of the reasons for this is the long process of assuring the quality and suitability of the material for clinical use. Furthermore, since the drug product of cell therapy is a live product that can react to changes in the process, the impact an alternative product could have on the characterization of the cell product is basically unknown. To mitigate the risk of single source materials, we created a cross-functional team composed of representatives from QA, supply chain, manufacturing, development, and QC. Using a risk assessment process, the team evaluates each material based on its risk to the supply chain and potential effect on product characterization. Based on that assessment, a mitigation plan begins to work on the highest risks.

If during the assessment of the alternative material gaps are detected in the level of quality, the QA team will work with the manufacturer to close these gaps. On the other hand, if gaps are detected on the operational level, customization of the product will be done. The work of the cross-functional team on the alternative material continues from the highest material risks to the lower ones in parallel to the product development steps, creating a continues process of supply chain risk reduction, and many times even cost reduction.

Apart from the risk of single source materials, additional risks to specific materials quality also come from the material transport and storage conditions. These risks, if not reduced, can have a significant impact on the process and product quality, reproducibility, and batch-to-batch consistency. Since our product is a live product that reacts to changes in the process, even small changes to the critical raw material specifications can have an impact on the product characterization. To mitigate these risks and increase the level of consistency and reproducibility, we test the critical raw materials and study how different storage and transport conditions (temperature, etc.) affect the product characterization. If gaps are detected, we work with the suppliers to adapt the storage and transport conditions.

This approach to raw material risk reduction increases our level of understanding and knowledge regarding the raw materials in use and allows us to better define the critical material attributes. During the COVID-19 pandemic we learned the importance of having this process well established, since it allowed us to search, test, approve, and source alternative raw materials when needed.

Q How have you sought to address any additional upstream supply chain issues that have been presented by the ongoing pandemic? And how will the pandemic change materials sourcing on an ongoing basis in your own particular sphere?

RG: Coronavirus 2019 (COVID-19) has caused significant disruption to the cell and gene therapy industry, which has generally encountered complexities in supply of materials, and logistics processes. The supply chains have had to face new challenges as the disease rapidly has evolved.

The first challenge comes from the shortage of supplies of materials to the cell and gene therapy industry. Disruptions were also observed for cell collection from patients (human-to-human contact), visits to medical centers, shipments of cell material to manufacturing sites, and transportation of products to administration centers.

The first manufacturing step at Pluristem is collecting donated placentae at the time of delivery of healthy, full-term babies, from elective cesarean operations. As cesarean operations weren't drastically limited during the pandemic, and the placentae were collected with minimal human-to-human contact, no disruption was observed in this field. In addition, as part of a risk-based approach adopted by our company in order to mitigate the main risks of COVID-19 related to drug safety or quality, screening of the donors for SARS-CoV-2 before giving birth and at the day of discharge from the hospital was added to the overall viral control strategy.

On the other hand, we experienced long delivery times for plastic components and biological supplies, and sometimes found ourselves short of manufacturing or laboratory equipment such as personal protective equipment, disinfectants for cleaning rooms, single-use consumables, and biological raw materials.

Consequently, the pandemic had led us to re-evaluate our supply chain and manufacturing strategies. We considered strategic partnerships with key suppliers and identified and qualified at least two potential suppliers for critical raw materials, rather than relying on just one.

In addition, because of travel restrictions and physical-distancing guidelines, we adopted digitalization tools and prepared our company for remote working during the early days of the pandemic. For example, digitalization of signing on documentation allowed for remote access and a reduced need for onsite personnel. Also, digitalization of our suppliers remote site inspections and virtual audits did not hamper suppliers' evaluation and qualification. Thus, trustworthiness and readiness for digitalization is valued highly during the current crisis when site inspections are restricted.

Q Stepping back for a moment, what are the most pressing priorities for the cell and gene therapy field as a whole in advancing the industrialization of raw material and consumables supply?

RG: One of the things the cell and gene therapy industry needs is more standardization of terms for quality statements.

A diversity of terms is used to describe raw materials and it would be great if standard terms (terminology) were harmonized. For example, statements such as: 'Laboratory grade'/'Research grade'; 'GMP'/'cGMP'/'manufactured under GMP'/'GMP-compliant'; 'GMP intended use for research only' or 'GMP intended use for further manufacturing'; 'Clinical–grade (approved drug)'/'for a specified intended use only'/'not approved for other "off-label" processing uses

 DOI: 10.18609/cgti.2021.166

without qualification and approval from regulatory agencies'.

Developing a clinical-grade product according to FDA or EMA guidelines involves various elements and having cGMP-compliant raw materials is one of the most crucial ones to ensure the safety of the cell and gene therapies and eventually, of the patients. Having high-quality research and cGMP raw materials options smooths the transition from process development through clinical trials and commercial manufacturing of cell and gene therapy products. However, cGMP-compliant raw materials are not always readily available.

> "...having cGMP-compliant raw materials is ... crucial ... to ensure the safety of the cell and gene therapies and eventually, of the patients."
>
> Ruth Goldberg

Suppliers do make efforts these days to perform validation of raw materials' manufacturing processes in order to meet robust specific specifications, which will predict a precise performance of the raw materials. Suppliers also make efforts to have quality systems that manage change controls, traceability, and investigations. They perform GMP QC analysis emphasizing sterility, impurities and other residuals testing. In order to be GMP compliant, regulatory certificates are also important to have, for example: certificates of analysis, certificates of origin, stability reports, extractable and leachable study reports, and others certificates depending on the raw material type.

The demand for single-use technology has also increased, which in turn has led to a greater expectation that suppliers should have an expanded single-use network that will help cell and gene therapies to scale-up from research and development to commercialization. Single-use solutions will provide productive strategies in effectively scaling up and reduce risks and costs. Single-use technology will also offer a more flexible and safer approach to sterile fluid handling (closed system solutions) in cell and gene therapy manufacturing compared to traditional methods in place today. Cell and gene manufacturing requires small batch sampling under aseptic conditions to preserve the limited material for the patients, whilst complying with regulatory standards and having representative results. Manufacturers will benefit from collaboration with suppliers to tailor single-use systems and technology to the individual manufacturing requirements in the sampling process, which will allow for even better efficiency and process security [1].

Continuous investment in improvement and partnerships is required, and suppliers can make valuable contributions to cell and gene therapy production based on their existing knowledge, technology capabilities, and obligation to provide solutions.

Q Where and how is progress being made in increasing consistency, scalability, and standardization – and reducing costs – of allogeneic cell starting materials?

LR: It has become apparent in the last few years that efforts need to be made to increase process consistency and reduce the cost of processing and manufacturing,

to make cell therapy products viable in the real world. Our aim as cell product candidate developers and manufacturers is to develop a process that will yield an active, viable, and affordable product, remaining cognizant of the fact that the cell manufacturing process and product quality are strongly affected by the quality and consistency of all starting materials. In allogeneic cell therapies, among all starting materials, the cells themselves have the highest impact on process reproducibility. Over recent years, an effort has been made throughout the industry to develop industrialized solutions based on closed systems, standardized protocols, and automation in order to increase the level of consistency, scalability, and reproducibility of cell collection steps. Furthermore, studies have been performed in order to learn how different conditions can affect the cell starting materials during transport. Based on the increasing body of knowledge, new approaches and technologies are being developed to increase the starting material stability. It will probably take a few more years to learn what the best conditions for each product type are, but the industry, the clinicians, and the cell collection sites understand the importance and the effect of the cell collection step and are willing to contribute to the learning effort. Once standardized, the process will even deliver cost reductions due to a reduced failure rate in the cell starting materials. At Pluristem, we collect the donated placentae directly from the hospitals and we manufacture the product in-house. Through the years, we have conducted various studies on the different parameters affecting the donated starting material stability and quality. Based on that work, close collaboration, and teaching the hospital staff, we were able to increase the fresh starting material stability to over 24 hours, which allows greater flexibility in our manufacturing.

Another recent effort has been made in the field of media and media components. It has been commonly accepted for years now that a shift to serum-free media is needed to reduce risk and increase the consistency of manufacturing compared to the use of fetal bovine serum. Based on this understanding, many off-the-shelf serum-free media were developed by different companies. The consequent increase in availability of serum free media allowed cell therapy manufacturers to test them, better understand what is important, and give feedback to the media developers, driving a process of continuous improvement. We have ultimately seen a process of quality and consistency improvement in parallel to cost reduction.

> "It will probably take a few more years to learn what the best conditions for each product type are, but the industry, the clinicians, and the cell collection sites understand the importance and the effect of the cell collection step..."
>
> Lior Raviv

Furthermore, since cell manufacturing companies understand the impact of media and media components on their product, we now see a trend of companies customizing their own media formulations using media components bought from different suppliers. Based on our experience at Pluristem, in-house formulation development increases consistency, creates a dramatic cost reduction, and allows us to have full control over the cost and the source of the media components. At Pluristem, in parallel to our media

DOI: 10.18609/cgti.2021.166

development efforts, we performed an extra step to reduce cost and switched from suppliers' custom solutions to in-house solutions preparation. Because we work in a closed environment and everything needs to be sterilized before entering the clean rooms, the standard approach is to work with the supplier to have custom designed packaging suitable for the process. This process increases the overall cost. In parallel to the development of serum-free media, we established a team that filters each solution we purchase in-house and adapts it for our process needs. This gives us the ability to buy any packaging for the raw materials that we need off-the-shelf and to design the container in-house. Designing the container in-house increases the availability of the specific raw material, which then increases our independence – thus, the risk of not having the raw materials available when we need them is reduced.

"In parallel to the development of serum-free media, we established a team that filters each solution we purchase in-house and adapts it for our process needs."
Lior Raviv

Q Concern over regulatory uncertainty and disharmony around requirements for raw materials seems to be on the increase. Are there any specific aspects that are considerations for Pluristem as you approach the challenge of ensuring regulatory compliance on a global basis?

RG: Raw materials used in cell and gene therapies are not common raw materials with monographs, made in GMP environments, and there are no compendia documents available for these materials.

Terminology is the first subject to take into consideration. There are various terms for materials used in manufacturing of cell and gene therapy products: 'materials' in EU Directive 2001/83/EC; 'ancillary materials' in ISO Standard ISO/TS 20399-1 Biotechnology; 'raw materials' in European Medicines Agency (EMA) guidelines; and 'ancillary materials' in USP <1043> (ancillary materials). As terminology varies in different countries, ICH terminology may be recommended as a good option to use, as their terms are internationally accepted and applied across the pharmaceutical, biotechnology, and cell and gene therapy industries. However, despite the differences in terminology, the definitions according to the U.S. FDA regulatory guidance, EU Directive, and ISO Standards are all consistent in stating that the RMs are not intended to be present in the final product.

Although there are regulations that describe both quality and regulatory requirements for the manufacture of cellular therapies, the regulations do not specifically describe quality requirements for RMs. However, they do provide a framework for strategies to control these RMs. Guidance on RM use is available from the U.S. FDA (USP <1043> and specific chapters for fetal bovine serum, cytokines, growth factors), US FDA directives in Title 21 CFR,

the International Conference on Harmonization (ICHQ10), the European Medicines Agency (EMA) (Annex I, part IV of Directive 2001/83/EC) and the EP 5.2.12. Most of the guidance is relevant to medicinal products (small molecules) and biologic drug products (blood or blood products), and does not apply directly to cellular and gene therapeutics. However, as the industry has grown, more specific standards and relevant cGMP regulations for cell and gene therapy RMs have arrived.

Ensuring that the biological RMs used are human/animal origin-free (AOF) is one of our main concerns. A certificate of origin (CoO) is much desired because it helps to reduce adventitious agents risk concerns, which are one of the main regulatory deficits for cell and gene therapy companies. A certified animal origin-free product allows us to not have to prove viral safety of biological-derived raw materials or their components used during manufacturing. What we do need is a consistent definition of AOF to be agreed by the regulatory agencies. Users should obtain AOF statements that include as much detailed data as possible relating to the supply chain of components involved in the manufacturing of biological RMs

A new ISO draft for Ancillary Materials ISO/CD 20399 (current ISO/TS 20399, 2018) is anticipated. This document will provide guidance to suppliers and users of RMs to improve the consistency and quality of RMs of biological (human and animal) and chemical origin used in the production of cellular therapeutic products and gene therapy products for human use. It will help the suppliers and users of RMs to achieve and maintain an appropriate level of documented lot-to-lot consistency in the aspects of identity, purity, storage and stability, biosafety, and performance.

As there are regulatory requirement differences – and some regulatory guidance may have more detailed requirements on viral safety testing or characterization, leading to confusion – Pluristem's objective is to choose well characterized, high quality RMs intended for use in cell and gene therapy manufacturing, which meet the current regulatory guidance in the major markets (such as USA and Europe). Whenever available, FDA- and EMA-approved GMP or Clinical Grade materials are used at Pluristem. The use of such materials should eliminate the need to make subsequent changes to materials.

Q How do you weigh up the pros and cons for in-house development and production of critical raw materials versus outsourcing? And do you see the balance changing in this regard?

LR: This is indeed a hot topic in cell therapy. Our approach is based on what will affect the 'day after approval'.

Pluristem works through the development stages of the company and the product with Phase 3 and market approval in mind. We will be measured not only on gaining market approval, but mainly on the day after when we need to deliver actual commercial cell therapy products to patients. If we are not able to supply the product, or the product is too expensive, this will affect our success. Pluristem is a cell therapy developer, and our aim is to invest our efforts in producing our products, not to develop RMs. However, wherever we saw that a RM

 DOI: 10.18609/cgti.2021.166

(or any other element needed by the manufacturing process) had the potential to disrupt our manufacturing, we decided to develop an in-house solution.

For every new product or process we are developing, we assess the criticality of the different starting materials and other RMs in terms of their availability, cost, consistency, complexity, and more. Using this assessment, we categorize the risk of this outsourced material and the related cost in commercial manufacturing. If we identify a potential risk, we develop a mitigation that will be either outsource or produce in-house. For example, during the development of our second product (PLX-R18), we realized that working with fetal bovine serum had a crucial impact on our ability to manufacture and on our product cost, so we implemented a project for switching our products to serum-free media. Once we started working with off-the-shelf serum-free media, we noticed that our cost of goods (COG) significantly increased. Assessing the risk, we implemented methods in our process development to understand the critical material attributes and we realized that we could design our own formulation of serum-free media. By doing this, we created a solution with full control of our sourcing material, costs, and the capabilities of the in-house serum-free media to support the process. At the end of the mitigation process, we managed to increase yield of our product, to reduce the cost, and to gain operational independence.

In the RMs field, we performed many development studies and gathered a lot of information and knowledge about what critical material attributes are. Therefore, if we have a specific component that we believe is needed for the manufacturing process and it has only one supplier, increasing our understanding allows us to potentially work with alternative suppliers and materials. By creating the ability to work with alternatives, you can reduce the cost and increase the availability of specific RMs.

Ultimately, there is no right or wrong answer to the question of 'outsource vs in-house'. At Pluristem, we believe that manufacturing, process development, quality, and product data collection and interpretation must all be in-house to allow us to fully understand the product. All other elements could be either in-house or outsourced, providing they don't increase risk and cost to the process compared to the alternative solution.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Sep 23 2021;* ***Publication date:*** *Oct 22 2021.*

DOI: 10.18609/cgti.2021.166

CELL & GENE THERAPY INSIGHTS

October 2021

Business Insights

CELL & GENE THERAPY INSIGHTS

Business Insights

October 2021
Volume 7, Issue 10

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Establishing gene therapies as a relevant, recognized and accessible therapeutic category

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CELL & GENE THERAPY INSIGHTS

BUSINESS INSIGHT: OCTOBER 2021

INTERVIEW

Establishing gene therapies as a relevant, recognized and accessible therapeutic category

TAY SALIMULLAH is Vice President, Global Head of Value and Access, currently building a 21st century integrated access function to transform rare diseases through gene therapies. He oversees translational access, gene pricing and contracting, geneconomics, real-world data, and public policy. He has diverse global market access, pricing, and commercial experience across biopharmaceuticals, consultancy, social sector and private equity. Since joining Novartis in 2013, Tay has led strategic and operational teams across various divisions in Pharma, Region Europe, Oncology, Cell and Gene Therapies, and Group Global Health with a focus on inspiring associates to think early and differently about patient access and reimbursement. Before Novartis, Tay spent over a decade with Pfizer where he held positions of increasing responsibility within commercial, market access, and strategy development. In 2009 Tay completed his Global Health Fellowship: in-field healthcare systems experience in Malawi. He has also worked for a private investment group to incubate healthcare access across Asia. He has lived and worked in Europe, Asia, Africa and the USA, and is passionate about breakthrough thinking to transform global patient access.

Cell & Gene Therapy Insights 2021; 7(11), 1393–1400

DOI: 10.18609/cgti.2021.183

Q Let's begin with some reflections on your career to date in market access. Firstly, tell us about your experiences around the initial strategic shift in pharma from small molecule drugs to biotherapeutics, particularly in the oncology space – what valuable high-level parallels can be drawn as further evolution takes place today in terms of the transition to commercial advanced therapies?

TS: In the past few decades, we moved from chemical-based small molecules to biologics, cell therapies, and one-time gene therapies. And today, we are witnessing the reemergence of vaccines and mRNA technologies, fast-forwarded through the recent pandemic. In an industry like ours, it's so difficult to develop novel medicines for broad access and I think it's a moment to pause and reflect on the incredible work organizations in the advanced therapy space are doing. They are defining what advanced medicines look like when you have the ambition to address the root causes of a disease, and to challenge the status quo by raising the bar of a standard of care.

We are dealing with the reality of these 21st century medicines in healthcare systems that were designed for ongoing chronic therapies. However, many of the foundational elements can be leveraged in targeted areas in rare diseases, where significant unmet needs still exist and development cycles can rapidly establish a benefit-risk profile. Trials are conducted in highly specialized centers of excellence, which will also become part of the early-stage commercialization and access pathways.

Q You were involved directly in establishing the value of Kymriah® in the eyes of HTAs and payers – with the benefit of hindsight, what worked and what didn't in that trailblazing project? And what are the 2–3 core lessons that you take forward from it?

TS: We learnt a lot about attempting to commercialize one-time cell therapies. First and foremost, we discovered that establishing the value proposition for treating and extending people's lives with an autologous product is no small feat.

It was an honor being part of the team that got the first cell therapy for acute lymphoblastic leukemia approved by the FDA, an inflection point for the industry. Then, to see players like Kite and Juno follow suit and rapidly create value for their acquisition partners, and most recently, Spark Therapeutics doing the same in the area of gene therapies marks an exciting time. In all my years in pharma, I cannot remember having had the potential to impact patients with such compelling data in a very short space of time. In the span of just a few years, the field released unprecedented data indexing over 90% response rates, with an ambition to transform the lives of children with relapsed and refractory forms of leukemia, and redefined the pricing and access model and strategy for these therapies.

By getting the target disease right and rapidly characterizing the unmet need, we were able to move forward with both the US FDA and EMA and converge HTA thinking with regulatory

DOI: 10.18609/cgti.2021.183

thinking on, for example, moving from overall survival to a totality of endpoints that includes MRD-negative, duration of response, and complete response. There was also a new openness to generate evidence through real-world data.

"...be open to the fact that pioneering work will include setbacks and requires a clear purpose and unwavering commitment..."

There were – and are – two major 'make or break' points or aspects: upstream, you have Cost of Goods (COG) and manufacturing – you've got to get that right. And downstream, the commercial go-to-market model and successfully characterizing the value of one-time therapies with a compelling value proposition, which can be quite a mindset challenge for a healthcare system built around chronic treatment.

In terms of the lessons learned from the experience with Kymriah: firstly, clarify the unmet need at the earliest possible stage, and build a robust natural history and real-world data to support that value assessment. We have a continuous challenge that is the reality of incomplete data in the continuum of care, which necessitates starting the regulatory and HTA discussions early and outside of your pivotal program in order to supplement that evidence. Closely aligning HTA and payer thinking with regulatory thinking and endpoints is critical.

Secondly, be open to the fact that pioneering work will include setbacks and requires a clear purpose and unwavering commitment, especially when you are trying to trailblaze and create a new modality for the industry.

Q How has your access strategy evolved in the past few years to help challenge the traditional go-to-market model?

TS: The access vocabulary has made its way into boardrooms. 'Access' is to some extent the most overly used word in biotech and pharma now – and rightly so!

Companies have over-indexed in past decades on the core technical skills – for example HEOR, HTA methodology, traditional launch sequences and willingness to pay pricing strategies. I think it's time to pivot now beyond the core technical roles and focus instead on access leadership. We need leadership to counter the reality and ambiguity of incomplete data, and uncertainty around how to build a robust value proposition. Advancing treatment with advanced therapies requires a mindset shift – willingness to reset and relearn from the past.

It's critical to think about access as an integrated core function rather than a series of standalone technical critical skills, because for advanced therapies in particular, it is integral to your commercial model.

There are a few questions that need to be raised here: How closely aligned is HTA/payer access thinking with regulatory thinking? And if you're in the biotech building stage, what does the percentage of clinical development investment look like and what percentage of that is allocated to access activities?

"When you have platforms like AAV, cell therapy, and RNA technologies, you've got to ensure you are plugged into the science at the translational stage..."

Why are these questions important? Because you need to work out how you can reach your peak ambitions by avoiding the need to try and retroactively apply your access solutions.

It's never too early to start building out an access strategy. When you have platforms like AAV, cell therapy, and RNA technologies, you've got to ensure you are plugged into the science at the translational stage to quantify the opportunity and the unmet need.

Q You are now leading Global Value and Access for Novartis Gene Therapies – can you tell us about your vision for driving increased access to advanced therapies for rare diseases such as spinal muscular atrophy (SMA), and the key pillars that will support/realize it?

TS: Again, there is a real compelling value proposition and shift in standard of care for what is a new modality. And it's a real privilege to be serving a purpose-driven team dedicated to using gene therapy to solve puzzles for patients. Our vision is to establish gene therapies as a valued and recognized therapeutic category. And why we exist in the organization's construct is simply to ensure that no patient is left behind, which is easier said than done.

We have deliberately focused in on five must-win capabilities:

1. Translational access to focus on key development targets. I would even say at the pre-proof-of-concept stage, in fact, as market access is assumed to happen after regulatory approval.

2. Gene pricing and contracting. These advanced therapies are going to require a new way to recognize revenue, which is going to require smart risk-taking from a contracting perspective and a focus not only on endpoints that you have studied in the clinical trials, but endpoints and outcomes that are being demanded by payers in the real world.

3. Gene economics and outcomes research. How do we change mindset and methodology for one-time therapies versus the incumbent methodology that was designed for chronic therapies, and then ensure that as an innovator we are rewarded for that?

4. Real-world data. Although many of these therapies are given once, we need to ensure we're in the business of sustainable access. Both your registry build and the ongoing tracking of your patients are imperative to support the ongoing reward and recognition for the transformative benefit from a one-time gene therapy.

5. Public policy and advocacy to underscore the above and help enable healthcare systems to seamlessly plan for one-time therapies. For example, in areas such as spinal muscular atrophy

DOI: 10.18609/cgti.2021.183

(SMA) where you have a continuum of care, it is important to help ensure countries are including SMA on their newborn screening panels for to help ensure an early diagnosis and ultimately the best possible outcomes for patients.

As a group, we are very proud that we have managed to achieve access for our gene therapy for SMA in almost twenty markets around the world to date, including in several emerging markets where no other gene therapy has been made available before. Three years since FDA approval, we now have approvals in more than 40 countries and we have treated more than 1,400 patients worldwide, providing a proof of concept for the industry on how to address the hurdles one-time therapies present. Indeed, we are on a journey to take gene therapies where no other advanced therapy may have gone.

Q Zalmoxis recently became the latest approved advanced therapy to struggle when it comes to commercialization in Europe – what is your view of the market access environment on the continent for ATMPs, and what the industry needs to do to ensure future products a) become established initially, and b) enjoy market longevity?

TS: We as a field have encountered some recent headwinds in Europe, but not only there – I think in the biotech space in general, we have been coming to a realization that both inside and outside the clinic, we have been learning about these advanced therapies and their development in real-time as there really isn't a 'gene therapies playbook'.

Whilst I cannot comment directly on the Zalmoxis case, there are some fundamental design questions and considerations at the biotech scoping stage.

For example, ex-US, do I go it alone or partner to accelerate and advance development and commercialization? In other words, do I go faster alone, or can we go further together?

Again, engaging early at the design stage to converge HTA thinking with regulatory thinking is going to be key in helping to develop tailored access solutions.

We also know that Europe is a heterogeneous environment which is why these discussions need to start as early as possible. For example, given the urgent need to treat spinal muscular atrophy (SMA), a rare and devastating genetic disease, we recognized the need for progressive solutions to enable rapid access to our gene therapy upon European approval.

We often say 'Time is Neurons' as SMA in its most common and severe form, SMA Type 1, typically manifests shortly after birth and leads to progressive muscle weakness, paralysis and, when left untreated, most children don't survive past the age of two.

This led to the launch of our 'Day One' access program. Designed to work within existing pricing and reimbursement frameworks, yet recognizing the novel nature of a one-time gene therapy for a devastating and progressive disease, the access program offers ministries of health and reimbursement bodies (in countries without pre-existing early access pathways) a variety of flexible options that can be implemented immediately at time of approval. The program is meant to ensure the continued integrity of the local pricing and reimbursement framework with options that can be customized for each country:

- Retroactive rebates ensuring early access costs are aligned with negotiated prices following local clinical and economic assessment processes;
- Deferred payments and instalment options allowing reimbursement bodies to manage budget impact during the early access phase;
- Outcomes-based rebates negotiated following clinical and economic assessments can be applied to patients treated during the early access period;
- Robust training for treating institutions on administration and follow-up care;
- Access to RESTORE, a global registry of patients who have been diagnosed with SMA that draws upon existing country registries.

As I mentioned earlier, in an industry like ours, it's so difficult to achieve and develop broad access for even one medicine. You have got to play the long game on access, like we are used to playing the long game on development cycles in our traditional models. You also have to be prepared for the fact that the launch sequence, compared to traditional therapies, will be disrupted.

In summary, what I'd say is start your scientific advice and engagement early. The need for education and co-creation with HTA/payers cannot be underestimated as this is how you can genuinely learn about and address their pain points well in advance of commercialization, and ultimately, fulfil your shared desire to enable access for patients.

Q What is your long-term vision for ensuring industry involvement in the rare and ultra-rare disease areas?

TS: I think that as an industry, we need to pivot from a focus on rare diseases in general to a focus on what is a net therapeutic gain for a specific disease on the continuum of care versus standard of care. We are seeing the development of therapies against neuromuscular diseases, lysosomal storage disorders, tumors, and you also have more and more cell and gene therapies targeting chronic conditions. So really, the question needs to be, what is the net therapeutic gain I can achieve versus standard of care in each disease area?

We need to move to a space of greater healthcare system acceptance of these advanced therapies as a viable alternative to chronic cumulative therapies for rare and ultra-rare diseases. For example, maybe there is something the field can learn from the fact that the level of cost scrutiny for potentially life-saving curative advanced therapies at large hospitals (that are used to purchasing high-value assets and equipment) is still greater than it is for more highly cost-associated procedures like heart or lung transplants.

Secondly, as an industry, we need to look at our early engagement models and look beyond the clinical trials to solve real operational, reimbursement, and other hurdles. These are critical to overcome if we are to successfully advance transformative therapies and ensure their rapid uptake in the real world.

DOI: 10.18609/cgti.2021.183

Q You have amassed experience over the past two decades across North America, Europe, Africa, Asia, and Oceania – in a year where expanding patient access to advanced therapies has been a key theme, what is your take on next steps in actually making this happen on a truly global basis?

TS: Globally, there are hundreds of advanced therapies in clinical trials and with the growing number of market approvals, proof points are emerging for gene therapies and cell therapies, and RNA is establishing itself as a treatment modality. I fully expect healthcare systems to adapt in the future to accommodate these new therapies and the transformative value they bring for patients.

At Novartis Gene Therapies we'll continue to leverage the principles that made our first gene therapy for spinal muscular atrophy successful. As we further commercialize in approved countries and pursue approvals in others, it's important to acknowledge that we are still learning as both a company and an industry. We'll remain committed to partnering with all stakeholders to adapt, change course, and continue to learn about how we can ensure every patient who may benefit can get access to transformational therapies.

As an industry, we'll need to continue to remain curious, innovative, and flexible, as well as drive earlier market access involvement in the development process to crystalize the clinical value proposition.

Identifying the right measurements for clinical trials will also be key. Advanced therapy companies should proactively plan early on in the trial process for the evidence that stakeholders might need. That means starting your natural history studies and real-world data constructs at the time of IND submission.

We know that there will be more and more cell and gene therapies coming to the market in future. Moving forward, progressive, value-based solutions that focus on the holistic value a therapy can provide is a reality that the industry is going to have to take the lead on. We must take the business model into the 21st century, moving away from the cumulative and transactional approaches of the past. Ultimately, we need to balance the systemic needs with the very real needs for patients who need treatment, often as quickly as possible – they should always remain core to everything we do.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Sep 22 2021;* ***Publication date:*** *Nov 10 2021.*

DOI: 10.18609/cgti.2021.183

CELL & GENE THERAPY INSIGHTS

BUSINESS INSIGHT: OCTOBER 2021

INTERVIEW

Analyzing trends and opportunities in the advanced therapy tool and service provider sectors

DAVID ANDERSON is a General Partner at Ampersand Capital Partners, Boston, MA. David joined Ampersand in 2010 and participates across many sectors in healthcare but with a special focus on CDMOs and biopharma services in the cell and gene therapy arena. David's current and past board seats include Accuratus, BioClinica, Arranta Bio, Brammer Bio, Elite One Source, Stage Bio, Cellero, Vibalogics, and Genezen. David holds a BSc from the University of Aberdeen, Scotland, a PhD in Cancer Immunology from the University of Sheffield, England and an MBA from Babson College, MA.

Cell & Gene Therapy Insights 2021; 7(10), 1253–1259

DOI: 10.18609/cgti.2021.167

Q What are your working on right now?

DA: As a growth equity investor, Ampersand Capital Partners is focused on the healthcare services market and within that, the CDMO sector is a particular favorite of ours. We have had a number of CDMO portfolio companies ranging from blood

products to medical devices to our topic for today, the advanced therapies market. I recently just returned from our newest investment in this space – Genezen, a lentiviral vector manufacturing business – and I also spend a lot of time with Vibalogics, which is a CDMO focusing on oncolytic viruses and viral vaccines.

This is a market that we pay particular attention to, whether it be monitoring products/therapeutics in development to know where the potential customers are, or tracking changes within manufacturing and process technologies and new, innovative approaches to manufacturing scale-up, scale-out and analytics. This is a very fast-moving market, in all respects. We monitor competitive dynamics but probably the thing that keeps us up at night is the speed and application of technological advances that may alter or negate the need for large-scale manufacturing.

We also continue to look at the periphery of this market. Other investors have jumped into the cell and gene therapy CDMO world of late. But I think that the periphery of this area remains somewhat neglected – there are other businesses in the cell and gene therapy market, whether they be instrumentation, reagents, collection services… There's a whole subset of peripheral support that goes into the advanced therapies market that we are also very interested in. We spend a lot of time trying to understand the whole supply chain, the whole value chain, to look for companies that are not just pure play CDMO assets.

Q There is an extraordinary amount of money flying around cell and gene therapy at the moment, with major IPOs for preclinical-stage biotechs becoming relatively commonplace. What's your view of the current field and market sentiment?

DA: As someone who grew up in the space in the '80s and '90s (I was doing retroviral T-cell immunotherapy work in the lab) I witnessed gene therapy hit some major speed bumps with severe adverse events. However, I think those are well behind us as the market has matured significantly. The technologies and applications have come in leaps and bounds since those early setbacks, and now gene and cell therapy constitutes a stable, focused area for medicine and therapies.

What's particularly exciting is that in many situations, these products are curative. These aren't chronic disease therapies, patients are often 'one shot and done'. I think that's what is driving a lot of the excitement around the field – not just in the orphan indications but even in some more mainstream diseases, we can do something that actually changes a person's life forever. I believe this market is here to stay, and it is why there is a ton of money being poured into it – it is changing the way medicine is delivered. The potential to change medical care is significant.

> "There's a whole subset of peripheral support that goes into the advanced therapies market that we are also very interested in."

Maybe the whole world hasn't woken up to it yet, but let's not forget that we did just run

DOI: 10.18609/cgti.2021.167

the largest clinical trial ever on a gene therapy! It involved a substantial portion of the world's population receiving genetic therapy in the form of a COVID-19 vaccine. Yes, there have been a few adverse events and some unfortunate incidents with a few of the COVID-19 vaccines but overall, the safety profile of the mRNA vaccines in particular is phenomenal, and the efficacy is also significant. I think that on the back of these vaccines' success, we are going to see an even greater move towards harnessing genetic therapies – and by inference, cell therapies – to be a real mainstay of the medical community. mRNA in particular is a very exciting field and we are seeing an explosion of clinical trials.

Q What have been the key recent trends in the cell and gene therapy CDMO space, for you?

DA: Coming from the investment side, we clearly follow deal flow – maybe even a little more than we follow the contracts that are being written between innovators or sponsors and CDMOs. We focus in on things like the recent purchase of Aldevron by Danaher, which I think caught everyone's eye. I think that deal just goes to show that the supply chain specialists are as important and as valuable as the actual CDMOs themselves.

We were fortunate enough to partner with Mark Bamforth and Richard Snyder to create Brammer Bio, at a time when there were no large-scale manufacturing assets focused on *in vivo* gene therapy. We built facilities and experience with our customers, and created a very successful company on the backbone of Richard and his team's decades of experience in the sector. Many others that were either on par with us or followed us – Paragon being one, which was purchased by Catalent – are also very scientifically and technically strong CDMOs. And there is still room for more. We see huge facilities going up; we see tons of investment going into CDMOs in the sector. And yet we continue to hear about a supply-demand imbalance – there still isn't enough supply to meet demand from innovators in this space.

I think that will continue, although it might not for much longer. If you look back at history with the monoclonals and some other methodologies, you see a huge influx of investment into building facilities and then an oversupply. The given field then comes back down to earth a little bit, at which point people try to sell those facilities. The bad ones get shut down and the good ones survive, and there is consolidation. I think we will be heading towards that situation in the next 3–5 years. But for now, there is still a significant opportunity to create CDMO assets, not least because biotechs like the virtual model. There are some that don't – some like to have it all in-house and to control everything – but in general, there has been a wholesale embracing of a virtual model of outsourcing manufacturing to experts. I believe the CDMO market is here to stay and that, especially in cell and gene therapy, there is going to be plenty of room for more growth on the CDMO side.

Q Are there any further trends you expect to see developing moving forward?

DA: As I mentioned, we like to keep an eye open for new technologies that are going to disrupt the traditional CDMO. At Brammer Bio, we established significant capacity by utilizing 2,000-liter suspension reactor systems. We installed multiple iCELLis® 500 systems for anyone who wanted adherent. I must admit that we did scratch our head as to why someone would still want to use an adherent system when suspension gives better yields at lower costs, but it was a case of don't change your process if it's already half-done, just get your product on the market – worry about transferring it to suspension later. There's still a little bit of that gold rush mentality in this field – just get the product to market, and for our next-generation products we will start thinking about how we can reduce cost of goods sold, how we can improve efficiency, how we can improve yield. I think that is where we are going to see the evolution coming in the CDMO space and across the cell and gene therapy market: solutions that can drive efficiencies in the process and address costs.

For example, I was talking to an electroporation company today. If you had said 'electroporation' to me 5 years ago, I'd have said "Great, 90% cell death and 10% viability". But they have solved a lot of those problems and now they are getting 90% cell viability after electroporation, making it a very efficient way of doing genetic transfection.

Better reactor vessels, better media, better downstream processing to reduce waste – I think all of these things are going to continue the drive towards smaller footprints and more efficient, concentrated manufacturing. The smart CDMOs are the ones that are going to keep up with it, constantly evolving by looking at their physical plant and facilities and adjusting as new technologies improve process efficiency.

Q Are there any trends or issues within the CRO sector that will significantly impact the advanced therapies field moving forward?

DA: One thing that is critical in the advanced therapies market is that clinical trials are very, very different.

Within our CDMOs we would get clients that were just about to go into Phase 1, which was really a Phase 1/2 … and then it was really a Phase 2b, and they weren't going to do a Phase 3 because there weren't enough patients … They could really turn the clinical trial paradigm upside down and push for earlier FDA clearance. And the FDA were on-board with that based on the data and the patient dynamics.

I think there's a very different way of running a CRO or services business that is focused on these advanced therapies. It's about speed and it's about access to the patients. But what's very critical is that it's also about long-term follow up. That's the unknown. There isn't a track record or long history with these new therapies. We don't have 30-year follow-up data. We're getting there, and it's so far, so good with one or two speed bumps. So I think in the CRO services space there is significant opportunity to address not just the clinical trial need but the long term follow up of patients into Phase 4 studies. We have one company in our portfolio that is focused on doing the 15-year follow up required to make sure these genetic elements we are introducing into people stay where they are supposed to stay and do what they're supposed to do.

DOI: 10.18609/cgti.2021.167

"I think ... the clinical paradigm will flip: instead of taking 14 years for clinical trials to get FDA approval and then doing a 12-month follow-up, it will be 12 months for FDA approval but a 14-year follow-up to make sure that the therapy is safe and efficacy is maintained."

I think that's where the clinical paradigm will flip: instead of taking 14 years for clinical trials to get FDA approval and then doing a 12-month follow-up, it will be 12 months for FDA approval but a 14-year follow-up to make sure that the therapy is safe and efficacy is maintained.

As for the CROs, I think this movement towards post-approval studies and long-term follow-up will ensure a whole different market emerges – a new opportunity for them. It's already there to a degree, of course – there are already post-approval studies for small molecule and large molecule drugs. But I think there's a different approach now to long-term follow up in genetic analysis that needs to be performed because of some of these advanced therapies.

Q Focusing on AAV-driven gene therapy in particular, it is a period of both great investment by tool and service providers, but also concern over recent clinical issues and long-standing challenges such as pre-existing immunity and redosing – what is your outlook on this field?

DA: I think the positive is that AAV rose to the top for multiple reasons. Payload, safety, integration, all those things. We have all seen the table that shows the different viral modalities and their various pros and their cons, and you basically pick the one that works best for you.

In terms of pre-existing immunity and re-dosing, the good news there is that so far, the clinical results are showing quite long-term effects. So it's not like you are saying to a patient, "This is only going to last ten years and then you're done because I can't re-dose you." You are getting a long enough duration of therapeutic effect for many patients.

I also believe that within the interim period, we will develop the ability to address some of these technical challenges and issues through new vector designs, new serotypes, new ways of looking at things, etc. We will see all the platforms evolve and developers switching vector, if required, to ensure they are using the best possible option for their therapy. There is constant research and development going into not only how we can make AAV better, but how we can also improve other virus types or even other delivery modalities to deliver the same sort of payload with the same or better efficiency that we get with AAV.

Q What are your expectations for the non-viral gene delivery field over the short-to-mid-terms?

DA: I mentioned electroporation earlier, and of course, those guys and others are very bullish on non-viral gene delivery because that's their business. But I do think there's a lot of promise in non-viral gene delivery, and there are lots of different applications or technologies out there now. They all have their place, they all have their pros and cons. If I'm a gene therapy developer, I'm just going to expand my table: I'm going to have my viral gene delivery platforms and my non-viral, and I'm going to cherry pick the best delivery mechanism for me, depending on the size of my payload, what cell types I'm targeting, what controls I want on it. I don't think any one platform, viral or non-viral, is going to capture 99% of the market. There is room for a lot of platforms – maybe not all, but there's room for a lot.

Regarding the short-to-mid-term, all of the non-viral gene delivery folks that I've interacted with believe it's a short-term opportunity. They believe that in a very short period of time, their technologies are going to become the preferred method, because the world wants a non-viral solution. And I would say that's correct from a philosophical level – you can see why someone would make that argument. Unfortunately, the field is faced with a harsh reality that is the practicality of gaining regulatory approval with such novel biotechnologies. Although a non-viral delivery platform might be better – it might be cheaper, faster, safer – if your gene therapy product is already spec'd in with an AAV or a lentiviral vector in the process, you are simply not going to change it. You might change it for the next generation, but you are going to let your therapies that are already in development or on the market run their course.

So I think there is a lot of room for the non-viral approaches, but their adoption is going to be slower than some of the technology providers would like it to be. When they do arrive, though, they will just add more ammunition to the arsenal. They won't replace viral gene delivery, but they will offer different options for developers who are looking for differentiation in the market based upon a perception of safety profile, or differentiation from a cost or an efficiency standpoint.

Q In light of the trends and developments we've discussed, what would be your key advice for cell and gene therapy tool and service providers seeking investment in today's market?

DA: If I was in the services business or tools business and looking at cell and gene therapy as a market, my first thought would be to focus on what the need is today. You want to create a slightly better mousetrap, because incremental innovation is what people are looking for currently. They want to create efficiencies, reduce costs, and increase yields.

The disruptive technologies all have a place, but I don't think developers are going to wake up tomorrow and suddenly decide to throw out the old way of doing something in favor of something brand new. From the investor point of view, we love disruptive technologies, but

DOI: 10.18609/cgti.2021.167

not ones that are so disruptive that nobody is going to adopt them right off the bat. We are looking for things that really apply to a need today. That's why in the cell and gene therapy space, I would come back to my point of looking for opportunities in the peripheral areas. There is a lot going on in transfection – not just electroporation but transfection reagents. New ways of doing things that represent incremental improvements on the current standard. However, if you add up incremental improvements to transfection efficiency, incremental improvements to downstream chromatography, to bioreactor efficiency, to media, etc., etc. – if you stack all of those small changes on top of each other, it really can have a major impact on manufacturing these products.

That's where we look, and that is where I would encourage tool and service innovators to focus moving forward. They don't have to change the world – they just have to change the one little piece they are working on and let others worry about the other pieces, because they do all add up in the end.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Anderson is a former board member of Brammer Bio and a current board member of Vibalogics, Genezen.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

Article source: *Invited.*

Interview conducted: *Jul 15 2021;* ***Publication date:*** *Oct 26 2021.*

CELL & GENE THERAPY INSIGHTS

October 2021

Clinical Trends

CELL & GENE THERAPY INSIGHTS

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INTERVIEW

Playing the long game in gene therapy clinical development

JONATHAN D SCHWARTZ, MD. Following an academic career marked by excellence in teaching, translational research and patient care, Dr Schwartz has demonstrated expertise in biopharmaceutical development. Specific strengths include comprehensive integration of scientific, clinical, operational, and regulatory issues, efficient translation of concepts into well-designed clinical programs, expert leadership of cross-functional teams, and mentorship of junior colleagues. Dr Schwartz oversaw the development of ramucirumab (CYRAMZA) from late Phase 1 into a multifaceted global Phase 3 program culminating in FDA, EMA and PMDA approval in stomach, liver, colorectal and non-small cell lung cancers. As a foundational executive officer of Rocket Pharma, Dr Schwartz was a core member of a leadership team that enabled growth from Series A start-up to a publicly traded, multiplatform gene therapy company (valuation >$2B) over 5 years, with 5 successful IND filings in 2018–20.

Cell & Gene Therapy Insights 2021; 7(10), 1261–1267

DOI: 10.18609/cgti.2021.168

Q What are you working on right now?

JDS: At Rocket, we often say that on any given day, one hour you will have your head in the clouds working on something very strategic and conceptual, and the

next, you will be knee-deep in the trenches, digging along with everyone else on very granular or logistic matters! And right now, I'm certainly involved in a real mix of things.

A big part of my current work is recruiting and onboarding new colleagues, and planning organizational growth that will enable us to fulfil and sustain our programs as they move further in development. I'm also overseeing some of the data cuts and presentation plans for disclosures across the pipeline that will be occurring in the remainder of Q3 and Q4 2021.

From a bigger picture standpoint, I recently worked on a lentiviral vector safety webinar that was hosted by Josh Schimmer, biotech analyst and innovative therapeutic expert at Evercore ISI. The aim was to provide a lot of critical data-focused points regarding the overall lentiviral vector safety profile to date, which demonstrated that the small number of unfortunate adverse events that have occurred have for the most part been sequestered in some very specific programs, and don't portend poorly for the entire field. It's likely that we'll be planning a subsequent session regarding AAV safety, with a similar emphasis on the data and the promise that continues to exist.

From a science and medicine perspective, I've been working with a number of colleagues in both US and European academic institutions on a review publication for pyruvate kinase deficiency, which is the target indication for one of our lentiviral vector programs. We have attempted to summarize much of the critical natural history results that have been presented and published in the last three or four years and their relevance, particularly in terms of how we think about gene therapy playing a key role in this disorder moving forward. We will also be doing some additional review articles on other topics across the lentiviral vector platform.

Finally and very importantly, on the regulatory front, we have two programs that are now in registrational trials – in Leukocyte Adhesion Deficiency-I and Fanconi anemia. I'm spending as much time as I can thinking about the timing and essential content for the potential BLA filings that we'll be hopefully progressing in the not-too-distant future.

Q Rocket's pipeline includes product candidates based on platforms that are very different in many ways (AAV *in vivo* and LVV *ex vivo*) – what are the key considerations for each one in terms of your own role and activities, and how do you balance the two?

JDS: The short answer is: "it ain't easy!" The more nuanced response is that at Rocket, while the platforms and product characteristics differ, the unifying features are that:

a. The disorders we target are in general life-threatening (sometimes during childhood or adolescence/young adulthood), and

b. Each therapy has the potential to transform or in some cases, cure the most dangerous aspects of these disorders.

DOI: 10.18609/cgti.2021.168

So, whether we are dealing with AAV or lentiviral programs, the focus is on minimizing potential risk while maintaining the potential for extensive efficacy. Regardless of platform, I'm always thinking about efficacy endpoints, what matters to patients and their caregivers, and how do we design programs that demonstrate the value of a therapy to both regulators and payers. For instance, do we have sufficient natural history data to enable us to articulate the program value, and how can we further those efforts to make sure that we are not only providing the best therapy, but also telling a meaningful story?

"I'm always thinking about efficacy endpoints, what matters to patients and their caregivers, and how do we design programs that demonstrate the value of a therapy to both regulators and payers."

But the balance is always difficult. In many respects, we are aided by having Chief Development Officers for each of the AAV and lentiviral programs, who can help shepherd the programs. They also nurture talent at every level and across disciplines, both within the medical ranks and all the other areas that either do or don't report through me, so that the teams are really empowered to make things happen – day-in and day-out.

Q Clinicians and clinical development professionals speak of the 'stack' as being a key challenge from the COVID-19 pandemic – the fact that all the COVID-related challenges are stacked on top of all the usual challenges they face. What have been the major issues this 'stack' has thrown up for you, and what has been your/Rocket's approach to keeping the clinical pipeline moving forward as far as possible?

JDS: The key is limited sleep! And on a more serious note, there have definitely been some stack issues that have necessitated a lot of vigorous workarounds.

One issue is patient travel – not so much for therapy but for follow-up. That was a particular challenge for some of the initial patients in our Danon disease program, where follow-up at the same center at which the baseline evaluation took place was especially critical: echocardiograms and other cardiac-focused evaluations really need to be in the hands of the same operator with the same equipment, otherwise you run the risk of comparing apples with oranges, which is never optimal. With the lentiviral programs, the critical endpoints are in the hands of very specialized, centralized laboratories, so even when the patients were able to get to the treatment center or a local medical center was able to procure blood cells and serum for testing, shipping the biospecimens was a real challenge. The number of flights going from one part of the US or Europe to another, or going across the ocean, were vastly diminished, which meant fewer collections. And even though these are specimens that may be on ice or in other special containers, there is still only a 48-hour window, perhaps, before the test results start to become suspect.

"We are really looking at hopefully lifelong and at minimum decades-long benefits, and I think that objective is increasingly backed up by the science."

So shipping was an enormous challenge and at times, the workaround was developing additional labs, assays, and facilities closer to the treatment centers. However, that introduces the requirement for subsequent comparability assessments, meaning that for every test, there are additional layers of complexity.

I think the important thing that we've needed to keep in mind throughout this testing period is that we are really playing the long game here. We believe that the value of our therapies is in their sustained efficacy – it is not as though we are looking for a tumor response at 3 months that's then confirmed at 6 months, and if we've done that, well, great – everything else is gravy. We are really looking at hopefully lifelong and at minimum decades-long benefits, and I think that objective is increasingly backed up by the science. So if we have had to wait a bit longer to confirm results at times, while it's disappointing and sometimes prevents us from having a nice presentation in the short-term, in the long-term it's not a deal-breaker.

But certainly, there has been no shortage of pandemic-related challenges on top of all the other challenges of working in rare diseases, and looking at the innovative and unique endpoints that these diseases demand.

Q Are there any less-obvious pandemic-related issues for clinical development that you would you pick out as significant?

JDS: Other than the shipping issue, I think the other thing is that while we might have our Zooms and our Microsoft Teams – our speed dials, emails, and all the other ways of communicating without leaving our domiciles – the in-person conferences are nonetheless invaluable, and I miss them greatly. Whether it's a gene therapy, hematology, immunodeficiency, or congestive heart failure cardiology conference, the chance to meet in person with our investigators, additional advisors, and investors – and to meet with potential new collaborators – is very special. There are also the chance encounters at these conferences that spur new ideas and new collaborations. And very importantly, there's the things that you learn and share between the sessions and the big meetings – those hallway conversations and the insights one gleans over coffee early in the morning, or at the bar after the last session is over; those are invaluable. Sometimes those details make all the difference. For me, it can't be too soon that we can get together again in person – I really can't emphasize that enough.

Q On the AAV side, it has been a challenging year or two for a space looking to deliver enhanced clinical safety and durable efficacy – what do you see as the most promising routes forward towards achieving these twin goals?

DOI: 10.18609/cgti.2021.168

JDS: That is the question of the moment in AAV, and I think that the answer in one word is 'perspective'.

By that I mean that in many examples both at Rocket and across the field, we are talking about life-changing and potentially curative therapies for devastating diseases. These are often disorders without viable treatment options, or current treatment options that are associated with sustained complications. And I think it's important to recognize that across medicine and science, many of the most meaningful therapies do come with side-effects. You can think of allogeneic hematopoietic stem cell transplants, for example – there are now tens of thousands of these procedures being done every year across North America and Europe, including several thousand pediatric transplants and a good number of transplants for inherited disorders, and these all are accompanied by adverse events. Nevertheless, the benefit justifies the risk. You can also think about the treatments that we offer for cancer where frequently the benefit is incremental, and yet there are very substantial side-effects that are sometimes nearly universal – but again, it's accepted. So the expectation that gene therapy will be toxicity-free really doesn't reflect our experience over the last forty years in drug development.

Our job is obviously to optimize our therapies by minimizing these side-effects while maintaining that potential for transformative benefit. I think the field has been doing an excellent job of that to date, and I'm optimistic that we'll do an even better job over the coming years.

Q Rocket specializes in targeting rare disease indications. How do you go about obtaining the required insights from the naturally limited patient populations and clinical data sets available in this area? What supporting or alternative data sources and tools prove most valuable in this regard?

JDS: I think the key is to keep listening and to commit to being a lifelong student. Don't trust anyone who claims, "I've got this all figured out." And listen to as many information sources as possible.

One thing we've tried to do is create forums for our patients and families to tell their stories, or where those forums already exist, make sure that we are able to participate and listen more than opine. It's very important to do this across the spectrum of each disorder, including the most and least severely afflicted individuals, and also across geographies – the most relevant concerns for someone in Germany might be differ to those for somebody in Spain, or somebody in a Pennsylvania Amish community, or a Romani community. But it all matters. We have trusted investigators who are amazing partners and other

"...it's important to recognize that across medicine and science, many of the most meaningful therapies do come with side-effects."

advisors as well, but it's always important to think about widening that circle and hearing additional voices that are relevant. And it takes time to uncover these voices.

Additionally, we read as much literature as one can. We read like someone's life depends on it, because it does. And then once we've read enough and listened enough, it's vitally important not to be frightened to put it all together. And whenever possible, we make sure that we are generating publications that are consolidating all those voices. Some of the most informative publications on these rare disorders came from data generated in the 1980s, before there were many meaningful therapeutic interventions, and so much of what the investigators could do was observe and describe. Today, the more prominent publications tend to be those that describe the interventions as opposed to the disease manifestations themselves, but all of it is important. So whenever we identify a need, we seek to then make sure that we're articulating the most relevant aspects of each disorder in ways that are incorporating all of the work that's been done over prior decades as well as recent years.

Q What would you highlight as some key best practices in long-term safety and efficacy follow-up study design and implementation for gene therapies against rare diseases?

JDS: That is becoming another question of the moment, but it's not just for this moment. Here, I think the importance is balance, a very careful balance, because in some cases we are designing studies for 12 or 15 years. We need to think really carefully about every test that's specified, because we are going to be doing it for a long time. And we are committing a patient and a medical center, and a study team, and financial resources for the duration. I've always thought of myself as someone who was in it for the long game, but I had never designed a 15-year study until very recently. It requires a lot of thought and a discussion for every component.

One important thing we can do is to put away cells, serum, and other critical materials and save them for things that we don't yet know about and haven't yet anticipated. One thing is for certain: in five or ten years, there will be new questions, new tests, and new methods that we're not even aware of right now. We want to make sure that those long-term studies are able to address or accommodate those future innovations. For example, we know that for the lentiviral vector programs we need to do integration site analysis on blood cells for the next 12 or 15 years, but it's likely there will be other things as well.

It's also key to bear in mind that it's not just about long-term safety. Long-term efficacy is going to matter as well. Especially for health authorities and payers, we need to be able to demonstrate that hopefully the benefits of these therapies are sustained. So far, the science appears to be backing that up, but it's our job to make sure that we're positioned to demonstrate this.

Q Finally, can you sum up your major goals and priorities in your work over the coming 12–24 months?

DOI: 10.18609/cgti.2021.168

JDS: Coming back to my initial comments, much of it will be about building the team and investing in new colleagues. For the registrational studies in Fanconi anemia and Leukocyte Adhesion Deficiency-I, we will be planning for thoughtful, detailed health authority submissions that articulate the value of the programs. And for the early-phase studies, it's about focusing on optimizing safety and also thinking carefully about the most meaningful endpoints as we prepare for Phase 2 registrational trials. Further up the pipeline, we'll keep thinking about and planning for the next generation of programs, because although we have five programs currently in clinic, we will always want to ensure that these are technologies that can be applied to a widening range of appropriately selected disorders.

It will also be important for me in my role as Chief Medical Officer to continue helping the broader community to focus on the actual data to date. What do we have? What have we seen? What are the benefits, what are the risks, and do the benefits justify the risk? So far, they have, but it's easy to lose sight of this. We need to make sure that the perspective for each therapy is maintained – to ensure that we are able to offer patients in most if not all circumstances a therapy that carries the promise of meaningful sustained benefit, with side-effects that are hopefully limited and less than those for the available standard of care.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Schwartz is a board member of and stock holder in Rocket Pharma.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

Article source: *Invited.*

Interview conducted: *Sep 28 2021;* ***Publication date:*** *Oct 22 2021.*

CELL & GENE THERAPY INSIGHTS

Clinical Trends

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INTERVIEW

Pioneering commercial CAR T cell therapy clinical development in India and South Asia

ANIL KAMAT is a recognized clinical leader in hematology, with extensive experience in both clinical and manufacturing contexts. He is head of clinical development at Immuneel Therapeutics, based in Bengaluru, India, and serves as a consultant hematologist for the UK National Health Service. Dr Kamat is a member and contributor to several societies, including the International Society for Cell and Gene Therapy, the American Society of Hematology, and the European Group for Blood and Marrow Transplantation.

Cell & Gene Therapy Insights 2021; 7(10), 1269–1274

DOI: 10.18609/cgti.2021.169

Q What are you working on right now?

AK: Immuneel is one of the first biotech start-ups focused on delivering cell and gene therapy products for the India+ region, and we want to bring these therapies at an affordable cost.

Our primary focus now is on getting ready for our CD19-targeted CAR T cell therapy clinical trial, ensuring we get everything ready for dosing our first patient.

Immuneel's study will be the first industry sponsored CAR T trials in India. Obviously, the challenges around that are significant.

Q Expanding patient access to advanced therapies on a global basis has been a key message at major virtual conferences and events throughout the course of 2021. Can you frame for us the scale of this challenge in India and South Asia, and how Immuneel is preparing to play a key role in addressing it?

AK: There are already four approved products for CD19 in the US/Europe, as well as one BCMA-targeted CAR T approved in myeloma. However, in India and in South Asia, we do not yet have access to these treatments. There are multiple issues. One is the availability itself. Second is the cost. And thirdly, the capabilities.

The plan for Immuneel is to commercialize CAR T therapy here and offer it at a world-class quality. We also want to make sure the costs are reduced by producing it in India. To do this, our focus is on building scalable ecosystems for the cGMP manufacture and delivery of cell therapies. As all in this field know, it is not as simple as opening a box, and there – you have a solution. We have to work on the entire chain in terms of how the cell and gene therapy is delivered.

So, we are trying to affordably provide these transformative immunotherapies to patients in India. There's a huge unmet need here: just as you see in the West, India also has a huge number of patients with hematologic malignancies such as acute lymphoblastic leukemia (ALL), B cell non-Hodgkin lymphoma (B NHL), and multiple myeloma (MM).

The rising uptake of these therapies, and an increasing number of publications showing that the real-world data for safety and efficacy is in sync with the clinical trial data, is very encouraging - especially for countries like India, because it means we now know that these technologies can deliver. The published data shows that six out of ten patients with ALL can have long-term remissions, while for B NHL, the data is four-to-six out of ten patients.

Initially, we are working on two tracks. One is that we have in-licensed a CD19 construct from Hospital Clinic de Barcelona. This particular construct has already been used in a Phase 1 study in Spain, showing 71.1% responses, which is as good as any of the approved products. Leveraging this data, we are going forward into Phase 2. Secondly, we are working on our own R&D in terms of constructs in various hematological malignancies and beyond as well.

> "The plan for Immuneel is to commercialize CAR T therapy here and offer it at a world-class quality. We also want to make sure the costs are reduced by producing it in India."

To deliver these therapies, we need a combination of people, science, and infrastructure. Infrastructure is absolutely critical over here because these are 'niche' personalized cell

DOI: 10.18609/cgti.2021.169

and gene therapy products. You also need certain cGMP conditions, and we haven't had any commercial manufacture of CAR T cell therapies in India to date. This will be the first time in India, and in South Asia at large, that we will be operationalizing a facility towards CAR T cell therapy for clinical trials and later commercialization, which speaks to the scale of the challenge – we have to develop an entire ecosystem, including personnel. And of course, there are the regulatory processes to consider.

Q What would you firstly pick out as they key trends in regulatory evolution in the region?

AK: The first CAR T cell therapy products approved in the US were approved in 2017, so we are four years down the line and regulators across the world have had some time to adapt. However, cell and gene therapy remains an area of deep learning for the regulators.

India has produced a few good guidelines, which were issued in late 2019 – we have a cell and gene therapy guidelines issued by the central regulator, as well as some new documentation as to the conduct of clinical trials. We also have the ICMR (Indian Council of Medical Research) that has issued updated guidance around bone marrow transplant units for quality control of these novel cell therapies. All in all, it is pointing in the right direction. The regulatory environment is now getting ready to approve these products. However, because we are undertaking the first industry sponsored study, it is still a steep learning curve for everyone involved.

There are several regulatory checkpoints in India. There are entities like the Gene Therapy and Advisory and Evaluation Committee (GTEAC), which is the advisory council. We have the RCGM (Review Committee on Genetic Manipulation), which is a council for gene materials. And then there is the central regulator itself, the Central Drugs Standard Control Organisation (CDSCO), which is the federal regulator for approvals. So here it is not a case of a 'single window' for approval – it is a multilayered regulatory process. The heart of the matter is that the product should be of world-class quality, which needs to be underpinned by the science, the Phase I safety and efficacy data, and of course, the quality control and release assays. We are focusing on having everything in place in terms of the cGMP manufacturing, the quality control processes, and the quality assurance surrounding it to enable our first clinical trial.

Q How does Immuneel need to tailor its trials as a result of this regulatory environment?

AK: It's a very pertinent question because if we look at the current approvals in the West, they are for specific diseases indications.

With regards to the scientific hypothesis, when you start a clinical trial, the background incidence of responses is very important. That is quite challenging here, because if you look at new drug approvals for CAR-T cell products in US/Europe, they are for second-line, third-line, and beyond – in other words, for multiple relapsed/refractory indications. And in India,

the really big challenge is that beyond first-line treatment, there is a natural reduction in the number of patients proceeding to further lines of treatment. This is for various reasons, including loss of fitness, economic reasons, and lack of access. Basically, you are not going to find too many third-line or fourth-line patients or those post-transplant going into cell and gene therapies. And even if you do find these patients, because they've already been exposed to so many treatments in the past, their fitness may not be at the level where you would be comfortable to recruit them into the study. Consequently, in the India+ region, we may have to look at more upfront treatment with cell and gene therapy. By upfront, I'm not talking first-line right now, I'm talking about second-line relapses or patients with residual disease post induction.

There is also the location of the study to consider. We already know the challenges of ultra-cold chain logistics from the COVID-19 pandemic and of course, the nature of autologous cell and gene therapy is that it is a personalized product with a complex supply chain. Immuneel is based in Bangalore. We are on the top floor of a very busy bone marrow transplant unit – the Mazumdar Shaw Medical Center. This means that our cGMP-compliant manufacturing will be one floor up and the logistics are downstairs, just one floor down. That is about as near as you can get to the patient. There are advantages to this strategic position – of course, we are also in the process of sorting out the logistics so that the product can reach other parts of the country as well, but the more you can cut down on the challenges of ultra-cold chain logistics the better.

Two of the main known adverse events with CAR T cell therapies are of course cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). There are quite a few elements to our approach to adverse event management. There is the preparation, in terms of making sure that drugs like tocilizumab are on hand. Then there is the education part, ensuring that all the stakeholders and investigators are fully aware. And importantly, there is also the patient selection component.

In a country like India, it is key to keep the adverse events to a minimum and avoid them as far as possible. For example, Fractionated dosing (i.e. splitting the total dose in separate infusions infused over different days) is one such option. Every little thing that helps to improve the experience for the patient, is important for us to adopt. And the trial needs to enable some flexibility – for example, if a given patient doesn't tolerate the second infusion, let's say, then they will not receive the third. We should adapt according to each patient's tolerance of the therapy.

To summarize, the key aspects include patient selection, the indication, the training, the balance of flexibility and scientific rigor of the trial, and how close the manufacturing is to the patient. That's how we are approaching this interesting challenge for ourselves and for India as a whole. We believe we can set a trend in terms of how clinical studies of these advanced therapy products can be done in the India+ region.

Q In what areas is the COVID-19 pandemic having its most significant impact on clinical development in the region, currently? And what have been the most valuable strategies and tools available to

DOI: 10.18609/cgti.2021.169

clinicians and trial sponsors in trying to minimize disruption?

"it has been critical to focus on the study schedule because one of the biggest disruptions during the height of the pandemic was logistics, patient travel and transport."

AK: Almost 2 years into the pandemic, we have not yet seen the last of COVID. It has been a huge disrupter for all, especially in the cell and gene therapy industry, and we at Immuneel have been affected as well. Our trials have been delayed, for example, which has disrupted everything from infrastructure to trial planning. But we are getting there. We are learning to live in the new normal, with COVID expected to transition to being an endemic rather than a pandemic disease.

Clinical trial practice and processes have been disrupted – and have now been adapted – in a number of ways. Firstly, let's address the conduct of the study. The practice today in both the West and India is for trial patients to get vaccinated against COVID as soon as possible. So, if you are a patient who is likely to be a candidate for a clinical or industry sponsored study, you would then be vaccinated, preferably with both doses. I know there is a bit of an uncertainty around immune response, but vaccination is definitely mandatory.

The second piece is the COVID-prompted behaviors that have now become standard – the use of PPE and so forth. And thirdly, and perhaps most importantly, it has been critical to focus on the study schedule because one of the biggest disruptions during the height of the pandemic was logistics, patient travel and transport.

These cell and gene therapy studies are designed in such a way that the patient stays in hospital for a few days or weeks initially, before being discharged and then followed-up subsequently at certain time points. However, this model can actually help in terms of improving compliance – you arrange transport to bring the patient to the hospital, or for a test that could be done at the patient's home, and that brings a measure of control and ensures the study stays on schedule.

Ensuring that manufacturing is as close as possible to the patient will minimize the impact of disruption to ultra-cold chain logistics.

Q Finally, can you summarize your chief goals and priorities, both for yourself in your own role and for Immuneel as a whole, over the coming 12–24 months?

AK: As I mentioned at the beginning, our mission at Immuneel is to provide affordable cell and gene therapies to India and the India+ region. Our main focus over this period will be to recruit patients to our upcoming studies. So, I would hope these studies will lead to approvals and increase access to these therapies for patients in the region – that's our goal. Of course, to get there, there's a whole process to navigate including ensuring the

cGMP manufacturing, the quality assurance and quality control systems, and the logistics are all in place.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Sep 7 2021;* ***Publication date:*** *Oct 22 2021.*

DOI: 10.18609/cgti.2021.169

CELL & GENE THERAPY INSIGHTS

Clinical Trends

-

EXPERT INSIGHT

Clinical applications of gene and cell therapies: case studies for the relevance of precision medicine

Oscar G Segurado, MD, PhD & Ruhong Jiang, PhD

Precision medicine, a medical modality focusing on tailoring medical decision-making to individual patients, is changing the way we think about, prevent, treat, and monitor many diseases, including those requiring gene and cell therapies. Both gene and cell therapies involve the therapeutic transfer of new genetic material into a target cell with the goal of treating disease. The fields of gene and cell therapies are growing, but there are many unknowns and reasons to be cautious remain. Selecting the right patient for the right therapy and monitoring that patient's response to the therapy is imperative. Biomarkers are tools that can facilitate selection and monitoring of gene and cell therapies, and their proper identification and application allows patients to be treated accurately, effectively, and safely. Several biomarkers of disease, immune, cellular, and molecular responses to gene and cell therapies are available, and the role of biomarkers will expand as gene and cell therapies continue to develop. With the rapid growth of gene and cell therapies, biotechnology and pharmaceutical companies face a call to action: we must establish proper selection and monitoring protocols to provide patients with the safest and most effective therapeutic options for genetic diseases. This article presents two case studies from a biopharmaceutical company's clinical programs for gene and allogeneic cell therapies and provides a primer for the relevance of precision medicine applications.

Cell & Gene Therapy Insights 2021; 7(10), 1325–1336

DOI: 10.18609/cgti.2021.175

INTRODUCTION

Precision medicine is a medical modality that focuses on tailoring medical decision-making to individual patients, and it offers an innovative, individualized approach to health care by considering a patient's genetics, lifestyle, and environmental exposures to tailor disease prevention and treatment [1]. Gene and cell therapies are part of precision medicine, and they are changing the way we think about, prevent, treat, and monitor many diseases [2]. Several modalities of gene and cell therapies involve the therapeutic transfer of new genetic material into a target cell. With gene therapy, only genetic material is transferred to a patient. The new genetic material changes how a cell expresses a gene and makes a targeted protein. This approach may include making more disease-fighting protein, less disease-causing protein, or an entirely new protein. With cell therapy, whole cells are transferred to a patient. The new cells restore or alter cells in the body or carry therapy to specific organs or tissues. Terminology related to gene and cell therapies is listed in Table 1 [3–9].

The fields of gene and cell therapies are growing at unprecedented rates and will change the future of health care, but there are many unknowns and reasons to be cautious remain. For gene and cell therapies to be effective, the body is challenged to do something that it does not normally do, such as express a new gene that synthesizes a protein or interacts with a foreign cell, or to do what it normally does in a different way or in a different quantity, such as producing more of a naturally occurring protein. These changes may result in immune response and toxicity concerns. Therefore, it is imperative to first select the right patient for the right therapy and, second, to monitor that patient's response to the therapy. Biomarkers are tools in the arsenal of selection and monitoring of precision medicine, and their proper identification and application allows patients to be treated accurately, effectively, and safely with gene and cell therapies. An expanded role for biomarkers is emerging as gene and cell therapies continue to develop.

Precision medicine is a fast-changing and variable field, and this article is not intended to be a comprehensive review of its relevance for gene and cell therapies. Instead, this article offers two representative case studies of gene and allogeneic stromal cell therapies targeting diseases with unmet clinical needs where precision medicines are essential components. Many clinical trials of gene and cell therapies are underway around the world and several comprehensive review papers of gene and cell therapies have been published; readers are encouraged to learn more about precision medicine applications by accessing these resources.

CLINICAL DEVELOPMENT OF GENE & CRISPR THERAPIES

Gene therapy involves transferring a new gene to a patient with the goal of treating a disease [10,11]. The new gene may be an addition to the host genome, replace a disease-causing gene, or correct or inactivate a defective gene. For example, hemophilia A is a monogenic hereditary disorder (meaning that it is caused by a single defective gene) that leads to deficient production of factor VIII, a key blood-clotting protein. The genetics of hemophilia A are well understood and, as such, hemophilia A has become a target for gene therapy that corrects the defective gene.

Currently, many clinical studies of gene therapy for hemophilia A use an adeno-associated viral (AAV) vector to deliver genes that encode production of factor VIII directly into target cells in the liver. The liver cells, in turn, become 'protein factories' that secrete factor VIII into the body's circulation. With this gene therapy technology, the host cell primarily retains the transgene sequences as episomes; that is, the AAV vector exists as extrachromosomal material and is able to synthesize protein independently from the host chromosomes (Figure 1). It is uncommon for episomes to integrate into the host

DOI: 10.18609/cgti.2021.175

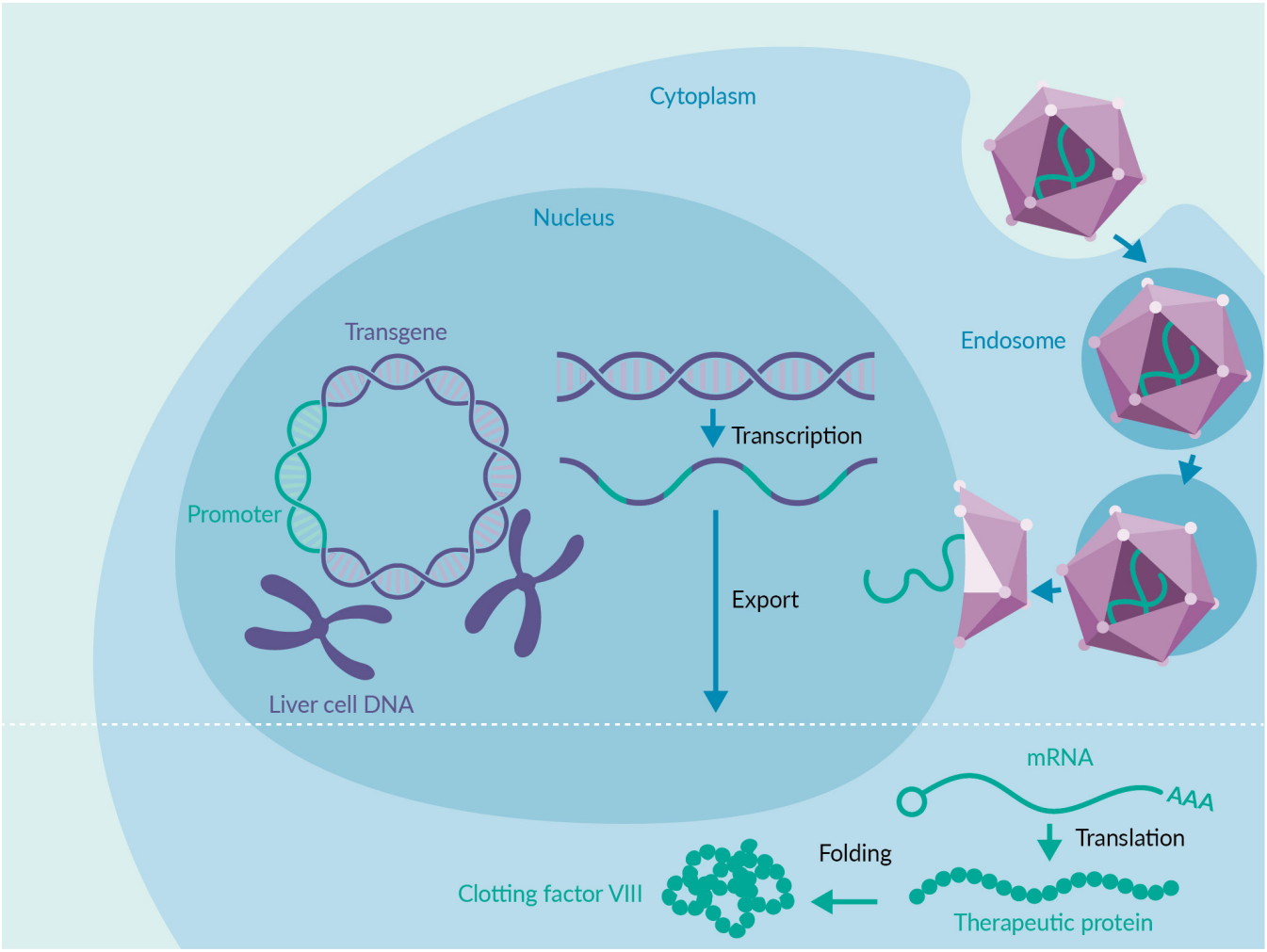
TABLE 1

Terminology used in cell and gene therapies [3–9].

Term	Definition
Adeno-associated virus (AAV) vector	An adenovirus that is used as a vehicle for genes, whose core genetic material has been removed and replaced by the dysfunctional gene
Antibody	Proteins that help fight infections
Biomarker	A measurable indicator of a physiologic state of an organism
Chromosome	A DNA molecule stabilized by proteins that carries hereditary (genetic) information (genes) of an organism
Cellular therapy	Transferring intact cells into a patient to cure a disease
CRISPR	Stands for clustered regularly interspaced short palindromic repeats; a gene-editing technique that is used to identify and modify specific DNA sequences in the genome of an organism
DNA	Deoxyribonucleic acid. One of two types of nucleic acids made by cells (the other being RNA); the molecules inside cells that carry genetic information and pass it from one generation to the next
Decidua stromal cells (DSCs)	Maternal stromal cells derived from the fetal membrane, more immunosuppressive than other types of stromal cells
Gene	The pieces of DNA that are passed from parent to offspring; genes contain instructions for making a specific protein
Genome	All the genetic information of a cell or organism
Gene deletion	The loss of all or part of a gene
Gene duplication (gene amplification)	An increase in the number of copies of a gene
Gene editing	The use of biotechnological techniques to make changes to specific DNA sequences in the genome of a living organism
Gene substitution	A type of mutation where one nucleotide is substituted for another
Gene therapy	A type of treatment in which altered genetic material is inserted into a person's cells to prevent or treat disease
Gene transfer	The insertion of genetic material into a cell
Genetic mutation	A permanent change or alteration in the DNA sequence that makes up a gene; it can be harmful, beneficial, or have no effect
Hematopoietic stem cells	Cells that can replenish themselves and produce cells that develop into a variety of mature types of blood cells
Hepatocytes	Liver cells
Immune response	The action of the immune system against foreign substances (antigens)
Mesenchymal stromal cells (MSCs)	Multipotent, non-hematopoietic stem cells that are present in adult and fetal tissues; capable of differentiating into various cell types, including adipocytes, osteocytes, chondrocytes, and cells in connective tissues
Protein	The major macromolecular constituent of cells; it is required for structure, function, and regulation of the body's cells, tissues, and organs
RNA	Ribonucleic acid. One of two types of nucleic acids made by cells (the other being DNA); contains information that has been copied from DNA. Several types of RNA exist, each with diverse functions that are important to normal cellular processes
Transgene	A gene that has been transferred from the genome of one species into that of another
Transcription	The process of synthesizing messenger RNA from DNA
Transduction	The process of transferring foreign DNA into a host cell using a virus or viral vector
Translation	The process by which the information from a sequence of messenger RNA is used to produce a protein
Virus	A simple microorganism that infects cells and may cause disease; can multiply only inside infected cells, so they are not considered to be alive
Vector	Viral DNA that is used to transmit genetic material to another cell or organism

FIGURE 1

Gene therapy for hemophilia A.



genomic DNA [12,13]. A key limitation of episomal genetic material is the inability to be maintained during cell division. This impacts the use of gene therapy in target cells in organs that continue to grow and develop through childhood. Therefore, gene therapies targeting liver cells are indicated only for adults.

Gene therapy is limited by high costs and challenges in the large-scale manufacturing of vectors, vector quality control and assay standardization, and immunologic barriers to gene delivery through viral vectors. Additionally, the purification of recombinant AAV particles is difficult and batch-to-batch variations in vector potency limit consistency [14]. One particular concern of AAV-based delivery is $CD8^+$ T-cell–mediated immune responses. These cells are able to eliminate vector-transduced cells, which induces an inflammatory response in the target organ and diminishes the potential benefit of the gene therapy. In AAV-based delivery in hemophilia A, $CD8^+$-mediated response has been identified against the viral capsid, which causes the loss of hepatocytes that express the therapeutic transgene [15–17].

CRISPR (which stands for clustered regularly interspaced short palindromic repeats) is an effective gene-editing tool for targeted gene therapies. The CRISPR technology requires two key components:

1. An RNA guide that identifies the target sequence; and
2. An enzyme that cuts DNA (usually Cas9).

DOI: 10.18609/cgti.2021.175

There are three different approaches currently in clinical development to treat monogenic diseases: *ex vivo*, *in vivo* gene deactivation, and *in vivo* gene replacement [3,18]. In contrast to standard gene therapies, CRISPR does not retain genes in the cell nucleus as episomes. With CRISPR, the gene is integrated into the host DNA and preserved during replication, meaning that it can be used in cells that are growing and dividing, such as the liver cells of children. For example, in hemophilia A, CRISPR has been used to insert the B-domain that is deleted from the *FVIII* gene, which directs factor VIII production, to restore factor VIII expression [19].

Despite its promise, CRISPR is associated with potential off-target effects. The consequences of the off-target effects are variable and depend on many factors. The risks of off-target effects, though also variable, may limit future uses of CRISPR technology [20–22].

A key consideration in the clinical development of gene therapies is defining the single therapeutic dose to be administered to patients. Initially, preclinical studies are conducted in animal models, and a starting dose is evaluated and adjusted. After the dose is established, the findings can be translated to dose-finding and safety trials (Phase 1/2). Next, large-scale Phase 3 clinical trials can be conducted to demonstrate safety and efficacy for the target population.

Biomarkers for patient selection & monitoring in gene therapies

Gene therapy for hemophilia A is limited by potential neutralization or inhibition of the transgene or vector by antibodies and cell-mediated immune responses. Additionally, a variety of patient characteristics can impact the distribution, uptake, and response to therapy. By identifying biomarkers that indicate potential safety or efficacy concerns, one can optimize therapy for patients with the highest likelihood of successful outcomes.

Neutralizing antibodies of the viral vector

Neutralizing antibodies to specific AAV serotypes are prevalent due to natural infection with wild-type AAV during childhood [4,23–26]. By neutralizing the vector, these antibodies reduce the efficacy of gene therapy for hemophilia A. Unfortunately, no tests for detecting anti-AAV antibodies have been standardized [13] and no strategies for overcoming the antibodies have proven effective so far [27].

INHIBITORS TO THE TRANSGENE PRODUCT

Traditional hemophilia A treatment consists of factor VIII replacement [28,29]. A primary complication to this approach is the development of inhibitors, which are antibodies that neutralize the replacement factor [29]. To date, there have been no reports of inhibitors to factor VIII in clinical studies of AAV gene therapy for hemophilia A treatment. However, clinical studies excluded patients who had any history of inhibitor presence and, therefore, have included only patients with a low risk of antibody formation [30]. Studies of patients with active factor VIII inhibitors are ongoing to determine the impact on safety and efficacy [26].

Functional biomarkers

Liver enzymes can serve as functional biomarkers of response to gene therapy. Asymptomatic increases in alanine aminotransferase (ALT) levels can be observed in patients receiving gene therapy for hemophilia A. Most ALT elevations are no more than 1.5- to 2-fold above the upper limit of normal and are transient in nature; as such, the ALT elevations are unlikely to be clinically relevant. However, hepatocyte death can occur after ALT increases [26].

Clinical data in hemophilia A show that the increase in ALT after gene therapy is

dependent on vector dose and, possibly, the number of CpG motifs (a cytosine linked to a guanine by a phosphate bond), but is independent of the AAV capsid, genome configuration, transgene promoter, and method of manufacture [25]. Long-term assessment of the health and function of liver cells is critical to understanding the safety and efficacy of gene therapy.

Structural biomarkers

Several imaging techniques can be used for screening purposes or for comparison of gene-therapy–related anatomical changes with baseline characteristics. For example, when the liver is the target organ, as in hemophilia A gene therapy, FibroScan® (Echosens; using transient elastography) or ultrasound is often employed to assess organ structure. Such investigations can identify patients who have any indication of risk for complications to the therapy, such as preexisting or worsening fibrosis, steatosis, or cancer [31,32].

Cellular biomarkers

The overexpression of a protein in a target cell, such as factor VIII in hepatocytes, may induce cellular stress in the endoplasmic reticulum [33,34]. The unfolded protein response is designed to protect the cell from this protein accumulation and minimize cellular stress [35–37]. The unfolded protein response is a particular concern in gene therapy for hemophilia A because the hepatocytes are forced to produce a protein they do not normally produce. In addition, traditional AAV-based gene therapies for hemophilia A use a B-domain–deleted factor VIII transgene [38,39]. Because the newly expressed protein differs from naturally produced factor VIII, the risk of misfolding or overexpression is high [38]. Although unfolded protein response should ideally be measured at the cellular level, biopsy samples of the target organ have allowed the description of a serum biomarker, glucose-regulated protein 78, also called binding immunoglobulin protein [40,41], which can predict cellular stress and hepatocyte damage in response to gene therapy.

ASC Therapeutics has developed ASC618, an AAV vector-encoding B-domain–deleted factor VIII for the treatment of patients with hemophilia A. ASC618 contains two components: a liver-directed promoter that minimizes the size of the vector and a bioengineered factor VIII molecule containing 91% human and 9% porcine sequences that offers increased biosynthesis, expression, and secretion efficiency compared with standard factor VIII transgene therapies [42–47]. The design of ASC618 allows for 10- to 100-fold increased protein expression because of limited interaction with the endoplasmic reticulum and attenuated unfolded protein response. The ASC618 clinical program for patients with severe and moderately severe hemophilia A received Investigational New Drug clearance from the United States Food and Drug Administration and an interventional clinical trial is currently ongoing (ClinicalTrials.gov identifier: NCT04676048; Table 2) [48].

Several different mutations in the gene encoding factor VIII are associated with hemophilia A [49]. Depending on the mutation, patients may have different levels of naturally occurring factor VIII and may respond differently to gene therapy [13,50]. Therefore, the sequencing of a patient's genes is an important element of gene therapy. Identification of the specific mutation can help predict response to therapy [51].

CLINICAL DEVELOPMENT OF ALLOGENEIC CELL THERAPIES

Cell therapy can work through several mechanisms, such as delivering new cells to a patient to replace damaged or diseased cells or tissues [2] or provide an immunoregulatory functionality [52,53]. Several types of cells can be used for cell therapy, including stem cells and stromal cells. One of the most common cell therapies is the transplantation

DOI: 10.18609/cgti.2021.175

TABLE 2

Gene and cell therapy clinical trial designs for ASC618 [48] and ASC930 [64].

	Population	Selection biomarkers	Monitoring biomarkers
ASC618 gene therapy	Severe hemophilia A (FVIII activity ≤2 IU/dL)	▶ Inhibitory antibodies to FVIII protein ▶ Total and neutralizing antibodies to AAV8 ▶ Liver function tests, including imaging and liver enzymes ▶ *FVIII* gene mutations	*Monitored up to 52 weeks* **Safety** ▶ On-target liver AAV infectivity, excluding off-target in other organs and tissues ▶ Total and neutralizing antibodies to AAV8; Cellular immune response (ELISPOT) ▶ FVIII inhibitor levels **Efficacy** FVIII activity
ASC930 decidua stromal cells (DSC)	Steroid-refractory acute GVHD	▶ Immune profiling of circulating T cells and cytokines ▶ Tissue-resident immune cells in the gut and skin ▶ *In vitro* effect of steroids and ruxolitinib in DSC mixed lymphocyte response	*Monitored up to Day 56* **Safety** ▶ Multi-omics predictors of immune-related adverse events ▶ Immune profiling with mass cytometry **Efficacy** DSC phenotyping and functional tests: MAGIC biomarkers

AAV: Adeno-associated viral; DSC: Decidua stromal cells; FVIII: Factor VIII; GVHD: Graft-versus-host disease; MAGIC: Mount Sinai Acute GVHD International Consortium.

of hematopoietic stem cells, which is currently used to treat hematologic cancers and diseases and is showing promise in other conditions.

Following allogeneic hematopoietic stem cell transplantation, graft-versus-host disease (GVHD) may cause considerable morbidity and mortality [54,55]. Simply, the donor blood cells, in addition to targeting the neoplastic cells, mount an immune response against cells and tissues of the host. Acute GVHD usually appears within the first 3 months after allogeneic hematopoietic stem cell transplantation and primarily affects the skin, gastrointestinal tract, and liver with rash, secretory diarrhea, and abnormal cholestatic liver function. Chronic GVHD usually appears more than 3 months after allogeneic hematopoietic stem cell transplantation and can affect any organ system in the body through tissue-damaging inflammation and dysregulation of immune response [56]. Typically, GVHD treatment consists of steroids with or without calcineurin inhibitors, but only about half of patients respond to treatment. Many second-line therapies have been developed for steroid-refractory GVHD, with mesenchymal stromal cells (MSCs) and decidua stromal cells (DSCs) being used successfully [54].

MSCs are multipotent, non-hematopoietic stem cells that have the ability to differentiate into a variety of cell types [4,33]. MSCs are present in adult and fetal tissues, as well as adipose tissue, peripheral blood, dental pulp, the endometrium, amniotic fluid, fetal membranes, the placenta, the umbilical cord, and other tissues and secretions [57–59], and are often isolated from bone marrow [4]. MSCs have immunomodulatory and anti-inflammatory properties and have therapeutic potential across a range of diseases. They avoid immune response because they do not express human leukocyte antigen, and they secrete

immune mediators and interact with T-regulatory cells, natural killer cells, and T-helper cells [4]. Specifically, the immunosuppressive abilities of MSCs in GVHD are based on the secretion of indoleamine 2,3-dioxygenase, transforming growth factor β, and interleukin-10, among others. MSCs also stimulate and induce T-regulatory cell differentiation; inhibit T-helper 17 differentiation; inhibit B-cell activation, proliferation, and immunoglobulin secretion; inhibit T-cell and natural killer cell proliferation; inhibit interleukin-2 production; and induce T-cell apoptosis [60]. However, while MSC transplantation reduces the risk of chronic GVHD, it does not change the risks of relapse or mortality and only slightly reduces the risk of acute GVHD [4,60].

DSCs are derived from the placenta, which is composed of cells and tissues of fetal and maternal origin, and are isolated from one of its key components, the fetal membrane. They have been shown to be safe and efficacious treatments for several diseases in both *in vitro* and *in vivo* animal models. DSCs have several advantages over MSCs and other stromal cells, including decreased production of interferon gamma and interleukin-17, increased secretion of anti-inflammatory interleukin-10, and higher expression of integrins [52,53]. DSCs also suppress alloreactivity, increase expression of programmed cell death ligands 1 and 2 [61], and increase the frequency of regulatory T cells [51,60,61]. They exhibit contact-dependent suppression of allo-activated immune cells, produce indoleamine 2,3-dioxygenase, and do not upregulate human leukocyte antigen-II after interferon gamma stimulation. Furthermore, DSCs have more potent immunosuppressive properties *in vitro* and do not display any differentiation potential [54]. The lack of capacity for differentiation amplifies the immune-regulatory potential driven by a stable phenotype [62]. Together, these features make DSCs ideal candidates for treating acute GVHD and, potentially, other diseases involving a compromised immune response.

ASC930 is under development by ASC Therapeutics as an allogeneic off-the-shelf cell therapy using DSCs for the treatment of steroid-refractory acute GVHD after allogeneic hematopoietic stem cell transplantation. A Phase 1/2 clinical study of DSCs in acute GVHD reported a 100% response rate at 4 weeks among patients with steroid-refractory acute GVHD, and no major long- or short-term safety events were noted [5,6,63]. The safety and efficacy of ASC930 will be evaluated in a Phase 2b, open-label, multicenter study (ClinicalTrials.gov identifier: NCT04883918; Table 2) [64].

Biomarkers for patient selection & monitoring in stem cell therapies

As stem and stromal cell therapies continue to be developed, more robust biomarkers are needed. Specifically, biomarkers of disease progression and response to therapy must be defined and optimized to minimize the risk and maximize the potential benefit of DSC therapy for acute GVHD.

Cell therapy biomarkers

Infused DSCs can be radiolabeled to measure their presence in various organs over time. In a study of three patients with GVHD after allogeneic hematopoietic stem cell transplantation, DSCs were labeled with ^{111}indium and the distribution of the DSCs was tracked for 48 hours. Compared with MSCs, DSCs have a higher expression of integrins, which are important for homing to inflamed and damaged tissues. However, DSCs did not show increased homing to organs affected by GVHD, including the intestine, esophagus, or skin, in the first 48 hours after treatment; instead, the DSCs traveled to the lungs, then to the spleen and liver [54]. This method of assessing the effect of DSCs should be applied to larger populations and used as a basis for further clinical study.

Immune-response biomarkers

To assess the safety of DSC therapy accurately, the patient's immune response to therapy

DOI: 10.18609/cgti.2021.175

must be measured. Flow cytometry is used to measure characteristics of cell populations and can be used to create a profile of immune cells and detect immunological biomarkers. Specifically, immune response to DSC therapy can be measured with mass cytometry, a variation of flow cytometry that uses mass spectrometry. Flow cytometry simultaneously identifies and quantifies cellular systems and measures cells' functional attributes at the single-cell level [65]. Additionally, proteomics, multiomics, and single-cell 'omics' are increasingly important in understanding gene expression in individual cells [66–68], and these technologies could be applied to the safety assessment of DSC therapy. The ideal biomarker will be able to identify and validate immune-related parameters to predict response and guide decision-making; standardization of immune-response biomarkers is important as the field of cell therapy continues to grow.

Disease biomarkers

Disease response in GVHD can be measured using surrogate safety and efficacy endpoints. Two biomarkers of long-term outcomes can be measured from whole blood: suppressor of tumorigenicity-2 and regenerating islet-derived protein 3-α. Both proteins have been identified in high concentrations in the blood of patients with GVHD and are predictors of increased mortality. Both biomarkers are incorporated into the MAGIC (Mount Sinai Acute GVHD International Consortium) algorithm probability [55], which is a tool for assessing mortality after GVHD treatment. In the study of ASC930, whole blood will be collected at regular intervals throughout the study and follow-up period to predict mortality and resistance to treatment [69].

CONCLUSIONS

Cell and gene therapies are extraordinarily costly and complex, and efficacy and toxicity vary according to individual patient characteristics. Therefore, it is important to select the right patients for these treatments; this is even more important than with standard therapeutic approaches. Also, comprehensive monitoring of patients is required to address inter-individual variabilities, even more variabilities than are observed with standard therapies. For example, as described in this article, for hemophilia A gene therapy, a patient's hepatocytes are forced to become 'factories' for factor VIII, and individual responses to therapy vary on immunological, cellular, and functional levels, such as quantities of naturally occurring factor VIII and patient risk factors for toxicity. When a patient's cells are repurposed through the administration of a transgene, there is little room for error. This underscores the need for careful patient selection and accurate and timely assessments of response in terms of both therapeutic benefit and adverse or unintended consequences.

With the rapid growth of gene and cell therapies, biotechnology and pharmaceutical companies face a call to action: we must establish proper selection and monitoring protocols to provide patients with the safest and most effective therapeutic options for genetic diseases. Several biomarkers of disease, immune, cellular, and molecular responses to gene and cell therapies are available, but most require further study and validation before they are routinely applied in clinical practice. As they are assessed and validated, biomarkers will continue to improve the efficacy and decrease the toxicity of gene and cell therapies. Trials are ongoing to clarify the role and utility of existing and new biomarkers and the future of precision medicine applications is strong.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Oscar Segurado and Ruhong Jiang are employees of ASC Therapeutics.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Aug 2 2021;* ***Revised manuscript received:*** *Sep 15 2021;* ***Publication date:*** *Oct 27 2021.*

DOI: 10.18609/cgti.2021.175