CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:

CELLULAR IMMUNO-ONCOLOGY 4.0

GUEST EDITOR: DR MARK LOWDELL, DIRECTOR OF CELLULAR THERAPEUTICS, UCL, UK

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EXPERT ROUNDTABLE

Immuno-oncology manufacturing: progress towards streamlined commercialization

ANTHONY DAVIES . Executive Chairman, Dark Horse Consulting

GUEST MODERATOR

Anthony founded Dark Horse Consulting in 2014, bringing 20+ years of leadership experience in product, process and manufacturing development to cell and gene therapy companies in need. Anthony has a proven track record in managing pharmaceutical pipelines, is a skilled liaison with international regulatory agencies, and has an intense familiarity with a wide range of biologics, and cell and gene therapies. He is a highly sought-after keynote speaker and chair of national and international conferences and seminars, noted for his provocative, thoughtful and sometimes contrarian presentations.

ØYSTEIN ÅMELLEM . Director of R&D at Thermo Fisher Scientific

Øystein is Director of R&D at Thermo Fisher Scientific. For more than 19 years, Dr. Åmellem has held different leadership positions in R&D, Product Management and Business in Thermo Fisher Scientific. In these roles he was responsible for development and commercialization of products and services, including for the cell therapy market. He received his PhD from the University of Oslo in the field of molecular cell biology. During his academic career, he focused on the study of physiological & molecular mechanisms of tumor cell growth and was involved in investigating the method of actions for a novel group of anti-cancer compounds developed by Norsk Hydro.

KNUT NISS . Chief Technology Officer, Mustang

Dr. Niss has served as Chief Technology Officer since March 2018. Dr. Niss joined Mustang in March 2017 as Vice President of Operations, where he initiated and oversees the establishment of Mustang's cell therapy manufacturing facility. Prior to Mustang, Dr. Niss was Cell Therapy Asset Leader at Biogen, where he oversaw CMC-related activities for gene-edited hematopoietic stem cell and lentiviral gene therapy programs for sickle cell disease and hemophilia, respectively. Earlier in his career, Dr. Niss was Senior Technical Project Leader at Novartis' cell therapy manufacturing facility in Morris Plains, New Jersey, where he directed the transfer and implementation of the CTL019 process from Penn to Novartis. He also served as Senior R&D Program Manager at EMD Millipore, where he established processes for the large-scale expansion of adult and pluripotent stem cells. Dr. Niss began his career in senior research positions in Pfizer's Regenerative Medicine and Immunology groups. He holds a Ph.D. in molecular biology from Humboldt University of Berlin, and an M.S. in microbiology from the University of Göttingen in Germany. Dr. Niss completed his postdoctoral research at Boston Children's Hospital and the Dana-Farber Cancer Institute.

SANJIN ZVONIĆ . WindMIL Therapeutics

In his current role at WindMIL Therapeutics, Dr. Zvonić leads the development of WindMIL's core technologies and pipeline products, while concurrently contributing to the organizational growth and development. In 2009, Dr. Zvonić joined PCT, where he focused on client engagement and technology transfer into PCT, giving him a comprehensive understanding of cell therapy development, manufacturing and commercialization requirements and strategies. In 2014, he joined Novartis Cell and Gene Therapy Unit, where he focused on the development and commercialization of Novartis C/GT pipeline products. In 2016 Dr. Zvonić returned to PCT, with a focus on driving the growth and development of PCT's clinical and commercial manufacturing business lines while integrating into Hitachi Chemical.

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AD: Let's begin by discussing the current state of cell therapy cancer IO manufacturing and the issues it faces. I'd like to the panel to begin by framing the current drivers and challenges as they see them, and discussing barriers to progress towards a desirable future state in each case.

OA: We need to start by celebrating some of the success we have had in the market which paves the way for future advancements. We are targeting a brand new market where cell and gene therapy is addressing a very real unmet medical need.

One of the challenges we see in the cell and gene therapy market is linked to the fact we have a very academic manufacturing process. The question will be how do we get from this academic model and into the future scenario where we see more industrialization and more cost-effective methods?

Another major challenge is deeper characterization. A lot of the biology here is still unknown and that's a very difficult starting point when it comes to manufacturing – therefore we need to continue to develop and deepen our understanding of the fundamental biology.

And finally, interlinked to the commercialization path for these therapies, there are a number of other challenges such as supply chain and the issue of reimbursement in order to get these products successfully into the marketplace.

"One of the challenges we see in the cell and gene therapy market is linked to the fact we have a very academic manufacturing process. The question will be how do we get from this academic model and into the future scenario where we see more industrialization and more cost-effective methods? "
- Oystein Åmellem

KN: One issue that is not discussed enough is workforce development. We have seen a lot of success in terms of clinical efficacy and a lot of companies are springing up but one problem we certainly face is developing a workforce. This is especially important in the areas of regulatory, quality control, quality assurance, and even business development. To deal with this, we need to start focusing on how we work with universities and colleges to influence education and build a future workforce that will help us for years to come.

SZ: I agree that is an issue we need to focus on. When I was in the contract manufacturing organisation (CMO) side of the business, one of the biggest barriers to growing the business itself was workforce development and talent acquisition. As I have transitioned onto the sponsor side, the challenge remains. In order to grow and develop our company we need to focus on workforce development. That's the only way to truly ensure scalability.

Another key challenge is the transition from bespoke academic type processes and procedures to transforming as a field to develop technological and procedural platforms that can make workforce development more universally applicable and therefore help us drive costs down. If each organization is developing this workforce to meet its unique needs, that's not true scalability, that's really just temporarily plugging the holes in our system. Therefore, to truly bring the field forward, it's about moving from bespoke to more universal processes, approaches and platforms.

AD: Improving the depth of characterization with robust methods are probably THE central issues we address day-to-day – how should we drive towards better tools and more standardized manufacture?

KN: Over the past few years we have focused on improving process technologies, working out where the gaps are and what we need to improve but we haven't spent much time on the analytical side, and I think that's a mistake. Right now the assays that we are using are what we would call 'academic assays'; by that I mean there is low throughput and the process is fairly hands on. There's room for a certain degree of automation and a move towards high throughput. I would say that in order to improve all this, we need to share information between various companies.

I'll give you an example: here at Mustang Bio, for our next phase, we have determined that having incubators in the cleanroom is a waste of space, and we really would like to take the incubators out. So we're developing our own incubator prototype together with an engineering company but we purposefully haven't made that that propriety and have left the IP open because we feel that if the incubator is a successful technology, we really want others to utilize it as well.

AD: What has your experience been with tech transfer and executing assays from potentially academic sources in order to translate them into an industrialised environment?

SZ: From a CMO perspective, I did not see this as a challenge but instead as an opportunity to provide value to the sponsors working with us. In a CMO we would get customers or sponsors whose analytical development, while appropriate for what they were working on or the stage of the trials it was supporting, was not necessarily set up as a platform for late stage development to allow them to progress to a commercial stage.

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Our task was to balance out immediate needs so we could proceed expeditiously to the next stage of trial, whilst actually taking a little bit longer to reposition those assays in a CMO environment to allow customers not only the ability to move onto their next stage but to create the platform that allows them to fully develop them and validate them for commercial use.

AD: How does the novel nature of the therapeutics and their individual complexities make it difficult to develop analytical methods into off-the-shelf forms for this industry?

OA: I think there's one element that really is not discussed enough which is the underlying biology of the drug. I think the complexity here is how are we finding the most critical quality attributes to control them. As mentioned earlier, one obstacle is secrecy surrounding each process. Everyone would like to protect their own knowledge base and that's a drawback from making progress.

AD: Sanjin could you describe your experiences of relationship management from the CMO and client side?

SZ: CMOs I believe, and I'll say that I'm somewhat biased given my background, are still a vital part of our entire ecosystem, and will become, in my opinion, more important as the field matures. Every sponsor entering into a CMO relationship should start with an introspective look to see what they are really looking to get from a CMO. Then this becomes a selection process that allows them to find the right partner.

From my experience, these searches start off from the technical side, with CMOs often very focused on technical competencies, without actually looking into how those technical competences are going to be applied and how they're going to be managed and integrated into the overall business of your company. A CMO should in my opinion not be viewed as a transactional entity but as more of a true collaborative partner. There's often not a very distinct or purposeful attempt to set up collaborations and maintain them.

"I would say I'm a 'build a bear' kind of guy. I don't believe in the GMP-in-a-box concept much, for several reasons. If you do a GMP-in-a-box, you put your entire process in the hand of one vendor. I'm not sure that's really where you want to be long term. ...However, if you do a modular approach...you have the opportunity to have a back-up on every process step."
- Knut Niss

With regard to comparability, this is not a CMO-specific issue. It's just an issue in general, of scale out, expansion, and growth, of any business, as you move from one manufacturing site to multiple manufacturing sites. Comparability is something that actually needs to be very thoughtfully and intentionally managed from the beginning. As part of the CMO relationship and competence evaluation, it is important for you as a sponsor to ask yourself at the end of this journey, let's say tech transfer to a CMO or another manufacturing site, how are you going to establish comparability, what are you actually measuring, how are you measuring it, and what are the capabilities necessary to achieve that? Then use that as the framework through which you evaluate and engage your partners, and then from there you can draw out the whole operation.

AD: When it comes to automation, there are two schools of thought at the moment, automate everything, GMP-in-a-box, versus focused automation of specific key unit operations, the "Build a Bear" approach. What are the pros and cons of each approach?

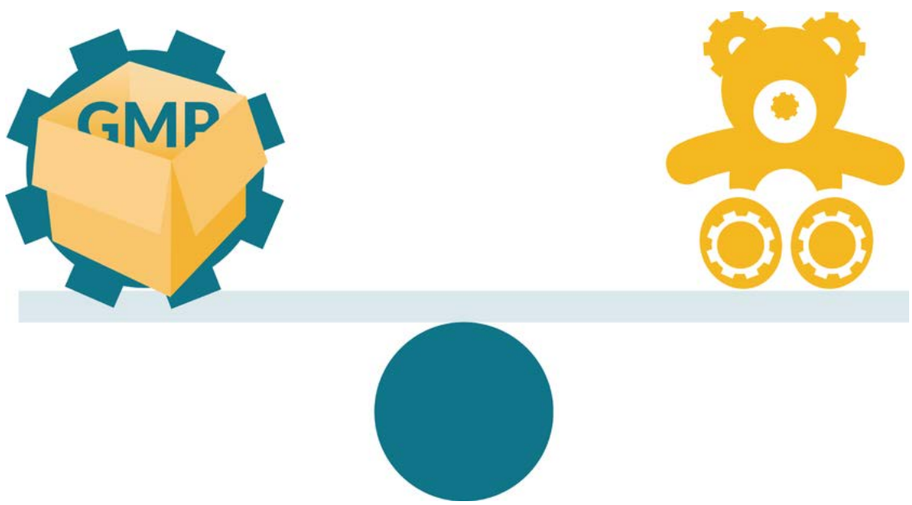
KN: I would say I'm a build a bear kind of guy. I don't believe in the GMP-in-a-box concept much, for several reasons. If you do a GMP-in-a-box, you put your entire process in the hand of one vendor. I'm not sure that's really where you want to be long term.

Here at Mustang we do use a GMP-in-a-box device, not for the entire process, but for certain processing steps. However, there are issues we're running into sometimes, such as getting a software update that's corrupt or a machine that's not 100% functional. The risk of these types of failures to me alone is a reason to think about not putting your entire process into one piece of equipment. If you experience a failure mode, your entire process is going down.

However, if you do a modular approach, the build a bear approach, you have the opportunity to have a back-up on every process step. For instance, if you use device X in your processing, you can spec in a device Y in case device X is not working.

SZ: Overall, it's situational and you have to first assess what you need to choose the right path. I would start with the build a bear approach as it's a great risk mitigation strategy. As someone who has come into this field as a true biologist, from the perspective of evolution, if things are still individual, it allows you to really respond to challenges. Everything that can go wrong will go wrong in development, so by using a modular approach you can respond to it much more flexibly.

I would say there is a time and a place for GMP-in-a-box. After refining the process through a build a bear approach, and I have really defined my design space, my parameters have good control of the system and its needs, then in order to make it more efficient and less costly, I would create a GMP-in-a-box version of my process. However, I would keep the blueprint very modular to continue to evolve my core technology.



OA: In order to really optimise and improve certain parts of the process I do believe that the way to go right now is to do a unit operation type approach. We did an exercise some time back and tried to go back in time to look at where we are today, and we were wrong 5 years ago. So 5 years ahead of us, will we accurately predict where we are? I'm not so sure. Because the rapid evolution of this market is going to take us places we don't even know about today.

As such, I think we need to keep a very flexible approach in order to test new technologies in certain parts of the workflow. I think this will be even more important when we work more on solid tumors.

KN: I think it's important to remember that if you go with a GMP-in-a-box, you really need to think about life cycle management of your asset. Ultimately if you end up with an all-inclusive device, you want to understand what the commercial impact is, what the license fee is and the technology fees you have to pay. You don't want to go through all of clinical development to learn half your revenue is going somewhere else.

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SZ: I would also say that the build a bear approach leads us as a field away from the bespoke nature of our manufacturing, which, as discussed earlier, is one of the root causes of the scalability and cost challenges we're facing as a community and field in general.

When we go with GMP-in-a-box, especially at the very beginning, it has to be by definition very bespoke, versus if we're working a unit operation process, we can as a field share those unit operations while still, for the purpose of our IP development and protection, protect let's say a very specific utilisation of those unit operation platforms. This way the ecosystem of device manufacturers and CMOs can still all have a universal palette of unit operation technological competencies that allow them to service the entire environment in a more effective and flexible way.

AD: A key element supporting renewed optimism for allogeneic approaches is the rise of gene editing. Can we talk about the economics of allogenic and gene editing's impact on this?

OA: I think we're at the very early stage of the transition into an allogeneic space. One of the things the market struggles with at the moment is using healthy donors, which does of course help with some of the economics of these therapies. I think we are now starting to see more of the end point coming into play, which is a more salon-based approach where you really can talk about scale. It's going to be a rough road because there are so many things you need to control. You need to have high efficiencies and you need to control the safety aspect of allogeneic. Particularly if you want to advance your programme in a clinical setting, it's going to be challenging, because the technology is very new, there are many things you need to manage from a safety aspect before we see a broad use in the clinic. I'm very much in favour of finding the right balance between autologous and allogeneic – both represent important modalities.

KN: The main issue from my perspective is clinical efficacy and safety. Part of the allo/auto discussion is also of course the reimbursement or price tag. If you look at the cost of goods of an allogeneic therapy, I'm not convinced yet it's that much cheaper than autologous. The reason for this is you have significantly more processing steps with gene editing. Besides this, you also have to have the mechanism of sorting out the non-edited cells. I haven't seen really convincing data to tell me that the allogeneic approach will be one tenth of the cost of an autologous approach. By not being significantly cheaper, the question becomes why would you prefer allogeneic over autologous, for example in the CAR-T approach, if it's roughly in the same ballpark price?

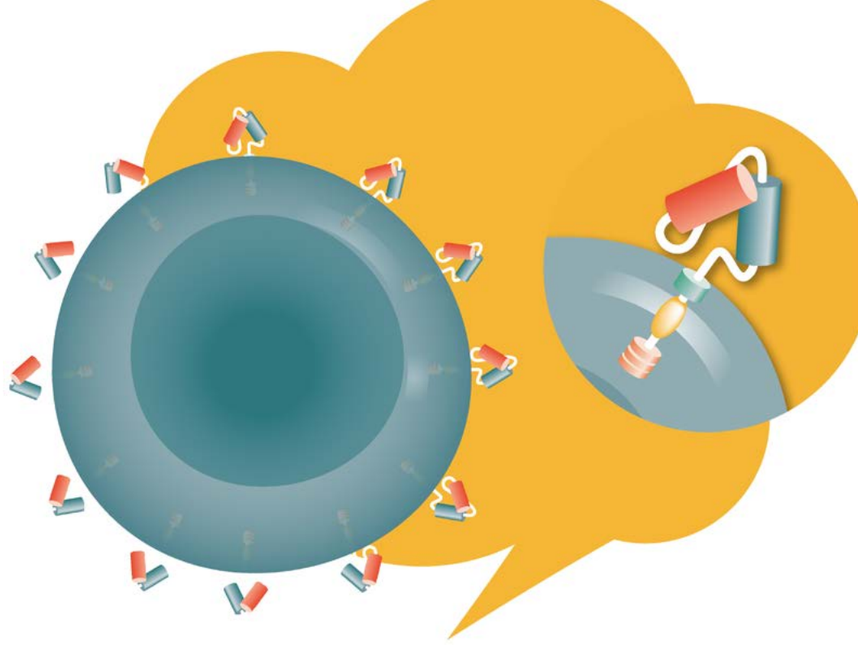
SZ: Ultimately for me, allogeneic is the future, but the future might be much further away than people are thinking about. The key issue to resolve for the future of allogeneic is around donors. We have to not think in terms of donors but in terms of cell lines. Once we get to the point where we have established lines, such that the source material is actually truly allogeneic, only then can we think about the downstream applications of various gene-editing technologies. At the end of the day, if I had a choice I'd much rather go with an autologous product just out of concern for safety and efficacy.

If you also look at how the reimbursement is working, Kymriah and Yescarta as well as a couple of other drugs that are now commercial are using performance-based reimbursement. So, in essence, if you have an allogeneic product that might start out at a lower price tag but is not as efficacious, the company developing it will not actually get as much reimbursement out of it as an auto product.

As someone working on autologous therapy, I ask myself, do I resolve the issue of cost of goods by going allogeneic or do I resolve it through technology to make my autologous product even better? I'm moving more towards the latter rather than the former.

"do I resolve the issue of cost of goods by going allogeneic or do I resolve it through technology to make my autologous product even better? I'm moving more towards the latter rather than the former." Sanjin Zvonić

AD: What are your thoughts on the safety profile of these products and the tests which will assure that safety?



OA: The issue is that as you increase the complexity of the product, you need to effectively manage genes and how they are transcribed. It's going to be important to remove all the impurities that each process will generate. There will be cells that escape the CRISPR or TALEN technology and they need to be removed. This will trigger new assays, new detection and in-process controls, and as such the complexity and risk will increase. Manufacturing will be far more complex than today, which is complex enough. It's an opportunity for companies like the one I represent, so we're ready to play and see where we can help.

AD: We haven't touched yet on capacity as a key bottleneck, which perhaps manifests most clearly today in viral vector supply – what does the panel make of recent trends in this regard?

OA: The typical footprint of an emerging area like cell and gene therapy is really around technologies and production capacity. Typically in emerging markets you see a lot of small players move in with niche technologies. The technologies that these companies have is often good, but the challenge for them is to increase their capacity and gain investment to drive technology advancement as well as advancement in manufacturing and quality.

It's only natural that the larger players start to react when they see the market mature. It's also an opportunity for larger companies to not only come in with their capacity, but also to leverage some of the technologies that are important to optimize new technologies like improved production. This is where the bigger companies can actually help advance the field more rapidly. It's only a natural thing, evolution of the market, and it's a good sign because it means the market is getting more mature.

KN: We have decided to build our own manufacturing facility, based on the assessment that with multiple programmes it becomes eventually more cost effective to do it this way. One problem we are seeing capacity-wise these days is there's a lot of facilities that are large, and there's a strong belief you need a large footprint to produce these kind of products. When you look at these facilities they look very much like biologics facilities with clean rooms and the infrastructure is geared towards what I would call biologics. However, really the way we look at it is if you design the facility around your process, you actually get more

capacity out of a smaller footprint, and that ultimately relates to cost reductions.

For example, lentiviral transduction is very short and requires a small footprint, so the cleanroom for that unit operation will be very small compared to a fill finish room. We're looking at an incubator that we don't put in the cleanroom but put outside, in a clean room space but not in the designed clean room, because that way you can separate products. I think going forward, smart facility design is one way of reducing the costs dramatically.

SZ: There's a lack of manufacturing capacity not because the current availability of manufacturing capacity in the field is a small footprint, or not a sufficiently large footprint of universally applicable capabilities and capacities, but it's actually because its rather too big a smorgasbord of loosely related pockets of capability.

What Knut just said really dovetails into the comment I really wanted to drive home here, which is that what we have to do as a field is in this theme of standardization, when all the facilities are organized in a way that universally fits everyone. In my previous role, in my portfolio at one time I had 12 customers who basically were doing 99% the same thing, but everyone was doing it in a completely different way. So of course, there's shortage of manufacturing capacity, because you have to reinvent the wheel for every car that comes into your garage. It's not just a matter of size, not just a quantitative question for me, it's more of a qualitative question.

AD: If you could wave a magic wand, and conjure up a single solution for any of the issues we've discussed today, what magic would you create?

KN: I think my wand would not go to the processing side, but to the QC side. I think if I could have my wish I would have an a fully automated FACS and PCR technology because that's where in QC we spend a lot of operator time, which again translates to cost.

OA: Since I am wearing an R&D hat today, I have to go for the solid tumor space. I see there is an opportunity to generate T cells regardless of donor that will be effective in a solid tumor environment. This would mean T cells trained for an enormously complex and hostile environment and this will require multiple technologies put together. We see now that might be possible, and that's my passion to make sure that happens.

SZ: I agree with Knut, and for me the focus is actually on quality control. I think if we can enhance the ways in which we can characterise, for example, the phenotype of the cells, understand how it actually aligns with their functionality, develop a broader range of functional assays that replicate the biological processes that are underlying the mechanism of action then we can unlock future problems. By having that greater understanding at earlier stages of trials, this would help us get in an improved automation of processes, drive manufacturing efficiency, and facility design because everything stems from understanding your product.

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EXPERT ROUNDTABLE

Gaining critical characterization insights for development of CAR-T Therapies for Solid Tumors

SADIK KASSIM . Executive Director, Kite Pharma, A Gilead Company

With a CV boasting the likes of Johnson & Johnson, the NIH, Novartis and Mustang Bio, Dr Sadik Kassim brings a wealth of experience and expertise spanning rare diseases, AAV-based gene therapy, immunotherapy, oncology, CAR-T cell therapies and CMC to his new role at Kite Pharma.

JANANI KRISHNAMURTHY . Senior Scientist, Atara Biotherapeutics

An expert in the fields of immunology, adoptive immunotherapy and neuroimmunology, Dr Janani Krishnamurthy enjoyed stints at MD Anderson, bluebird bio and TCR2 Therapeutics before taking on her current role leading EBV+ve CAR T cell pre-clinical initiatives for targeting solid tumours at Atara Biotherapeutics.

TAMARA J LASKOWSKI . Senior Research Scientist – Immunotherapy, Allison Group Department of Immunology, MD Anderson Cancer Center

Having originally joined Dr Laurence J.N. Cooper's laboratory as a fellow, where her work focused on engineering stem cells with the goal of generating off-the-shelf NK and T-cell immunotherapies for targeting solid tumor malignancies, Dr Tamara Laskowski recently transitioned to Dr. James Allison's Immunotherapy Platform at MD Anderson Cancer Center. In her new role Dr. Laskowski's work primarily involves immune-monitoring of patients undergoing clinical trials in Immunotherapy and development of novel immunoassays.

JOHN O'ROURKE . Head of Product Development, Cell Analytics, Intellicyt, A Sartorius Company

John O'Rourke completed his Ph.D. in Biochemistry from The Ohio State University where he studied cancer biology and gene regulation. During his postdoctoral training at Nationwide's Children's Research Institute and the University of New Mexico, he continued his studies in cancer biology along with the development of viral and nanoparticle-based therapeutics. John O'Rourke completed his MBA at the Andersen School of Management at the University of New Mexico and joined IntelliCyt in 2017.

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The ability to genetically engineer T cells with synthetic molecules, such as chimeric antigen receptors (CAR), and redirect the cells to user-defined targets on cancer cells marks the beginning of a new era in medicine.

In the context of the broader immuno-oncology therapy revolution, CAR-Ts differ from therapies such as checkpoint inhibitors in that they can sense and respond to their microenvironment. This makes these cell-based therapies very challenging to manipulate, manufacture, and control.

Although the success of the CD19-directed, FDA-approved CAR-T cell therapies, Kymriah and Yescarta, has been remarkable, the field still awaits a clear demonstration of clinical efficacy in solid tumors – a challenge which is becoming the defining issue in cellular immunotherapy as a new decade approaches. CGTI recently put a series of related questions to an Expert Roundtable panel comprising leaders from the academic, biopharma and enabling tool provider communities: where are we today, what are the most intriguing new approaches on the horizon, what are the biggest hurdles we need to overcome, and how can we address these challenges from both technological and clinical standpoints?

Q What do you see as the greatest challenges in achieving the same sort of success with cellular immunotherapy approaches in solid tumors as we have seen in liquid tumors?

TL: The greatest consideration and point of differentiation for solid tumors compared to haematological malignancies is the tumor microenvironment (TME). It is so much more challenging in the solid tumor realm.

The cells encounter a number of barriers to tumor infiltration in the way of resistance mechanisms. For example, one of our groups here at MD Anderson published recently on the role of transforming growth factor-beta (TGF-β) in impairing NK cell function in glioblastoma. We know NKs do not thrive well in the TME, especially due to hypoxia, but this study demonstrated that blocking TGF-β enhances NK function inside a solid tumor.

That's an interesting method of manipulating the microenvironment to sustain or allow for better functioning of immune cells once they penetrate those TEM barriers. Certainly, one of our biggest considerations as a research team is how our cells can thrive in inhospitable environments such as the TEM.

Persistence is something we must consider, too: what are the mechanisms that may act against our cells and decrease their ability to persist once they are active *in vivo*, or actually within the solid tumor?

Another important factor is immune modulation – not just focusing on the TEM itself, but on the immune cells themselves: what kind of challenges can these immune cells encounter through the process of activating, differentiating and then encountering the tumor antigen?

The potential of combinations of CAR-T immunotherapies and PD-1 checkpoint inhibitors to overcome the TEMs immune blockades is a major focus for us right now.

JK: In order to develop a better T cell therapy for solid tumors, we need to cultivate a better understanding of the kind of solid tumor we're working with.

For example, there are many different factors involved with hypoxia in the TEM, and still more of lactate-fueled respiration. There is also much to learn about the metabolic profile on these kinds of cancers.

I agree fully that we have to employ a multi-pronged therapeutic approach with solid tumors, not just a single agent. Apart from utilising checkpoint inhibitors in combination, we should be very creative in figuring out how to overcome immune suppressors such as TGF-β, and issues such as hypoxia and metabolic profile. For example, the role immature dendritic cells and macrophages play in causing immune suppression in the TEM could potentially provide a target.

JO'R: I look at this from a slightly different angle – how we can develop better CAR-T cell therapy targets for these solid tumors.

One of the key aspects of solid tumors is that most of the targets are also present on other, normal cells. The question becomes, then: how do we develop CAR-Ts that specifically target the tumor cells and leave normal cells alone?

We are looking at this in many different ways by tuning affinity of the CAR-Ts, employing Boolean logic to develop a CAR-T that binds to the target on the tumor, but that is inhibited in terms of its impact on normal cells.

"One of the key aspects of solid tumors is that most of the targets are also present on other, normal cells. The question becomes, then: how do we develop CAR-Ts that specifically target the tumor cells and leave normal cells alone?"
- JO'R

SK: Just to build on what John is saying, I think the major issue with solid tumors really is the target. Chris Klebanoff (Memorial Sloan-Kettering Cancer Center) did a really good analysis a couple of years ago, which looked at the monoclonal antibodies that have been approved for cancer and classified them into different categories. He concluded that while there have been roughly twenty monoclonal or radioisotope conjugated antibodies approved for cancer indications, not one of the targets involved have been truly tumor-specific. Instead, they tend to be targets like CD20, which is the lineage marker on B cells that happens to be expressed on lymphoma cells, or CD33 and CD38 for multiple myeloma.

I think what we're finding is that in liquid tumors, there is some leniency in targeting lineage-specific antigens that also happen to be expressed on the malignancy. However, in solid tumors you don't really have that flexibility, and the same targets that have been pursued have resulted in tremendous toxicity within the CAR-T context. I think Boolean logic is definitely a good way to go if we want to find more druggable solid tumor targets for CAR-T cell immunotherapy.

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Q What else can be gleaned from the limited success of clinical applications in solid tumors to date to help guide future approaches?

SK: I read a really interesting metareview recently in Transfusion Medicine Reviews, where they looked at over 550 clinical patients that had been enrolled in CAR-T trials. They broke it down into 3 buckets.

The first bucket was hematological malignancies targeting CD19. The complete response rate in that context was something like 54%. The second bucket included seven non-CD19 CAR studies – so hemalignancies again, but not targeting CD19. The response rate there was lower – 24%.

The third bucket was all other clinical CAR-T experience. This was across a total of 86 evaluable patients and they found the response rate was somewhere in the order of 4%. So that means 4% of patients who have undergone a CAR-T trial in a solid tumor setting have experienced some sort of clinical response.

There's clearly quite a bit for us to learn from this. These were really one-off patients, but I there was some success, albeit very limited. The question is, what can we learn from those patients who did respond?

I think one of the most remarkable recent incidences of a complete response was described in a paper in the New England Journal of Medicine, by City of Hope's Christine Brown, Stephen Forman and Behnam Badie. They targeted IL-13 receptor alpha 2-targeted glioblastoma multiforme in an end-stage patient. The patient was dosed regionally with the CAR-T construct and underwent a durable, complete response for seven months.

Getting back to John's point, I think the target really made an impact in that case. There was homogenous expression of this particular target within the GBM microenvironment and the authors also reported that the patient had some underlying inflammation. The tumor was somewhat inflamed to begin with – in other words, it was a hot tumor.

So infusing the CAR-T within that kind of microenvironment is probably going to lead to more durable responses in solid tumors. That's one thing we can learn from that particular case.

Memorial Sloan-Kettering have also achieved dramatic results with intrapleural administration of a mesothelin-targeted CAR-T cell therapy. They've treated around a dozen patients, at least one of whom has undergone a completely response that is still ongoing.

Again, what we can learn from this clinical experience is that the nature of the target really makes a difference. If it's a somewhat unique target that is expressed in a homogenous fashion on the tumor, then we can see quite a large impact.

Additionally, the route of administration would seem to be significant. The two examples I just described both involved regional rather than systemic administration.

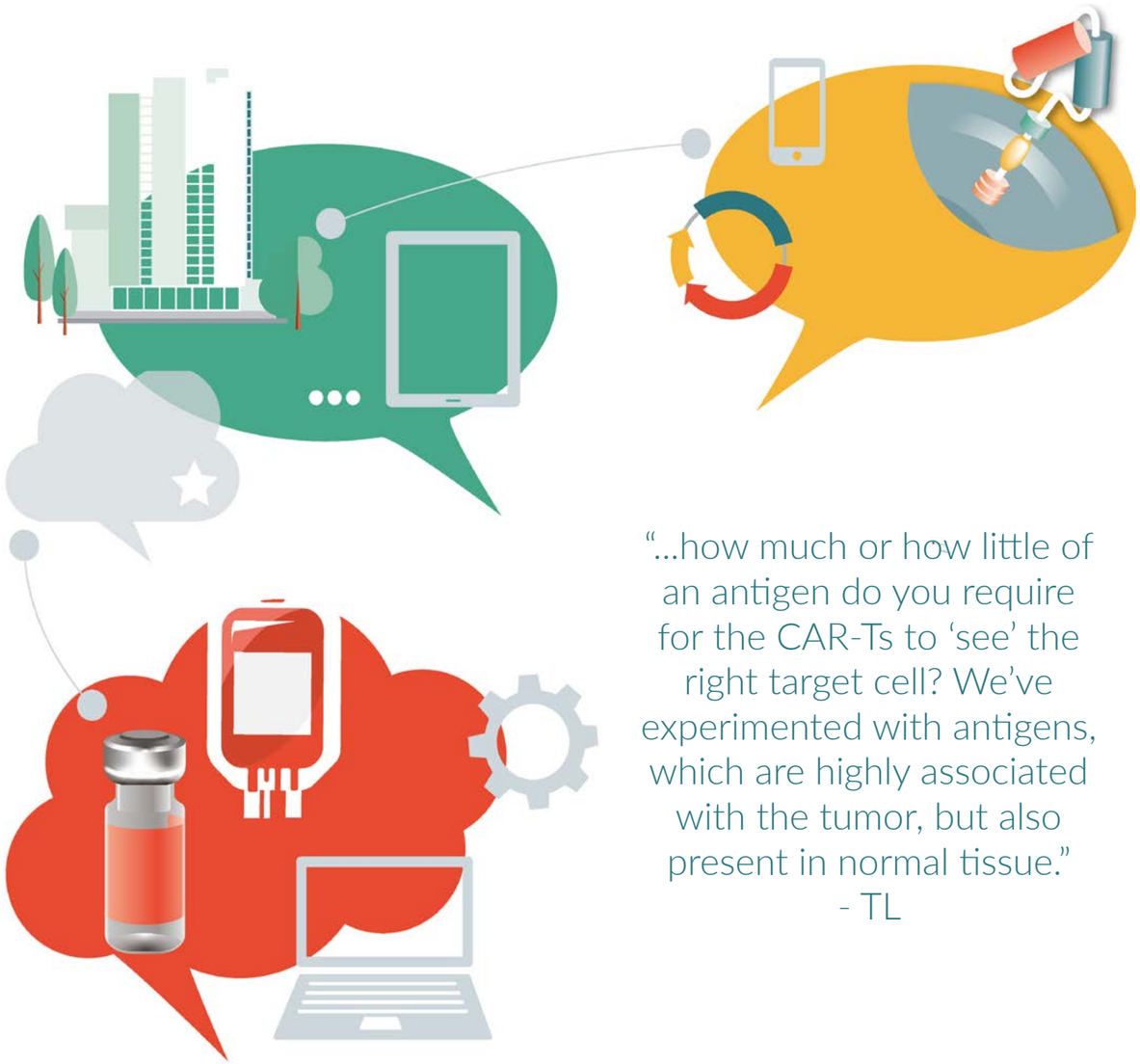
TL: I agree completely. I think a lot of the negative outlook from trials to date in solid tumors is the result of undesirable off-target effects that were not necessarily predicted.

There have been examples in breast cancer, for example, where CAR-T trials have been shut down as a consequence of off-target effects. It's a very serious consideration. As has been pointed out already, it's incredibly difficult to find a tumor-specific targets in solid tumors – it's a far more challenging scenario than for hematological malignancies, antigens such as CD19 make great targets because because they are not essential to life. With solid tumors, many of the potential targets are also present in healthy tissues and in other vital organs. A lot more work needs to be put into identifying antigens could potentially be highly specific, but there are already some great approaches targeting tumor-associated antigens.

For instance, we have experimented with affinity-tuning CARs, modifying scFvs, trying to introduce switches to turn CARs on and off as needed. Steven Rosenberg's group at NCI just recently published a breast cancer study – a single case, but where there was a phenomenal response from a nearly terminal patient with metastatic disease who was treated with tumor-infiltrating lymphocytes (TILs). TILs are essentially T cells that have found their target in the tumor – in this particular case, they sequenced the TILs, identified what the mutations were, expanded them, and infused them back into the patient, whose tumor was cleared. Again, that just shows you that if you have the right target, the probability of success is great.

It brings us back to the question of how can we predict these targets? I think that's essential. For us, there's always that concern: how much or how little of an antigen do you require for the CAR-Ts to 'see' the right target cell? We've experimented with antigens which are highly associated with the tumor, but also present in normal tissue. Key considerations then relate to the differences in density, pattern of expression, stringency of targeting. Tuning or controlling CAR may help with that challenge. In fact, at a recent research exchange in Washington, DC, a group from California discussed a dual-targeting approach, where they require that two separate antigens be present in a cell to decrease off-target activity. This is one of the many very clever approaches to overcoming these issues.

JK: I agree. Identifying a unique target is very important, but so too is fine-tuning the specificity or affinity. There have been many different approaches to this – of late, people have



"...how much or how little of an antigen do you require for the CAR-Ts to 'see' the right target cell? We've experimented with antigens, which are highly associated with the tumor, but also present in normal tissue."
- TL

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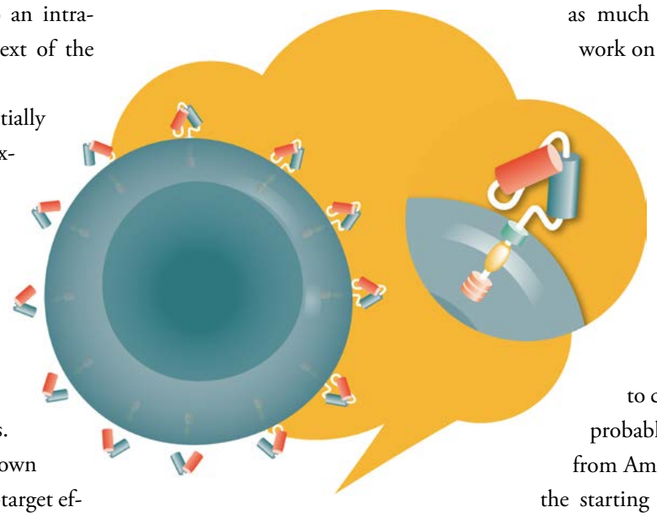
trended towards humanised CARs, which come with a slew of both advantages and disadvantages. The signalling part must also be considered; whether you want to go with the 28z CAR or the BBz CAR. All of this plays into the therapeutic potential of a product.

Also, returning to the Boolean logic approach brought up earlier, Memorial Sloan-Kettering have done some work combining a first-generation CAR, which is targeted to a relatively abundant antigen that is also present in the tumor, with a second-generation CAR aimed at a more tumor-specific antigen. I see this sort of dual-targeting approach as the future.

JO'R: One of the disadvantages of using CAR systems is we're generally looking for extracellular targets. One of the exciting aspects I'm looking at is can we use T cell receptor-like antibodies in CARs, or in other words, finding antibodies that can bind to an intracellular peptide in the context of the MHC molecules.

I'm interested in potentially using that approach to expand our toolbox in terms of looking for neoantigens and other aspects – taking advantage of the CAR constructs and the ability to genetically manipulate the CAR, but also increasing the number of targets. Could that help further on down the line with some of the off-target effects we're seeing?



Q Turning to the manufacturing side for a moment, how does the panel view the various bioprocess tools, steps and strategies currently employed? And what do you think the future holds in this area, with cost reduction being such a prominent driver for the field?

JK: We're dealing with a live drug – it's a T cell, it's not synthetic. That comes with a slew of problems.

Manufacturing has been a difficult task for most of the companies involved in this space. Beginning with starting material, we firstly must decide whether we want to use PBMCs or T cells. Then, most of the apheresis product we get in the autologous setting has to be screened, because if it's contaminated with a lot of PMNs, they could serve as nets for trapping the T cells.

So that needs to be taken care of and then the incoming patient-specific material must also be screened to ensure it doesn't include any tumor cells, although that will be less of an issue with solid tumors, of course. We must then decide how we want to activate the T cells – whether to go the APC route, to use beads, or to use colloids. And what cytokine do we want to use? People have used IL-2, IL-7, IL-15, IL-21, and also combinations of these.

That brings is to transduction, which I think is one of the key bottlenecks in manufacturing, especially using viral vectors. You certainly want to use really high-quality vector, especially in the GMP setting. However, there are alternative strategies to explore, such as electroporation with the Sleeping Beauty system, or PiggyBac – the transposon-based plasmids.

Additionally, we must decide what kind of phenotype we desire. In this field, we generally require an early memory phenotype, and there are manufacturing ideas such as small molecule and AKT-inhibition that could be added to you process to help the T cells grow in that sort of phenotype. Then there is QC and release criteria, which by themselves add about 15 days to the process, currently.

I think that one of the things that would definitely make things easier and cut costs would be a closed, fully automated system. Right now, there's quite a bit of manual labour involved. Humans are prone to error, so it's better to reduce that as much as possible. We could also work on product release criteria.

Most of the points I have talked about would be cost efficient if you took the allogeneic route. You would definitely have more flexibility and choices in your starting material, also in terms of how long you want to culture the cells. The dream is probably to order your cell therapy from Amazon, right? But we're just in the starting phases of that programme. We'll see how the clinical data pans out for it.

SK: I think that hits the key points right on the head. It's amazing to think that both Kymriah and Yescarta were approved within the context of a single arm trial looking at 90 to 100 patients, roughly. That's a very limited patient data set. As these products go commercial, you're opening up the treatment to new patients who haven't been enrolled in the initial clinical trials. The initial biomarkers that were discovered may not really hold true when you open up to a larger patient population, because the clinical trial patients were mainly stage 4, end-of-life patients who had already undergone multiple lines of treatment. As we treat patients who are at earlier and earlier stages of disease progression, we must consider whether the findings we discovered regarding starting material, target product profile, etc. during the trials are going to hold true. I think that's something to keep in mind.

I do think manufacturing timelines are reducing, overall, but QC testing is certainly still a major bottleneck. A lot of the methods used there are yet to be automated – even if you can make a product within 2 days, you still need to wait a week or more to release it. Reducing that timeframe needs to be a major focus for the field moving forward.

JO'R: To follow up on transduction methodology, I certainly think there are non-viral integrating methodologies such as Sleeping Beauty that show promise, but the state-of-the-art today remains lentiviral vector. And one of the biggest issues with lentiviral production is trying to isolate functional lentiviruses. You can make lentivirus at very high levels, but by the time you've finished the purification process, you're down to 25% functional virus. So developing new filtration and isolation technologies is going to be critical for that particular area.

A further issue is that you would typically think that low Multiplicity of Infection (MOI) would work for lentiviral transduction, but it's just not the case. You have to make a lot more lentivirus than you would think you might need. So there's a need for technology that allows you to use less lentivirus but still have high transduction efficiency – for example, there are new microfluidics techniques out there to reduce the amount of lentiviral vector needed for transduction. There are also new receptor-targeted lentiviruses that specifically transduce CD4, CD8 cells, which again might reduce the amount of LVV required.

As you increase the number of patients treated, you're going to have to increase lentiviral vector production. I think there will have to be a significant step forward in technology to allow us to produce enough of the virus of the right quality to treat all these patients, which is another reason to pursue the allogeneic route.

TL: Cell source is going to be a big consideration in this field. We've focused a lot on creating an 'off-the-shelf' approach, which makes cells available to the patients as and when they need it. I think that in itself will play a major role in decreasing the overall cost of therapy.

When you think of an idea, you have to think about how that can come to fruition. How can you generate a source that lends itself to off-the-shelf cell therapy? Many have looked at stem cells and what type might be most applicable. Once that is decided, the next step is to look at specific approaches that fit a particular disease or patient population of interest.

Another benefit on an off-the-shelf source is that it allows you to standardise product characterisation. We modify these cells. let's not forget that – we modify them with CRISPR or TALEN or LV, or whatever the choice of tool may be – and it's incumbent upon us to also investigate whether the desired modifications are are the only modifications that happened in the genome. This kind of question can be better addressed with a source that can be fully characterised then stored to be used

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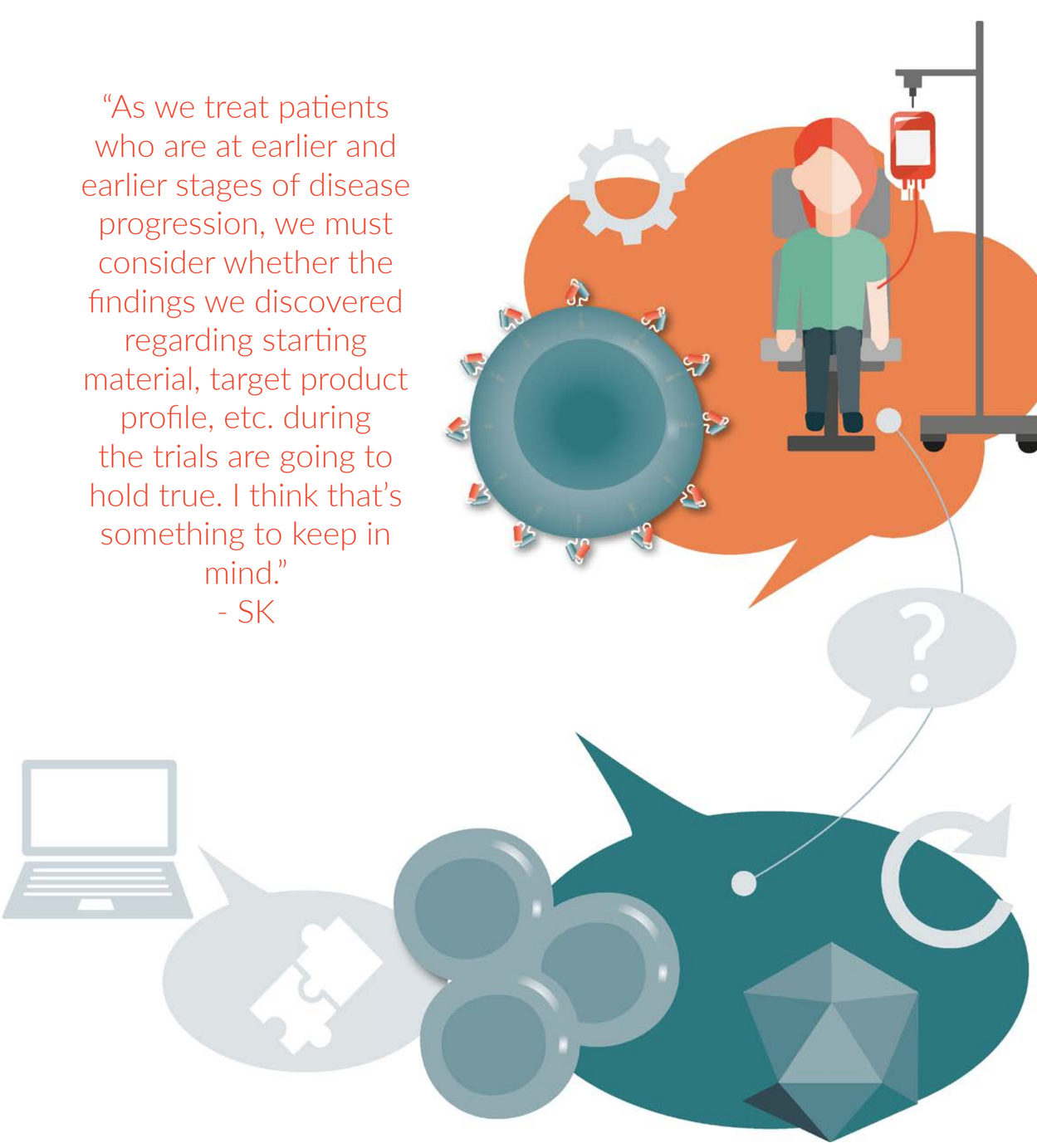


as and when required, as opposed to going through all those characterisation steps with each and every batch produced.

Logistics will also become easier if the field does continue to gravitate towards the off-the-shelf approach.

Q Let's turn our attention to today's tools and technologies and how they're helping the CAR selection process and our understanding of the TME. Firstly, what is the panel's assessment of the utility and predictive qualities of current ***in vitro*** screening assays?

JO'R: I think one of the biggest challenges we see in early CAR development is how do we develop high throughput analysis to look at different CAR constructs, and show that in vitro test predictions do map to in vivo functions?

What has typically been done, at least early on, is to do short-term cell killing assays looking at very high effector-to-target cell ratios. And it's been shown – especially when you're just changing specific parameters such as hinge length, or altering stimulation domains – that that's not very predictive, especially in the short-term.

So what you're seeing now is people doing more of what they're calling 'stress tests'. These stress tests are looking at very low E:T ratios, something a CAR-T would see in a solid TME, and again, repeated antigen stimulations.

For example, some of the newer cold culture techniques, whether it's a 3D tumorsphere or monolayer, involve letting the CAR-Ts go for two days. You then take those T cells off the culture and and put them onto fresh tumor cells, and you go beyond that, looking at it 2, 3, 4, 5 times.

What these techniques are showing, at least in their predictions, is that the cell exhaustion or some of the cell death that occurs through Fas ligand often doesn't happen until you have the second or third antigenic stimulation. The question is, how do we go about incorporating these sorts of *in vitro* assays but in a high throughput situation, when you may want to test lots of different variables? Additionally, how do we get the high content data needed for the T cell characterisation of those datas, looking also at secreted cytokines which could be a predictor of *in vivo* success, also in a high-throughput manner? I think these are key challenges we need to address.

JK: Just in the CAR-T process itself, there are several steps where I wish we had better *in vitro* tools.

The first would be characterisation of the virus. Getting a functional titre on the virus itself takes a bit of time, and then there is characterising it and seeing the functional lenti/retro particles... I wish I could just take a pipette, stick it in, and it would give me a functional titre!

As John has pointed out, stress test is something that is being done in all labs. It's either through repeated stimulation in a cold culture setup at a really low E:T, or doing a low E:T and just monitoring the cell life over an extended period of time. Something that also needs to be better understood in product characterisation terms is the metabolics of the T cells in culture.

These days, we do a lot of large data handling analysis to better understand these products we're putting out. That's been very helpful in recent years and I can see the trend continuing where we'll be using it more and more. So yes, the field is developing, but there's definitely room for better tools out there as well.

SK: The analytical repertoire and tools are definitely expanding. However, I think that one of the wild cards we need to keep in mind here is irrespective of the tools we have, we still lack a mechanistic understanding of what makes these CAR-Ts or cell therapies work *in vivo*.

I'm thinking of the recent publication by Jos Melenhorst's group at UPenn, where he identified a single clone – a single CAR-T cell – where the CAR was inserted in the TEK2 gene, and this was below the limit of the detection of any analytical assay. This particular cell was below the limit of detection of the infusion bag, but it was infused into a CLL patient and it took a long time for this one cell to expand and to eventually result in a complete response.

When you have one-off, random, anomalous results like that, it brings up the question of even if we have the best analytical tools, at the end of the day, how predictive are they going to be when you have patient-based studies like this, where an individual cell below the limit of detection of anything that we know expands and leads to a durable complete response that's still ongoing today?

JK: Single cell analysis is taking off, which could help in that sense, but again, it's a lot of data to handle and it's a little like looking for a needle in a haystack. It's a hard thing to do, but I know that a lot of groups are looking into single cell analysis, at both gene level and protein level, to answer some of these questions.

SK: That's a great point. It's not just generating the data, but how can you analyse it in real time fashion to really make actionable decisions? Maybe we need to bring in Amazon there, too, with their big data warehouse service to enable big data analysis as well!

TL: Everything is starting to move towards single cell analysis and I agree completely with Sadik that eventually you may just create a lot of noise, making it difficult to distinguish what is relevant from what irrelevant. You might be following up on a particular mutation that is never going to elucidate any problems. It's going to bring about a need for additional thinking in terms of these studies: what exactly do we need, what are the things we are going after, and how can we use these tools to find these specific functional phenotypes?

It's an insurmountable amount of data we need to sift through and make sense of – that in itself creates another hurdle. But I think it's all going in a good direction, overall. We're learning from a different angle.

One of the things we ask – when we modulate, when we add a checkpoint blockade – is what does that do to the cells? Why are they now functioning better than they were before? What are some genetic changes happening in these cells? That's a whole new set of questions that we can investigate, from the gene expression perspective as well.

Q Regarding ***in vivo*** modelling of the TME, what useful information have we been able to glean to date from the tools we have available?

JK: *In vivo* modelling is difficult, especially for solid tumors. Basically, at this point we're using either cell lines that have the antigen expression which is the best fit for the CAR, or we using PDX models, which are patient-derived tumor cells that have been grown a bit *in vitro*, or passaged in mouse in order to make them grow a bit more.

However, there is a discrepancy between the *in vitro* and *in vivo* assays. We could do a 2-day culture of the tumor cells before testing them, and the antigen expression could differ in a 3D versus a 2D culture. Sometimes you see excellent CAR-T activity in an *in vitro* 2D culture, but once you go *in vivo* with the same cell line you don't see anything. That's probably because when the tumor cells are growing in 3D postures, the antigen expression is either lost or decreased. There's certainly the possibility of it changing.

"It's not just generating the data, but how can you analyse it in real time fashion to really make actionable decisions?" - SK

Additionally, many of the solid tumors are actually shed antigens, so that's another curve ball in the whole *in vivo* system.

Regarding other *in vivo* models for predicting TME, we mostly use immune-deficient mice, such as NSGs, to test the CAR-T activity. However, these mice are actually deficient in IL-6- and IL-1-producing macrophages, so they don't really represent a typical TME that you might see in a person.

There are a few indirect methods of using *in vivo* models for situations such as trying to predict cytokine release syndrome (CRS). There's an Italian group that used xenotolerant human CARs that do not respond to mouse antigen, and they managed to figure out that monocytes are one of the key players for CRS. There's a further group in New York that used immunotolerant beige mice, but they infused CAR-Ts through the IP route only in those mice with high tumor burden in order to produce a CRS effect. In their case, they saw macrophages were involved in producing the CRS effect. But again, each of these models come with a whole lot of criteria that must be met for the model to work in this setting, so in that sense, it's not a direct evaluation.

It is a critical drawback in the field that we don't have a good *in vivo* model to test these CAR-T responses.

"...cross-reactivity is a major limitation of mouse models. You cannot take an immune-compromised mouse and look at cross-reactivity against human tissues – any CAR-Ts that may react to antigen that would be expressed at a low level in a human cannot be explored." - JO'R

TL: I think another thing we often consider, but which is incredibly difficult to model *in vivo*, is that patients come to immunotherapy having endured multiple different procedures or protocols beforehand. The path that has led them to the immunotherapy protocol will likely have caused changes to their immune system and will likely cause differences in how they're going to respond to the immunotherapy itself. We know there are variables, we just can't necessarily replicate them accurately in our models.

I do think some of our models give us some notion of what we're dealing with. For instance, the TME challenges. We know that different solid tumors originate in different organs, different cell types. They evolve differently, they create different internal structures to deal with. We have found in the course of our studies that when you dissect through a renal carcinoma versus prostate cancer or breast cancer, you see differences in the immune infiltrate. That tells you there are inherent differences in each of these tissues that lead to them accommodating more or less of a different cell type, or allowing cells to become activated or not, or exhausting cells more or less rapidly.

There are so many different things we may be able to learn from animal models by replicating these TMEs and studying them. But at the same time, there are many questions that we are just unable to answer with today's tools.

We glean the best we can from what we have. In other words, we do our due diligence: investigate as far as you can, and then you've got to take it into clinical trials. You've got to see what it looks like when you begin to do this in a human. But we always joke that you see beautiful things in mice. I once gave a presentation where I pulled up the number of preclinical trials for solid tumors – it's just incredible. Then you look at the other side of that equation and how many have actually moved on into the clinic. They are comparatively very few and far between. People are trying. You can see there are many, many beautiful preclinical mouse models. They just do not translate very well.

I think that comes with the territory. But we're making progress, which is great news. A lot of what we see in the animal models does give very solid evidence of what we can work with when moving into human application. So it's not a waste of time and resources – it's an incredible application of the resources we have. It's just that it has limitations, like many other things we work with.

JO'R: Great points brought up by all of the panellists.

What I look for is predictivity of efficacy and toxicity and in this regard, cross-reactivity is a major limitation of mouse models. You cannot take an immune-compromised mouse and look at cross-reactivity against human tissues – any CAR-Ts that may react to antigen that would be expressed at a low level in a human cannot be explored further in a mouse model. Therefore, you can't really look at these on-target off-tumor effects. You also can't really look at those rare cases where you're just binding to an unrelated protein.

So the prediction of toxicity is a real issue. Although we have become better with some models looking at CRS, there is still a long way to go before we can reliably use these models to predict toxicity before the clinic.

Q Shifting focus to the endgame of cellular immunotherapy commercialisation, what emerging tools could really make an impact in terms of delivering time and cost savings, and where specifically do you see the greatest need for further innovation in this regard?

SK: I think that today, we're in a much better place than we were two years ago, on multiple fronts.

For example, a few years back there was maybe only a handful of academic centres that had any experience with apheresing a patient for this type of therapy. Today, there's a whole network of centres that have been trained by the Novartis's, the Celgene's and the Kite's on the proper way to apheresis a patient, store that apheresis, then ship it to a central manufacturing site. That's one thing I think has been largely worked out.

Moving forward, if allogeneic ends up being a feasible path forward, we're going to need to identify the characteristics of the starting material that are going to be the most amenable to commercial application. With allogeneic cell therapy as it is today, even though one cell line can potentially treat more patients, you're still depending on a normal, healthy donor to manufacture a batch of many vials. I don't think we're at the point just yet where people are using iPSC-derived cell banks to generate allogeneic cells.

So in the mid- term, we're going to have to identify those characteristics of the starting material, and be able to reproducibly isolate that starting material from healthy donors in order to make a commercial product. I think that's something that is missing today that we're going to have to become much better at doing.

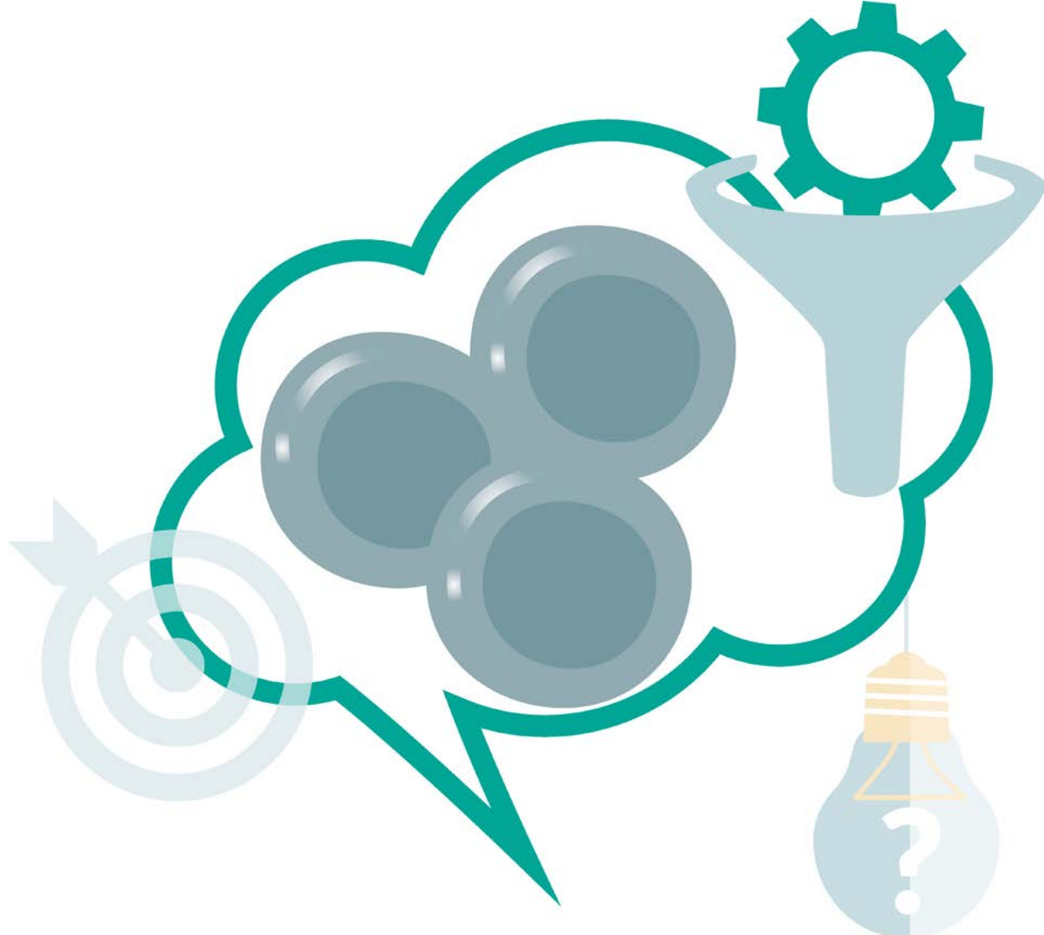
Longer term, there are going to be alternative cell types beyond T cells that are going to emerge as commercially viable options. NK cells, for instance – the data from MD Anderson with CD19 CAR NK cells is very compelling. Gamma delta T cells are emerging as another therapeutic modality. However, I would say the infrastructure we have today has largely been designed specifically for alpha beta T cells. So the question is, do we need to reinvent the wheel, or are there things we can tap into for the emerging infrastructure that can enable the quick adaptation of scale-up and commercialisation methods and models for these new immune cell types?

JO'R: I also look at it largely in terms of manufacturing. Can we find the specific starting material we need? Can we find a process and miniaturised assay that can be reproducible in the type of T cell one is using, all the way through to product release? Do we do functional analysis – again, on a very small amount of material – prior to release? And what are the best cell types to infuse back into the patient?

Developing assays and other aspects during the cell manufacturing process that use very little material is key, because you frequently can't generate a large amount of material. And can we identify more predictive cell types? We talk about stem cell memory and central memory – can we harness that?

Finally, with the shortening of the vein-to-vein timeframe in mind, there have been some recent studies that suggest as few as three days of ex vivo expansion would help enhance efficacy in patients. So again, we are talking about very small amounts of material – developing technologies that can use very small amounts of material to standardise our infusion products will be very important, I think.

"From my perspective, it will be innovation in target discovery that is going to drive this field forward. Above all, we need new, better targets."
- JK



JK: From my perspective, it will be innovation in target discovery that is going to drive this field forward. Above all, we need new, better targets.

Innovation on the processing side would also be welcome, of course. Better methodologies to transduce T cells, figuring out the assays, shortening the timeframes, making it much easier and more automated, if possible.

TL: I agree that processing is a major bottleneck. We spend a lot of our time on it. But I agree with Sadik's point, too, that the cell source is going to be a big player here. Fate Therapeutics is looking at iPSC-derived immune cells and moving that platform into trials – I'm really excited to see how that plays out, because it's a very viable source for this off-the-shelf idea. If they begin to demonstrate the feasibility and safety of that cell source, it's a game-changer. You could potentially do all sorts of characterisation of the product and generate a product that's isogenic, that's completely known.

I think once these elements are figured out, then time from bench to bedside will be reduced, along with cost – time and labour are extremely costly things.

And this helps us with other types of strategies, too. Just recently, Cell Medica published some of their CAR-NKT data, which seems very promising. So that's yet another player in the immune cell field that might be interesting to interrogate.

The field is moving really fast, but technology is catching up. It's nice to see that happening. But as John pointed out, introducing high throughput analysis and miniaturising assays, where you could quickly take a little bit of the product and harvest as much information as possible from it, will be very helpful. Being able to do that in 384 well plates and characterising all these conditions in one go – all these things will be relevant.

Then, of course, there is the ability to replicate these assays. The ability to know with confidence that what you see in that miniaturised analysis is consistent with what you see in a larger scale analysis will be crucial.

All these things are being worked out – I think we're moving in that direction. I would say it looks good!

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Featuring the top 5 questions from our live webinar audience

Q Dr Rosenberg's study used extremely high numbers of TILs. Please could the panellists comment on why these high doses were used and if they will continue to be necessary as TMEs are better understood or as combined therapies are implemented?

TL: That's a really great question, and one that I'm sure is always at the forefront of the minds of the groups working with adoptive cell therapies.

With respect to TILs specifically, we have T cells that are harvested from the TME that already recognize specific targets that they have found in that environment. These cells can be expanded ex vivo in culture, under defined culture conditions, and what has been shown is that they have the power to penetrate the tumor barriers, to infiltrate the tumor, and to destroy it, once they have been reinfused into the patient.

Oftentimes, there might be a screening (through sequencing of the TCRs, for instance) during this process to look for specific subtypes and identify what they're targeting in the tumor It might be desirable to in fact infuse a combination of these cells, with specificities for multiple antigens present in the tumor. Perhaps the increased number of cells in a large dose would favour that approach – you may have one type of cell that is fighting the tumor based upon antigen A, and another fighting the tumor based on antigen B, for instance and they may be present at different frequencies By infusing a combination of specificities into the patient, it may actually confer an advantage. The high cell doses may help in this aspect of the therapy.

There are concerns, however, with the expansion time these cells spend in culture before they go back into the patient – that they might lose their potential: they might exhaust, terminally differentiate, somehow lose be more limited in what they can achieve once back into the patient. So I think there are a lot of different factors that play a role in the decision to use high doses. I do think that as we learn more and as our tools become more defined and specific – allowing us to really identify cell populations that we think are of the right type, targeting the right antigen – then we will see a change towards more specific and more precise approaches, where these larger dosing volumes not be required.

JK: All TILS are T-cells taken from the tumor site and many of them could be exhausted already. They are grown at high doses too, which could potentially add to the exhaustion profile as well. Trafficking is also a component to it. All of this combined requires the TILs at a higher dose, to see any sort of efficacy and make them expand to the number you want.

SK: In terms of TIL cell therapies, the requirement for high doses relates to the poly-functionality or poly-clonality of the product. We know it recognises multiple antigens but we don't know which of the driver antigens is actually leading the TIL response. As we get a better understanding of the somatic mutations within the tumor you can refine and have a more pure product that selectively targets the mutation. As this happens, the hope will be that we dose with pure cells.

Q Is it possible to utilize any targeting approaches with specific biomarkers to attack solid tumors, so that T cells demonstrate specificity?

SK: The PD-L1 experience shows that if you have certain levels of PD-L1 expression, then you can almost stratify patients into response versus non-response. However, with CAR-T therapies and TCR therapies we haven't quite achieved that level of refinement yet. The only real biomarker that's being tested right now in terms of the apheresis product is the study from Jos Melenhorst at University of Pennsylvania, where he shows that if you have a high frequency of CD27-positive PD-L1-negative 45RO-negative cells in the apheresis and starting material, there's almost a minimum threshold that determines response within the context of CLL patients.

People are now building up a body of data of the biomarkers that translate to a response, such as reduced tumour burden. However, we don't yet have defined biomarkers like the checkpoint world; they are a little ahead of the CAR-T world.

JK: It is possible to use a variety of biomarkers but in order for it to be used in a context of CAR-T, these biomarkers need to be overexpressed on the surface of the tumor cells, not internally. TCR specificity cells can overcome this, where they can actually target intracellular antigens and biomarkers.

There is a group of thought that maybe we should look into cancer stem cell markers, rather than just tumor biomarkers and that it may drive a better response but that's still under early investigation. Something to keep in mind with this avenue is safety and off-tumor, on-target toxicity.

TL: That is the million dollar question we're all asking! What makes solid tumors so much more challenging is there just aren't as many antigens that are exclusively found in the tumor. There's always concern that the antigen will be also present in normal tissue. If you have a drug targeting a given antigen present in both normal and tumor tissue, this drug may destroy an unintended target. There are, in fact, cases reported in the literature describing fatal occurrences due to off-target effects.

Is it possible? Yes, I think so. We already have the tools for targeting. We need to find the antigens. This quest is still on – we're continually looking. With help from recent technology advances in single cell sequencing, we can actually study the tumor in greater depth now, and learn the variety of mutations and the differences in expression levels of specific antigens in the tumor cells. These might reveal potentially targetable antigens.

In the absence of a true tumor-specific maker, Perhaps a more feasible strategy is to identify targets that exist in both tumor and healthy cells, but which are slightly modified or more highly expressed in the tumor cells. Take for instance the carbohydrate GD2 which is highly expressed in neuroblastoma, and is therefore being pursued as a CAR-T target for this disease .

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Q What are some of the ongoing efforts for accelerating throughput of analytical methods?

TL: We and others employ high throughput flow cytometry as one such method. You can now miniaturize assays and test the activity of the immune. This can be done very quickly, assaying hundreds of combinations at a time. Immunologists rely a lot on flow cytometry to interrogate cells and so that's a tool we certainly use a lot.

There are also technologies that allow for single cell sequencing, giving you a detailed view of the genetic signature of a given tissue or sample. For instance, You can study post-therapy samples of patients who have responded versus those who have not responded, then identifythe differences in the immune cells foreach patient. Single cell cytokine analysis is another-tool for identifying cells that are functional. Take a situation in which you find a particular tumor is infiltrated by a number of T cells and NKcells. It appears as though there's an immune response against the tumor, and yet the patient doesn't benefit. We may now be able to answer the question of whether cells in the tumor are functional or not.

Q While targeting multiple tumor antigens will be really helpful, do you believe we really need to rethink about targets that we are going after? Perhaps choosing functionally relevant (like cancer stem cell markers) markers might be better as opposed to biomarkers that are not always expressed on the treatment resistant tumor cells.

SK: Going after cancer stem cell markers would be ideal but there's a lot of controversy in the field about what really defines a cancer stem cell. By extension, if there's no clear consensus on what a cancer stem cell is, there's no clear consensus on what a good target would be for cancer stem cells.

One cancer stem cell target people are researching within the context of multiple myeloma, is CD19. There are a few groups going after dual hit CD19 VCMA CAR for multiple myeloma. The idea being that VCMA targets the majority of the malignant cells and CD19 targets the stem cells within the myeloma compartment. This is a 'two birds with one stone' approach but although there's some anecdotal evidence that will work, it hasn't been tested in enough patients yet. When completed, that particular trial will be very informative about the value of going after stem cell markers.

TL: In many cases, what we see with cancer therapies is that we eliminate the bulk of the tumor but leave behind the cells that were not sensitive to the = agents administered as first-line therapy. Those cells then go on to reform a tumor, and that second tumor is characteristically different from the first one – perhaps even more aggressive , and often times resistant to the therapeutic agent that eliminated the majority of the tumor to begin with. There is of course the idea that this minor percentage of cells that remain alive and well after the initial therapy are these 'stem cell-like' cancer cells. There are efforts underway to tackle this particular population, but the issue is that because it is a minor population, it's one that's harder to identify.

I think novel approaches such as single cell sequencing, which offer the ability to interrogate heterogeneous tumor cell populations and the differences between each cell type within them, are really going to pro vide some incredible insights into the characteristics or the pathways that are regulated in these cells. I believe they will eventually tell us why it is that certain drugs only kill a number of the tumor cells and leave behind a population that resists and becomes incredibly hard to treat.

So it's a really insightful question – one we think about a lot – but it's also a hard one to tackle. Finding the right cell population to target – cancer stem cells being a great candidate – and finding the right antigen to target are very important considerations As we learn more this new knowledge will be reflected in the new therapeutic strategies developed. CAR-T and CAR NK cells can be powerful tools to access and destroy these rarer, therapy-resistant cells.

Q What does each panellist think of the various therapy combinations being touted as potential answers to the solid tumor conundrum – which combination(s) hold the greatest promise, for you, and what will be the repercussions of such combinations for characterisation?

JK: Recently some groups have been utilizing Pembrolizumab, or PD-L1 inhibitors, in combination with CAR-Ts, and have demonstrated good efficacy. However, the problem is the need to pre-screen these patients for PD-L1 expression. Nevertheless, there have been cases where even in the absence of PD-L1, Pembrolizumab has been demonstrating promising efficacy, which is driving us to try to understand the mechanism of action.

There are also other options being tested, including Ipilimumab, which is a CTLA4 inhibitor. This could be a be promising target of the tumor microenvironment in combination with a small molecule, for example something that targets TGF-beta along with a CAR-T.

TL: I think it's a great question and a very timely one, because following the initial success of CAR-Ts for hematologic maligancies, we're beginning to see that the challenges we're facing with solid tumors are more complex. We're dealing with resilient cells, inhospitable microenvironments, and attempting to through these barriers

We know T-cells, NK cells, macrophages get into the tumor. We are able to isolate these cells and analyze them. Learn about their function and phenotype. We can expand and enhance their function *in vitro* (as it is done with TILs), or we can block inhibitory signals and empower these immune cells to invade and destroy the tumor (as we have seen with checkpoint inhibitors). We can also isolate healthy T-cells and NK cells and genetically-modfiy them ex vivo to generate populations with greater tumor-killing potential. Other types, as mentioned, include gamma-delta T-cells, NK T cels, macrophages, each possessing unique properties that may be redirected to kill cancer.

Just like the immune system, containing a variety of cell types all working together to that eliminate threats, I believe a combination of approaches that may enhance the overall response against cancer may be a way to solve some of the challenges we have with solid tumors. We already see some of these strategies playing out. There are combination approaches involving target therapies (which often target a specific feature on the tumor in order to weaken it) and immunotherapy (which enhances immune function).

There have been so many amazing discoveries in the immuno-oncology field and many incredible leaders continue to advance the field through innovative research. We already cure a lot of diseases we did not cure ten years ago. We are getting closer and closer and the future is looking brighter

WATCH THE FULL ROUNDTABLE NOW

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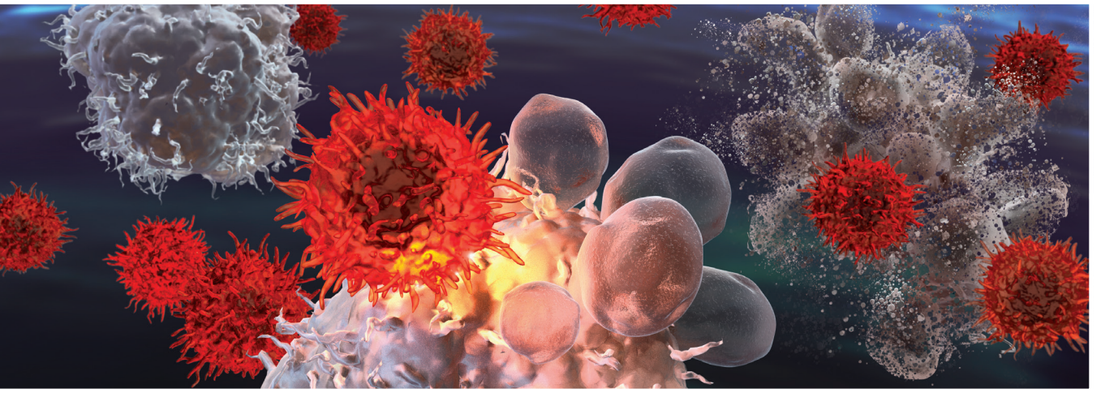
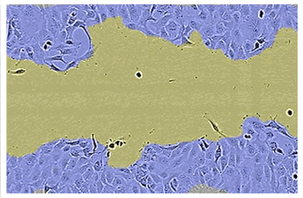



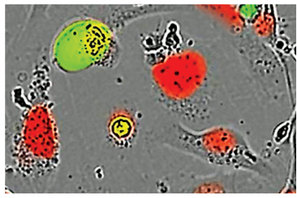

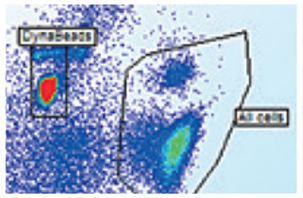


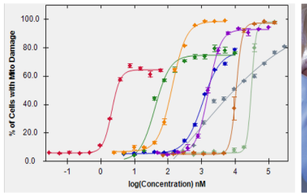

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INTERVIEW with Lindsey Clarke, Cell Therapy Product Manager at Bio-Techne

"The fact that we are seeing cell therapies really making a difference to people's lives and we're even daring to talk about them being curative, suggests the beginning of a whole new era of medicine."

Building Robustness and Scalability into the Immuno-Oncology Supply Chain

Lindsey joined Bio-Techne at the end of 2018 as EMEAs Cell and Gene Therapy Product Manager. Overseeing the portfolio of tools, technologies and instrumentation applicable to Cell and Gene Therapy her role to date has been focused on building the team to support Bio-Techne's customers Cell and Gene Therapy applications, planning the roll out of a host of new innovations into the European market and developing strategic partnerships within the industry. Prior to this she spent 8 years in Miltenyi Biotec's Cell and Gene Therapy team, working closely with numerous process development and manufacturing teams to assist them in translating their varied cell

therapies to the clinic. During this time she contributed significantly to the national strategy for the successful roll out and market positioning of the CliniMACS Prodigy and GMP products for Cell Therapy manufacturing within the UK. Lindsey holds a First class degree in Pharmacology (MPharmacol) from the University of Bath and a PhD in Immunology from UCL. Her PhD research focused on surrogate markers of endothelial inflammation and repair in paediatric vasculitis and led on to a postdoctoral position at Imperial College in the Department of Bioengineering investigating atherosclerosis.

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Q Can you tell us about your background leading up to your current role at Bio-Techne?

Lindsey: I started out as a typical academic scientist, following an initial degree in pharmacology with a PhD in immunology at UCL and then moving into a post-doc position. When that was coming to an end, as with many post-docs, I wasn't sure what I wanted to do next. I liked talking about science and I was good at trouble shooting, so I ended up taking a position in the UK Cell and Gene Therapy account management team at a biotechnology company

That was about 8 years ago now and I feel like I can claim I've been working in cell therapy since before it became popular! The last 5 years have just seen phenomenal changes and it's been a real privilege to be involved in the industry and working with a number of big groups within the UK, helping translate their processes into the clinic.

Q What are you working on specifically in your role at Bio-Techne?

Lindsey: I joined Bio-Techne 7 months ago and we've been making some substantial investments in technology in the cell and gene therapy space. My role is to be the person on the ground who oversees the strategy of how we support customers and how we launch these technologies in Europe.

I'm focused on building the right team. I think it's critical to have the right people working with cell and gene therapy manufacturers, who really understand what the pain points in manufacturing are, but also understand the science of the cell processes they're working with. That way it allows us to develop solutions that can address the needs of the industry.

Things in this space have changed very rapidly and we need to be agile to meet new requirements. If you asked me 5 years ago what the biggest challenge was, it was always about GMP. Now the question manufacturers are asking is very different; it is not just, "can I make this GMP?" its "can I make this GMP AND at scale?"

> "I feel like I can claim I've been working in cell therapy since before it became popular! The last 5 years have just seen phenomenal changes and it's been a real privilege to be involved in the industry and working with a number of big groups within the UK, helping translate their processes into the clinic."

Q Talking about scale, how can Bio-Techne help build robustness and scalability of the cell and gene therapy supply chain?

Lindsey: One thing I've learnt over the years is that very small changes to a process can make a significant difference to how it can be delivered at scale. Particularly with regard to autologous manufacturing, where you have a really complex supply chain and you have to be in complete control of the process because, ultimately, you have one chance of getting this product to a patient.

Everyone talks about automation and closing the system, and those are critical, but we're starting to see bottlenecks around raw materials that we weren't necessarily expecting. So one thing we're looking at closely is the preparation of medias or buffers that go into your manufacturing process. Even though you might have a completely automated process, this element still requires a manual and open step to supplement things like cytokines or HSA. Without finding a solution that simplifies and de-risks this, scaling up and out is going to be very, very difficult.

So as well as developing exciting and innovative new products we're also working on problems such as this and thinking about how you can de-risk the raw materials coming into the process from the supplier side to enhance scalability. Innovations will of course be part of the answer but a very simple solution that we already offer for example is filling vials with the exact quantity of a reagent that a manufacturer needs. This then takes away from the manufacturer the risk to the process associated with measuring out exact quantities or aliquoting themselves. A simple change but one which can positively impact on their ability to deliver at scale.

Our other key focus at the moment is on raw material supply. Security of supply is critical and we know we need to be flexible because of the scale of change between trials. For instance, the number of patients that people are treating can quickly escalate from one patient a month to

potentially thousands of patients a year. As a manufacturer of raw materials we have to anticipate that and be ready when our customers need us to be. How do we do it – it has to be by working closely with the manufacturers of cell and gene therapies so there's no surprises around their needs if their early trials are successful.

Q As clients move through to clinical trials and commercial scale, do you get the impression that they have a strong awareness of what the implications of that scale will be?

Lindsey: Some people are starting to think about these things but often when you're a commercial organisation you've got to move as quickly as possible to derive your phase 1 data, to open the door to further funding. I think people can be focused on getting to the clinic as fast as they can and sometimes they're not planning for the five thousand patients a year that may be around the corner.

Some of the companies that are further ahead in commercial development are thinking about scale from the very beginning of their next trials. But they're learning from their first generation products which ultimately confers some advantages.

Q What excites you most about the immuno-oncology sector at the moment and where do you see on-going challenges that need to be addressed in order to catalyse more products into the clinic?

Lindsey: CAR-T has been a real game changer in this immuno-oncology space, with the results from some of the early trials really ushering in a new era of cancer care. However, these are very complex products to make and again, autologous manufacturing is difficult. There are just so many elements people are having to juggle around raw material supply and patient scheduling to meet the clinical need.

Nevertheless, it's a fantastic space to be in. There's a myriad of exciting new cell types coming through that may be less of a challenge to manufacture. What excites me about this space is we're just at the beginning; CAR-T has set the precedent, but there's such a wave of innovation and technology that is set to evolve the immuno-oncology field further.

"CAR-T has been a real game changer in this immuno-oncology space, with the results from some of the early trials really ushering in a new era of cancer care. However, these are very complex products to make and again, autologous manufacturing is difficult."

There have been recent reports about the efficacy of CAR-T therapies in solid tumours and whilst it is early days in that respect, it is incredible to think that cancer may not be such an insurmountable disease in ten years' time. Then we need to start looking beyond that, and thinking what's next? There is potential for new cell and gene therapy approaches to chronic illnesses and autoimmune disorders, and perhaps even cancer being eradicated.

Q How can collaboration help optimise and resolve current challenges in the raw materials area?

Lindsey: It's essential to collaborate both with people manufacturing the cell therapies but also with other suppliers as well. Nobody has expertise in everything and so to really drive the industry forwards, we need to harness individual knowledge and play to our strengths.

At the moment our focus is on raw materials and, as we don't have instrumentation platforms for manufacturing, it's been really beneficial for us to work with device manufacturers and map out the best ways to collaborate.

Standardisation around components coming into the process such as the weldable tubing on consumables and medias, and buffers is an area we as an industry need to work on. There are still issues regarding how we transfer cells between pieces of equipment and the only way we're going to overcome some of these is by working together, and defining what the standards are, to which manufacturers and suppliers can then adhere to.

Ultimately it's about getting the best therapy to the patients and that's going to be cell and process dependent as to which platforms and raw materials fit best which makes it the responsibility of the process development team to assess a variety of solutions in order to make the best cell product.

By working closely with providers of tools and technologies like ourselves, manufacturers are at an advantage too, we always have things in the pipeline that people may not know about. By working closely with us, we can provide early access to these new products, which we are keen to do as our collaborators can actually shape how we develop our products and ensure that what we deliver is what they actually need

Q You started in this sector before the glory years we're experiencing now. As more therapies get

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closer to commercialisation do you sense less openness and collaboration for fear of losing property benefits?

Lindsey: Because the intellectual property (IP) is in the flavour of the cell, the actual manufacturing challenges are common, regardless of where the IP sits, and so actually I think this is an industry where people are willing to talk about challenges. Obviously, they can't give away confidential information but people are willing to share information about how they solve a problem.

In meetings where you bring together technical experts, you see a lot of sharing of information. In the academic sector, collaboration is commonplace and because the early stage spinouts are still very closely tied to research, I think we're potentially a lot more open in this industry than others.

Particularly in the UK, we are seeing the work of advanced treatment centres and the collaborative environment around that, and it is clear that companies as well as academics are working together to further the field.

> "The challenges of meeting the scale of demand for these therapies is what is going to be keeping the industry busy for the foreseeable future."

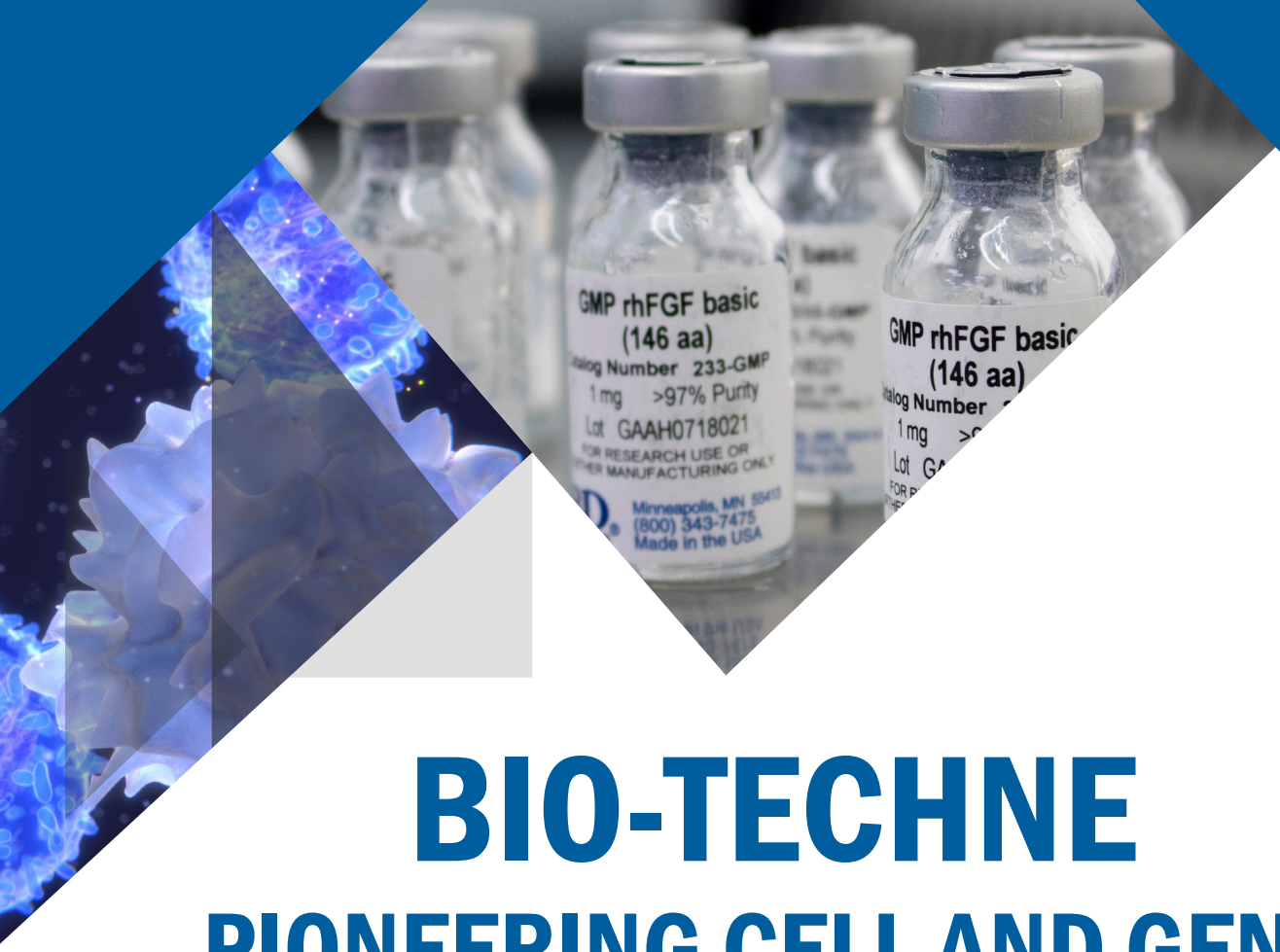
Q What's your vision for the future of the cellular immunotherapy space, and cell-based therapies in particular? Do you anticipate a more platform agnostic future as the sector matures?

Lindsey: I think as the sector matures, everyone is looking to the biologics sector and the way that they managed to bring the cost of goods down massively from where they started. I hope we will see this kind of progression in the immunotherapy space.

The challenges of meeting the scale of demand for these therapies is what is going to be keeping the industry busy for the foreseeable future. Are we going to move towards allogeneic products and off the shelf? Suddenly that's a whole new model of manufacturing. The autologous products are what we've seen efficacy with and are potentially safer because they're self-derived, but allogeneic is a more typical pharma model, and there is potentially more money to be made there. That is a factor which will drive the industry. Moving towards allogeneic is where I think the space is going, but whether we can achieve the same level of success as we have done to date with autologous therapies is unclear.

I've been talking with a few people lately about the vision of an "instant coffee pod style" kit for cell therapy, where the patient needs their specific CAR-T, presses a button, the kit arrives, you add cells put it into a machine and out pops the treatment. That would be amazing. We're not there yet but that's the vision I would love to see come to reality!

All I can say is we're just at the beginning. Everywhere I go people are working on the most amazing new technologies, new processes, smarter cells with clever targeting, switching on/off ability and improved efficacy. It really is an amazing environment to be working in and I feel privileged to be part of it. The fact that we are seeing cell therapies really making a difference to people's lives and we're even daring to talk about them being curative, suggests the beginning of a whole new era of medicine.



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SPOTLIGHT

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Novel equipment and process changes: implications for your manufacturing strategy

Nina G Bauer

With Cell and Gene Therapies transitioning through clinical trials and into commercial approvals at an unprecedented speed, process optimization steps are often considered late in the process. Alongside this fast-moving clinical development, tools providers are working hard to optimize equipment to better serve these new modalities. Evaluation, and implementation, of new or novel equipment is therefore, an ongoing process across the industry. Once INDs (or CTAs) have been filed, and first patients have been treated, onboarding new equipment constitutes a major manufacturing change. Such changes need to be appropriately communicated to the regulatory bodies, and thus managed from a data submission and risk profile perspective. In this article, we outline the main considerations from a broad GMP equipment compliance perspective, as well as indicating key resources and referencing guidelines for both Europe and the United States on how to navigate the regulatory aspects of manufacturing changes.

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We recently discussed the need for tailor-made equipment to suitably serve the cell and gene therapy (CGT) industry and its growing manufacturing needs [1]. At the core of this statement lies the need for high quality cells, in sufficient doses, in order to provide seriously sick patients with the best possible treatments.

Current manufacturing approaches, both during clinical trial

stages, and in some of the recently approved commercial treatments, tend to still be labor intensive [2]. They are based on a range of technologies typically found in a discovery laboratory environment, paired with technologies that have been proven useful in biologics manufacturing and blood processing. This approach has worked for small patient numbers, where manufacturing capacity and experienced manufacturing personnel were not limiting factors. Improving manufacturing processes has, however, become a recurring theme now that the field is seeing indication expansions, and more importantly applicability of CGT treatments in larger indications, thus mandating significantly higher numbers of doses.

One fundamental aspect to solving some of the manufacturing issues is a better understanding of the product in general, and, more specifically, understanding the critical quality attributes. Novartis has been reported to address this challenge with an Industry 4.0 approach, banking on data and Artificial Intelligence [3]. Once a product is better understood, so the thinking goes, there is a potential that smaller but more potent doses will be just as effective (if not more so) as the current approaches; think University of Pennsylvania's finding that 94% of Emily Whitehead's treatment success can be traced back to one single clone [4].

In addition to reducing the required cell numbers as one approach to countering manufacturing constraints, there is broad consensus that having suitable tools and equipment to further enable the manufacture, of what are after all living organisms, will be key. With a range of new 'made for purpose' tools entering the CGT space, we routinely encounter the question of the ideal timepoint to implement new equipment to improve manufacturing outcomes. In this article, we discuss the different considerations when introducing novel manufacturing technologies, with a focus on commercial and regulatory implications.

THEORY VS REALITY

It is widely recommended to start with the end goal in mind, i.e., what would a process need to look like in order to supply a commercial market with the predicted patient numbers? To put it more broadly, when designing a manufacturing strategy, it is critical to consider quality, cost of goods, distribution, sustainability and scalability right from the start. With the right strategy and long-term view, costly and often difficult and time-consuming manufacturing changes can be avoided at a later stage. In an ideal world, this would mean integrating the appropriate manufacturing technologies at the preclinical stage, optimizing them through the early clinical phase, and locking down a process for the pivotal trial – the so-called Development by Design approach [5] and similar concepts.

While this strategic approach is broadly accepted and makes rational sense, the reality is impacted by a range of external factors: Typically, CGT developers are small to medium sized companies in a pre-revenue situation. While being small allows them to be nimble and flexible, their funding milestones tend to be based on clinical achievements such as Clinical Trial Application (CTA)/Investigational New Drug (IND) application filed, or first patient treated,

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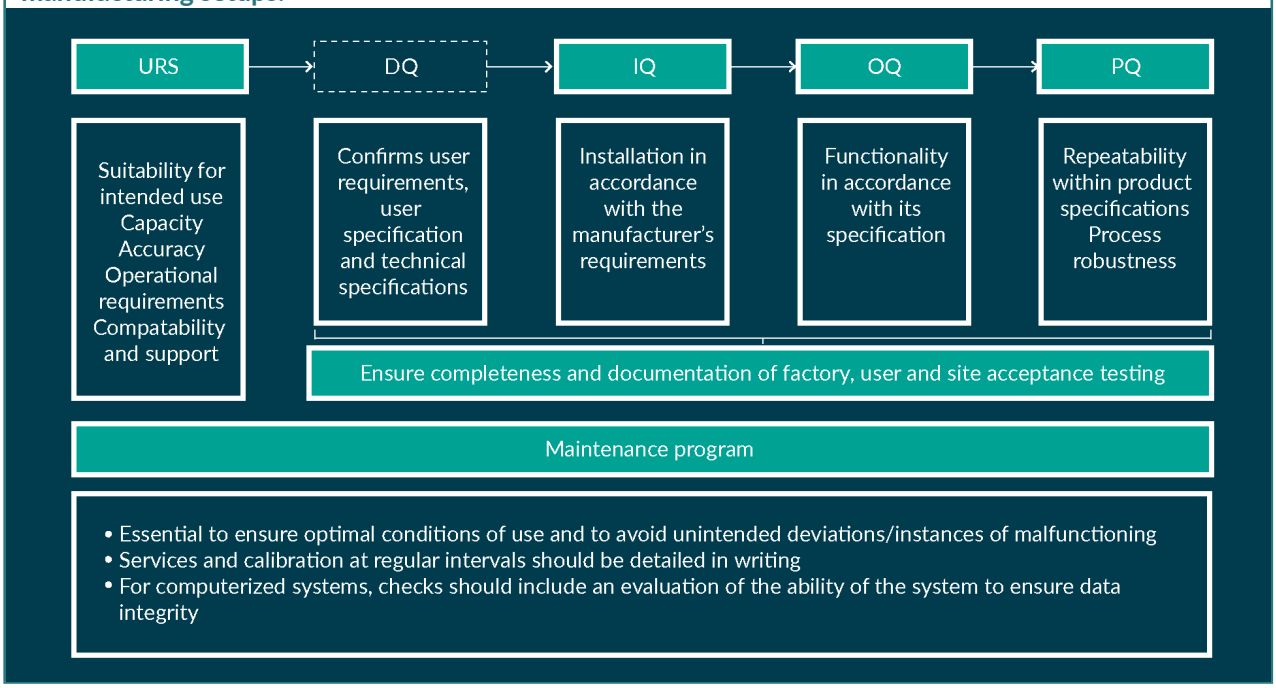
FIGURE 1

Outline of qualification activities and maintenance program requirements to for GMP compliant manufacturing setups.



etc. This inevitably means that timelines need to be weighed up against process improvements, more often than not with the outcome that relatively manual processes will enter the clinic and are even carried through to a pivotal trial. In other words, and to quote an industry colleague: "Science usually trumps process." It is not uncommon to introduce more appropriate technologies late in development or even post-approval.

The drivers that ultimately trigger such a process change tend to vary from company to company but can broadly be summarized in three categories: manufacturing robustness, scalability and cost. (i) Manufacturing robustness will be the main consideration for manual processes: they tend to be marred by operator variability, as well as the risk of contamination due to open manipulation steps. Where available, automation through suitable equipment can be a remedy, and therapy developers will always be on the lookout for novel equipment that could provide a solution to close a process. (ii) Scalability will be the driver for medium to large indications. While early phase trials tend to address limited patient numbers, most modalities will ultimately experience an indication expansion to increase the return on development investment. This will lead to an increase in dose numbers, and manufacturing processes that were initially designed for a smaller addressable market may no longer be suitable. Equipment can be one approach to enable both scale-up and scale-out. (iii) Finally, in an industry where treatment costs can reach the million-dollar mark, the cost of goods has been under tight scrutiny [6]. Investigating the individual cost drivers would be another article in itself; however, it is safe to say that novel equipment is expected to play an integral part in reducing cost, by enabling a reduction in hands-on time (labor cost), more efficient

use of space by combining and automating unit operations (facility cost), automating testing (quality assurance cost) and reducing the regulatory burden by lowering the facility classification requirements through novel closed and fully automated equipment (monitoring cost), to name but a few [7].

Depending on the developmental stage, different regulatory aspects need to be considered. When equipment is introduced during the pre-clinical stage, developers have a broad portfolio of technologies to choose from. Once an IND filing for the USA, or an CTA filing for the EU, is prepared, the contents for the Chemistry, Manufacturing and Controls (CMC) section start to get populated: the initial focus during the early clinical phases will be on the manufacturing process itself. While manufacturing technologies are referenced, their full documentation, and suitability for larger scale manufacturing, will not be tightly scrutinized until a pivotal phase with line of sight for commercialization is initiated. To avoid unexpected pushback due to unsuitable equipment or missing documentation, we have outlined some of the quality and regulatory considerations for equipment selection. While they are not intended to be exhaustive and will not replace direct conversations with regulators, we hope to contribute to a broader understanding of the interplay of manufacturing processes, equipment and regulations.

EQUIPMENT CONSIDERATIONS & GOOD MANUFACTURING PRACTICE

Equipment is intrinsic to and a key consideration when manufacturing CGT products. Its understanding is as important as the nature of the product itself, and the numerous

BOX 1

GMP Equipment Check List

- ☑ The equipment is designed, located and maintained to suit its intended purpose.
- ☑ Repair and maintenance operations do not present any hazard to the quality of the products.
- ☑ The equipment is designed so that it can be easily and thoroughly cleaned. It should be cleaned according to detailed and written procedures and stored only in a clean and dry condition.
- ☑ Washing and cleaning equipment is chosen and used in order not to be a source of contamination.
- ☑ Equipment is installed in such a way as to prevent any risk of error or of contamination.
- ☑ The equipment does not present any hazard to products. Parts of equipment that come into contact with the product must not be reactive, additive or absorptive to such an extent that it will affect the quality of the product and thus present any hazard. In addition, parts of the equipment that come into contact with cells/tissues should be sterile and of appropriate quality for the purpose.
- ☑ Balances and measuring equipment of an appropriate range and precision be available for production and control operations.
- ☑ Measuring, weighing, recording and control equipment be calibrated and checked at defined intervals by appropriate methods. Adequate records of such tests should be maintained.
- ☑ Fixed pipework is clearly labelled to indicate the contents and, where applicable, the direction of flow.
- ☑ Defective equipment, if possible, be removed or at least be clearly labelled as defective.

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TABLE 1

Relevant GMP Guidelines.

European Union	
Two key legal instruments on the principles and guidelines of cGMP for medicines, specifically for active substances and medicines for human use	Regulation No. 1252/2014 [17] Directive 2003/94/EC [18]
Overall interpretation of these guidelines including a rich annex with further details and examples	EU cGMP guidelines [19]
GMP considerations for ATMPs, specifically	Regulation (EC) No 1394/2007 [21] GMP Guideline for ATMPs [22]
United States	
Guidance for all pharmaceutical products	21 CFR Part 211 [24]
Guidance for biological products	21 CFR Part 600 [25]
Guidance for cell and tissue-based products, specifically	21 CFR Part 1271 [23]
Global	
Good manufacturing practice guide for active substance manufacture, recommended for adoption in the EU, USA, and Japan	ICH Q7 [20]

variables that factor into the manufacturing process. The impact of changing equipment once a drug product is authorized by regulatory agencies for clinical use needs to therefore be considered carefully: any change to the equipment will require qualification to confirm the equipment is fit for purpose and does not impact the process or the product quality.

When choosing new equipment, it should be of a suitable size and construction to facilitate cleaning, maintenance and proper process operations for any given therapeutic product **(see our GMP checklist for equipment Box 1)**. The ability for adequate cleaning and disinfection of the equipment is required to ensure aseptic conditions for processing. Fail safe modes should be built into automated equipment design and associated computer systems to ensure no compromise to the process/product. Clearly defined User Requirement Specification (URS), potentially a Design Qualification (DQ), as well as detailed Installation, Operational, Performance Qualification (IQ/OQ/PQ), relevant acceptance testing, and maintenance procedures are further prerequisites (see **Figure 1** for further explanations). The European Commission has established detailed guidelines for GMP, Eudralex Volume 4, in particular Chapter 3, and similar regulations, such as 21 CFR Subpart D (Equipment), are in effect in the USA, established by the Food and Drug Administration (FDA). **Tables 1 & 2** detail regulatory frameworks relevant to equipment currently in effect in Europe and the USA, for further guidance.

Even if equipment and the intended manufacturing context is considered appropriate from a regulatory standpoint, and all relevant documentation to underpin this has been compiled, it needs to be validated in the context of the specific manufacturing process. Elements of validation apply as soon as a Phase 1 is initiated; requirements for validation will increase as the product nears the market and will need to be appropriately documented if novel equipment is introduced. Qualification should take into account all

TABLE 2

Relevant Guidelines and Recommendations for Equipment Changes post-Approval.

European Union	
ICH Q5E: Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process [12]	
Note for Guidance on Biotechnology/Biological Products Subject to Changes in Their Manufacturing Process [29]	
EMA Questions and Answers on Gene Therapy [30]	
EMA Guideline on the Quality, Non-Clinical and Clinical Aspects of Gene Therapy Medicinal Products [31]	
United States	
Reporting of equipment changes post-BLA	21 CFR 601.12 [33]
Changes need to comply with a range of regulations	▶ Section 501 of the Federal Food, Drug, and Cosmetic Act (FD&C Act) (21 U.S.C. 351a(2)(B)) [34] ▶ 21 CFR Parts 210, 211, 600 through 680, and 820, as applicable
Requirements for making and reporting manufacturing changes to an approved BLA, and for distributing a licensed product made with such a change	Section 506A of the FD&C Act (21 U.S.C. 356a) [35]
For further context	▶ Changes to an Approved Application; Final rule (62 FR 39890, July 24, 1997) [26] ▶ Supplements and Other Changes to an Approved Application; Final rule (69 FR 18728, April 8, 2004) [27] ▶ Guidance for Industry: Changes to an Approved Application for Specified Biotechnology and Specified Synthetic Biological Products [28]
Global	
Recommended Reading: ISPE Good Practice Guide, Applied Risk Management for Commissioning and Qualification [32]	

critical factors, for example equipment sanitization and the integrity of the equipment. Equipment should be re-evaluated at appropriate intervals to confirm that it remains suitable for the intended operations. When using computerized systems, their validation should be proportionate to the impact on the quality of the product; consideration should be given to GAMP 5 [8], EU GMP Vol 4 Annex 11 [9] and to the FDA guidance 21 CFR Part 11 [10].

Lastly, equipment cleaning procedures and cleaning reagents should be chosen carefully. In most cases, equipment providers will recommend tried and tested approaches; equipment materials and design will ideally have been chosen in a manner that is safe and appropriate for GMP implementation. To simplify changeover and cleaning procedures between runs, most providers in the CGT space have taken lessons from the bioprocess industry and apply a single-use concept, thus minimizing the risk of (cross-) contaminations [11]. In comparison to stainless steel containers with their cleaning requirements, single-use consumables come with their own set of considerations: made from various types of plastic or polymer films, sterilization technologies such

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as gamma irradiation need to be implemented and validated. Extractables and leachables data sets should provide proof that the chosen material is fit for use – this is particularly important for material that comes into contact with cells, media and buffers [12]. And last, but not least, single-use means exactly that: these production consumables will have to be disposed off after one use, which not only generates questions around the environmental impact, but also concerns over potentially hazardous material left in bags, tubing or other containers.

BOX 2

Case study

FloDesign Sonics customer Alpha*:

- Mid-sized US start-up company, venture-funded in a series B, working towards finishing their clinical phase 1/2a milestone in the US, which will trigger the next funding round and a move into phase 3/pivotal with the aim of applying for a US BLA.

Product and current process*:

- Allogeneic MSCs grown on microcarriers, currently in a 3 L stir tank bioreactor, with the long-term aim of expanding to up to 200L. To scale, the open, semi-manual manipulations in the biosafety cabinet and using a centrifuge are going to be replaced with the novel ekko™ platform. The proposed changes are also expected to improve yield and some quality aspects.

Proposed process changes:

- Introduction of a novel equipment (ekko™), which will close the process, and fully automate the major unit operations outside of the bioreactor.

Resulting product changes:

- Demonstrated increased uniformity of MSC coverage on microcarriers during culture, still within IND-listed range.
- Demonstrated increased viability due to removal of non-attached single cells, still within IND-listed range.
- Demonstrated increased efficiency in microcarrier residual removal, well below IND-listed range.
- Demonstrated change in surface marker expression, while no morphological changes were noted.

Regulatory steps to consider:

1. File an investigational IND quality information amendment, containing:
   - Details of process changes
   - GMP-relevant documentation and data sets for novel equipment (unless Master File reference is available and contains all relevant data sets)
   - Timelines of process change implementation
   - Comparability data
   - Existing product knowledge details confirming continued product quality despite changes
2. Request Type C meeting to discuss severity of surface marker changes.
3. Expect additional toxicity data requirements, and potential potency data to confirm equivalence of the new quality profile.
4. Potential need to add more patients to the phase 1/2a to demonstrate efficacy.

*Fictitious customer and process, any and all similarities are coincidental.

DATA CONSIDERATIONS FOR PROCESS IMPROVEMENTS

Assuming the equipment satisfies GMP requirements and meets all other regulatory standards, a change in equipment once clinical studies have been initiated may signify a 'substantial' manufacturing change. At the very least, comparability runs will need to be performed, which should demonstrate that the resulting product conforms to the same quality specifications, and to demonstrate equivalence of batches. If the outcome of these runs shows differences, the existing product knowledge should be sufficiently predictive to ensure that this has no adverse impact on the safety or efficacy of the therapeutic, as per guidance ICH Q5E [13].

The extent of the comparability requirements during the clinical development phase depends on the specific stage of development, the availability of analytical procedures, and the extent of product and process knowledge. Consequently, comparability testing during early development (up to Phase 1/2) tends to be less extensive than for an approved product, with the focus being on safety. It should be noted that, if changes are introduced in late development, and no additional clinical studies are planned to support a Biologics License Application (BLA) in the USA, or a Marketing Authorisation Application (MAA) in the EU, the comparability requirements could be as comprehensive as for an approved product. For interested readers, Dr Joslyn Brunelle from the FDA presented a range of case studies in a talk titled "FDA recommendations for comparability studies to support manufacturing changes" [14]; while the modalities considered are mostly small molecules and biologics, it provides a good overview on the increase in data requirements in relation to the clinical development stage.

Overall, it is recommended to implement any manufacturing process and/or equipment changes during early phases of clinical studies (i.e., prior to initiating a Phase 3/pivotal study). These changes should be communicated to the FDA through an investigational IND quality information amendment, or in the EU as an amendment to the clinical trial authorization application to the appropriate member state. The extent of the changes should be clearly outlined, with a projected timeline as to when these changes will be introduced into clinical manufacturing. In the USA, a Type C meeting might be warranted to discuss the comparability exercise; for the EU, advice can be sought through the Scientific Advice procedure with a regulatory or scientific advice meeting.

Should changes post BLA or MAA be warranted, these changes need to be reported according to regulatory requirements. A draft guidance summarizing these regulations has been issued by the FDA at the end of 2017 [15] and is currently being finalized. In the EU, any change to the MA will be through Variation submission [16]. Please also see Tables 1 & 2, which references the most relevant regulations.

IN SUMMARY

A clear focus when moving a cell or gene therapy through clinical development should be on designing the process with manufacturability and commercial market supply

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in mind. This includes raw material selection, supply chain and logistics considerations, and the appropriate manufacturing equipment to ensure the best possible drug product quality. Any changes introduced after a product has already been used in humans tend to be costly and time consuming. However, despite all the outlined requirements and considerations that come with manufacturing and/or process changes, process improvements should generally be encouraged. Changes that can be shown to: (i) enable reliable manufacturing; (ii) underpin overall supply assurance; and/or (iii) improve the quality profile of a given CGT product, may be perceived as favorable and have seen a good level of support from regulatory agencies.

From an equipment supplier perspective, we encourage our customers to have an open dialogue about clinical timelines. This allows both sides to ensure that all GMP-related documentation can be assembled as needed, and comparability studies can be properly supported.

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NB is an employee of FloDesign Sonics but otherwise has no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in this manuscript. No writing assistance was utilized in the production of this manuscript.

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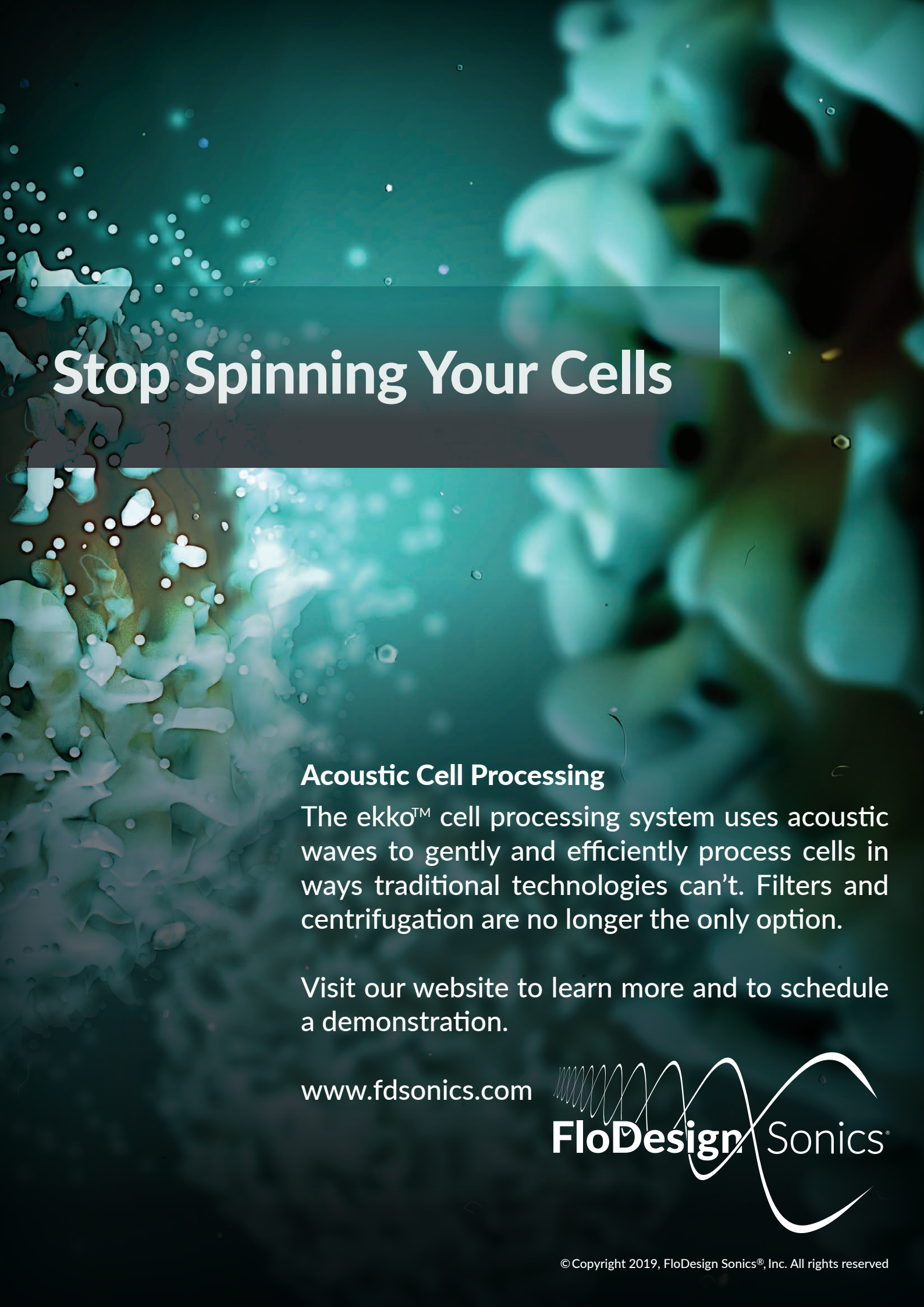
AFFILIATION

Nina G Bauer

Chief Commercial Officer,
FloDesign Sonics Inc.,
380 Main Street, Wilbraham,
MA 01095, USA



CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

EXPERT INSIGHT

Gene editing platforms for T-cell immunotherapy

Xiuyan Wang & Isabelle Rivière

Genome editing holds the remarkable potential to transform medicine as a new therapeutic modality enabling correction of genetic defects and customization of genetic addition or ablation. The discovery and optimization of gene editing tools such as meganucleases, ZFNs, TALENs and CRISPR/Cas9 are advancing the prospects of clinical applications. Clinical trials using gene-edited T cells have been conducted or are in progress for the treatment of patients with HIV infection and various cancers. T cells engineered using combinations of gene editing and gene transfer technologies are also being investigated. The development of off-target detection methodologies and the establishment of efficient manufacturing platforms are essential to bring gene-edited T cells to the forefront of novel immunotherapies.

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INTRODUCTION

T lymphocytes play an essential role in cell-mediated adaptive immunity. The adoptive transfer of T lymphocytes has led to remarkable clinical outcomes in patients with various cancers. The three major categories of adoptive T-cell transfer rely on tumor-infiltrating lymphocytes (TILs), transgenic T cell receptors (tTCRs) and chimeric antigen receptors (CARs). TIL therapy depends on the isolation and *ex vivo* expansion of tumor antigen specific T cells from surgically resected tumor samples. tTCR therapy and CAR therapy are genetically-engineered T-cell therapies that redirect the antigen-specificity of T cells against tumor-associated antigens. Early clinical trials have

shown the efficacy of TIL therapies for metastatic melanoma. In a series of trials (NCT00096382, NCT0031416, NCT00513604), objective response rates of 49, 52 and 72% have been observed, respectively, using TIL therapy in conjunction with nonmyeloablative preparative chemotherapy regimen, alone, or with 2Gy or 12 Gy total body irradiation [1]. Despite promising outcomes, the logistical requirements for TIL manufacturing, such as limited number of TILs isolated from the resected tissues, manufacturing time of 5–6 weeks and labor intensive screening and release assays [2], have hindered their broader application. Among the several tTCRs being clinically tested, NYESO-1 directed tTCR therapy has shown the greatest clinical benefit in multiple myeloma (MM) patients. In a recent report, 16 out of 20 patients with advanced MM responded to the therapy with a medium progress-free survival of 19.1 months [3,4]. In the clinic, CAR T cell therapies have demonstrated the most remarkable success in CD19 targeted hematological malignancies such as acute lymphoblastic leukemia (ALL), non-Hogdkin's lymphoma (NHL) and diffuse large B-cell lymphoma (DLBCL) [5]. FDA has recently approved two CAR T products: Tisagenlecleucel (Kymirah) for the treatment of pediatric ALL and DLBCL, high-grade B-cell lymphoma; and Axcabtagene (Yescarta) for the treatment of NHL and DLBCL.

Most of the adoptive T-cell therapies to date are largely autologous. The logistics and associated cost of manufacturing T-cell products for each patient limits their more rapid dissemination. Additionally, the quality and fitness of T cells harvested from heavily pretreated patients potentially negatively impact their *in vivo* efficacy. These challenges could be circumvented by the use of allogeneic T cells. However, the major hurdles of using an unmodified allogeneic T-cell source include: 1) graft-versus-host disease (GVHD) caused by recognition of host's antigens by the donor's TCRs; 2) possible rapid rejection of the donor T cells by the host immune system. In recent years, the discovery and evolution of gene editing tools and technologies has made it possible to avoid these difficulties by specifically targeting and interrupting genes that mediate self versus non-self-recognition such as the TCR. Seminal research and clinical studies using gene-edited T cells are quickly moving forward [6–8].

GENE EDITING TOOLS

In eukaryotes, the knock-in and knock-out of specific genes by homologous recombination have shown low efficiency until the emergence of engineered nucleases with site specificity. The occurrence of double stranded DNA breaks (DSB) lead to the activation of endogenous gene repair through one of two mechanisms: homology-directed repair (HDR) in the presence of suitable DNA template, or non-homologous end joining (NHEJ) in the absence of template. Four major engineered site-specific DNA-editing endonuclease systems have been developed so far.

Meganucleases

Meganucleases are homodimers that recognize 14–40 nucleotides

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palindromic DNA sequences, and lead to double stranded DNA cleavage with 3' cohesive 4 bp overhang which favors HDR [9]. Meganucleases have been described to efficiently correct the RAG1 gene in hematopoietic stem cells [10] and to correct the XPC gene in skin cells [11]. However, the overlap between DNA-binding and cleavage domains and the complexity of engineering meganucleases in order to target new sequences have limited their broader applications.

Zinc finger nucleases

Zinc finger nucleases (ZFN) are hybrid nucleases derived from DNA binding domains of zinc fingers and Fok I nuclease cleavage domain [12]. ZFN functions as a dimer with each zinc finger DNA recognition domain consisting of approximately 30 amino acids and recognizing approximately 3 bp. The sequence specificity of ZFN is mediated by an array of zinc fingers recognizing up to 18 bp in the genome with high affinity. Fok I subsequently cleaves the double stranded DNA which results in DNA repair mechanisms. ZFNs have been effectively used in several experimental organisms [13]. Clinical trials using ZFNs are in progress (Table 1). The broader application of ZFN is limited by its reliance on protein engineering for site specificity and affinity and its context-dependent sequence recognition [14].

Transcription activator-like effector nucleases

Transcription activator-like effector nucleases (TALENs) are another type of hybrid nucleases composed of transcription factor like effector's DNA binding domains and Fok I cleavage domain. TAL protein consists of multiple 33–35 amino acid tandem repeats and can specifically recognize a single bp of the genome in a context-independent fashion [15]. When compared to ZFN, TALEN technology is more user friendly, cost effective and displays lower genotoxicity [16]. TALENs have been successfully used in multiple species [17] and clinical trials (Table 1). Their relatively larger size, however, poses a challenge for delivery into target cells.

Clustered regularly interspaces short palindromic repeats (CRISPR)/Cas system

Different from ZFN and TALEN, the CRISPR/Cas system is a nucleotide-mediated gene editing tool. It is derived from the bacterial antiviral adaptive immunity. Bacterial viral DNA segments inserted into the CRISPR loci, and form the CRISPR-short viral segment-protospacer adjacent motif (PAM) complexes. These complexes are transcribed and processed into CRISPR RNAs (crRNAs), which forms a complex with the CRISPR-associated protein (Cas). Upon recognition of the homologous protospacer and PAM DNA sequence, the crRNA/Cas complex enacts a double stranded break, which invokes the downstream DNA repair machinery [18]. Short guide RNA (sgRNA) has been introduced to serve as crRNA in combination with Cas9 to establish a simpler and more efficient sequence specific gene editing platform. The CRISPR/Cas9 approach

TABLE 1

Characteristics of the major tools used for *in vivo* gene editing machinery delivery.

Delivery strategies	Cargo type	Expression duration	Reported gene editing tools delivered	Usage setting	Pro and con
Lipid, polymeric, and inorganic particles, synthetic particles	sgRNA/Cas RNP complexes, plasmid DNA, mRNA	Transient	Reviewed in [62]	*In vivo*: i.v., i.t., i.m.	**Pro:** easier to scale up and can be chemically functionalized with ligands for targeting specific cells *in vivo*; low frequencies of off-target events **Con:** biodistribution; need to increase ability to specifically target cell types *in vivo*
Non-viral physical methods (such as microinjection, electroporation)	Nucleic acid, protein, RNP	Transient	ZFN mRNA [63], TALEN mRNA [37] Cas9-gRNA RNP [38,62]	*In vitro* and *in vivo*	**Pro:** mechanical deformation or electroporation can be used to create holes in cell membranes to allow nucleic acid and proteins to enter cells **Con:** the number of cells that can be handled at a given time can be limited; special equipment is required
Non-viral vectors	Nucleic acids, protein, RNP	Transient	Cas9-sgRNA RNP [64]	*In vitro* and *in situ*	**Pro:** less toxic and immunogenic than viral vectors **Con:** presents inherent challenges for delivery, negatively charged nucleic acids need to be complexed to cationic materials, such as nanoparticles in order to be endocytosed by cells and to enter nucleus
Integration-defective lentiviral vectors (IDLVs)	Nucleic acid	Transient (depends on cell proliferation rate)	ZFN [65], and donor template [56]	*In vitro*	**Pro:** broad tropism of the vector; no risk of genotoxicity due to the non-integration defective feature **Con:** limited capacity of pay load (5kb); transient expression in dividing cells; challenges in the manufacturing of IDLV
Adenoviral vectors (AVs)	Nucleic acid	Long term in quiescent cells (years)	ZFN [66,67], CRISPR/cas [68]	*In vitro* and *in vivo*	**Pro:** efficient delivery to dividing and nondividing cells, large capacity (8kb to 36kb) **Con:** High immunogenicity, high cost
Adenovirus associated viral vectors (AAVs)	Nucleic acid	Long term (weeks to month)	ZFN [69], CRISPR/cas, donor template [38]	*In vitro* and *in vivo*	**Pro:** capable of transducing both dividing and non-dividing cells **Con:** Existing immunity to AAV may lead to rapid clearance of the vector; limited capacity (4.5kb), high cost, saturated market for large-scale cGMP grade AAV manufacturing

i.v.: Intravenous; i.m.: Intramuscular; i.t.: Intratumoral; RNP: Ribonucleoprotein. [60–62].

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has risen as the tool of choice for genome editing due to its simplicity of use and efficiency [19]. Other CRISPR proteins such as Cpf1 are also being investigated [20].

APPLICATION OF GENE EDITING TECHNOLOGY FOR T-CELL IMMUNOTHERAPY

The therapeutic potential of gene editing technologies are being evaluated in various cell types and various organisms [21]. To date, gene-edited T cells have been generated and applied to three major areas of immunotherapy.

Generation of HIV resistant T cells

Infection with human immunodeficiency virus (HIV) leads to the loss of cellular immunity and the development of acquired immunodeficiency syndrome (AIDS) [22]. $CD4^+$ T cells are the primary targets of HIV infection. The C-C motif chemokine co-receptor type 5 (CCR5) is the primary entry point for the majority of HIV strains. Early studies in HIV-infected mouse models demonstrated that treatment of these mice with CCR5-knockout ZFN-mediated gene-edited $CD4^+$ T cells significantly reduced the viral load and increased the number of circulating $CD4^+$ T cells, suggesting that the infusion of HIV resistant $CD4^+$ T cells could be a promising treatment [23]. Several clinical trials were initiated using autologous $CD4^+$ T cells with CCR5 knockout mediated by ZFN gene-editing technology with a 11–28% knockout efficiency [24]. These studies proved to be safe and could elicit transient antiviral activities in some patients [25]. It has been hypothesized that the biallelic knockout of CCR5 gene and simultaneous knockout of C-X-C chemokine receptor (CXCR4), another important HIV entry co-receptor, could improve the effectiveness of this therapy [26]. Moreover, removal of integrated HIV provirus from infected patient genome may be needed to further reduce the latent HIV reservoir. To this end, encouraging findings have been reported in cells lines using ZFNs, TALENs and CRISPR/Cas [27–30]. However, efficient delivery of these site-specific nucleases into HIV infected cells *in vivo* still awaits further investigation and innovation.

Generation of off-the-shelf universal CAR T cells

Despite remarkable clinical outcomes using CAR T cells in hematological malignancies, this autologous therapy requires complex and time-consuming manufacturing and one-product-one-patient lot release. Generation of off-the-shelf universal CAR T cells could overcome these inherent limitations and allow lymphopenic patients to become eligible for these therapies. In order to generate off-the-shelf universal CAR T cells, third party allogenic T cells need to undergo the following engineering steps: 1) Elimination of endogenous TCRs to avoid GvHD. This goal has been achieved by knocking out the TCRα chain by targeting the TRAC gene using either ZFNs [31], TALENs [32], or the CRISPR/Cas [33,34] systems; 2) Elimination of endogenous MHC I molecule to

avoid or delay the rejection of transferred T cells by the host immune system. To this end, β2 microglobulin (B2M), the molecule that is essential for the surface expression of MHC I has also been successfully targeted by both ZFNs and CRISPR/Cas technology [31,34,35]. Knockdown of MHC I may trigger the host NK response to the donor T cells, and one possible remedy to this side effect is to further engineer the donor T cells to express HLA-G or HLA-E [36]. Two pediatric patients with refractory $CD19^+$ B cell ALL were successfully treated with TALEN CD19 CAR-T cells lacking TCR and CD52 molecule [37]. Researchers have also developed more sophisticated systems, such as one-shot CRISPR system that can efficiently and simultaneously knockout four genes (TRAC, TRBC, B2M, PD1 or FAS) [34,35]. Site-specific integration of a CAR at the TRAC locus by combining gene editing and viral vector-based cell engineering technologies has also been tested *in vivo* in an animal model and not only resulted in TCR disruption, but also led to a uniform CAR expression, enhanced T-cell potency and decreased terminal T-cell differentiation and exhaustion [38,39].

In either the autologous or the allogeneic T-cell immunotherapy setting, the continuous exposure to antigen stimulation under conditions such as chronic infection or cancer often leads to T-cell exhaustion indicated by the expression of exhaustion markers such as TIM-3 and LAG-3, and constitutive activation of immune checkpoints, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1). Immune checkpoint blockade using monoclonal antibodies has been shown to reinvigorate the function of exhausted T cells [40,41]. Gene editing technologies have recently been applied to interrupt T-cell exhaustion pathways and to block immune checkpoints. Current efforts are focused on testing the safety of PD-1 knockout using CRISPR/Cas in the context of TCR or CAR engineered T cells or EBV specific T cells (Table 2).

DEVELOPING GENE EDITING PLATFORMS FOR T-CELL IMMUNOTHERAPY

Genome editing holds tremendous promise for both research and clinical applications. Gene editing is rapidly being developed as a next-generation therapeutic approach to treat a wide range of diseases. To enable this approach, all components of the genome editing platform must be delivered inside the target cells. If HDR-mediated gene editing is required, a donor template that is typically in the form of single- or double-stranded DNA must be delivered to the target cell in addition to the site-specific nuclease.

Gene editing tools

Delivery of the gene editing components can be mediated by non-viral and viral vector methods (Table 1). The non-viral delivery methods of Cas9 and sgRNA include electroporation, microinjection, microfluidic-based mechanical cell deformation, lipid nanoparticles, cell penetrating peptide, DNA nanostructure, polymer nanoparticles and gold nanoparticles. Viral vector delivery methods are mainly carried out by three types

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TABLE 2

Clinical trials* using gene editing tools in T cells.

Nuclease	NCT identifier	Delivery method	Diseases	Trial title	Trial location
ZFN	NCT00842634	Adenovirus	HIV	Autologous T-Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases SB-728 for HIV	University of Pennsylvania, USA
ZFN	NCT01044654	Adenovirus	HIV	Phase 1 Dose Escalation Study of Autologous T-cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases in HIV-Infected Patients	Sangamo biosciences, USA
ZFN	NCT01252641	Adenovirus	HIV	Study of Autologous T-cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases in HIV-Infected Subjects	Sangamo biosciences, USA
ZFN	NCT01543152	Adenovirus	HIV	Dose Escalation Study of Cyclophosphamide in HIV-Infected Subjects on HAART Receiving SB-728-T	Sangamo biosciences, USA
ZFN	NCT02225665	mRNA electroporation	HIV	Repeat Doses of SB-728mR-T After Cyclophosphamide Conditioning in HIV-Infected Subjects on HAART	Sangamo biosciences, USA
ZFN	NCT02388594	mRNA electroporation	HIV	A Phase I Study of T-Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases SB-728mR in HIV-Infected Patients	University of Pennsylvania, USA
TALEN	NCT02808442	mRNA electroporation	Replased/ refractory B ALL	Study of UCART19 in Pediatric Patients With Relapsed/Refractory B Acute Lymphoblastic Leukemia (PALL)	Institut de Recherches Internatio-nales Servier, UK
TALEN	NCT02735083	mRNA electroporation	ALM	A Study to Evaluate the Long-term Safety of Patients With Advanced Lymphoid Malignancies Who Have Been Previously Administered With UCART19	Institut de Recherches Internatio-nales Servier, UK
TALEN	NCT02746952	mRNA electroporation	ALL	Dose Escalation Study of UCART19 in Adult Patients With Relapsed / Refractory B-cell Acute Lymphoblastic Leukaemia	King's College Hospital, London, UK
TALEN	NCT03190278	mRNA electroporation	ALM	Study Evaluating Safety and Efficacy of UCART123 in Patients With Acute Myeloid Leukemia (AML123)	Weill Cornell Medical College, USA
TALEN	NCT03203369	mRNA electroporation	BPDCN	Study to Evaluate the Safety and Clinical Activity of UCART123 in Patients With BPDCN	MD Anderson Cancer Center Houston, Texas, USA
CRISPR/ Cas9	NCT02793856	N/A	Metastatic non-small cell lung cancer	PD-1 Knockout Engineered T Cells for Metastatic Non-small Cell Lung Cancer	Sichuan University
CRISPR/ Cas9	NCT03399448	mRNA electroporation	Multiple myeloma, melanoma, synovial sarcoma, myxoid/round cell liposarcoma	NY-ESO-1-redirected CRISPR (TCRendo and PD1) Edited T Cells (NYCE T Cells)	University of Pennsylvania, USA
CRISPR/ Cas9	NCT03545815	N/A	Mesothelin positive solid tumor	Study of CRISPR-Cas9 Mediated PD-1 and TCR Gene-knocked Out Mesothelin-directed CAR-T Cells in Patients With Mesothelin Positive Multiple Solid Tumors.	Chinese PLA General Hospital Beijing, China
CRISPR/ Cas9	NCT03081715	N/A	Esophageal cancer	PD-1 Knockout Engineered T Cells for Advanced Esophageal Cancer	Hangzhou Cancer Hospital, China
CRISPR/ Cas9	NCT03166878	mRNA electroporation	B-cell leukemia B-cell lymphoma	A Study Evaluating UCART019 in Patients With Relapsed or Refractory CD19+ Leukemia and Lymphoma	Chinese PLA General Hospital Beijing, China
CRISPR/ Cas9	NCT03747965	N/A	Solid tumor	Study of PD-1 Gene-knocked Out Mesothelin-directed CAR-T Cells With the Conditioning of Paclitaxel and Cyclophosphamide in Mesothelin Positive Multiple Solid Tumors	Chinese PLA General Hospital Beijing, China
CRISPR/ Cas9	NCT03044743	N/A	Stage IV gastric carcinoma, stage IV nasopharyngeal carcinoma, T-cell lymphoma, Stage IV/IV adult Hodgkin lymphoma, Stage IV diffuse large B-cell lymphoma	PD-1 Knockout EBV-CTLs for Advanced Stage Epstein-Barr Virus (EBV) Associated Malignancies	The Comprehensive Cancer Center of Nanjing Drum Tower Hospital, Nanjing, China

*Information in the table is based on clinical trial registered in http://clinicaltrials.gov
BPDCN: Blastic plasmacytoid dendritic cell neoplasm; NCT: National Clinical Trial.

of non-integrating viral vectors: integrase-defective lentiviral vectors (IDLVs), adenoviral vectors (AVs) and adenovirus associated viral vectors (AAVs) [33].

Therapeutic gene editing can be administered through delivery of either a gene-editing nuclease *in vivo* or to cells engineered *ex vivo*. *In vivo* gene editing presents the potential to drive gene modifications in multiple tissues. However, the tissue specificity, biodistribution, pharmacokinetic profile and potential side effects of this approach warrant further investigation. *Ex vivo* gene editing requires a relatively elaborate manufacturing procedure prior to cell transplantation. Nevertheless, this approach has the advantage of allowing the use of technologies such as electroporation applied to either autologous patient cells or healthy donor cells as the starting material and the possibility of generating large quantity of off-the-shelf therapeutic cellular product. Various means of *in vitro* and *in vivo* gene editing strategies are summarized with their advantages and disadvantages discussed in Table 1.

Building a gene-editing platform for T-cell immunotherapy

As for most cellular therapies, the manufacturing of gene-edited T cells starts from the collection of PBMCs from a patient or healthy donor, followed by T-cell selection. Selected T cells are then subjected to gene editing through the delivery of gene editing machineries through either a nonviral or viral vector mediated approach. The gene-edited T cells may be further modified to express additional molecules, such as a CAR or a tTCR. After the completion of all the desired modifications, these T cells can be expanded. Further selection of the desired phenotype and formulation are performed prior to cryopreservation. After meeting all the release criteria, the cells can be released for infusion (Figure 1).

Potential concerns of gene editing for T-cell immunotherapy

Gene editing technologies hold great promise to further enhance the scope of T-cell therapies but poses novel safety concerns. Despite their design for accurate target gene editing, unintended off-target interactions between nucleases and the genome sequence do occur [23,42]. In addition, in the rare occasions potential chromosome rearrangement events, such as deletion, inversion and translocation could also occur among the on-target and off-target events [43]. Certain off-target effects potentially could be the major concern for clinical applications of gene editing therapeutics and various assays are being investigated to quantify these events. Both cell based off-target detection strategies such as HTGTS, BLESS/BLISS, GUIDE-seq [44–49], and *in vitro* detection methods such as CIRCLE-seq, Digenome-seq and SITE-seq have been reported and compared [50,51]. A number of efforts are focused on improving the specificity of gene editing and minimizing off target effects by for example engineering Cas9 to increase targeting specificity [51–54]. Understanding how to bias the repair mechanisms to homology-directed repair (HDR) away from NHEJ

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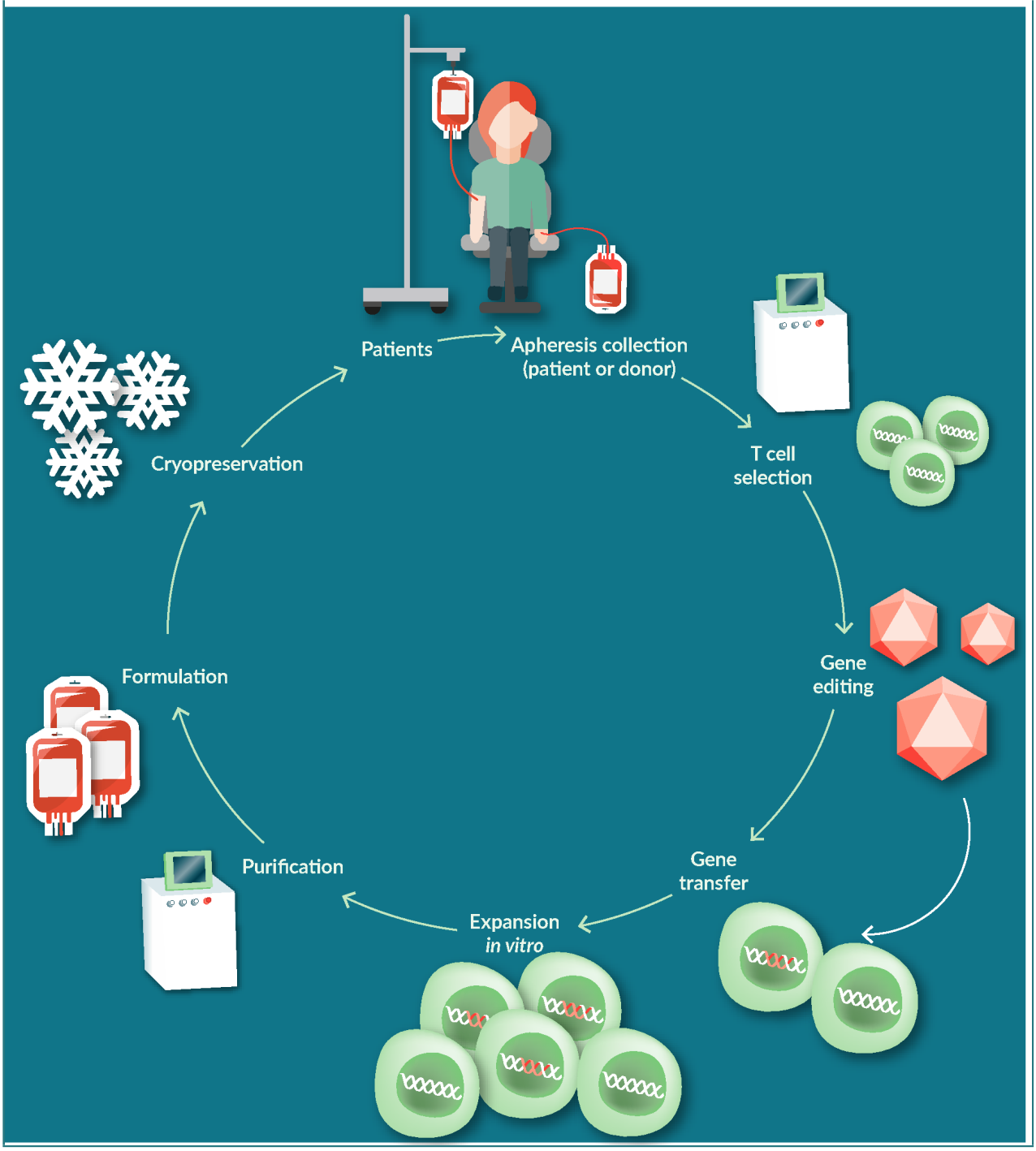
FIGURE 1

Ex vivo **T-cell gene editing and gene transfer manufacturing platform.**



may also help decrease genome changes [55,56].

With the understanding that off-target studies are warranted to address safety concerns related to gene editing, we should also bear in mind that there are naturally occurring and well-tolerated genome changes due to endogenous and environmental conditions throughout of our life cycle. Therefore, assays specific for the detection of off-target events are especially important. The level of tolerance for off-target events for each specific clinical application remains to be determined

and will likely depend on risk/benefit assessments based on disease characteristics and stage.

TRANSLATIONAL INSIGHT & CURRENT STATUS OF T-CELL BASED CLINICAL TRIALS USING GENE EDITING APPROACH

The transduction of human T cells with viral vectors such as gamma-retroviral and lentiviral vectors is a more mature technology that has resulted in durable complete remissions in patients with refractory, relapsed leukemias and lymphomas, leading to the approval by the FDA of two CD19-specific CAR T cell products [5]. Although the follow-up time is still limited (<5 years in a majority of patients), this approach has so far proven to be safe from the genetic standpoint, with the exception of unintentional CAR transgene integration into leukemic B cells occurring during T-cell manufacturing with lentiviral vectors, which results in loss of CD19 expression and resistance to CAR T cell therapy [57].

One approach to circumvent the risk of insertional oncogenesis associated with semi-randomly integrating vectors in hematopoietic cells is to use targeted gene editing in combination with homology-directed repair (HDR) templates. Several experimental approaches have already reported success in preclinical models and feasibility in early clinical trials. There are a growing number of clinical trials using gene-edited T cells for HIV infected patients and cancer patients (Table 2). In clinical trials for HIV patients, ZFNs were introduced in T cells by either AVs or mRNA electroporation to target CCR5. In clinical trials for various cancer indications, either TALEN or CRISPR/Cas9 were used as the most common method in combination with mRNA electroporation to target TCR and/or PD1 disruption in T cells. Gene editing approaches were further combined with CAR gene transfer using lentiviral vectors or AVs to generate universal CAR T cells in a few other trials (Table 2). It has been reported that the CCR5 gene edited T-cell therapy was safe but resulted in limited clinical benefit [25]. The clinical outcomes of cancer patients treated with gene-edited T cells are not yet mature. Their safety profile still remains to be fully ascertained over time in the clinic.

Although the advent of gene editing technologies offers additional prospects for enhancing T-cell therapies for the treatment of cancer and viral infections, pre-clinical and clinical evaluation are limited at this time by their high cost, the paucity of manufacturing sites and expertise resulting in restricted availability of the clinical grade and cGMP reagents required for their implementation at academic centers in early phase clinical trials. Lower production costs are needed to evaluate their full potential [58].

The expending array of gene editing tools creates novel prospects that will facilitate the targeting of genes and regulatory sequences. The wider adoption of gene editing technologies will also require better characterization, quantification and measurement standardization of off-target activities. Currently, efficiency, specificity and levels of off-target events are being investigated in case by case studies which will hopefully help shape and delineate criteria for the development

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of regulatory guidelines. As these technologies may generate heritable editing, they will require strict regulatory and ethic criteria with stringent oversight [59]. The scope of clinical applications should be limited to the treatment and prevention of diseases. There is broad consensus that gene editing technologies should not be used to select or alter human characteristics.

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AFFILIATIONS

Xiuyan Wang
Cell Therapy and Cell Engineering Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
and;
Center for Cell Engineering, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
and;
Molecular Pharmacology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Isabelle Rivière
Cell Therapy and Cell Engineering Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
and;
Center for Cell Engineering, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
and;
Molecular Pharmacology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

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CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

INTERVIEW

Leading the charge: driving allogeneic CAR T cell immunotherapy towards clinical and commercial success

DAVID CHANG is the President, Chief Executive Officer and Co-Founder of Allogene. He previously served as Executive Vice President, Research & Development, and Chief Medical Officer of Kite, a Gilead Company. He has an industry-leading track record of innovation in the field of oncology drug development, including the development of Yescarta™ (axicabtagene ciloleucel), the first CAR T therapy approved for non-Hodgkin lymphoma. From 2002 to 2014, he held senior leadership roles at Amgen, including Vice President of Global Development and Head of Hematology-Oncology. During this time, David spearheaded personalized therapy strategies underlying the success of Vectibix® (panitumumab). He also provided therapeutic area leadership to pivotal programs for Blincyto® (blinatumomab), a bispecific T cell engager antibody in acute lymphocytic leukemia and for IMLYGIC™ (talimogene laherparepvec), a first-of-its-kind oncolytic immunotherapy in melanoma. Prior to joining Amgen, David held dual appointments as Associate Professor of Medicine and of Microbiology, Immunology and Molecular Genetics at the David Geffen School of Medicine at the University of California, Los Angeles. He obtained a BS in biology from the Massachusetts Institute of Technology and MD and PhD degrees from Stanford University. David completed an internship and residency in internal medicine at Brigham and Women's Hospital and a fellowship in medical oncology at Dana-Farber Cancer Institute at Harvard Medical School, where he was a Howard Hughes Medical Institute postdoctoral fellow.

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Q What are you working on right now?

DC: At Allogene, our primary focus is to lead the development of allogeneic CAR T therapy, the next chapter in cell therapy.

We have seen tremendous excitement in the field over the past 4 years with the successful development of autologous CAR T therapy. While that output has shown remarkable efficacy in leukemia as well as lymphoma, we believe the field must to move on to the next chapter, which means allogeneic cell therapy that could make this life-saving therapy more widely accessible.

In our case, we're using cells derived from healthy donors. We're estimating that from each manufacturing run, we may be able to treat approximately 100 patients.

Q Can you summarize for us Allogene's particular platform and approach, and why it holds such promise for the cancer immunotherapy field?

DC: I think we've all learned to appreciate the power of using chimeric antigen receptor therapy to direct T cells against leukemia and lymphoma. As we go from autologous to allogeneic, however, there are two critical issues we have to address.

The first is overcoming immunological phenomena that distinguish self from not-self, which could lead to Allo CAR-T cells reacting against the healthy tissue of the recipient. This is known as graft-versus-host disease. The second is the immune system of the patient recognizing AlloCAR T cells as foreign and rejecting them before they have a chance to exert their anti-tumor activity.

These are two very important topics and what's allowing us to address this issue very effectively is the advent of gene editing. The platform we use is TALEN, which was pioneered by Cellectis.

"Because cell therapy is so potent, you do need to make sure the target is expressed only in tumors and not in normal tissues..."

To address the first challenge, we edit out a T-cell receptor – specifically, TRAC Locus, which is one of the essential genes for the T-cell receptor to be expressed. This results in the final product not having the T-cell receptor which reduces the potential of graft-versus-host-disease from occurring. Early clinical data coming from the UCART19 program, led by our development partner, Servier, appears to substantiate this

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scientific hypothesis. In data to date, we have seen only a limited number of patients experiencing very low-grade graft-versus-host-disease limited to the skin only.

The second challenge is how to prevent the patient's own immune cells from rejecting AlloCAR T cells. Here we augment the lymphodepletion, which is an essential part of CAR T therapy, by adding an anti-CD52 antibody. Anti-CD52 antibodies deplete the lymphocytes, thereby creating a window during which our AlloCAR T cells can expand and carry out anti-tumor effects.

CAR T cells also express CD52, which means anti-CD52 antibodies can indiscriminately deplete both AlloCAR T cell as well as the patient's immune cells. However, we can overcome this through gene editing technology and edit out CD52 in our AlloCAR T cells, which then makes them insensitive to anti-CD52 antibodies.

Q In addition to these advances on the safety side, what are the key factors which justify recent renewed optimism that allogeneic cell therapies can become successful commercial products?

DC: Number one with regard to advances is certainly manufacturing. This is an area in which we are investing heavily, not just in fundamental bioprocessing methods but also in building the infrastructure to manufacture AlloCAR T cell therapies at scale. We have done a considerable amount of the work and are in the process of expanding our capabilities further within Allogene.

Beyond manufacturing, I think there are two elements for AlloCAR T therapy to be useful: first, getting the necessary depth of response in broad patient populations, and second, making sure that this response is durable, as has been seen with anti-CD19 CAR T therapy in the autologous setting.

It will be critical to see a deep minimum residual disease (MRD) response with AlloCAR T therapy. In terms of durability of response, you really have to follow the patient. What has been seen in anti-CD19 autologous CAR T therapy is that if you have patients who are in complete remission at 3 months, they tend to stay in remission at 6 months, 12 months and up to 2 years. As such, we'd very much like to see in our ALPHA study with ALLO-501 a high initial complete remission rate that is maintained. The ALPHA study, Allogene's first sponsored study, is being studied in patients with relapsed and refractory non-Hodgkin's lymphoma – specifically, those with diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma (FL). ALLO-501 shares the same construct as UCART19 but utilizes a different manufacturing process.

Q What translational R&D learnings or considerations relating to the cellular immunotherapy field as a whole can Allogene draw upon for the development of your pipeline candidates, such as ALLO-501?

DC: First, we want to understand what are the critical quality attributes (CQAs) within the cell product that we can optimize. This is something that needs to occur not just for us in the allogeneic field, but also in autologous cell therapy. Once that degree of insight has been achieved, you can then improve the manufacturing process and try to optimize those CQAs.

Second, I believe that CAR T cell therapy, can move to more of a precision medicine approach. Improved understanding of other attributes will enable us to identify those patients who may have optimal response. This is why translational research is key. Traditionally, translational research has been done in the context of linking research to clinical sciences – trying to see what happens in patients through biomarkers and other means to improve how we optimize the product. But now, I believe translational research in the field of cell therapy must expand to include manufacturing.

So instead of just a two-way discussion between the research group and clinical team, this becomes a triumvirate approach encompassing research, manufacturing and clinical development. That is precisely how we've constructed our translational research group, which is a focal point for our efforts to continuously improve our products, and potentially identify patients who may receive the most optimal, durable benefit from the CAR T cell therapy.

Q Can you share your thoughts on likely optimal approaches to cracking solid tumors with CAR T cell immunotherapies?

DC: So far, there has been very limited success in advancing CAR T therapy into solid tumors. Because cell therapy is so potent, you do need to make sure the target is expressed only in tumors and not in normal tissues, which is essential if we are to maintain the therapeutic index.

Moving the field into solid tumors begins with finding the right targets. Then, as we study the CAR T in those targets, we must understand what is needed to overcome the tumor microenvironment, which can sometimes be suppressive to the immune system. There's obviously a lot of research ongoing in solid tumors, which can be actualized either as

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combination therapy, or engineering that combination into the CAR T cells themselves.

From that perspective, I believe AlloCAR T is really key to the future. Multiple levels of engineering - making CAR T cells that recognize multiple targets, enabling them to overcome immune suppression, allowing them to expand better – can really only happen in an allogeneic platform. I believe that is precisely what will be required to achieve high rates of durable clinical response in solid tumors.

> "AlloCAR T therapy truly has the potential to be the next big breakthrough in cell therapy with solid tumors on the horizon."

If we can get there, it will really revolutionize how we deliver therapies to patients with cancer.

Q Can you tell us about any particular lessons you can take from Kite Pharma's success in bringing Yescarta to market to help prepare an early stage biotech like Allogene for the clinical, regulatory and manufacturing challenges to come?

DC: The number one lesson I've learned from Kite Pharma is that manufacturing is the key. This is not simple manufacturing of small molecules or biologics. It is complex and any player in the field has to own the manufacturing and excel in how they engineer and manufacture their CAR T therapies. As I've said before, this is an area where we're expending a lot of effort and resources at Allogene.

At the strategic level, we identify a clinical situation with a high unmet medical need and try to address that using the cell therapy approach. That's a little bit different to the traditional technology-based approach employed by biopharma, where you have a certain technology and you're trying to find the applicable clinical situation for it. In many ways, I would say that we are technology-agnostic – we're willing to go after different technologies as long as they can lead to solving the clinical problem we want to address.

While the field of CAR T therapy has exploded, it is still a relatively nascent field. We have to focus on continuously generating supporting data as we interact with regulators with an evidence-based approach. I think that is something that we were exposed to while at Kite and it has to be one of the key components in terms of how we operate moving forward. The entire allogeneic CART field will certainly have to do something similar in order to advance this very exciting approach into the commercial sphere.

Q Finally, what are the key priorities and goals both for yourself and Allogene over the course of 2019/20?

DC: We just passed our 1-year anniversary, which means there's still a lot of company building, hiring and maintaining of the company culture to be done. At the same time, we need to build our manufacturing capability. Those two are requiring a lot of our time and effort and on top of that, we want to maintain the momentum of our clinical programs.

Specifically, we have initiated our clinical study in ALLO-501, which is our anti-CD19 AlloCAR T program for the non-Hodgkin's lymphoma indication, and we expect to begin our Phase 1 trial for ALLO-715, our anti-BCMA CAR T program, later this year.

While we've not talked specifically about 2020 goals, there is preclinical research we are continuing to advance including our next generation ALLO-501 which is devoid of a CD20 safety switch and AlloCAR T programs for FLT3 in AML and two solid tumor targets, CD70, which can be used for renal cell cancer, and DLL3 for small cell lung cancer.

The latter two programs are very interesting because by all accounts these are very clean targets and may demonstrate the potential for AlloCAR T therapy in solid tumors. AlloCAR T therapy truly has the potential to be the next big breakthrough in cell therapy with solid tumors on the horizon.

AFFILIATION

David Chang
President, Chief Executive Officer and Co-Founder,
Allogene

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CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

EXPERT INSIGHT

Current state of genetically engineered macrophages for anti-cancer therapy

Nicole AP Lieberman, Shannon Kreuser, Ciana Lopez, Katherine Brempelis, Harrison Chinn, Courtney A Crane

As immunotherapy becomes an increasingly important modality for the treatment of several types of tumors, current approaches that enhance T-cell function, including checkpoint inhibition and Chimeric Antigen Receptor (CAR) expression, have revolutionized the way that cancer is treated. Clinical trials are revealing obstacles in some types of tumors, however, suggesting that transforming the immunosuppressive tumor microenvironment (TME) may be a pivotal event to improve patient outcomes. Anecdotal clinical data suggest that toll like receptor agonists and heat shock proteins may support immune responses to tumors, although underlying mechanisms are poorly understood. Recent studies that improve our understanding of the TME in difficult to treat solid tumors have expanded the options for cell and gene therapy platforms, including the use of engineered dendritic cells and macrophages tailored to improve the recruitment and functions of cytotoxic tumor infiltrating lymphocytes. The development of engineered macrophages is in its infancy, but preliminary studies suggest that they are long-lived cells capable of producing stable and titratable doses of transgenes. Herein, we provide a brief overview of this field, focusing on technical challenges in the study of immunologically 'cold' or immunosuppressive solid tumors, and the early data supporting further development of genetic engineering of macrophages to support immune based therapies in patients who have exhausted treatment options.

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INTRODUCTION

Immunotherapy has become a mainstay in the treatment of many cancers, including metastatic melanoma, non-small cell lung cancer, and acute lymphoid leukemia (ALL). In the last decade, the vast majority of new Food and Drug Administration (FDA) cancer immunotherapy approvals have been for T-cell-related processes. Many of these approvals have been for immune checkpoint blockade, encompassing anti-CTLA4, anti-PD-1, or anti-PD-L1 therapy, which removes the 'brakes' from T cells and allows them to overcome tumor-mediated exhaustion and eliminate transformed cells. More recently approved are chimeric antigen receptor (CAR)-modified T cells, which are patient T cells engineered *ex vivo* to express a synthetic receptor that redirects their cytolytic activity towards a protein on the surface of the cancer cells (reviewed in [1]). With rare exception, however, these single agent approaches are realizing limitations in the elimination of solid tumors, in part, as a result of the complex microenvironment created by many types of solid tumors.

The complexity of the cellular components of the TME are highlighted in **Figure 1**. In addition, many solid tumors are immunologically 'cold', remaining invisible to the immune system due to insufficient recruitment of immune cells and low neo-antigen burden on tumor cells. Soluble and cellular factors in the TME also result in insufficient signals to activate T cells locally, or active suppression of pro-inflammatory responses. These tumor defense mechanisms are typified by glioblastoma (GBM, reviewed in [2]), a grade IV astrocytoma with a bleak 5-year overall survival of about 10% [3], for which T-cell-based immunotherapies have had only limited success to date in spite of over 100 completed and ongoing clinical trials. Anecdotal clinical successes using activators of tumor resident macrophages suggest that engaging the innate immune system will be critical to improved patient outcomes [4,5], and may translate to a number of other types of solid tumors sharing similar features in the TME that prevent T-cell focused approaches from eradication of a tumor in patients [6].

INNATE IMMUNITY

In contrast to the adaptive immune system, which comprises B and T lymphocytes, the innate immune system does not evolve primarily in response to antigenic exposure. Rather, upon sensing environmental signals, innate immune cells of the myeloid lineage, including dendritic cells (DCs) and macrophages, coordinate an appropriate response. For example, lipopolysaccharide is detected by tissue macrophages, they activate a pro-inflammatory immune response that includes pro-inflammatory cytokine secretion and recruitment of neutrophils to engulf and eliminate the invading bacteria (reviewed in [7]). Similarly, when viral nucleic acids and neo-antigens are taken up by dendritic cells, they release interferon and initiate antiviral (Th1) T-cell responses that result in the elimination of virally-transformed host cells. In several clinical and pre-clinical studies employed myeloid cells to direct an appropriate adaptive immune response for a number of successful cancer vaccines. Under pro-inflammatory differentiation conditions, macrophages increase

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antigen presentation, and produce pro-inflammatory cytokines that stimulate and support T-cell survival, activation and proliferation. By contrast, differentiation using M-CSF and IL-4 *in vitro* results in the production of anti-inflammatory cytokines [8], prevents the production of cytokines required to support tumor-specific cytotoxic immune cells [9]. Tumor resident macrophages are well-documented suppressors of effective anti-tumor immunity [10], which is initiated by various components of the TME, including tumor, stromal, and infiltrating immune cells [11].

Thanks to an increased understanding of myeloid cells and their roles within the TME, there is a newfound interest in utilizing the genetic engineering toolkit developed for T cells and expanding it to myeloid cells, allowing the effects of targeted transgene delivery alongside innate immune activation. Engineered myeloid cells may act as effectors unto themselves [12], or as potentiators of pleiotropic anti-tumor immune responses within combination immunotherapies. From an engineering perspective, there are several advantages to working with myeloid cells. These include the relative abundance of blood monocytes, which can be differentiated *in vitro* to either macrophages or DCs, the fact that differentiated myeloid cells do not divide *in vivo*, minimizing some of the safety concerns associated with rapidly expanding CAR T cells, as well as the persistence of macrophages in tissue [13]. In this article, we highlight the unique challenges and opportunities specific to innate immune cells, focusing on macrophages and DCs, as well as showcasing examples of engineered innate cells currently in development.

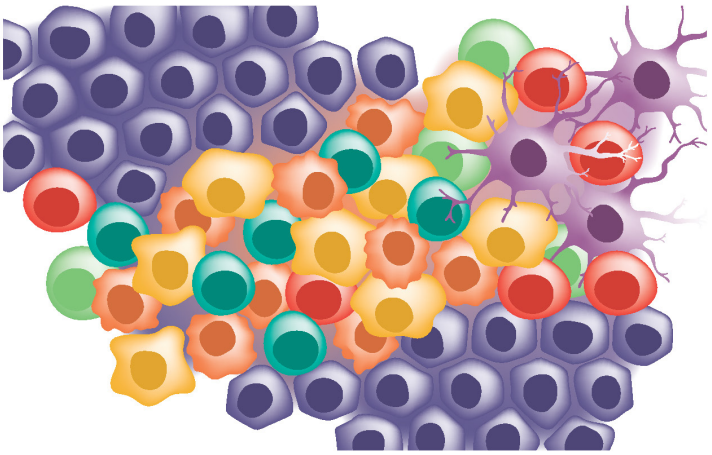
CHALLENGES IN STUDYING MECHANISMS OF MACROPHAGE BIOLOGY

Diversity

In recent years, the increased spatial resolution afforded by techniques such as laser capture microdissection and single cell RNA sequencing has enabled an increasingly nuanced understanding of the role of myeloid cells, particularly tumor-associated macrophages (TAMs) in solid tumors. Recent flow cytometric and single cell RNA sequencing data in glioblastoma [14,15] and breast cancer [16] has revealed that tumor associated macrophages exist on a polarization continuum, and that many individual macrophages exhibit both pro- and anti-inflammatory characteristics. Recent gene expression analysis using patient samples identifies macrophages infiltrating GBM as distinct from *in vitro* MCSF and IL-4 polarized macrophages, showing instead that GBM patient macrophages share more similar gene expression with 'M0' macrophages [17]. These data suggest that that even in tumors, macrophages are sensitive to signals that affect their functions, despite terminal differentiation [17]. This has upended previous dogma, which suggested that for most types of cancer, myeloid cells within the TME had been polarized towards an anti-inflammatory, pro-tumor phenotype. Differences in macrophage phenotype as a function of location within the tumor are also being revealed by this method [18], and suggest changes in phenotype and function in response to environmental cues [19]. Recent single cell transcript analysis in animal models and human cells [20–22] demonstrates that this approach

FIGURE 1

The solid tumor microenvironment.



Heterogeneous antigen expression by tumor cells (orange), healthy and often irreplaceable tissues (purple), immunosuppressive cells and the proteins they express (regulatory T cells [Treg, turquoise], tumor-associated macrophages [TAM; yellow], and tumor cells [ornage], dysfunction of antigen presenting cells [dendritic cells, pink, and TAMs, yellow]) collectively suppress T-cell, NK cell and CAR T cell activation and anti-tumor immunity.

can identify regulatory genes capable of controlling transitions in cell states that are linked to functions, despite being governed by complex networks [23]. Studies such as these performed on human patient macrophages and cells isolated from humanized mice bearing solid tumors represent an exciting new frontier in our understanding of the diversity of tumor infiltrating macrophages in different disease settings. A significant number of these types of studies will be necessary to define the complex regulatory networks governing macrophage biology in tumors.

Species variability

Many fundamental features of macrophages significantly hindered by differences in mouse and human innate immune systems, rendering traditional mouse models insufficient to predict the behavior of human myeloid cells. For example, the preparation of macrophages for downstream assays varies significantly, including the source material. For mouse studies, thioglycolate elicited peritoneal macrophages or bone marrow progenitors are typically the source of macrophages for immune functional analysis, as opposed to peripheral blood monocytes that are isolated from human donors. In addition to variability in the source material used to derive macrophage populations, many studies use mouse models that depend on classification of macrophages based on gene clusters that are absent in human macrophages [24]. In contrast to animals held in a sterile barrier facility, human donors also have significant variability in genetic and environmental factors that can influence the dynamic and sensitive monocyte populations in circulation. Tumor bearing patient samples often have other confounding conditions, such as steroid treatment, that our

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group recently showed can directly impact macrophage phenotypes and functions [25].

Plasticity

Macrophage accumulation correlates with poor prognoses in patients with many types of solid tumors [26–28]. Although a hallmark of many tumors, including GBM, is an accumulation of macrophages, these tend to be either tissue resident macrophages or monocytes that have migrated from the blood into the tumor and differentiated upon arrival. Although many *in vitro* studies have demonstrated the plasticity of macrophages when treated with strong polarizing factors such as LPS, it is challenging to draw consistent conclusions based on the disparate species, source materials, methods of isolation, and culture conditions for macrophages in these studies. We isolated CD14 expressing cells from GBM patient tumors and compared with macrophages differentiated under a variety of conditions that influence inflammatory function, and performed Nanostring analysis for gene expression. Consistent with prior studies [29], our data suggest that *in vitro* cultured macrophages are significantly different at the gene expression level than those isolated from patients (Figure 2). In addition, gene expression differs significantly between individuals, suggesting that stratification of patients based on molecular features of their tumors, prior treatment history, and concurrent therapies, which we have found can significantly impact the expression of immune regulatory genes and proteins should also be considered [25].

ENGINEERING MACROPHAGES & DENDRITIC CELLS

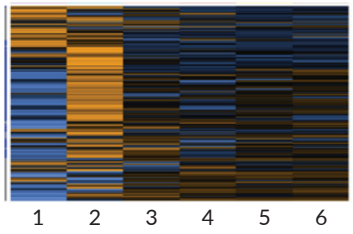
To date, the vast majority of myeloid cell engineering has been performed using DCs in an effort to improve their ability to activate T cells, generally by genetic manipulation of DCs to express tumor antigens or costimulatory molecules. A wide range of techniques have been tested *ex vivo*, including electroporation of naked nucleic acids and several types of viral transduction, as outlined in a recent comprehensive review [30]. However, due to the multiple mechanisms by which myeloid cells can eliminate foreign nucleic acids [31–33], these methods are limited to transgene expression for a few days. Although this timeline is generally long enough to activate patient T cells following adoptive transfer of DCs, the incremental increases in immunogenicity do not consistently result in improved patient outcomes, and come with an enormous price tag. For this reason, *ex vivo* engineering of DCs to improve antigen presentation has largely supplanted by vaccination strategies that activate DCs in situ, either by DC-tropic viruses or through injection of naked antigens along with an adjuvant [34,35]. However, advances in T-cell engineering have reduced both the cost and complexity of *ex vivo* manipulation and adoptive transfer for cellular immunotherapy, sparking renewed interest in dendritic cell engineering.

Potential

Our group has demonstrated that human macrophages can secrete a variety of lentivirally encoded proteins following transduction with gene expression vectors (Figure 3)

FIGURE 2

Gene expression in GBM patient Mφs are not similar to Mφs differentiated *in vitro*.



CD14+ Mφs were selected from glioma patient tumors (1&2). Healthy donor monocytes were differentiated with GM-CSF (3), GBM cell line supernatants (4&5), or M-CSF (6). Nanostring 'myeloid inflammation panel' gene expression.

[13]. This may be an especially appealing approach for treating patients with treatment refractory solid tumors, which is discussed in greater detail in the following section. Perhaps equally intriguing is the possibility to leverage this platform to understand mechanisms underlying the behavior of macrophages in a targeted fashion, allowing overexpression or deletion [13] of genes that may affect macrophage functions in a variety of diseases. Although the artifacts of *in vitro* culture will remain, this approach will allow dissection of complex networks *in vitro* and *in vivo* using primary, human macrophages. This may reveal novel signaling pathways, interactions with stromal or immune cells, or migratory requirements to reveal novel therapeutic targets.

Potential clinical use

Lentivirally modified, adoptively transferred macrophages may also represent a novel approach that could be used as an adjuvant to improve the efficacy of existing immunotherapies, or as a standalone treatment that supports endogenous immune cell functions in the TME [13]. The antigen independence of this approach is resistant to neo-epitope loss and antigenic drift, as activating engineered macrophages enhance the recruitment and activation of the full repertoire of innate and adaptive cytotoxic immune cells. In addition to retaining their inherent biological functions, engineered macrophages may serve as vehicles that can locally deliver of a wide array of therapeutic proteins, including cytokines, scFvs, and full-length antibodies (Figure 3) alone or in combination. Genetically Engineered Macrophages (GEMs) could therefore be used alone or as an adjuvant to enhance the efficacy of existing strategies, including CAR T cells, bispecific T-cell engagers, therapeutic antibodies, or small molecule inhibitors.

Outside of oncology, engineered macrophages may be useful to treat patients with a variety of diagnoses, broadly including regenerative medicine, enzyme replacement, and autoimmunity. Like most tissue differentiated macrophages, engineered macrophages don't divide *in vivo*, and may serve as a repository for long-term secretion of therapeutic proteins. In the current state, the use of engineered macrophages as tissue resident therapeutic protein factories will depend on payload and indication, as macrophages retain functional plasticity even after engineering. This is an important consideration in the context of immune modulating proteins, which may be insufficient to keep macrophages in the desired functional state, leading them to contribute to disease pathology. Several groups are currently working to better understand the functional commitment of macrophages and ways to regulate it.

TRANSLATIONAL INSIGHT

There are several advantages of using engineered macrophages as a therapeutic cell, both in terms of improved and novel properties compared to current immune cell therapies, and studies to improve out understanding the inherent biology of macrophages are essential to developing the most useful and versatile generation of engineered

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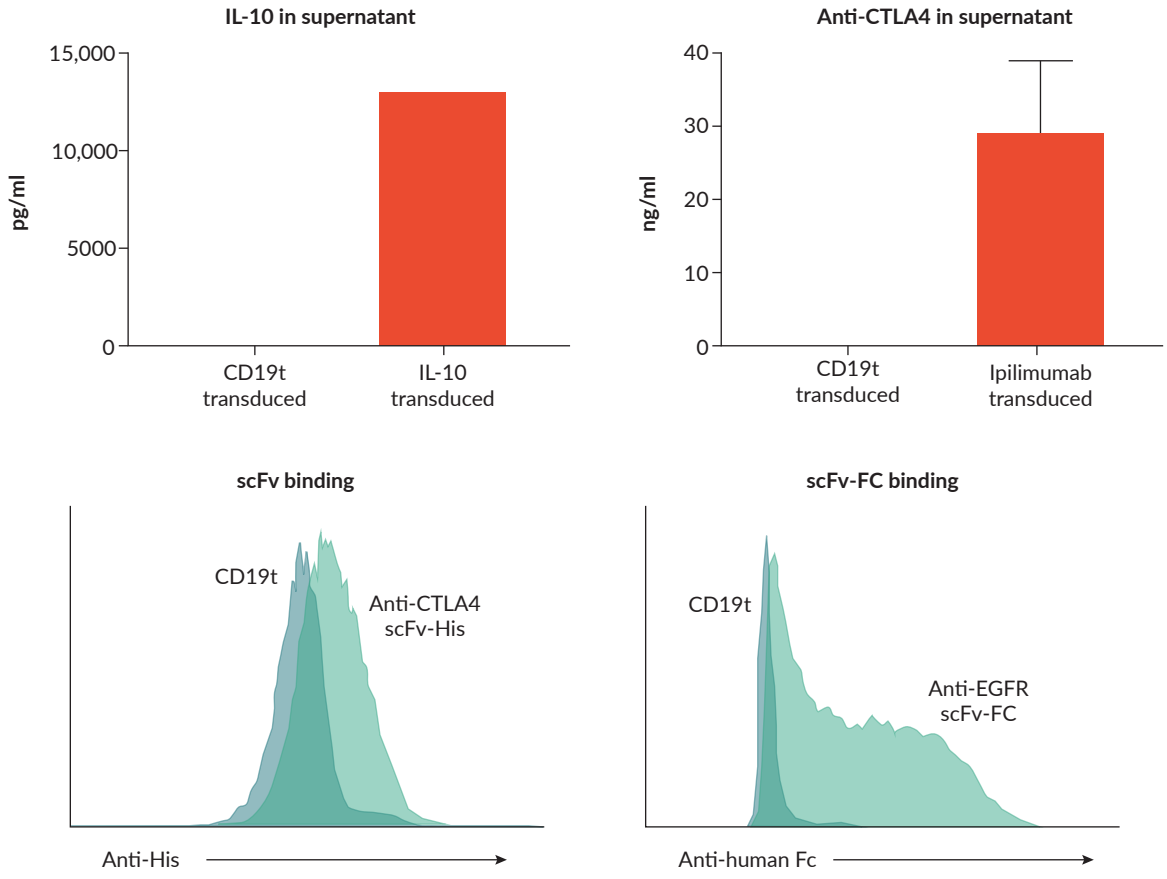
macrophages for patients. For example, the difference source material to include stem cells or bone marrow as opposed to peripheral blood is an important concept that may overcome obstacles in any type of immune cell therapy to increase the number of doses per produce, allow effective therapeutic protein titration, allow single donor off the shelf treatments, as well as enhance *in vivo* persistence and immune functions of the final macrophage product. Additional comparative analysis of the effects of source material on cell therapy are underway, and may reveal critical improvements to future iterations of cell therapies.

1. Macrophages naturally infiltrate tumor tissue [36], addressing tumor penetration challenges that have limited efficacy of cellular, protein, and many small molecule targeted delivery approaches.

2. Macrophages can present a multi-faceted anti-tumor response that is hard to achieve with single target agents, including initiating a

FIGURE 3

Lentiviral transduction of human monocyte derived macrophages.



CD14⁺ monocytes were selected from healthy donors and differentiated with GM-CSF, followed by transduction with 250 lentiviral particles of lentivirus encoding the gene for IL-10 or a sequence encoding a CTLA4 scFv-FC under control of the constitutively active EF1alpha promoter in the epHIV7.2 lentiviral backbone. Supernatant was collected 24 hours later to validate protein expression by ELISA (A&B) and flow cytometry (C&D) of secreted IL-10 cytokine (A), full-length recombinant anti-CTLA4 antibody (B), anti-CTLA-4 scFV (C), or anti-EGFR scFv-FC (D).

pro-inflammatory cascade, presenting antigens, killing tumor cells through release of reactive oxygen species and phagocytizing dead cells.

3. Lentivirally modified macrophages persist in the tumor environment, thereby reducing the frequency for clinical intervention

4. Engineered macrophages do not divide *in vivo*, allowing linear titration of lentivirally encoded proteins.

5. Macrophages in the TME reduce chemokine receptor expression reducing the likelihood that they will be recruited away from the tumor site if locally delivered. Engineered macrophages will then locally produce therapeutic proteins for improved anti-tumor response with reduced systemic toxicity.

Regulatory considerations

The characteristics of differentiated myeloid cells themselves present both technical and regulatory hurdles. Because macrophages and DCs possess several families of viral nucleic acid receptors, including STING, RIG-I and TLRs 3, 7, 8 and 9, the introduction of naked RNA or DNA by electroporation or transfection generally results in potent antiviral responses and the rapid degradation of cytosolic nucleic acid, limiting their utility to sustain the expression of the desired transgene [33]. Furthermore, because macrophages and DCs do not long divide, most retroviruses cannot stably integrate into the genome. This limitation does not affect lentiviruses, a genus of retrovirus; however, myeloid cells are naturally relatively resistant to lentivirus integration due to their expression of a restriction factor, SAMHD1, which sequesters and degrades the dNTPs necessary for lentiviral reverse transcription and insertion into the host genome [37]. SAMHD1 restriction can be overcome by viruses, such as HIV-2 and SIV, that express a protein called Vpx, which targets SAMHD1 for degradation [38,39]. A novel lentiviral packaging system that incorporates the Vpx protein into the assembled virus allows macrophages and DCs to be successfully transduced at low viral doses and transgene integrated into the genome [40].

CONCLUDING REMARKS

Although very early in its development as a cellular immunotherapy, engineering of macrophages to restructure the solid TME offers numerous advantages. First, in contrast to an antigen-dependent approach, the ability to activate a patient's own immune system may allow a broad applicability irrespective of diagnosis, or rapidly evolving molecular features of a tumor. Their natural recruitment and infiltration of the TME also offers the potential to deliver therapeutic proteins to areas that are highly restrictive to other cell types. Their long-term persistence *in vivo* and sustained lentiviral gene expression suggest that a single cell infusion could deliver therapeutic proteins in safe doses to globally restructure the suppressive TME to support anti-tumor immunity, in a way that can be titrated due to their lack of expansion after delivery.

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The use of engineered macrophages may be evaluated as a standalone therapy to determine the effects on recruitment of immune cells and impact on their functions. Importantly, however, this approach may have the greatest effect on patient outcomes when used in combination with other immunotherapy approaches ranging from checkpoint blockade and cell therapies to standard of care radiation and chemotherapy.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

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AFFILIATIONS

Nicole AP Lieberman, Shannon Kreuser, Ciana Lopez, Katherine Brempelis, Harrison Chinn, Courtney A Crane[1]

Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute, Seattle, WA, USA

[1] Department of Neurological Surgery, University of Washington, Seattle, WA, USA
courtney.crane@seattlechildrens.org
Phone: 206-884-4084
Mail: 1100 Olive Way Suite 100, Seattle WA 98101, USA

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CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

EXPERT INSIGHT

Genetically modified natural killer cells: an 'off-the-shelf' immunotherapy for cancer

Subhashis Sarkar, Cillian O'Ceallaigh & Michael O'Dwyer

The success of chimeric antigen receptor (CAR)-T cell-based immunotherapy is currently revolutionizing the treatment of hematological malignancies. However, there are numerous hurdles in developing this as an 'off-the-shelf' therapeutic for cancer treatment, including cost-of-goods, non-availability of allogeneic T cells, treatment induced toxicities, and disease relapse due to tumor antigen escape. Natural killer (NK) cells may provide a suitable platform to develop a safe 'off-the-shelf' cellular immunotherapy, while addressing many of the limitations of CAR-T cells. However, to elicit a successful clinical response using CAR-NK cells, a multi-factorial genetic engineering approach is necessary. In this review, we first discuss how NK cells are biologically different from T cells and summarize the different NK cell sources available for clinical application. Thereafter, based on our understanding of NK cell biology, we describe a list of critical genetic modifications, which in our opinion, are necessary to exploit the maximal therapeutic efficacy of genetically modified CAR-NK cells.

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INTRODUCTION TO NATURAL KILLER CELLS

Natural killer (NK) cells are innate immune cells that act as a first line of defense against a multitude of pathogens and malignancies. Most NK cells circulate in the blood, liver, spleen, bone marrow and lymph nodes [1,2]. Based on the expression of two cell surface markers, CD56 and CD16, the distributions of NK cells differ among these tissues. $CD56^{Dim}$ $CD16^{Bright}$ NK cells are the most abundant population found in peripheral blood, whereas $CD56^{Bright}$ $CD16^{Dim}$ cells predominately reside in tissues and secondary lymph organs [1,3].

NK cells are unique to their T cell counterparts in the fact that they respond rapidly to malignant and infected cells, without the need for prior sensitization. Instead of expressing rearranged antigen-specific receptors, cytotoxicity is mediated by a balance of signals from an array of germline encoded activation and inhibitory receptors (Figure 1) [4]. These receptors generally recognize classical or nonclassical major histocompatibility complex (MHC) class I or MHC class I-like molecules expressed on the surface of cells. Inhibitory receptors play a pivotal role in preventing NK cell killing of healthy cells [5,6]. In virally infected or malignant cells, MHC Class I molecules tend to be downregulated. This reduces inhibitory stimulation and shifts the balance towards an activation phenotype, inducing NK cytotoxicity of targeted cells [7]. Engagement of inhibitory receptors by MHC Class I molecules during functional development of NK cells is thought to be important for their optimal effector function, a process referred to as NK licensing [8].

Once activated, NK cells can exert cytotoxic effects through several diverse mechanisms. Primarily, NK cells secrete perforin and granzyme to induce tumor cell apoptosis [9]. NK cells also express the FasL (CD95L) and TNF-related apoptosis-inducing ligand (TRAIL), which can directly induce apoptosis in target cells that express the Fas or TRAIL receptors DR4 or DR5 [10,11]. TRAIL induced death receptor signaling has recently been shown to be important for antigen independent chimeric antigen receptor (CAR)-T cell killing of tumor cells [12]. Lastly, NK cells secrete cytokines, such as IFN-γ and TNF-α, which further strengthen the adaptive arm of the immune system during viral infection or malignant transformation [9].

RATIONALE FOR NK CELL BASED CELLULAR IMMUNOTHERAPIES FOR CANCER

Immune cells can be specifically redirected to target and eliminate tumor cells by expressing recombinant chimeric antigen receptors (CARs). CARs are primarily comprised of an antigen binding scFv domain derived from an antibody, which are linked to a hinge, transmembrane and activating domains. The single chain variable fragment (scFv) region binds a specific target antigen, and thereafter triggers effector cell activation through the activating domains of the CAR construct [13]. Until now, autologous CAR T cells have been at the forefront of immunotherapy for

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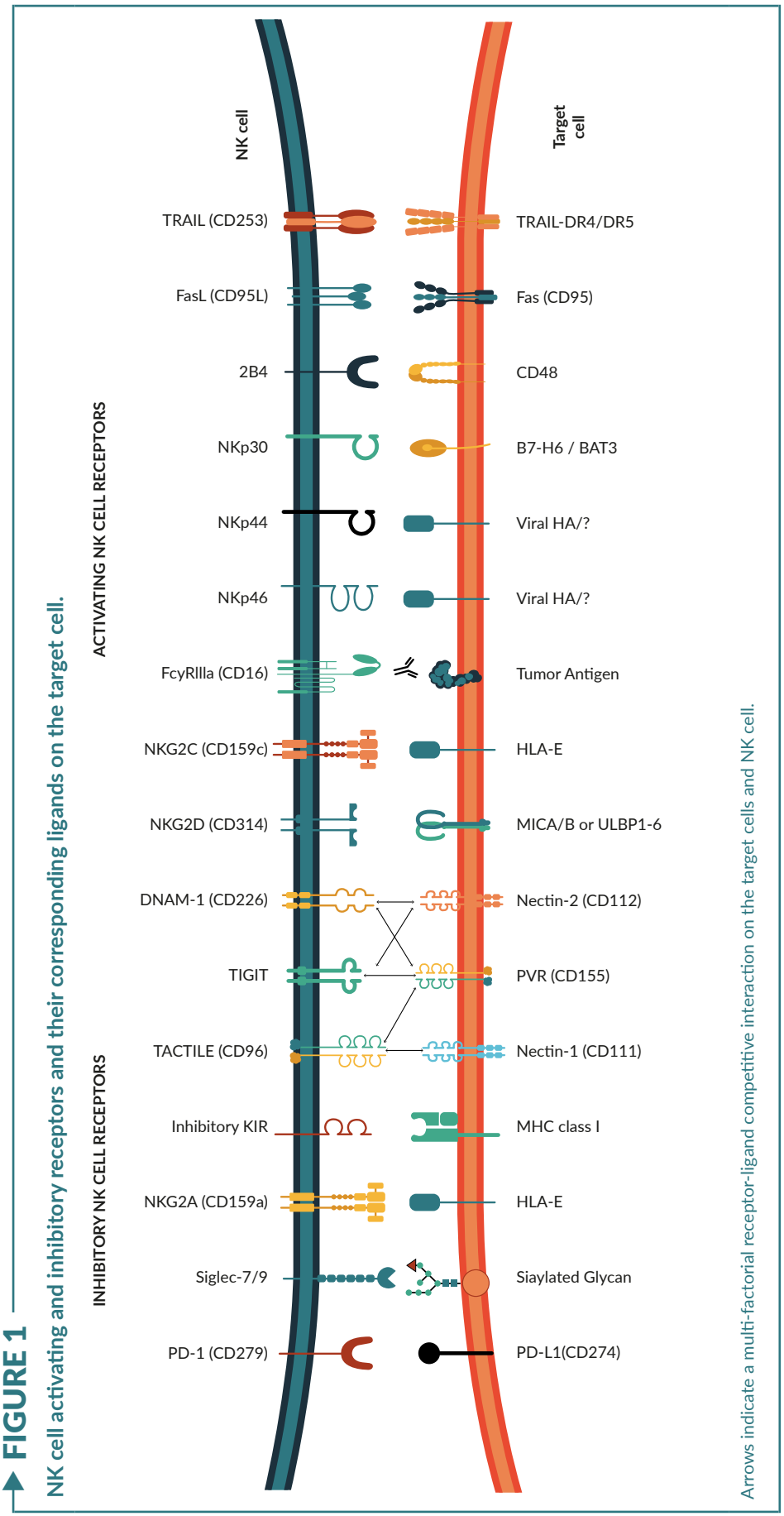

FIGURE 1
NK cell activating and inhibitory receptors and their corresponding ligands on the target cell.

hematological cancers [14–17]. The approval of Kymriah and Yescarta, both CD19 CAR-T cell therapies, have shown significant efficacy in patients with B cell malignancies [18]. CAR-T cells are an attractive therapeutic option for advanced disease patients who have failed other treatment modalities. However, in a pivotal trial with CD19 CAR-T cells, 18% of enrolled patients who had apheresis product collected failed to receive their autologous CAR-T cell (Tisagenlecleucel) infusion due to product-related issues, death, and other adverse events [19]. Generating CAR-T cell products can also be challenging if patients have pre-existing lymphopenia from prior cytotoxic therapy. The CAR-T manufacturing process from pediatric patients with solid tumors might be even more challenging, and the potential of CAR-T product further declines with cumulative chemotherapy in these patients [20]. The use of allogeneic CAR-T cells, even if HLA-matched, poses serious risks of causing life threatening graft-versus-host disease (GvHD). However, generating allogenic CAR-T cells with gene-editing technologies have proven useful in overcoming these limitations [21,22]. Nevertheless, as with autologous CAR-T cells [23,24], cytokine release syndrome (CRS) has been observed in a significant proportion of patients undergoing allogeneic CAR-T cell therapy (UCART19), including one incidence of death in context of severe grade 4 CRS and neutropenic sepsis [25]. Furthermore, the loss/downregulation of the tumor cell antigen, a phenomenon described as "tumor-antigen escape", has been shown to cause disease relapse in patients receiving CAR-T cell therapy [26]. Therefore, there is an unmet need to identify other cell therapy platforms to improve on the initial success of CAR-T based therapies.

Over the past 15 years, donor derived allogeneic NK cells have been shown to be safe and well tolerated in several clinical trials [27,28]. Allogeneic NK cells can exert a graft-versus-leukemia/tumor (GvL/GvT) effect with minimal risks of GvHD [29,30]. This makes administration in an 'off-the-shelf' manner, from an allogeneic source, at high doses of up to 5 x 10^9 cells/m^2 feasible [31]. Furthermore, as genetically engineered NK cells retain their complete array of native receptors, they have the potential to eliminate tumors through mechanisms other than that triggered by the specificity of the CAR. In theory, NK cells could thus mitigate the risks of relapse due to loss of the CAR-targeted antigen [32]. This is potentially a distinctive advantage of using CAR-NK cells especially in the context of solid tumors, which are known to have a highly heterogeneous landscape of targetable tumor antigen(s) [33–35].

NK CELLS PLATFORMS AVAILABLE FOR THERAPEUTIC APPLICATION AGAINST CANCER

Several cellular sources are available for the generation of genetically modified immunotherapies. Adoptive transfer of *ex vivo* activated autologous NK cells, although a safe and easy approach, have failed to elicit any significant clinical responses [36]. Allogeneic NK cell sources include peripheral blood

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(PB) [29], umbilical cord blood (UCB) [37], induced pluripotent stem cells (iPSCs) [38–40], and NK cell lines [41]. Adoptive transfer of haploidentical donor-derived NK cells in combination with high dose Cyclophosphamide and Fludarabine was able to induce complete remission (CR) in 5 of 19 poor prognosis acute myeloid leukaemia (AML) patients [29,42]. A more readily available NK cell source for developing 'off-the-shelf' cellular products is NK cell lines, such as NK-92 and KHYG-1. These cell line-based approaches offer a potentially economical, homogeneous and scalable platform for developing CAR-NK based immunotherapies [41]. Although initial clinical trials with NK-92 have shown safety, they have failed to show much therapeutic efficacy in patients [31]. Nevertheless, clinical trials with genetically modified CAR-NK-92 are currently ongoing (NCT03383978) and the results from these trials will be invaluable in shaping the future of 'off-the-shelf' NK cell therapies [43,44]. Furthermore, KHYG-1 is an alternative NK cell line that has shown promising pre-clinical results with CAR-KHYG1 cells targeting folate receptor 1 (FOLR1), TRAIL-R1 and EGFRvIII tumor antigens [45–47].

Another unique source of allogenic NK cells with distinct advantages over adult donors and cell lines is UCB. UCB is readily available without the need of invasive harvesting techniques or irradiating the cellular product prior to infusion [37,48]. The availability of HLA-typed UCB units make them an attractive option for developing 'off-the-shelf' CAR-NK cells, while still harnessing the 'alloreactive' nature of killer-cell immunoglobulin-like receptor (KIR)-mismatched NK cells in the event of tumor antigen escape. The NK cells from HLA-typed UCB units can be isolated and expanded on feeder cells such as K562-mbIL-21-41BB. Using this approach Rezvani K. *et al.* have successfully demonstrated the pre-clinical activity of CD19 targeting CAR-NK cells against B-cell malignancies [48]. Interestingly, the vector construct (iC9/CAR.19/IL-15) also contains gene elements for ectopic expression of interleukin (IL)-15 to promote NK cell proliferation *in vivo*. Additionally, the construct has an inducible Caspase-9 suicide gene element as an added layer of safety. These CAR-NK cells are currently in a Phase 1 Clinical Trial at MD Anderson, USA [49]. Alternatively, NK cells can be derived from hematopoietic CD34+ progenitor cells using feeder-free culture systems [37,50]. Furthermore, in a Phase 1 clinical trial, infusion of CD34+ progenitor derived NK cells from UCB units have shown safety and were well tolerated in a cohort of acute myeloid leukemia (AML) patients [51]. Interestingly, following infusion the NK cells acquired KIR and CD16 expression, highlighting the transition from an immature to mature phenotype. Although the long-term persistence of UCB derived NK cells remains to be proven, preliminary results show that UCB derived CAR-NK cells with IL-15 gene in the vector construct can have long survival and persistence *in vivo* [49; Unpublished Data].

NK cells can also be generated from iPSCs, as demonstrated by Kaufman *et al.* and Wang *et al.* [38–40]. More recently, Kaufman *et al.*

have been successful in developing potent iPSC CAR-NK cells targeting mesothelin [52]. Although iPSC derived CAR-NK cells have potential to become a truly 'off-the-shelf' cellular product, these cells have a more 'immature' NK cell phenotype with low KIR and CD16 expression, and high NK-G2A expression [39,52]. Ongoing clinical trials will unravel whether these cells also gain a more 'mature' NK cell phenotype upon infusion in patients as shown with UCB derived NK cells. Additionally, malignant transformation due to p53 mutations in pluripotent stem cell derived cellular products is an additional concern which needs detailed long-term characterization [53]. Nevertheless, several adoptive NK cell therapy studies are demonstrating the safety of using NK cells as a source for developing cellular immunotherapies [29,42].

OPTIMAL DESIGN OF GENETICALLY ENGINEERED NK CELLS FOR IMMUNOTHERAPY

NK cell persistence & proliferation *in vivo*

To achieve a durable and long-term response with NK cell-based therapies, a broader approach of genetic modifications is imperative. In the first instance, NK cells should be modified to ensure long term survival and persistence *in vivo* with appropriate cytokine stimulation. IL-15 is one of the key cytokines necessary for NK cell proliferation *in vivo* and its use is preferable to IL-2, which can lead to expansion of regulatory T cells. Therefore, the incorporation of an IL-15 producing gene element in the CAR construct, as demonstrated by Rezvani K. *et al.* [49] could be of significant clinical benefit in generating effective NK cell-based therapies. Another way to achieve this would be by knocking out the CISH gene in the NK cells. CISH encodes for cytokine-inducible SH2-containing protein (CIS), which is a critical negative regulator of IL-15 signaling in NK cells. Deletion of CISH in murine models has been shown to render NK cells hypersensitive to IL- 15 and promote NK cell proliferation *in vivo* [54]. Similarly, human donor derived activated NK cell with CISH knockout have shown enhanced proliferation in response to low doses of IL-15 [55].

NK cell homing to the tumor site *in vivo*

Successful homing and infiltration of NK cells to the site of the tumor is crucial for achieving potent therapeutic response. Evidently, high infiltration of NK cells has been shown to be an independent predictor of progression-free survival (PFS) in solid tumors [56]. One attractive and safe approach to achieve this would be to electroporate chemokine receptor m-RNA into the NK cells just prior to infusion, allowing homing to the tumor site. Carlsten *et al.* demonstrated the feasibility of this approach by electroporating CCR7 chemokine receptor m-RNA into allogenic NK cells which could then migrate more efficiently toward lymph node associated chemokine CCL19 [57]. In another approach, Lee *et al.* showed that NK cells can acquire chemokine receptors such as CCR7 by trogocytosis upon

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co-culture with a feeder cell expressing CCR7 (K562.Clone9.CCR7). This transient acquisition was stable up to a period of 72 hours and resulted in increased lymph node homing in athymic mice [58].

Optimal CAR construct design for potent on target NK cell cytotoxicity

CAR constructs should be designed to achieve potent 'On-Target On-Tumor' cytotoxicity and have minimal 'On-Target Off-Tumor' effects. This is critical when the target antigen is highly expressed on the tumor cell surface (e.g., CD38 in Multiple Myeloma) but is simultaneously expressed in low or moderate levels on normal healthy cells. A logical way to achieve this would be to design affinity optimized scFv constructs which can target only tumor cells with high expression of target antigen, while sparing normal healthy cells with low expression of the same target. In one such approach Mutis *et al.*, using 'light-chain exchange' technology, generated CD38 CAR-T cells consisting of scFv's with 1,000-fold reduced affinity. This construct effectively lysed $CD38^{high}$ Multiple Myeloma cells but spared CD38low healthy hematopoietic cells in vitro and *in vivo* [59]. Nevertheless, such an approach may necessitate the use of a bi-specific dual targeted CAR to prevent tumor escape due to antigen downregulation [22,60,61]. Currently, our lab in collaboration with VUMC, Netherlands, are investigating the therapeutic activity of this affinity optimized CAR in NK cells against Multiple Myeloma [62]. Furthermore, careful consideration of co-stimulatory and signaling domains in the vector constructs of CAR-NK cells could be of paramount importance for treating patients with solid tumors. The hostile tumor microenvironment consisting of hypoxic conditions and infiltrating immune suppressor cells (MDSC's, T-reg's) makes targeting of solid tumor by NK cells even more challenging [63]. Therefore CAR-NK cells harboring NK cell co-stimulatory domains such as 2B4 and transmembrane domain derived from NKG2D could be more potent than conventional T-cell-derived signaling domains such as CD28 [52].

Gene-silencing of (checkpoint) inhibitory receptors on NK cells to eliminate 'high-risk' tumor clones *in vivo*

Despite developing optimally designed CAR constructs for NK cell-based therapies, 'high-risk' clones may evolve at the tumor sites which may have downregulated the target tumor antigen, and/or upregulated key NK cell (checkpoint) inhibitory ligands, such as PD-L1 and HLA-E. Both upregulation of PD-L1 and HLA-E has been associated with poor prognosis in several tumor types [58,64]. To ensure elimination of these 'high-risk' tumor clones, which could eventually cause relapse, we would need to rely on the intrinsic 'alloreactive' characteristic of NK cells. Most NK cell sources, such as NK cell lines, progenitor derived NK cells, and iPSC derived NK cells lack the expression of KIR inhibitory receptors on their cell surface [38,40,65,66]. However, the majority of NK cells from these sources have high expression of NKG2A, and upregulate PD-1 during expansion [39,51,67]. Interferon-γ

production by NK cells can also up-regulate PD-L1 expression in tumor cells [68]. Therefore, it would be ideal to genetically silence these (checkpoint) inhibitory receptors prior to NK cell infusion to elicit an immune response against 'high-risk' tumor clones. This can be done by a CRISPR-based gene editing approach, as shown by knocking out PD-1 in NK cells which subsequently enhanced the potency of edited NK cells *in*

TABLE 1

List of clinical trials ongoing with genetically modified natural killer cells.

NCT number	NK cell source	Target antigen	CAR structure	Disease	Phase	Location
NCT03579927	Umbilical cord blood	CD19	†scFv + CD28 + CD3ζ	B-cell lymphoid malignancies	Phase 1/2	USA
NCT03056339	Umbilical cord blood	CD19	†scFv + CD28 + CD3ζ	B-cell lymphoid malignancies	Phase 1/2	USA
NCT02892695	NK-92	CD19	scFv + CD8αTM + CD28+ 4-1BB + CD3ζ	B-cell lymphoid malignancies	Phase 1/2	China
NCT01974479	Haploidentical donor NK cells	CD19	scFv + CD8αTM + 4-1BB + CD3ζ	B-cell acute lymphoblastic leukemia	Phase 1	Singapore
NCT00995137	Expanded donor NK cells	CD19	scFv + CD8αTM + 4-1BB + CD3ζ	B-cell acute lymphoblastic leukemia	Phase 1	USA
NCT02839954	N/R	MUC1	N/R	Solid tumors	Phase 1/2	China
NCT03415100	Autologous or allogeneic NK cells (m-RNA electroporation)	NK-G2D ligands	N/R	Solid tumors	Phase 1	China
NCT02742727	NK-92	CD7	scFv + CD28 + 4-1BB + CD3ζ	Lymphoid malignancies	Phase 1/2	China
NCT02892695	NK-92	CD19	scFv + CD28 + 4-1BB + CD3ζ	B-cell acute lymphoblastic leukemia	Phase 1/2	China
NCT02944162	NK-92	CD33	scFv + CD28 + 4-1BB + CD3ζ	Acute myeloid leukemia	Phase 1/2	China
NCT03383978	NK-92	HER2	scFv + CD28 + CD3ζ	Glioblastoma	Phase 1	Germany

CAR: Chimeric antigen receptor; HER2: Human epidermal growth factor receptor; iCasp9: Inducible caspase 9; MUC1: Mucin-1; NK: Natural killer; NKG2D: NK group 2 member D; N/R: Not reported; scFv: Single-chain variable fragment; TM: Transmembrane domain.
† Vector Construct includes iCasp9 + IL-15 gene elements.

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vivo [55]. Another elegant approach is to restrict the expression of inhibitory receptors to the cytosol of NK cells and prevent them from being expressed on the NK cell surface. Campana et. al. recently showed the feasibility of this technique by generating NKG2A^{null} NK cells using scFv derived from an anti-NKG2A antibody linked to endoplasmic reticulum-retention domains [69].

CLINICAL TRIALS ONGOING FOR GENETICALLY MODIFIED NK CELLS IN CANCER

Most of the work on CAR-NK cells has been largely pre-clinical. However, there are currently more than a dozen clinical trials registered to test the efficacy of CAR-NK cell therapies in both hematological and solid tumors (Table 1). Since 2016 China has been at the forefront of CAR-NK cell production with ongoing clinical trials focusing on NK cell lines. One of these trials has demonstrated the safety and feasibility of CD33-CAR-NK-92 cells in patients with relapsed and refractory AML [44]. While many of these trials are still at an early stage, initial results from a pioneering clinical trial at MD Anderson (USA) using UCB-derived NK cells expressing CD19 CAR has shown promising initial results [Unpublished data]. Ultimately, the outcomes of ongoing CAR-NK cell trials will shape the future of NK cell-based immunotherapies for cancer.

CONCLUSION

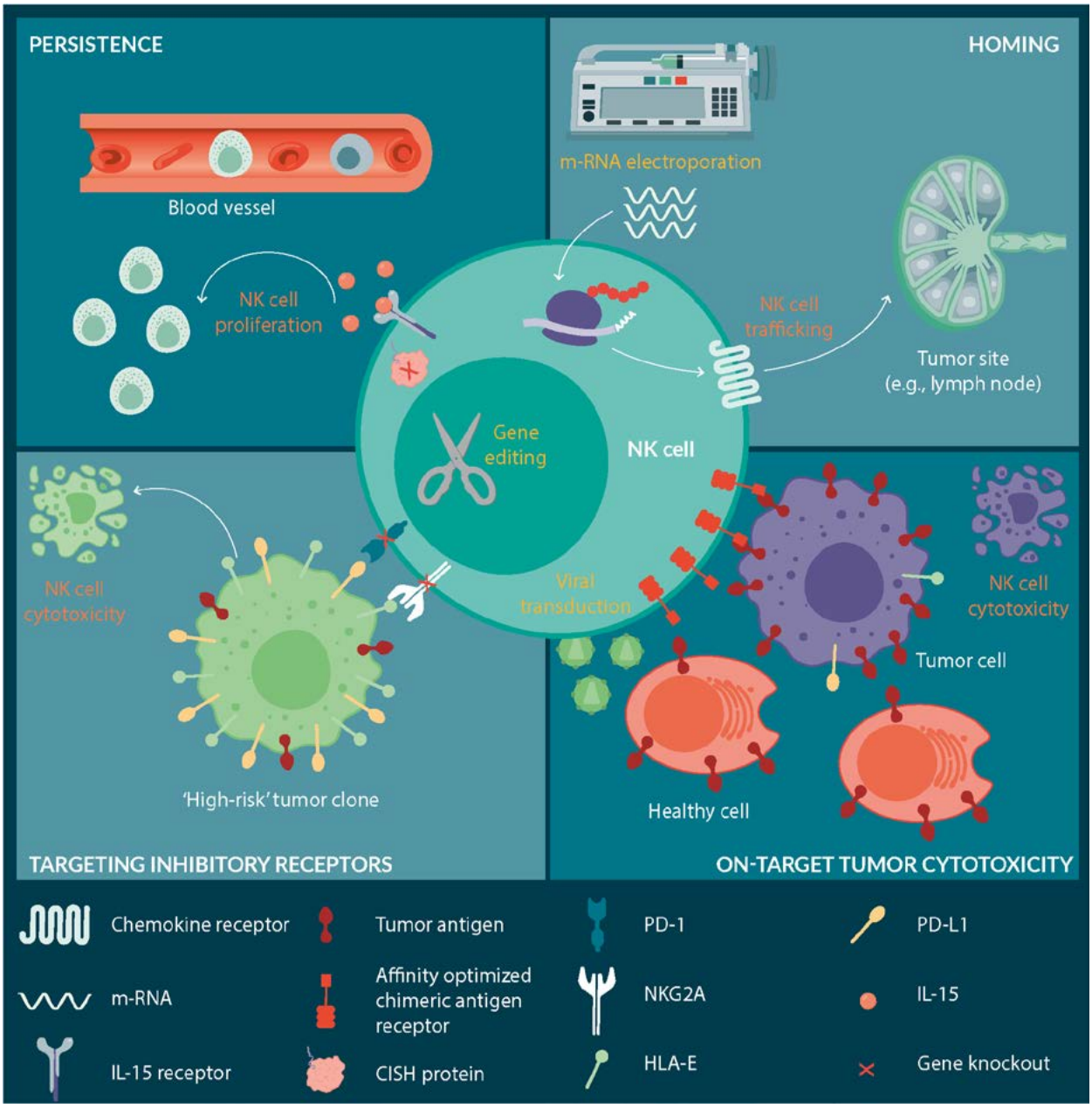
As a new era of cellular immunotherapy dawns, there are exciting opportunities to treat previously untreatable malignancies. CAR-T cells have so far paved the way for the initial wave of cellular immunotherapy by demonstrating strong and efficacious results in B cell malignancies. However, several factors, as discussed in the review, limits their wide-spread use as a therapeutic candidate. NK cells are now starting to come of age, with the number of investigators and clinicians who acknowledge their therapeutic potential growing exponentially. While we can learn several lessons from the development of CAR-T cells, NK cells have their own intrinsic properties and capabilities, which need to be considered as we develop genetically modified NK cells for therapeutic use. What works well for T cells may not necessarily be optimal in NK cells, which goes beyond the choice of CAR machinery. A thorough understanding of basic NK cell development, trafficking, persistence, intra-cellular signaling, cytotoxic machinery, and cytokine production will be key ingredients for a successful recipe [70]. In conclusion, we envision future generations of genetically modified NK cells that will not be restricted to the introduction of CAR's, but will encompass a broader armamentarium to confer long term persistence, improved homing to the tumor site, and the ability to eliminate clonal heterogeneity (Figure 2).

FINANCIAL & COMPETING INTERESTS DISCLOSURE

Subhashis Sarkar: Onkimmune: Research Funding; Michael O'Dwyer: Abbvie: Membership on an entity's Board of Directors or advisory committes;

FIGURE 2

Graphical representation of a comprehensive approach for genetically modified natural killer cells to achieve potent therapeutic efficacy.



Orange text: NK cell biology or effector function;
Yellow text: Different genetic modification approaches.

Celgene: Research Funding; BMS: Research Funding; Glycomimetics: Research Funding; Onkimmune: Equity Ownership, Membership on an entity's Board of Directors or advisory committees, Research Funding; Janssen: Membership on an entity's Board of Directors or advisory committees, Research Funding. All other authors have no conflict of interest.

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AFFILIATIONS

Subhashis Sarkar
National University of Ireland Galway, Galway, Ireland; and Onkimmune Ltd., Galway, Ireland,

Cillian O'Ceallaigh
National University of Ireland Galway, Galway, Ireland

& Michael O'Dwyer
Author for correspondence
National University of Ireland Galway, Galway, Ireland; and Onkimmune Ltd., Galway, Ireland
michael.odwyer@nuigalway.ie

CELL & GENE THERAPY INSIGHTS

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Taking on solid tumors with genetically engineered macrophages

STEVEN KELLY has been the President and CEO of CARISMA Therapeutics since February 2018. Before leading CARISMA, Mr Kelly was the founding CEO of Pinteon Therapeutics, an early stage Oncology and CNS development company. Prior to this he held a number of leadership positions in the biotechnology industry including: CEO, Theracrine; CCO, BioVex; CEO, Innovive Pharmaceuticals; as well as various commercial and manufacturing roles at Sanofi, IDEC Pharmaceuticals and Amgen. Mr Kelly holds a BS from the University of Oregon and an MBA from Cornell University.

MICHAEL KLICHINSKY is a co-inventor of the CAR Macrophage technology and a scientific co-founder of Carisma Therapeutics Inc. In his role as VP of Discovery Research, he oversees the research and discovery efforts of the company. Michael developed CAR Macrophages during his doctoral thesis under the co-mentorship of Saar Gill and Carl June at the University of Pennsylvania. Michael's scientific expertise is in the intersection of immunology, synthetic biology, cancer immunotherapy and translational pharmacology. Michael previously earned a Doctor of Pharmacy degree from the University of Sciences in Philadelphia, and a PhD in Pharmacology from the University of Pennsylvania.

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Q What are you each working on right now?

SK: Carisma Therapeutics is a pioneer in the discovery and development of engineered macrophages, and our current emphasis

is on the development of CAR macrophages as oncology therapeutics. Specifically, we're exploring the utility of CAR macrophages in the treatment of solid tumors.

To do so, our priorities are threefold. Firstly, to take our first product, a HER2-targeted CAR macrophage, into the clinic where we hope to demonstrate or achieve a few things: that we can make it consistently and reproducibly; that we can treat patients safely with a CAR macrophage; and to establish some early Proof of Concept data related to trafficking, tumor killing, adaptive immune response, etc.

> "...we took a step back and thought 'is there an alternative immune cell that's perhaps better suited for the solid tumor microenvironment?"

Our second corporate priority is to continue to refine and optimize our discovery platform.

Thirdly, we're developing an integrated research and development team that can accomplish these first two objectives. Those are the overarching corporate priorities today.

MK: At Carisma we're focused on developing novel approaches to expand our ability to genetically fine-tune the macrophage and take advantage of its unique cell biology to improve disease. Our approach is quite unique and novel from a scientific point of view. There are numerous technological approaches where other immune cell types are being genetically manipulated for cancer therapy – such as T cells and NK cells – but we are the first company to develop genetically engineered macrophages. As you might imagine, that comes with much excitement but also a lot of challenges.

We are having to develop and build a lot of the systems from the ground up – systems which in other, more mature areas such as CART are by now quite well established. Questions that might be taken for granted in the CART field today – what is the best method to introduce genetic material? What are the most appropriate assays to evaluate how the cells are functioning? These questions and many others still need to be worked out in the macrophage field – and that's exactly what we're doing.

The discovery team's focus is on enhancing Carisma's platform – the genetically engineered macrophage. We use myeloid cells because we think they're better suited for the solid tumor microenvironment. These are cells that tumors go out of their way to attract, whereas they often seek to exclude other cells, such as lymphocytes, from gaining access. We think we can take advantage of this with a Trojan Horse mechanism: the tumor actively recruits macrophages in a business-as-usual way, but they're

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genetically manipulated such that when they enter the tumor, they can start to exert anti-tumor function in a targeted fashion.

Q Can you go a bit deeper on why Carisma's CAR macrophages approach holds such promise for the 'holy grail' of cellular immunotherapy: targeting solid tumors?

MK: Based on the remarkable data with CAR T cells, we know that cell therapies have incredible anti-tumor potential. Unfortunately, as tremendous as they've been in the hematology setting against leukemia and lymphoma, CAR T cells in their current state have so far proven to be ineffective in solid tumors.

There are no clear answers as to why CAR T cells aren't working in solid tumors, clinically speaking, but it's safe to assume that it's a blend of a lack of trafficking into the tumors, an issue of antigen heterogeneity within the tumors, and lastly, some form of immunosuppression within the tumor microenvironment itself.

There are many institutions looking to overcome these challenges by engineering T cells in additional ways. However, we took a step back and thought 'is there an alternative immune cell that's perhaps better suited for the solid tumor microenvironment?'

We looked through an RNA sequencing database that was made publicly available by Stanford – they took the gene signatures of tumors from thousands of patients and asked the question – which immune cells do we find in these tumors, and what are their relative frequencies?

Across the board, the macrophage signature is about 3 to 5 times higher than that of T cells (and sometimes much more than that). In fact, there are many more macrophages present than any other type of immune cell in most human tumor types.

We know, then, that tumors go out of their way to recruit macrophages. The reason they do this is because macrophages are phenotypically plastic cells, which have the potential to be bad actors and promote tumor growth in some settings. Tumors therefore tolerate macrophages, but they do not tolerate T cells, because T cells have the potential to reject mutations.

"Process development is often not considered 'discovery research', but I would challenge that notion..."

Outside of the tumor microenvironment macrophages are actually sentinel cells of the innate immune system. They're often the first responders to various kinds of

infection. They're potent killers: they kill through the process of phagocytosis and others. Additionally, they're professional antigen presenting cells, meaning they can prime and initiate an adaptive immune response by presenting antigen to T cells.

The idea is that tumors will recruit these cells, they will come in engineered with a CAR, the CAR will engage with tumor antigen, and the macrophage will kill the tumor cells and shrink the tumor through the process of phagocytosis. Because we can finetune and control the phenotype of these cells *ex vivo*, they are coming in polarized towards a phenotype that induces them to produce a myriad of beneficial pro-inflammatory cytokines, such that they can warm up a cold tumor microenvironment and help recruit other immune infector cells like T cells and NK cells. And because these are professional antigen-presenting cells, after they phagocytose and kill the tumor through their CAR, they can actually process and present antigen, directly leading to an adaptive immune response by priming T cells against mutated peptides that were picked up through the process of tumor phagocytosis.

Q What are the particular challenges you anticipate meeting as you make the transition to the clinic, and how are you preparing now to be able to address them?

SK: With every new technology you're going to encounter challenges as you move forward, relating to manufacturing, clinical trial design, measurement of outcomes, etc.

I think there are a couple of broad parameters in terms of how to address such challenges. Firstly, we're recruiting a very experienced development team – people who have been through the process, albeit not with CAR macrophages, but with other biologic approaches. They have gone through this exercise on either a preclinical, clinical or manufacturing basis. It's getting the right team in place that is truly essential.

The second thing we're doing is to try to anticipate all the potential pitfalls and risks along the way and plan for them, then check them off the list. If there's going to be an issue in one area, you check it off and move on. It becomes an iterative process where you progress towards a goal, encountering and removing the potential risks as you go along.

"we're [trying] to anticipate all the potential pitfalls and risks along the way and plan for them, then check them off the list."

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Q What will be the chief manufacturing considerations for your platform as you continue through scale-up, and what are the key decisions you've made from an overall business model perspective in this regard?

"...it's all about making sure you have sufficient capital to get to your next value inflection point..."

MK: The manufacturing process is somewhat similar to those for other cell therapies, except that it's rather accelerated. Right now, our start-to-finish timeline is 7 days. Patients come in for apheresis, we isolate their monocytes, we turn those into macrophages, and we genetically modify those cells with a viral vector. Those cells can then be cryopreserved, shipped, and ultimately will be infused back into the patients when we start our Phase 1 clinical trial.

I would add that when it comes to cell therapies, you often hear the phrase 'the process is the product'. Process development is often not considered 'discovery research', but I would challenge that notion and suggest that it's very much a critical part of developing a successful cell therapy. All the little things - how to best grow the cells, what are the right nutrients and cell densities and vessels, etc. – have a real impact on what your cell therapy looks like at the end of the process. We have consequently spent a lot of time carefully measuring and optimizing each step of this process.

It's certainly not just true for macrophages, but all cell types you work with in this space. I think taking the time to optimize the process is vitally important in this field.

SK: We've made a conscious decision to use contract manufacturing for the early stage of development, for both our viral vector and cell manufacturing. Part of that decision was financial, part of it was due to internal expertise. Obviously, the capital requirements to build a facility on our own would be prohibitive, especially for a Series A company, and it would be at risk prior to our Proof of Concept data.

Similarly, to recruit an experienced manufacturing team that incorporates operators and technicians, QA and QC, etc. would be financially challenging, as well as taking some time to pull together.

Contract manufacturing helps us overcome these two issues and allows us to take advantage of people who, while they haven't done CAR macrophages, have done viral vector development and engineered cell development and manufacturing. We can leverage the expertise that is resident there.

As we look further forward, through scale-up, Phase 2 and beyond, we will have to carefully consider whether that outsourcing approach makes the most sense from a financial perspective, from a control perspective,

and in terms of overall outcomes and what we're hoping to accomplish as a company. At this point, I wouldn't guarantee we will stay in a contract mode, nor would I say we'll one day build our own facility. It's a decision we will have to take during our early clinical development, based upon what will make the most sense for us at the pivotal study stage and beyond.

Q Carisma's approach is one of a number of novel, next-gen approaches in what is becoming a crowded cancer immunotherapy arena – can you talk about how you go about planning at this comparatively early stage of R&D for a commercial future in such a rapidly evolving, competitive space?

SK: As I look at immunotherapy and specifically, engineered cell types, I think of the fact that we have an immune system. It's not individual T cells, or NK cells, or macrophages – there's a system there. What we are doing is to look at one component of the immune system, the macrophage. For a variety of reasons, we think we have a very viable approach, especially as it relates to solid tumors. However, we certainly recognize that a lot of great companies and great people are working on ways to make T and NK cell approaches succeed in solid tumors. Clearly, there are going to be multiple approaches on the market moving forward.

What we have to demonstrate as a company is that our own approach works – that we can mimic the innate immune system to generate a true adaptive immune response. If we can demonstrate that with our myeloid cells we can get into the tumor, we can phagocytose, we can present antigen and we can start to generate an adaptive immune response, then we're accomplishing what we need to do, and it will set us up for success as one of the many different avenues to treat cancer.

Obviously, we're hopeful we can get broad application in a number of different tumors and that we can achieve memory and maintain durable responses, but we have to prove it all. It's really up to us to ensure that we have solid science and we have very rigorous development programs with measurable outcomes that are meaningful to patients and to payers. If we succeed, I think we will have a viable commercial future in the cancer space.

Q Steve, you bring a great deal of experience across a range of biopharma organizations and biotechnology areas to Carisma – can you distil that into a few specific points of strategic focus that you regard as key for any fledgling biotech in today's sector?

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SK: Biotechnology is a capital-intensive endeavor and certainly to my mind, fundraising should be top of the list for any fledgling biotech. Whether it's seed, Series A or beyond, fundraising is obviously central to successfully driving a viable therapeutic through development to approval.

I think in order to obtain the level of fundraising required to make that journey, there are a couple of key factors. Firstly, you have to tell a story that is able to demonstrate an advance over both existing marketed approaches and competing approaches that are currently under development. I think your strategy to pursue that goal has to be clear, realistic and obtainable. And I think the results that you achieve along the way have to be measurable, grounded, and they have to really drive a value inflection point.

It is perhaps a function of where I currently stand but to me, it's all about making sure you have sufficient capital to get to your next value inflection point – about how to craft the story and make sure you deliver the results along the way that will enable you to realize it.

Q Finally, what are your specific priorities and goals for Carisma Therapeutics as we progress through 2019/20?

SK: Firstly, to take our lead program, the HER2 CAR macrophage, into the clinic. Our hope is to file an IND by the end of this year and start demonstrating biologic Proof of Concept.

That's a big driver for the organization. In parallel, as Mike has discussed, we are really looking at refining and optimizing our discovery platform, and then deploying it more broadly so we can start to build a portfolio of different CAR macrophages against other antigen targets.

MK: As Steve mentioned earlier, in order to achieve these goals, we have to build the team to do so. We are spending a lot of time assembling a really strong group of scientists on the discovery side to come in, bring ideas, and design and execute experiments to help build both the platform and the pipeline at Carisma.

AFFILIATIONS

Steven Kelly
President and CEO, CARISMA Therapeutics Inc.

Michael Klichinsky
Scientific co-founder, CARISMA Therapeutics Inc.

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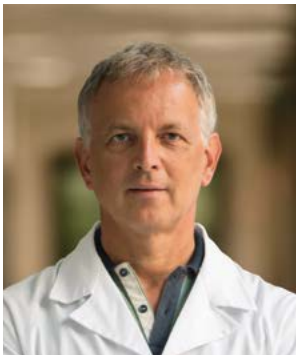
The promise of tumor-targeted gene-based delivery of immune-activating cytokines



LUIGI NALDINI is the Director of the San Raffaele Telethon Institute for Gene Therapy and Professor at the San Raffaele University and Milan, Italy. He has pioneered the development and applications of lentiviral vectors for gene therapy and contributed to advance targeted genome editing in cell and gene therapy. He is member of EMBO, past President of ESGCT, and received the Outstanding Achievement Award from ASGCT in 2014 and from ESGCT in 2015, the Beutler Prize from ASH in 2017 and the 2019 Jeantet-Collen Prize for Translational Medicine.

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Q Could you give us some background on how and why Genenta Science was founded?

LN: We started from nearly a decade of clinical development of hematopoietic stem cell-based gene therapy in rare inherited diseases at the San Raffaele Telethon Institute for Gene Therapy. Through this work, we proved both the safety and efficacy of the approach, as well as the feasibility of clinical deployment up to the point of market access for these personalized and complex new therapies.

"A further advantage of gene-based delivery is sustained expression of the cytokine."

This work was also undertaken in collaboration with big pharma, which helped to establish the roadmap for advanced clinical development and market access of these gene therapies.

Based on this success, we reasoned that one could go beyond the gene replacement design – essentially, replacing a malfunctioning gene in an inherited condition – and consider the possibility of gene addition: to instruct the hematopoietic progenitors with new functions and then take advantage of their progeny, which can migrate throughout the body to deliver specific biotherapeutics in different tissues.

A major input into this came from our experience with the treatment of a neurodegenerative disease, metachromatic leukodystrophy (MLD), due to inherited deficiency of a lysosomal enzyme involved in myelin catabolism, through hematopoietic stem cell gene therapy. This was an odd concept, on paper – why would you treat a brain disease with blood stem cells? But it worked because some of the stem/progenitor cells would travel to the brain, become microglia, and then locally release the missing enzyme. Although these cells were relatively few in number, they actually made a huge difference in the clinic – they were essentially responsible for effectively treating the condition.

The success of that model prompted us to consider whether we could deliver biological products to other tissues or targets using a similar strategy – to tumors, for instance.

Those were the scientific considerations. On the business side, we decided to again leverage on our previous experience of gene therapy clinical development and take all the steps ourselves up to First in Human clinical studies, retaining full control of the process and project so that we could best steer towards our goal and create the greatest amount of value possible ahead of licensing out. We therefore explored the possibility of obtaining venture capital to fund the required stages of R&D, which we could then control in the form of a spin-out company – Genenta Science.

Q Can you tell us more about the Genenta Science platform/approach and R&D pipeline?

LN: Genenta is essentially aiming for tumor-targeted gene-based delivery of immune-activating cytokines.

Tumor-targeting means selectively delivering biotherapeutics to tumor sites – both the main tumor and potentially, to its metastases as well. 'Selectively' means that we seek to spare most of the body from systemic exposure

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to the molecule. Instead, we achieve biologically and therapeutically effective concentration at the disease site only.

This can be achieved through cell- and gene-based delivery: rather than systemic administration of a cytokine, we infuse hematopoietic stem / progenitor cells which contain an engineered gene for that cytokine. That engineering is such that the gene is highly preferentially expressed at the disease site. The stem cell progeny behave as smart vehicles, which home in on the disease site through the tumor signal which is designed to recruit myeloid inflammatory cells. Once they reach the tumor site, these inflammatory cells will then turn on the engineered gene, releasing alpha-interferon.

The safety advantages of selective delivery are clear, of course – by sparing the rest of the body exposure through systemic delivery and the associated side effects, we benefit the therapeutic index.

A further advantage of gene-based delivery is sustained expression of the cytokine, which is very important because if you do a standard pharmacological administration, you go through peaks and troughs of expression. This can often have a desensitizing effect and it can also induce side effects through the expression being either too high or too low. On the other hand, experimental models show that sustained release by cell delivery leads to a durable response within a normal physiological range.

All of this constitutes a platform: this is not a tumor-specific therapy, but rather it's a platform targeting gene-based cytokine delivery to the tumor microenvironment. As such, it has potentially broad application across many tumor types, because the microenvironment is a typical feature of all tumors and some of its features, like immunosuppression, are particularly relevant for most tumor types. And it is precisely this feature that we target through our immunotherapeutic cytokine.

Q Speaking of the tumor microenvironment, can you go a bit deeper on the challenges that presents and how Genenta Science is aiming to tackle them?

LN: There is increasing evidence of the relevance of the tumor microenvironment in shaping tumor growth and its response to therapy.

The tumor microenvironment is made up of normal host cells, which are part of the tumor mass – it comprises connective tissue stroma, vessels (which are newly formed) and a lot of immune cells that are recruited to the tumor – both innate 'inflammatory' cells of myeloid lineage and adaptive immune cells such as T cells.

The last component is supposed to be a defense mechanism of the host – the innate and adaptive cells should play together to build a protective

immune response against the tumor. However, it turns out that the inflammatory innate immune cells recruited to the tumor actually play a role for the tumor – they are co-conspirators in tumor growth, because they promote tissue remodeling to accommodate the tumor, stimulate new vessel formation (angiogenesis) and essentially disable adaptive immunity. This is a normal feature of these cells in the body because they would usually help with tissue growth and regeneration in other situations, and the tumor viciously exploits these properties.

This immunosuppressive microenvironment appears to be a major inhibitor both of the natural immune defense against the tumor and of the cellular immunotherapies developed to date. There is increasing evidence that if you can counteract this immunosuppressive activity by enhancing immune effectors, you can achieve a high rate of response to the tumor. This is essentially what the whole field of immunotherapy is trying to achieve.

Our strategy is to achieve this immune activation by tumor-targeted delivery of a key cytokine in immune activation, which is interferon alpha – a cytokine naturally released by activated innate immune cells, which promotes antigen presentation and deployment of effector T-cell activity. The presence of an interferon response gene signature within the tumor is associated with effective tumor response – achieving an interferon-like response through various strategies is one of the key lines of development in the search for more effective therapies against solid tumors. Our strategy is a novel cell- and gene-based approach, which leverages on the development of cell and gene therapies to date, and it will hopefully achieve an important effect – we have data in experimental models that is supportive of its potential efficacy and safety as well.

Q You've touched already upon the importance of extending the durability of response – can you share more details on how you will approach this very current issue for the cellular immunotherapy field?

LN: While immunotherapies, whether based on checkpoint blocking drugs or CART, are probably the first therapeutic strategies to achieve substantial rates of complete response in at least a fraction of patients in some tumors, they are still vulnerable to this immunosuppressive microenvironment. So you have a fraction of patients who do not respond to them in the first place, and then a further significant fraction of patients who are relapsing – their responses are not durable.

The main culprit for these relapses is usually this immunosuppression, which builds up in the tumor and inhibits immune activation, thereby

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"[We] should be moving away from the concept of a stem cell transplant ... and move instead towards a mini-transplant or cell therapy design..."

allowing the tumor to escape from the adaptive immune response. This is especially the case where the adaptive immunity is directed against a single tumor antigen, as is typically the case with CART, for instance, which are designed to target a specific molecule associated with a given tumor type. They are very efficient killers, but if the tumor can manage to silence presentation of that single antigen, the tumor cells will escape.

Our strategy stimulates presentation of the endogenous tumor antigen repertoire without needing prior knowledge of it: essentially, we uncover the full spectrum of antigens present in a single tumor through the immuno-stimulatory activity of interferon. This likely leads to deployment of an immune response against multiple tumor-associated antigens, facilitating what is called antigen spreading of the response. In those circumstances, because the tumor is impacted at many different points, it's unlikely that tumor escape can occur. For this reason, this concept of antigen spreading is central to today's strategies for overcoming tumor evasion of immunotherapy, including our own.

Q What does the remainder of 2019 and 2020 hold for Genenta Science? What are your key goals and priorities?

LN: This is a key period for Genenta because we are entering clinical testing right now. We have two initial tumor targets, one solid and one hematopoietic: glioblastoma and multiple myeloma.

These two tumor types have been selected from the panel of tumors for which we have evidence of activity in preclinical tumor models, because they should provide the best therapeutic index and risk–benefit profile for First in Human testing.

Clearly, what we primarily aim to see is the safety of our strategy once deployed in the clinic, and the response to escalating dose. But we also want to see indications of its efficacy, both in biological terms through evidence of induced immune activation at the tumor site, but also hopefully in terms of actual therapeutic activity.

Typically, such trials in cell and gene therapy are designed as Phase 1/2, because they are performed in patients, not volunteers (due to the complexity of the treatment and the many unknowns, of course) and because part of the goal is to achieve some biological and therapeutic activity even upon administration of the first starting dose. We of course hope to achieve as

much benefit as possible through these studies, but beyond this, what these two concurrent studies in different tumor types should provide between them is a comprehensive analysis of the host response to our gene-based interferon delivery. Myeloma represents a hematopoietic tumor through which we can investigate the hematopoietic system in depth – that is important both for the activity but also the potential toxicity of our strategy. Glioblastoma, on the other hand, is a hard-to-reach deep brain tumor. Through that study, we can hopefully best demonstrate the capacity of our engineered cells to act as a smart vehicle to target the activity of interferon into this remote site.

Q Looking further ahead, what is your vision for Temferon's™ ongoing clinical development and commercialization, particularly in terms of its potential as both a first line therapy and a component in a combination therapy strategy?

LN: Obviously, all of this depends on us getting validation of our strategy through the early trials, but there are unique elements of our approach that I think we could exploit compared to other current cell-based gene therapies.

One of these is the fact that we don't need to replace all of the hematopoietic cells in the body. We will do a dose escalation study, but we already know from our experimental models that we only need a small fraction of engineered hematopoietic cells to deliver activity to the tumor site. Eventually, I think we could and should be moving away from the concept of a stem cell transplant, which requires conditioning prior to the treatment and which is consequently much more complex to deliver in the clinic, and move instead towards a mini-transplant or cell therapy design, which essentially will only administer engineered progenitors and require minimal conditioning. This will broaden the applicability of the strategy to many patients without the burden and limitation of the conditioning approach. If we can prove safety and efficacy in our first trial, I can see a pathway towards first line treatment due in part to this comparative ease of administration.

"...in the clinic ... CART approaches may be effective but usually don't achieve durable responses."

Regarding improvement of efficacy, we of course hope to see as much activity and efficacy as possible in the clinic, but it's always difficult to imagine that a single agent can achieve a full and complete durable response against cancer.

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However, we know from preclinical studies that our strategy synergizes with other immunotherapy approaches. It can work in combination to achieve full immune activation – both checkpoint blockade and CART are highly empowered in the presence of this interferon mediated reprogramming of the microenvironment. We know that in the clinic, including in the tumors we're working on like myeloma and glioblastoma, CART approaches may be effective but usually don't achieve durable responses. The indication is that they will need antigen spreading to become more effective at overcoming immunosuppression. So, I think the combination of our strategy with those strategies is probably the best way to go.

And last but not least, we will continue working at the bench-to-bedside level to further implement our second-generation platform. That will be even more versatile and potentially tunable than the current platform, because it will involve the ability to adjust the level of expression of the cytokine and also to switch it on and off. We will also potentially be able to add additional cytokine payloads, making it even more efficient and tunable in the long-term. We have that work ongoing at the bench level at the current time and hope to bring it to the clinic in the future, as we deploy our strategy further.

AFFILIATIONS

Luigi Naldini
Director, San Raffaele Telethon Institute for Gene Therapy; and Professor at the San Raffaele University,
Milan, Italy.

CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

COMMENTARY

Immunotherapy with iPSC-derived dendritic cells brings a new perspective to an old debate: autologous versus allogeneic?

Paul J Fairchild, Timothy J Davies, Christopher Horton, Kumaran Shanmugarajah & Marcelo Bravo

The advent of induced pluripotency has raised the prospect of personalized therapies based on the derivation of induced pluripotent stem cells (iPSC) derived from a patient's own somatic cells. Such bespoke cell products may successfully circumvent issues of rejection by the recipient's immune system but raise questions of affordability, the costs of generating patient-specific cell lines and their subsequent differentiation under cGMP conditions, proving a challenging business model. However, principles that have guided the decision between autologous and allogeneic cell products in the past may prove less reliable when considering the therapeutic use of dendritic cells (DC) differentiated from iPSC, whose role in the immune system would be adversely compromised in a fully allogeneic setting. Here, we review the immunological concepts that inform the debate between autologous and allogeneic cell therapies and discuss whether recent breakthroughs might provide a novel solution to this long-standing issue, paving the way for the widespread adoption of DC-based immunotherapy and increasing its reach from immune oncology (IO) to the induction of immunological tolerance.

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INTRODUCTION

The field of regenerative medicine has begun to mature over recent years, fueled by advances in reprogramming technologies, optimization of protocols for the directed differentiation of iPSC and the establishment of significant manufacturing capacity. These advances have resulted in on-going clinical trials for disease states as distinct as age-related macular degeneration, diabetes, Parkinson's disease, myocardial infarction and spinal cord injury [1,2]. Furthermore, the refinement of differentiation protocols for the production of more-specialized cell types continues to offer new avenues for subtle intervention in rare conditions that constitute unmet medical needs. Given that iPSC may be generated from the somatic cells of any patient, the opportunity to develop personalized therapies, tailored to the needs of the individual, remains an alluring prospect but one responsible for rekindling the debate as to whether autologous or allogeneic stem cells should ultimately be pursued for clinical applications.

This debate has traditionally revolved around two issues that are naturally in tension with one another. The production of an allogeneic cell line that serves as an off-the-shelf product for the treatment of numerous patients is clearly attractive but risks immunological rejection of the very cells required to restore the function of affected tissues [3,4]. So-called 'alloreactivity' that underlies allograft rejection, is precipitated by the recognition of products of the major histocompatibility complex (MHC), a series of highly polymorphic proteins that define an individual's immunological identity (Box 1): by resembling a molecular barcode, MHC molecules mark tissues as belonging to an individual while simultaneously identifying those from a donor as foreign to the body with no legitimacy to remain. The successful use of an allogeneic source of iPSC is, therefore, dependent on the judicious use of immune suppression, the long-term risks of which may paradoxically outweigh those of the very disease state being treated, making such a strategy ethically contentious. Under such circumstances, the production of autologous iPSC as a source of cells that would be accepted indefinitely without recourse to immune suppression would clearly be preferable, were it not for the inevitable time lag involved in creating appropriate cell lines and the current costs of manufacture which threaten to undermine the economic viability of such an approach. In most cases, companies producing cell therapy products have opted for an allogeneic source in the unproven anticipation that the transient application of immune suppression may secure long-term survival of replacement tissues. While the veracity of this assumption has yet to be fully determined for the variety of cell types and tissues currently in use, the arguments on which such decisions are based are eclipsed by issues of efficacy when considering DC differentiated from iPSC for immunotherapeutic purposes.

HARNESSING THE POTENTIAL OF iPSC-DERIVED DC

DC are attractive vehicles for immunotherapy since they are responsible for setting the underlying tone of the

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immune system, either establishing and maintaining a state of self-tolerance or breaking the *status quo* to initiate protective immune responses. These diametrically-opposed outcomes are equally dependent on the presentation of antigenic peptides via products of the MHC (Box 1), the outcome of antigen recognition by responding T cells being determined by the balance of auxiliary signals supplied by the DC in the form of cell surface receptors and secreted cytokines (Figure 1). Provision of peptide-MHC complexes in combination with the co-stimulatory molecules CD40, CD80 and CD86 and the pro-inflammatory cytokine IL-12, provokes a potentially destructive immune response. In contrast, circumstances that encourage expression of inhibitory receptors by DC, such as PD-L1/2 and ILT3/4, together with their secretion of the anti-inflammatory cytokine IL-10, favor tolerance through the polarization of responding T cells towards a regulatory phenotype (Figure 1). While the use of DC to re-establish a tolerant state to self-proteins implicated in autoimmunity or to induce tolerance *de novo* to therapeutic proteins remains largely in its infancy [5], more than 200 clinical trials to date have exploited the properties of DC for vaccination to defined tumor associated antigens (TAA) for the treatment of melanoma, glioblastoma, prostate cancer and renal cell carcinoma [6].

DC used in clinical trials are conventionally derived from the patient's own peripheral blood monocytes (moDC) for ease of access, however, this preferred source may help explain the disappointingly low objective response rates reported so far: by lacking appreciable capacity for the cross-presentation of TAA to $CD8^+$ cytotoxic T cells (CTL), the ability of this population of DC to effect tumor regression is inevitably limited.

Box 1 The Major Histocompatibility Complex

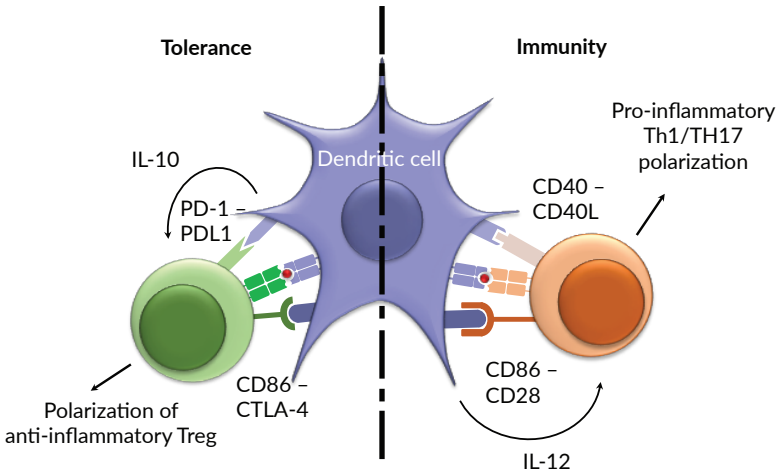
The Major Histocompatibility Complex (MHC) represents a large genetic locus on chromosome 6 in humans containing genes encoding so-called MHC molecules. In man, these molecules are referred to as human leukocyte associated antigens (HLA) and are of two types known as class I and class II. Although class I and II molecules differ in their structure, they share a peptide binding groove which confers on them the capacity to bind epitopes derived from foreign antigen and present them to the T-cell repertoire: indeed, the T-cell receptor (TCR) is inherently MHC-restricted, preferentially recognizing peptides bound to self-MHC molecules. MHC class I determinants are responsible for the presentation of epitopes to $CD8^+$ cytotoxic T cells and are expressed by all nucleated somatic cells. By contrast, epitopes bound to MHC class II molecules are recognised by $CD4^+$ Th cells and Treg and are far more restricted in their pattern of expression to dedicated antigen presenting cells, of which DC are uniquely capable of eliciting a primary immune response. In man, there are three loci encoding MHC class I molecules, HLA-A, HLA-B and HLA-C, and likewise three class II loci known as HLA-DR, HLA-DP and HLA-DQ. Each of these loci is highly polymorphic, existing in thousands of different allelic forms within the human population: given that each individual co-dominantly expresses two alleles at each locus, up to 12 different MHC molecules may be expressed by an individual, defining their unique MHC 'haplotype'. While diversity within the MHC is critical for establishing herd immunity to emerging pathogens, it creates a significant barrier to the success of tissue and organ transplantation, allogeneic MHC molecules marking tissues as foreign to the body. Indeed, a high precursor frequency of T cells is capable of recognising allogeneic MHC molecules, irrespective of the peptides bound, eliciting polyclonal T-cell responses that prove highly damaging to transplanted tissues. It is the balance between the roles played by MHC molecules in allograft rejection and the physiological function of DC that must be held in tension when seeking to develop a DC product for downstream clinical applications.

Under these circumstances iPSC offer a credible alternative source of DC that circumvents many of the difficulties encountered previously [7]. For instance, an appropriate iPSC line provides a permanent and scalable resource conducive to genome editing and the provision of an unlimited supply of DC proven to be safe and efficacious in animal models of IO [8,9]. More importantly, however, iPSC provide access to rare yet desirable subsets of DC previously beyond the reach of clinicians, including plasmacytoid DC that facilitate anti-viral responses [10,11] and the elusive CD141$^+$ subset whose unrivalled capacity for antigen cross-presentation is essential for anti-tumor immunity [12]. Furthermore, by subtly altering the conditions for their differentiation, DC committed to tolerance induction may be readily obtained. In the mouse, these so-called regulatory DC (DCreg) carry a tolerogenic signature defined by constitutive expression of inhibitory receptors and IL-10 secretion which elicits potent Treg responses *in vivo* [13,14]. Human iPSC, cultured under similar conditions, have likewise been shown to spawn DC that share with DCreg from peripheral blood [15], a CD141$^+$ phenotype and capacity for copious IL-10 synthesis [16]. While the access afforded by induced pluripotency to functionally-distinct populations of DC offers unparalleled opportunities for their use in immunotherapy, it raises, once again, the question of whether an autologous or allogeneic source would be preferable.

THE PROS & CONS OF AN ALLOGENEIC DC PRODUCT

FIGURE 1

Dendritic cells determine the outcome of antigen recognition by T cells.



Antigen specificity of the immune response is conferred through recognition by naïve T cells of peptide epitopes from foreign antigen bound to MHC molecules. T-cell receptor (TCR) engagement leads to T-cell activation when accompanied by ligation of the co-stimulatory receptors CD28 and CD40L and provision of the pro-inflammatory cytokine IL-12. In contrast, antigen recognition accompanied by ligation of inhibitory receptors in an environment replete with IL-10 polarises responding T cells towards a regulatory phenotype involved in the establishment and maintenance of tolerance.

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There is little doubt that economic considerations would favor an allogeneic source of DC, the generation of an off-the-shelf product available to a broad spectrum of recipients, justifying the significant financial investment required for the derivation of an iPSC line under cGMP conditions. However, unlike any other cell type whose efficacy *in vivo* is unrelated to its MHC haplotype, MHC molecules play an essential role in antigen presentation by DC and are inextricably linked to their physiological function, making the cost–benefit analysis rather more nuanced. Most importantly, a fully allogeneic source of DC would have no capacity to interact productively with recipient T cells in an antigen-specific manner: the debate between autologous and allogeneic sources therefore strikes at the very heart of efficacy of the DC product itself.

Given that fully allogeneic DC are physiologically impotent, such a cell therapy product would fail to fulfil the very function for which it was intended. Consequently, as a minimum requirement, the source of DC would need to be semi-allogeneic, sharing with the recipient one or more MHC class I loci through which TAA could be productively presented to the $CD8^+$ T cell repertoire. Given that some MHC class I loci, such as HLA-A*0201, are particularly prevalent, being expressed by approximately 27% of the US Caucasian population [17], a source of iPSC derived from an HLA-A*0201^+ donor would be compatible with a significant proportion of the population. Indeed, this reasoning has already led to the development of a plasmacytoid DC product based on a leukemic cell line derived from an HLA-A*0201^+ patient [18]. Ensuring provision for the remainder of the population expressing alleles other than HLA-A*0201 would, however, require the generation of iPSC lines relevant to progressively smaller cohorts of potential patients, rapidly invoking the law of diminishing returns. Importantly, patients with rare MHC haplotypes poorly represented within the population would be unlikely to ever have access to treatment, raising ethical issues of equitability. But although a semi-allogeneic source may potentially fulfil the economic advantages of a fully allogeneic product, the downstream technological risks are far from insignificant. While presentation of TAA may occur through the shared MHC class I molecules, the allogeneic MHC determinants would inevitably provoke the polyclonal activation of antigen non-specific alloreactive T cells. Given that the phenotype of DC renders them uniquely immunogenic, such alloresponses are especially dramatic, engaging an estimated 7% of the entire T-cell repertoire [19], and are, therefore, responsible for the ultimate demise of the administered cells. Consequently, while semi-allogeneic DC may theoretically succeed in provoking a TAA-specific response, they inevitably set in motion a race against time to vaccinate the recipient before they themselves are actively targeted for destruction.

While the ultimate demise of semi-allogeneic DC is inescapable, it has been argued that the allo-response elicited against them may, paradoxically, contribute to the concurrent activation of TAA-specific CTL by mimicking the activity of an adjuvant [20]: indeed,

DC have often been described as 'nature's adjuvant' to reflect their inherent capacity to provoke potent inflammatory responses. It is feasible, for instance, that the polyclonal activation of $CD4^+$ helper T cells (Th cells) through recognition of allogeneic MHC class II molecules, may provide bystander help in the form of secreted IL-2 and IFN-γ to CTL engaged in the cognate recognition of TAA (Figure 2). That such a pathway may operate *in vivo* is evidenced by the induction of alloantibody responses to vascularized organ allografts which has been shown to be wholly dependent on DC carried over within the graft eliciting $CD4^+$ T cell activation as a potent source of B-cell help [20]. More direct evidence in support of this notion comes from studies in mice of DC differentiated from ES cells [8]. Administration of DC loaded with a nominal TAA to semi-allogeneic recipients induced antigen-specific responses that restricted tumor progression *in vivo*, despite the simultaneous induction of significant alloreactivity [8]. That the secretion of pro-inflammatory cytokines was fundamental to this outcome was suggested by the findings of Martín-Fontecha and colleagues who injected recombinant TNF-α subcutaneously to mice followed by administration of TAA-laden DC at the same location. Prior exposure to TNF-α induced the up-regulation of the chemokine CCL21 by local lymphatic endothelium resulting in a 40-fold increase in the numbers of DC reaching the draining lymph nodes [21], a highly-relevant finding given that less than 5% of injected cells are normally expected to reach the site of T-cell activation [22].

These encouraging findings have recently gained further traction from studies of cancer immunotherapy in man which likewise suggest that on-going inflammation at the site of DC administration may substantially increase the efficacy of cancer vaccination. By using tetanus/diphtheria toxoid (Td) as a potent recall antigen, Mitchell *et al.* induced local inflammatory responses in patients with glioblastoma multiforme at the same site to which they subsequently administered DC pulsed with the TAA, pp65. This regimen showed significantly enhanced accumulation of DC in the draining lymph nodes, as assessed by Indium-111 labelling of the administered cells, which correlated with enhanced progression-free and overall survival of patients [23]. The role played by $CD4^+$ T cells as the principal source of pro-inflammatory cytokines was subsequently confirmed in mice, their depletion abrogating any beneficial impact of prior conditioning with Td [23].

Although these findings support the notion that alloresponses by $CD4^+$ T cells elicited by semi-allogeneic DC may, paradoxically, enhance concurrent activation of TAA-specific CTL (Figure 2), enthusiasm for this strategy must be tempered by two important considerations. Firstly, the polyclonal activation of $CD4^+$ alloreactive T cells inevitably leads to a broad repertoire of memory T cells capable of evoking far more dramatic responses upon subsequent exposure to the same inoculum. Indeed, careful experiments evaluating the survival of TAA-pulsed DC in mice revealed their greatly accelerated clearance following prior immunization with the same source of DC [24]. Consequently, the desired effects of DC vaccination in a semi-allogeneic setting would need to be achieved

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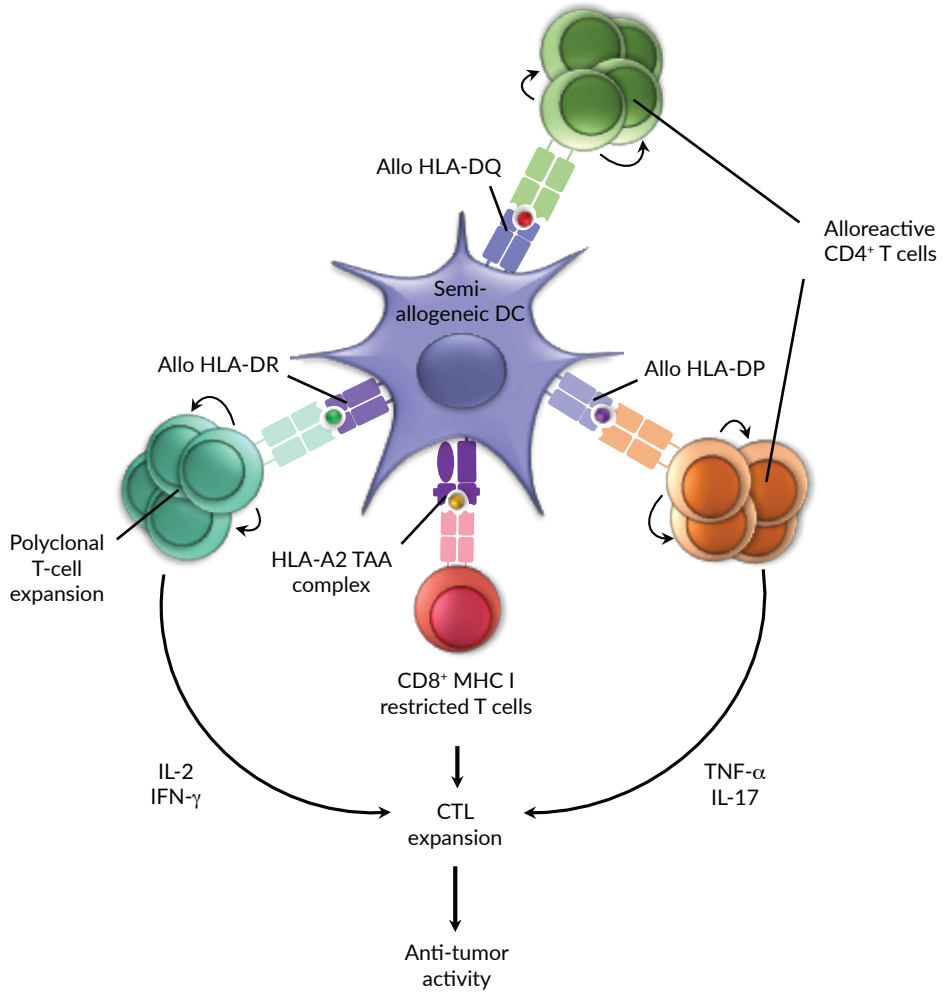
FIGURE 2

Alloreactivity among CD4⁺ T cells may serve to bolster anti-tumour immunity.



DC semi-allogeneic to the recipient have the capacity to present epitopes from TAA to CTL via shared MHC class I molecules. The simultaneous recognition of allogeneic MHC class II molecules by alloreactive CD4⁺ T cells may enhance anti-tumor immunity by the provision of pro-inflammatory cytokines and bystander help for the activation and clonal expansion of TAA-specific CTL.

through the administration of a single inoculum, since subsequent doses would be rapidly destroyed by an anamnestic response. Importantly, such a regimen runs counter to current evidence suggesting that the efficacy of DC vaccination correlates positively with the number of doses given, successive inocula serving to boost immunity over time. For instance, Teramoto and colleagues demonstrated that 1-year survival of patients with refractory non-small cell lung cancer increased from 25% in patients receiving 1–2 injections of autologous MUC-1-pulsed DC, to 39% in those receiving six or more vaccinations. Furthermore, the median survival time increased from 2.7 to 9.5 months, strongly supporting the expediency of progressively augmenting immunity over time [25]. Secondly, while the pro-inflammatory microenvironment elicited by semi-allogeneic DC may be compatible with vaccination protocols, it would doubtless prove profoundly antagonistic

to the induction of immunological tolerance, the release of inflammatory cytokines inhibiting the induction of Treg and most likely favoring aggressive Th17 responses instead. It is, therefore, challenging to envisage how semi-allogeneic DC could ever be re-purposed for tolerance induction, greatly limiting the reach of DC-based immunotherapy to IO. Such constraints naturally raise questions as to the feasibility of working towards an autologous cell therapy product instead.

THE PROS & CONS OF AN AUTOLOGOUS DC PRODUCT

The scientific mandate for an autologous DC product is beyond dispute. In the absence of confounding alloreactivity, the use of DC to establish or reinforce immunological tolerance becomes a far more realistic prospect [5]. Such a strategy would pave the way for the potential use of DC to establish tolerance to defined protein antigens serving as biological therapeutics, such as the recombinant enzymes required for the treatment of lysosomal storage diseases or clotting factors such as Factor VIII for the treatment of hemophilia A. The recent demonstration of pre-existing immunity to the bacterial enzyme Cas9 [26,27] may threaten the very future of *in vivo* gene editing, suggesting that new targets continue to emerge for which the establishment of immunological tolerance is necessary [28]. Furthermore, the role played by DC in autoimmunity and allograft rejection suggests that these indications may also serve as potential, albeit ambitious targets for the future establishment of tolerance [29].

In the context of IO, the availability of autologous DC would likewise prove a significant advantage since DC sharing all MHC class I loci with the recipient would be able to present a broad spectrum of epitopes generated from an appropriate TAA, provoking a polyclonal yet antigen-specific $CD8^+$ T-cell response against an established tumor. In contrast, the token expression of a single MHC class I allele in common between semi-allogeneic DC and recipient, would necessarily restrict the response to the small number of epitopes presented by the relevant MHC molecule. Given that the number and diversity of tumor infiltrating lymphocytes (TIL) serves as a biomarker of favorable prognosis [30], diversity in the immune response is an important goal with significant implications for efficacy. Furthermore, the absence of foreign MHC molecules that would provoke potent non-specific alloreactivity paves the way for the delivery of multiple doses of an autologous DC product over an extended period of time, a strategy that might establish and progressively augment immunity to the desired TAA. The administration of multiple small doses of DC is also preferable since it is less likely to provoke adverse reactions, such as cytokine release syndrome, than the delivery of a single large inoculum that a semi-allogeneic product would necessitate. Such considerations are clearly important, since, to date, the vast majority of clinical trials have made use of autologous moDC, on the basis of which, this form of immune intervention has been deemed safe and well-tolerated by patients [6]. The use of a semi-allogeneic source would, however, involve stepping into the unknown, the safety and efficacy data that have been acquired

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over the past decade bearing limited relevance to this new scenario.

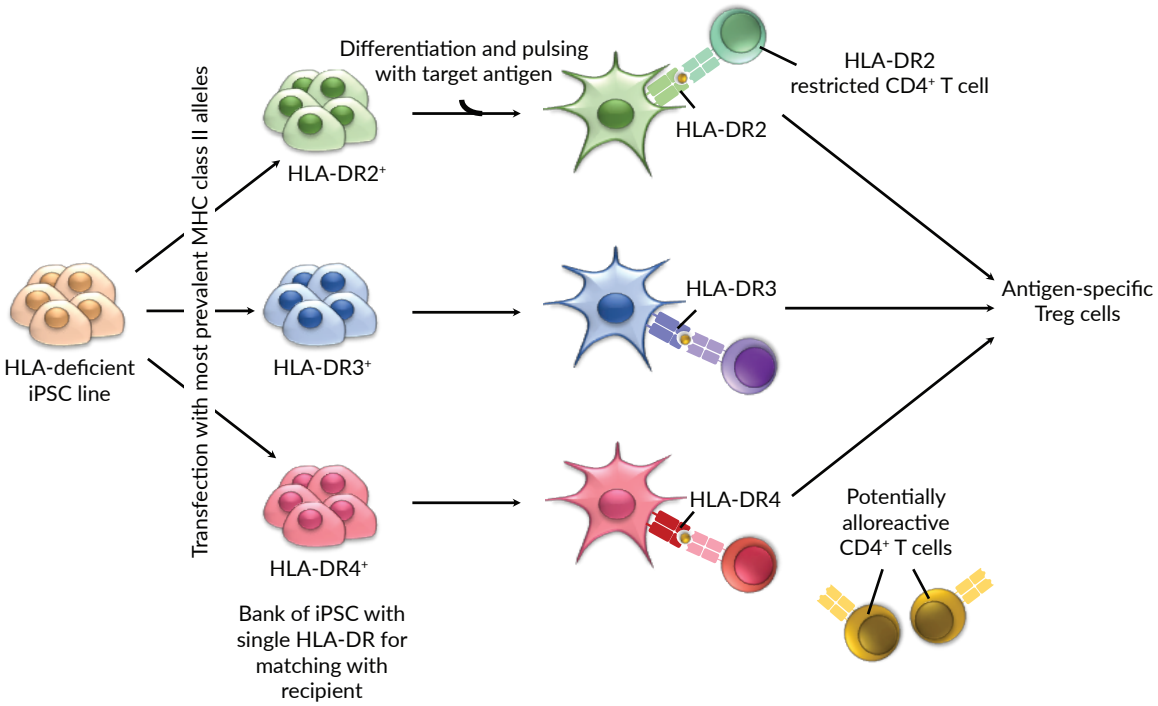
While the scientific credentials of an autologous DC product are indisputable, the economic argument is undoubtedly rather less persuasive since the more bespoke a treatment, the greater the cost of manufacture is likely to be: a fully autologous product is clearly at one end of the spectrum taking little advantage of the economies of scale [31]. Nevertheless, there is little doubt that the costs of manufacture of a cGMP-compliant product are likely to fall substantially in the future, fueled by the increased success and consequent uptake of cell therapies, the introduction of competition into market forces and the streamlining of regulatory pathways. Furthermore, in the context of tolerance induction, many indications may be considered orphan diseases with few alternative treatment options, greatly increasing the likelihood of reimbursement and altering the cost–benefit analysis [31]. Given that predictions of decreasing costs of manufacture have yet to be realized, however, it is doubtless pertinent to ask whether recent developments in the iPSC field might suggest solutions to the issue of alloreactivity that are compatible with the development of an off-the-shelf product.

TOWARDS A UNIVERSAL DC PRODUCT

Arguably the greatest impediment to the use of a semi-allogeneic DC product is the restriction that

FIGURE 3

Design of an off-the-shelf product for tolerance induction.



MHC-deficient iPSC may be transfected with the most commonly expressed MHC class II alleles to create a bank of lines, each expressing a single MHC class II allele. Administration of antigen-pulsed DC differentiated from such lines to appropriately-matched recipients may permit the expansion of antigen-specific Treg cells in the absence of confounding alloreactivity.

alloreactivity imposes on the number of doses that can be administered. One approach to circumventing the anamnestic response might be to derive multiple iPSC lines from different donors for each MHC haplotype, each of which could be administered in turn. For the most prevalent haplotype based on HLA-A*0201, for instance, six unrelated iPSC lines might be derived, each expressing the HLA-A*0201 allele but differing at all other loci. Repeated exposure to HLA-A*0201-restricted epitopes derived from an appropriate TAA would, therefore, be expected to establish robust anti-tumor immunity over time without provoking memory responses to allogeneic MHC molecules which would elicit only primary T-cell responses on each occasion. Such a strategy would build on the demonstrated success of using multiple doses of a DC vaccine [25] while also preserving the potentially beneficial adjuvant effect of a semi-allogeneic product [20]. The obvious disadvantage of such an approach is the associated costs of deriving multiple iPSC lines for each MHC haplotype, significantly weakening the economic arguments for such a semi-allogeneic product.

An alternative strategy might be to exploit recent efforts to generate so-called 'universal' iPSC lines, compatible with all patients, irrespective of their MHC haplotype. Various groups have succeeded in the genome editing of PSC lines to render the cells deficient in MHC class I. For instance, Gornalusse and colleagues targeted the β_2-microglobulin gene, a structural component of all MHC class I molecules, but protected the differentiated products of the resulting cells from Natural Killer (NK) cell lysis by the forced expression of minimally-polymorphic HLA-E molecules that actively engage inhibitory receptors expressed by NK cells [32]. A more refined approach has since been reported which targets HLA-A and HLA-B alleles while preserving expression of HLA-C. This serves the dual function of facilitating residual antigen presentation to MHC class I-restricted CTL while pacifying NK cells through the ligation of KIR receptors [33]. Given the lower levels of polymorphism at the HLA-C locus, Xu *et al.* have calculated that as few as 12 iPSC lines could be immunologically compatible with more than 90% of the global population [33].

While such developments hold promise for the generation of numerous cell types for the purpose of regenerative medicine, DC pose a greater challenge by virtue of their constitutive expression of MHC class II molecules as well as class I. To generate a universal DC product would, therefore, require the additional targeting of all class II loci, perhaps through disruption of the gene encoding the class II transactivator (CIITA) that controls all MHC class II expression. Notwithstanding the additional complexity of targeting CIITA in cell lines already devoid of MHC class I, such iPSC would provide a blank canvass in which to express individual MHC alleles prevalent within the population. TAA-pulsed DC differentiated from iPSC solely expressing HLA-A*0201 could be administered to all HLA-A*0201$^+$ patients with impunity and as often as necessary to build up anti-tumor immunity over time without the confounding influence of alloreactivity. Furthermore, DC differentiated from iPSC lines uniquely expressing some of

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the most prevalent MHC class II loci, such as HLA-DR2, could present target antigens to naïve T cells potentially polarizing them towards a Treg phenotype. Antigen-specific Treg cells might be expected to help establish and maintain a state of immunological tolerance that would otherwise be sabotaged by activation of alloreactive $CD4^+$ T cells (Figure 3). Such an approach might, therefore, provide the potential for the development of the first off-the-shelf product fully compatible with tolerance induction.

TRANSLATION INSIGHT

For most cell therapies based on the differentiation of iPSC, the debate between autologous and allogeneic sources has already been determined primarily by economic drivers, the advantages of an autologous product being eclipsed by the high costs of manufacture. The arguments on which these decisions have been based are, however, rather less persuasive when considering a DC therapy for which the very gene products that normally provoke rejection of tissues are essential to the physiological function of the very cells themselves. The stakes are, therefore, uncomfortably high, the development of a semi-allogeneic product potentially compromising efficacy and curtailing any further investment into an otherwise promising field. Under such circumstances, recent developments in the generation of broadly compatible iPSC lines through genome editing may provide a starting point for the rational design of off-the-shelf DC products suitable either for IO or indications requiring tolerance induction.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

Paul J Fairchild, Timothy J Davies, Christopher Horton hold intellectual property relevant to the differentiation of DC from iPSC but otherwise have no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in this manuscript. The other authors have no conflicts of interest to declare.

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AFFILIATIONS

Paul J Fairchild[1†], Timothy J Davies[1], Christopher Horton[1], Kumaran Shanmugarajah[2] & Marcelo Bravo[3]

[1]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, UK

[2]Department of Surgery, Massachusetts General Hospital, 55 Fruit Street, Boston, 02114 MA, USA

[3]Kellogg College, University of Oxford, Oxford, OX2 6PN, UK

[†]Author for correspondence:

paul.fairchild@path.ox.ac.uk

CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

COMMENTARY

How biomarkers can be used to optimize the clinical development of dendritic cell vaccines in immuno-oncology

Marcelo Bravo

Numerous clinical studies with dendritic cell (DC) vaccines to treat cancer have been conducted in the past two decades. While DC-based therapies have been shown to induce immune responses and to be safe, clinical outcomes have been disappointing. Nonetheless, emerging research suggests DC-based treatments might improve survival and there is renewed interest in next generation DC-based vaccine approaches, particularly in combination with other emerging immunotherapies such as checkpoint inhibitors. This article explores how predictive or prognostic biomarkers, either to select patients or to guide treatment, could be applied to improve outcomes of this novel therapeutic approach. Specifically, we discuss two main approaches: establishment of eligibility criteria based on confirmation of expression of the tumor-associated antigens used in the vaccine, and implementation of a delayed type hypersensitivity test to screen responders so as to extend treatment.

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A NEED FOR IMPROVED ENDPOINTS

For over two decades, dendritic cell (DC) vaccines have been used in clinical trials for a range of cancers. As summarized by Garg *et al.*, DC vaccines have been applied against various malignancies in over 200 clinical trials with the four most targeted cancer types being melanoma (>1000 patients), prostate cancer (>750 patients), glioblastoma (GBM; >500 patients), and renal cell carcinoma (RCC; >250 patients) [1]. This extensive body of clinical trials has shown that DC-based immunotherapy is safe and can induce anti-tumor immunity, even in patients with advanced disease. However, clinical responses have been disappointing, with objective tumor response rates rarely exceeding 15% [2]. As other emerging immunotherapies such as immune checkpoint inhibitors and CAR T cells started delivering breakthrough results, the interest in DC therapies waned.

Some recent reviews and new clinical data, however, have shed new insights that are putting the field back into the spotlight. The review by Anguille *et al.* for example proposes that the assessment criteria used as the primary endpoint in most of these early trials was simply not appropriate. Typically, the primary endpoint used in this extensive body of trials was the classic response assessment criteria such as RECIST, which is a measure of tumor 'burden'. Anguille *et al.* were able to demonstrate that an increasing number of trials that had survival secondary endpoints indicate that DC therapy could confer a survival benefit. Specifically, an increase in median overall survival of at least 20% has been documented in most studies that had a secondary survival endpoint. Although many of these trials were early phase and not designed primarily to measure survival, the results obtained are nevertheless noteworthy, especially in view of the fact that the bar for establishment of a clinically meaningful improvement in median overall survival is generally set at 20% [2]. Thus, the authors concluded that new clinical trials should either use overall survival as the primary endpoint or surrogate endpoints for clinical effectiveness. This absence of association between objective response and overall survival has been also reported with the use of other immunotherapies and as a result, RECIST criteria and improved endpoints for cancer immunotherapy have received significant attention [3].

But using survival as the main endpoint does nothing to help select patients that could respond best to therapy nor does it helps guide clinical treatment. What could be biomarkers or surrogate endpoints to guide DC therapy? As the investment in the field has dwindled after these initial set of clinical trials there are only a handful of recent publications exploring the subject of how to use biomarkers to achieve better outcomes with DC therapies. Nonetheless, there are some emerging directions that will be discussed below.

LOOKING FOR CLUES IN THE IMMUNE RESPONSE

The first port of call is the immune response itself. The mode of action of a DC vaccine is to induce an immune response in the form of clonal

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expansion of antigen-specific T cells that then need to infiltrate the tumor and exert a cytotoxic action. Therefore, as discussed by Lesterhuis *et al.*, as clinical responses were not obvious or did not occur in the majority of patients, researchers have looked for validated assays that can monitor immunological outcome. Most studies have focused on monitoring of antigen-specific T-cell responses in peripheral blood, which proved difficult as often it required in vitro re-stimulation due to very low precursor frequencies. Tumor tissue and lymph nodes would be more interesting compartments to monitor these responses but unfortunately, lymph nodes and the tumor site itself are not always readily accessible for monitoring purposes [4] and the early technologies to detect antigen-specific T cells were based on MHC class I tetramer staining which is limited by the sensitivity required to detect low frequency events [5]. Therefore, monitoring of antigen-specific T-cell responses was not adopted as a practical biomarker of response to treatment.

Several groups have explored other immune related measures or events as predictive biomarkers of an ongoing response. For example, Boudewinjs *et al.* evaluated the correlation between side effects and immunologic and clinical outcomes in stage III and IV melanoma patients. For this, a retrospective analysis of 82 stage III patients and 137 stage IV patients vaccinated with an autologous DC vaccine loaded with gp100 and tyrosinase tumor-associated antigens was conducted. Treatment-related adverse events occurred in 84% of patients of which flu-like symptoms (74%) and injection site reactions (50%) were the most common and both correlated with the presence of tetramer positive $CD8^+$ T cells. In stage III melanoma patients experiencing flu-like symptoms overall survival (OS) was not reached (median follow up time was 54.3 months) versus 32.3 months for patients not experiencing flu-like symptoms. Median OS in patients with an injection site reaction was not reached versus 53.7 months in patients without an injection site reaction. Superior clinical outcomes were also observed for stage IV melanoma patients [6].

Others have also established this correlation. Teramoto *et al.* have explored immune-related adverse events and presence of peripheral lymphocytes as possible predictive biomarkers. Specifically, the research group evaluated the effectiveness of a MUC1-targeted DC vaccine in patients with refractory non-small cell lung cancer. For this, forty patients were treated during a period of 10 years between August 2005 and May 2015. The median survival time (MST) after the initial vaccination was 7.4 months while the 1-year survival rate was 39.3%. Given that following vaccination it may take several months for activation of an anti-tumor response [3], Teramoto *et al.* explored the relationship between the number of vaccinations that patients received with survival outcome and established that the group that received six or more vaccinations achieved significantly higher MST and 1-year survival rate than those that received fewer vaccinations. It is noteworthy that the authors also evaluated the anti-tumor response via conventional RECIST criteria which showed no response, confirming the analysis of Anguille *et al.* and suggesting that new

endpoints are required to assess the clinical response to DC vaccines. Predictive biomarkers of a clinical response were then explored in the patients that received more vaccinations. In this cohort, patients who experienced immune-related adverse events, including skin reactions at the vaccination site and fever, had significantly longer survival times compared with patients without such immune-related adverse events (12.6 vs 6.7 months; p = 0.042). Longest survival times were also noticed in patients whose peripheral white cells contained over 20% lymphocytes (12.6 vs 4.5 months; p = 0.014). Importantly, MUC1-specific cytotoxic responses were achieved in all seven patients analyzed who received at least six vaccinations. Based on this, the authors concluded that immune-related adverse events and a higher percentage of peripheral lymphocytes prior to vaccination are useful to predict clinical responses [7]. It is important to note also that Teramoto *et al.* (and others) established that the robustness of the patient's immune system correlates with clinical response. In other words, patients that have a healthy presence of peripheral lymphocytes have better treatment prognosis. This has been interpreted to suggest that DC vaccines are best used early as advanced stage cancer patients frequently have weak immune systems showing low percentages of lymphocytes in the peripheral blood [7]. This has been noted before, for example, Aartzen *et al.* observed that an intact and proper functioning immune system seems to have a higher potential to react to immune therapy and concluded that "we might take better advantage of the unique capacity of DC to direct the immune response by exploiting DC-based cellular therapy earlier in the disease course" [8].

Using immune-related adverse events as a biomarker is of limited use, however. Typical adverse events are skin redness/swelling/rubor and fever. These measures are highly variable and subject to other confounding factors. Fever in particular may be affected if the patient is taking analgesic/antipyretic medicines such as ibuprofen.

GOING A STEP FURTHER: THE DTH TEST

A number of other groups have explored immune-related skin irritation further: specifically, the association between a positive reaction for the skin-delayed type hypersensitivity (DTH) test and clinical outcome. Escobar *et al.* claim to be the first to report a significant correlation between DTH positive reaction against tumor antigens and an increase of short-term progression free survival. In this study, 20 patients with malignant melanoma in stages III or IV were vaccinated with autologous DCs pulsed with a melanoma cell lysate, alone (n = 13) or in combination with low doses of subcutaneous IL-2 injections (n = 7), to assess toxicity, immunological and clinical responses [9].

To analyze the tumor cell lysate-specific reactivity, patients were evaluated using 400 µg/ml of tumor cell lysate in 200 µl aqueous solution, injected intradermally at a separate site in a volume of 100 µl. Saline solution was used as a negative control. At least 5 mm of induration or erythema, read 48 h after intradermal injection,

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were required to score a skin test as positive. This evaluation was made 1 month after the end of therapy. The group found significant correlation between DTH positive responder patients and a longer stability of disease, and also a longer post-vaccination patient survival. In the study, 8 stage IV patients who showed a positive reaction showed a median TTP of 13.4 months while the group of 8 stage IV patients who did not show a DTH reaction to tumor cell lysate had a median TTP of 2.4 months. The post vaccination survival was also significantly longer in DTH responder patients (17.3 months) than in non-responders (8.6 months) [9]. Subsequently several groups have also established this correlation. Okamoto *et al.*, for example, conducted a retrospective analysis of 255 patients with inoperable pancreatic cancer who received standard chemotherapy combined with peptide-pulsed DC vaccines. The median OS from diagnosis was 16.5 months and that from the 1st vaccination was 9.9 months. The authors report that survival time of the patients with positive DTH was significantly prolonged as compared to that with negative DTH [10].

The DTH test clearly provides for a controlled assessment of the skin reaction which has advantages versus using skin-related adverse events to identify responders. Subsequent to the study discussed above, the same research group has continued to use DTH to establish response to their proprietary tumor cell lysate pool derived from metastatic melanoma cell line, TRIMEL, used in their DC vaccine TAPCells product [11]. This group also established that positive DTH and prolonged patient survival correlates with increased proinflammatory cytokine profiles. Specifically, Duran-Anioz *et al.* determined that peripheral blood lymphocytes from melanoma patients have an increased proportion of Th3 ($CD4^+$ $TGF\text{-}\beta^+$) regulatory T lymphocytes compared with healthy donors and that DTH positive patients showed a threefold reduction of Th3 cells compared with DTH negative patients after DC vaccine treatment. Furthermore, in this study it was also observed that DC vaccination resulted in a threefold increase of the proportion of IFN-γ releasing Th1 cells and in a twofold increase of the IL-17-producing Th17 population in DTH-positive compared to DTH-negative patients. The authors concluded that increased Th1 and Th17 cell populations in both blood and DTH-derived tissues may be related to a more effective anti-melanoma response [12].

The DC vaccine TAPCells is now being used commercially and a publication by Lopez *et al.* describes the use of DTH testing to assess response criteria ("patients were defined as immunologic responders if they displayed activity against TRIMEL in DTH assays"). The authors report that more than 60% of patients showed a positive DTH reaction to TRIMEL and that stage IV DTH-positive patients had a median survival of 33 months compared with 11 months observed for DTH-negative patients [13]. This approach has limitations, however. A study conducted by Dillman *et al.* concluded that DTH to autologous tumor cells (irradiated tumor cells) was neither prognostic for survival nor predictive of benefit in their MACVAC trial. This was a 5-year follow-up of a randomized Phase

2 trial of autologous DC vaccines versus autologous tumor cell vaccines in metastatic melanoma [14]. The main difference versus other approaches appears to be that the DTH test was conducted before and shortly (1 week) after completing treatment while Escobar *et al.* specifically reported that the evaluation was conducted 1 month after the end of therapy, suggesting there is a time gap for response to the DC vaccine. It could also be that irradiated tumor cells are altogether different from the tumor cell lysates used by the TAPCells group or single peptide antigen as used by Okamoto *et al.*

USING THE DTH TEST TO MONITOR THE T-CELL RESPONSE

Coming back full circle, some research groups have gone beyond the DTH test to specifically investigate infiltrating T cells in biopsies. Lesterhuis *et al.*, actually explored this approach with success as early as 2005 and concluded that biopsies from DTH sites after DC vaccination of melanoma patients represent a convenient approach to detecting antigen-specific T-cell responses that highly correlate with clinical outcomes in stage IV melanoma patients [15]. In a subsequent study with colorectal cancer patients, Lesterhuis *et al.* reported that DTH testing provided superior results in the monitoring of antigen-specific T-cell responses compared with peripheral blood. Specifically, in none of the patients could they detect an increase of CEA-specific T cells in unstimulated peripheral blood by direct tetramer analysis, while in 7/10 patients CEA-specific infiltrated T cells were detected by tetramer analysis in DTH biopsies. These T cells were also able to be evaluated for functionality. Unfortunately, given small patient numbers and short duration of the study (the trial had to be stopped due to lack of funding) the authors could not establish correlation with clinical outcomes [4]. The conclusion of the authors was that skin testing provided superior results in the monitoring of antigen-specific T-cell responses compared to peripheral blood, lymph nodes and tumor tissue. It appears, however, that this line of reasoning has not been pursued by other groups that progressed with the translation of DC vaccines.

Clearly, being able to identify responders early during treatment can be a tool that helps clinicians improve outcomes. The ability to detect and assess the functionality of infiltrating T cells in DTH test biopsies would justify the continuation of treatment for responders and possibly improve outcomes. Thus, I would favor further exploring and validating the approach introduced by Lesterhuis *et al.*

GOING BEYOND DTH TESTING: LOOKING FOR CLUES IN MOLECULAR SIGNATURES

There is now a vast literature of reported 'molecular signatures' of disease progression due to the advent of new 'omics' technologies including gene sequencing, high throughput technologies, etc. However, there appear to be very few studies trying to identify

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molecular signatures in response to DC vaccination.

The exception appears to be the group responsible for developing TAPCells. This group is routinely treating patients and has established the use of DTH testing to identify responders to treatment. In a recent publication they reported that the DTH reaction was associated with the presence of distinct cell subpopulations in peripheral blood and have conducted molecular studies to identify gene expression markers that might serve as potential molecular biomarkers. Specifically, Garcia-Salum *et al.* used microarray analysis to profile the transcriptome of patients during treatment. Researchers identified 17 genes over-expressed in responder patients after vaccination relative to non-responders, from which ten were linked to immune responses and five were linked to cell cycle control and signal transduction. In immunological responder patients, increased protein levels of CXCR4 and CD32 were observed on the surface of $CD8^+$ T cells and B cells and the monocyte population confirming gene expression results. The clinical use of these findings as biomarkers, however, requires further investigation [16].

PATIENT STRATIFICATION TO IMPROVE CLINICAL OUTCOMES

Can patients most likely to respond to DC therapy be selected at the very start of the trial so as to maximize clinical benefit? The approach to 'arming' the vaccine may be a good place to start.

Most DC vaccines tested have been loaded with single or simple recombinant/synthetic antigenic peptide cocktails, usually targeting well-established tumor-associated antigens (TAAs) such as CEA, MUC1, gp100 or with tumor cell lysates prepared via various treatments ensuring 100% cancer cell death [17].

For DC vaccines that are 'armed' with defined TAAs, the obvious first port of call for a stratification strategy should be based, where viable, on confirmation of expression of the TAA in question. While this seems obvious, it was not routine practice in most of the early trials and perhaps partly explains why outcomes have fallen short of expectations. Teramoto *et al.* consider that selection of patients with high expression of target antigens on cancer cells is critical [7]. In the specific case of their MUC1-loaded DC vaccine, Teramoto *et al.* report that their immunohistochemistry data demonstrate that the expression of MUC1 on more than 60% of adenocarcinoma cells occurs in only about 40% of patients. Expression of MUC1 on more than 60% of adenocarcinoma was, in fact, a key eligibility criterion in their trial.

For DC vaccines that are 'armed' with tumor cell lysate, the picture is more complicated. In the case of TRIMEL, the allogeneic proprietary tumor cell lysate pool derived from metastatic melanoma cell line used in TAPCells, main antigens can be characterized and eligibility criteria can be established based on threshold levels of these in the patient's tumor tissue, if that is available. That is definitely applicable in certain clinical scenarios where tumor biopsies are available, but

not all. There is, however, a need to identify universal biomarkers that could be used to identify responders at the very beginning.

TRANSLATIONAL INSIGHT

Putting it all together, there are clear learnings that can be implemented at patient selection and during treatment to maximize clinical outcomes with DC vaccines:

- Recruit patients with robust immune systems, i.e., in early stages of cancer progression and by means of eligibility criteria based on % of peripheral lymphocytes, i.e., 20%.
- In DC treatments where tumor biopsies are available and where the antigen source is either a single defined TAA or a cocktail of defined TAAs or an allogeneic tumor cell lysate source, establish eligibility criteria based on confirmation of expression of the TAA in question. There is an argument that DC treatment may not be the best approach in clinical settings where this cannot be established.
- Implement a DTH test including analysis of biopsies to detect antigen-specific T-cell responses and use this to screen responders so as to extend treatment. The proven correlation between DTH-positive testing and improved outcomes [12,13] and the ability to detect and assess the functionality of infiltrating T cells in DTH test biopsies [15] would justify the continuation of treatment for responders which is desired as Teramoto *et al.* established that receiving more vaccinations improves outcomes.

Clearly there are many open questions:

- What should be thresholds to establish eligibility criteria either in terms of disease stage or in terms of % of peripheral lymphocytes?
- For specific TAAs or main antigens in a tumor lysate pool used to arm in a vaccine, what should be the appropriate thresholds of expression to establish eligibility criteria?
- What should be specific criteria in infiltrating T-cell composition and functionality that would warrant continuation or adjustment of treatment?
- For how long and with what frequency should vaccination continue and what should the clinician look for in the analysis of DTH biopsies to guide this?

It is noteworthy that, while the median number of vaccinations for the patients that received more than six vaccinations in the Teramoto *et al.* study was 10, the range was very wide, 6 to 42 vaccinations in total [7]. These were given bi-weekly, so patients had treatment that ranged from three months to 24 months. The correlation of presence and functionality of antigen-specific T cells in DTH biopsies with outcomes could be used to provide guidance to treatment duration. In other words, this biomarker(s) might be used to establish how much time it takes for a DC therapy to mount an effective anti-tumor

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immune response and establish T-cell memory.

The answers to these questions can only be explored in the clinic. The ability to conduct retrospective or meta analyses in this field is limited so shedding light on these questions will require prospective clinical work, most likely in the form of Phase 2 trials. A good example of this is the Phase 2 trial reported by Lopez *et al.* and Escobar *et al.* Specifically a Phase 2 trial with survival primary endpoints, eligibility criteria based on disease state and immune state, and ongoing monitoring of target T-cell responses via the DTH test with analysis of biopsies. A complexity to be considered in trial design, and outside the scope of this article, is that future DC trials will most likely be in the context of combination with other immune therapies, i.e., checkpoint inhibitors. This will certainly provide for more complex trial designs so as to read the effect of each therapy alone before assessing the effect of the combination therapy.

While there is still an unmet need to have robust validated assays to monitor the immunological outcome of DC vaccination in order to predict response and guide treatment, there are some basic approaches to implement and further explore in future trials which can help improve outcomes, even at an exploratory stage.

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AFFILIATION

Marcelo Bravo
Kellogg College,
University of Oxford,
Oxford, OX2 6PN, UK
marcelo.bravo@kellogg.ox.ac.uk

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CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

INTERVIEW

γδT cells and the future of drug resistant immunotherapy

LAWRENCE LAMB As Executive Vice President and Chief Scientific Officer, Dr Lamb currently directs clinical and translational research strategy and operations for Incysus Therapeutics. Dr Lamb was first to describe the association between relapse-free survival and γδT cell recovery in bone marrow transplant patients. For 26 years, most recently as Professor of Medicine and Director of the Cell Therapy Laboratory at the University of Alabama at Birmingham, Dr Lamb developed the scientific support for γδT cell-based immunotherapies. Today, this work has resulted in FDA approval for both the first clinical trials for allogeneic γδT cell therapy in leukemia patients undergoing haploidentical stem cell transplants and the first gene-modified γδT cell therapy for high-grade gliomas.

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Q What are you working on right now?

LL: We have a number of projects in various stages of R&D. On the clinical side, the first one coming out will be a study looking at enhancing immune recovery following haploidentical peripheral blood stem cell transplant. This trial will be done for leukemia and lymphoma and will use the Johns Hopkins protocol, as it's popularly known: a conditioning regimen followed by infusion of a T-cell-replete graft, after which we will administer Cyclophosphamide (60 mg/kg) on the third and again on the fourth day following transplant. The Cyclophosphamide serves to kill T cells in cycle, which would probably be alloreactive T cells at that early stage – that's in an effort to reduce or prevent graft-versus-host disease as the patient recovers.

The transplant does induce a fairly long period of immune reconstitution and there is a significant risk for relapse and for infection during that period. We will infuse an escalating dose of expanded and activated γδT cells from the patient's haploidentical donor into the recipient within 5 days after neutrophil engraftment, which usually occurs between day 9 and 14. We anticipate that these γδT cells will have no effect on graft-versus-host disease since the cells don't recognize transplant antigens, and that they will serve to provide protection from leukemia recurrence and perhaps some viral infections as well.

This project is based on original observations made some 26 years ago, where we showed that a subset of patients infused with a haploidentical transplant α/βT cell depleted graft could achieve homeostatic reconstitution of γδT cells in significantly higher numbers as opposed to others who recovered their immune systems in the usual way. The trial has been given the go-ahead by the FDA and we're scheduled to recruit the first patient in this summer at the University of Kansas. Two of the physicians running the trial there – Dr Joe McGuirk and Dr Sunil Abhyankar – were with me in South Carolina when we made these original observations, so they are already familiar with the study and are highly motivated.

The second trial we hope will accrue sometime around the same time – mid-2019. It's a little bit different – this is a trial in patients with glioblastoma multiforme. The trial will be for primary glioblastoma, not recurrent, and it will be layered on top of the Stupp protocol.

We know that we can upregulate stress antigen receptors up to 200- to 400-fold on brain tumor cells by administrating chemotherapy – in this case, Temozolomide. This makes the tumor a much better target for the T cell but in a normal situation, the chemotherapy would also either kill or impair the T cell's activity, meaning that at the point in time when the tumor is at its most vulnerable, the effector T cell is gone or not fully functional. We address this issue by inserting a gene into the T cell that makes a gene product called MGMT (O-6-methylguanine-DNA methyltransferase). MGMT renders the T cell resistant to Temozolomide chemotherapy. (It's actually the same mechanism that the brain tumor uses to confer resistance to chemotherapy, and the primary reason why most tumors reoccur after high-dose chemotherapy).

In this trial, which will take place at the University of Alabama at Birmingham, surgeons will resect the tumor and place a little tube into the tumor cavity called a Rickham shunt, which comes out as a little button under the scalp. Then the patient will recover from surgery over the next 4 weeks and right before they start their chemotherapy and radiation, they will undergo apheresis and from that apheresis product we'll manufacture the drug resistant γδT cells. The cells are then divided for dosing, and then frozen and stored in liquid nitrogen until they are used as drug-resistant

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immunotherapy (DRI). During this period, the patient receives 6 weeks of chemotherapy and radiation, has a 4-week rest period, and then starts maintenance therapy.

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Maintenance therapy would normally begin with 5 days of Temozolomide chemotherapy. This would be the first 5 days of a 28-day cycle, with the patient resting for the remainder. The cycle is repeated six times in total. Patients enrolled in this trial, in addition to the usual Temozolomide chemotherapy, will return for the first day of maintenance to the clinical research unit and receive a dose of expanded activated drug-resistant γδT cell therapy directly into the tumor cavity through the Rickham shunt we inserted when we took the tumor out. The cells will therefore be present and resistant to the chemotherapy during the period when the tumor will be most vulnerable. For the first three patients we will only do one cycle – we'll expand the number of cycles as we continue to gather safety data.

Looking further up the pipeline, we are looking at a few more things. One of these is combining checkpoint inhibitors with drug resistant immunotherapy. We have shown a little bit of data from that project at the Society for Neuro-Oncology meeting in a patient-derived xenograft model, which shows we can actually upregulate PD-L1 using intravenous Temozolomide, and that we can increase γδT cell function by ligation of either or both CTLA-4 and PD-1. (Editor's note: the final data from this xenograft study was presented at the AACR Annual Meeting in April 2019).

Finally, we have just started work on a syngeneic immunocompetent model testing the effect of γδT cells and checkpoint inhibitors together in a fully immunocompetent mouse with a syngeneic glioblastoma.

We're also looking at our manufacturing, of course, in order to be able to generate more cells. That's one of our pipeline projects – to be able to improve both the number of cells per dose and the number of patients dosed.

Q γδT cell therapy is one of the hottest emerging fields in cellular immuno-oncology today. As a pioneer in the area, can you give us some background as to why this is the case? What are the inherent advantages?

LL: I think part of it is just practical. I've been in this field long enough to know that it has a herd mentality: someone will discover one thing and everyone will run off to write grants and start companies on

it! We've been through dendritic cells, mesenchymal stem cells, regulatory T cells and all of those are still relevant, but the enthusiasm rises then plateaus.

"...we have just started work on a syngeneic immunocompetent model testing the effect of γδT cells and checkpoint inhibitors together..."

I think people are looking at the CAR T issues now, especially in heterogeneous solid tumors, and starting to figure out that you really can't make CARs sufficiently diverse to kill a whole tumor.

It's hard. Chemotherapy in certain instances has worked great. There have been CAR Ts that have worked great as well, such as the CD19 that have produced cures in patients who ordinarily would have been hopeless cases. But for solid tumors, it's a little different. You don't have a bright, shining target out there like you had with CD19. And so I think people are looking for the next thing.

γδT cells fall into that slot for a number of reasons, not least of which is that they recognize naturally expressed stress antigens that are pretty much ubiquitous on tumors and tumor stem cells, or can be upregulated with chemotherapy. They give you an opportunity to destroy the tumor with greater efficiency.

On the flip side, γδT cells were originally designed to be first responders – to come in and respond first to a microscopic tumor or an infection and then, as the adaptive immune system takes over during the next 7 to 10 days, to just float away. So we are trying to make a cell do what it's not naturally designed to do.

However, if we can be successful in doing this, it does seem to work – at least from what I've seen. It looks like this might be a significant weapon in the cancer arsenal, combined with others. I don't think it's going to be the lone ranger by any stretch of the imagination. That's not to discourage other therapies like CAR T cells, small molecules, chemotherapy, radiation, antibodies, bispecific antibodies – I believe all of this stuff works, but we need to get it synced together in order to affect a complete remission.

Q Can you go a little deeper in terms of key targets for γδT cell therapy?

LL: The surface targets are primarily NKG2D ligands: MICA, MICB, UL16-binding proteins and others. There are also tumors that elaborate inositol pyrophosphates due to defects in the metabolic pathway, which attract Vγ9Vδ2 γδT cells as well – that's a small molecule-activating target and also a surface target. Those are the main ones being explored at this point.

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Q Can you elaborate on some of the key challenges that will need to be addressed before the full clinical potential of γδT cell therapy approaches can be realized?

LL: The biggest is manufacturing. I think that is what has held big laboratories back for a long time.

I've been really pretty fortunate because we made this observation 26 years ago and as an academic, I have managed to remain pretty well funded ever since then, even though I was working in this tiny area. Part of the reason for that was because the study reviewers could see nobody else was doing it (or that what little activity there was, was mostly taking place in Europe or Japan rather than the USA). We would take it to some people who would ask, "well, how come nobody famous has figured this out?" And the reason is because these cells are hard to make in large numbers. They are not like standard CD4 or CD8 cells where you can throw in with some IL2 or IL5 and some CD3/CD28 beads and make a jillion of them. Manufacturing has been THE impediment. I would perhaps add one other thing, which is that if you have a big laboratory going in one direction, like an aircraft carrier, it takes a lot of time and money to turn it around and go somewhere else.

The γδT cells tend to be tricky as far as activation-induced cell death. The more resistant ones tend to come along slowly… It's just not that easy. I believe we were actually the first laboratory to be able to manufacture a gene-modified γδT cell at clinical scale. That is no small thing – it's taken years for us to get there. We have an industry partner in Miltenyi Biotec and we use their bioreactor, the Prodigy, which has a lot of features that have allowed us to do this. I'm sure there are others that will come along as well once these features are better understood.

Q Could you tell us more about the challenges in combining novel cellular immunotherapies with checkpoint inhibitor antibodies as you see them, and how the field as a whole might address them?

LL: Firstly, I would mention that there has been a problem with combination drug therapy trials for years, of course, simply because each company wants to market their own drug and not have to market it with someone else's. I think cooler and wiser heads have prevailed in immunotherapy, though, because people now understand

there's not going to be a single immune bullet that will knock out cancer – everybody will have to work together.

I think the main challenge at this point is regulatory. That is going to be the main thing that is going to have to be overcome – the data has to be clean enough, especially the safety data, for the FDA to be able to work through it. And I think that when you write the INDs, they will have to be very clear.

Personally, I believe that γδT cells constitute the end game. This is the cell you're going to get when you've done the other things necessary to reduce the tumor.

The two main projects that I described earlier – the leukemia study and the patient-derived xenograft data out of our brain tumor studies – are showing us that these cells work very well when the tumor is small and approachable. That's why we're focusing initially on circulatory and also enclosed tumors, such as those in the brain. If you want to use your imagination, you could probably guess the other things we're looking at as well that have a similar situation, and then ultimately, down the road, we may migrate into systemic tumors. However, I don't think I'll ever be able to present a slide where I show you a huge tumor on an MRI and the next day, show you a hole where the tumor used to be because I treated it with γδT cells. That's just not the biology of this approach. It's reflected in the rationale behind our trials: you will notice that both of them are upfront trials on primary tumors before they become significantly resistant, and they're being done in a setting of minimal disease.

You are therefore going to have to have therapies that come before, whether that is chemotherapy, radiotherapy, CAR T therapy, antibody therapy, etc. You have to have something to get the tumor mass down to next to nothing.

> "There are also tumors that elaborate inositol pyrophosphates due to defects in the metabolic pathway, which attract Vγ9Vδ2 γδT cells as well..."

The other thing I believe is it's not going to be as easy as just introducing the γδT cells. We're going to have to expand our drug resistant immunotherapy program to the point where we can actually force expression of high numbers of NKG2D ligands, even on therapy-resistant tumors. As an aside to that, Temozolomide doesn't kill a Temozolomide-resistant brain tumor, but it does hurt it. The way I explain it is it's like a hammer. If I hit you on the thumb with it today, it's going to hurt and you're going to scream, but in 2 weeks' time, your thumb will be back to normal and it will be like it never happened. The same thing is true with the brain tumor: we hit it hard and it 'screams' by popping out 400 times more stress antigens that it was

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expressing before, making a nice target for us. I think the next phase of our work is to throw in a couple of checkpoint inhibitors to boost the effect and make the tumor even more vulnerable. It might take something like that, in a patient with minimal residual disease, to close it out.

And that is really what I'm going for. We'd all love to see treatments that extend life for 6 months, a year longer, but my goal is total eradication.

AFFILIATION

Lawrence Lamb
Executive Vice President and Chief Scientific Officer, Incysus Therapeutics

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SPOTLIGHT

EXPERT INSIGHT

Natural killer cells may be scaled and engineered as a next generation, off-the-shelf cell therapy for cancer

James B Trager

The advent of immunotherapies for cancer, and more recently of cellular immunotherapies, has substantially altered the treatment landscape. In hematological malignancies, complete response rates to CAR-T cells can exceed 80%. These responses are often durable in nature and have attracted considerable excitement and investment in the development of cell therapies for an expanded range of indications, particularly in solid tumors. The continued progress of cell therapies depends on overcoming key obstacles that include a lengthy and costly manufacturing process, a high degree of product variability, risks of severe adverse events, and limited available targets. Natural killer (NK) cells have the potential to overcome these limitations. NK cells are highly potent lymphocytes that target cancer through multiple broadly expressed activating ligands; they can be used allogeneically without posing a risk of graft-versus-host disease (GvHD). In recent years, technologies have been developed that allow their efficient expansion and engineering. We will describe the current status of development of NK cell therapies as targeted, off-the-shelf, allogeneic cell therapies for cancer, highlighting the different approaches that have been taken for their effective exploitation, and will outline remaining obstacles to the advancement of the field.

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ADVANTAGES & LIMITATIONS OF CAR-T CELL THERAPIES

Cancer immunotherapy has assumed a prominent position in therapeutic development. The monoclonal CTLA-4 antagonist ipilimumab was the first immmuno-oncology agent to show an overall survival benefit in advanced melanoma, followed closely thereafter by the PD-1 antagonist nivolumab. Antagonists of the PD-1/PD-L1 signaling axis have since made major inroads in the treatment of melanoma, non-small cell lung cancer, renal cell carcinoma, and hepatocellular carcinoma among other indications [1]. Though impressive, overall response rates to these agents have varied widely, and as single agents are rarely greater than 40% and typically closer to 20%. More recently, results obtained in B-cell malignancies using CD19 and BCMA-directed chimeric antigen receptor (CAR) T cells have been dramatic, with overall response rates of 80% being observed in some settings [2]. While these results have galvanized the field, the challenges associated with CAR-T cell therapies are also daunting [3,4]. Toxicities associated with CAR-T cells can be serious and even fatal. The severity of these adverse events has in some cases limited the clinical sites at which they can be safely administered to those which have the training and infrastructure in place to recognize and respond to emerging symptoms of cytokine release syndrome and neurotoxicity, whose onset and progression can be rapid. Just as widely understood is the cost associated with the manufacture of these products, which to date have required expansion and engineering of autologous T cells drawn from the patient. The autologous nature of the products also means that it can typically take several weeks before a patient's product is available for infusion, with attendant risk of progression during that time frame. Just as important, the quality of the product can vary considerably between patients [5], and manufacturing failure rates of up to 13.2% have been reported [6]. All these factors may limit the widespread adoption of autologous CAR-T cell therapies. There is thus an increasing demand for the development of allogeneic therapies which can be produced at large scale and made available off-the-shelf. Natural killer (NK) cells are well suited to this approach. As we will discuss below, NK cells are potent and safe in allogeneic settings; they can be engineered efficiently and produced at large scale. While the biology of these cells is generally well characterized, production methodologies for clinical application are still maturing, and new methods continue to emerge.

BIOLOGY OF NK CELLS & APPLICATION TO CELL THERAPY

NK cells are uniquely well suited for development as cancer therapeutics (Figure 1). NK cells discriminate naturally between healthy cells and virally infected or transformed cells [7,8]. They recognize a variety of inhibitory signals expressed in healthy cells. One of these signals is mediated by expression of HLA class I molecules in most cells. HLA class I subtypes, responsible for presentation of peptides to CD8+ T cells, are recognized by a polymorphic family of killer-cell immunoglobulin-like

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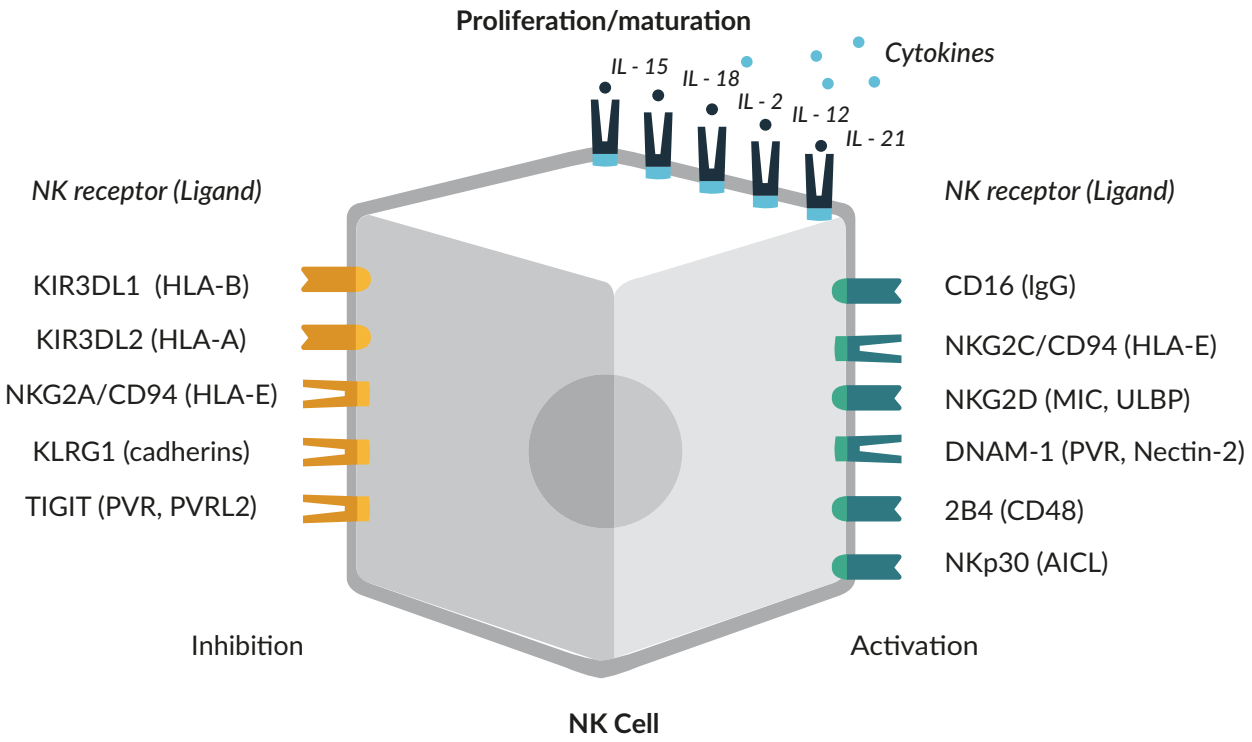
FIGURE 1

NK cells recognize and respond to a wide variety of activating and inhibiting ligands that allow them to distinguish between healthy and diseased cells.



Shown here are receptor (ligand) pairs of particular relevance in determining NK responses to tumor cells.

receptors (KIRs) on NK cells, and can act to suppress their cytotoxicity [7]. Loss of class I expression, or 'Missing self' has been recognized as a driver of NK cell recognition of impaired cells [9]. Loss of class I expression is frequent in tumor cells, abetting their evasion of T-cell recognition at the cost of allowing their targeting by NK cells [10].

NK cell activation is also driven by their expression of receptors capable of binding a variety of ligands frequently upregulated in cells undergoing aberrant DNA replication or protein misfolding, a common characteristic of cancer cells [11,12]. Multiple activating ligands may be expressed in any given tumor cell, giving NK cells multiple opportunities for target recognition [13]. This feature may be particularly important in solid tumor settings, where antigens recognized by T cells or monoclonal antibodies may have incomplete penetrance.

Moreover, unlike T cells whose recognition of tumor cells is restricted by the HLA class system, activating ligands of NK cells are broadly shared between individuals. The NK cells of any given donor can recognize the ligands expressed in a recipient's tumor. Indeed, NK cell activity is greater in allogeneic settings, as matched HLA class I acts as an NK inhibitory signal through the KIR receptor system discussed above.

Just as importantly, NK cells are not promiscuously activated by HLA mismatches with neighboring cells. For this reason, NK cells can be safely administered across HLA barriers without triggering graft-versus-host disease; a recent review of clinical use of NK cells documented no cases of GvHD in 17 different studies, comprising over 300 patients, using allogeneic NK cell in a non-transplant setting [14]. Thus, NK cells are extremely well suited to allogeneic application. Unlike T cells, which require extensive modification for safe allogeneic administration, NK cells can be safely administered to haplo-matched or completely unmatched recipients without serious toxicity – in fact the lack of KIR-mediated inhibition may enhance their potency in these settings [15].

Tumors suppress immune responses by multiple mechanisms that include secretion of inhibitory factors such as TGFβ, a hypoxic tumor microenvironment, infiltration by regulatory T cells, M2 macrophages, and myeloid-derived suppressor cells, and others [16]. The immunosuppressive tumor microenvironment has been an obstacle to successful therapy with either immune checkpoint inhibitors or CAR-T cells. Overcoming this barrier will likely require combinatorial approaches, and this creates an opening for applications of NK cells that may be synergistic with current standards of care. NK ligand expression is frequently sensitive to cancer therapeutics of all classes: radiation, chemotherapies, hypomethylating agents, and various targeted therapies (including proteasome inhibitors, histone deacetylase inhibitors, and tyrosine kinase inhibitors) have all been shown to upregulate activating NK cell ligands [17,18], creating many potentially synergistic opportunities for combining NK cells with existing standards of care. Moreover, NK cells will combine quite naturally with many marketed monoclonal antibodies, which frequently operate through antibody-dependent cellular cytotoxicity (ADCC), which is mediated primarily by NK cells through the low-affinity FcγRIIIA receptor CD16, which triggers potent NK cell activity [19,20]. Many such combinations have been demonstrated *in vitro*; a systematic exploration of potential combinations in animal models and eventually in clinical settings may ultimately be key to unlocking wider success of cellular immunotherapy for cancer.

BRIEF HISTORY OF NK CELL ADOPTIVE TRANSFER

As detailed by Veluchamy *et al.*, over 30 studies incorporating adoptive transfer of allogeneic NK cells have been published, representing well over 500 hundred patients treated [14]. Many of these studies have focused on the use of NK cells to support hematopoietic stem cell transplants (HSCT) in patients with hematological malignancies. In these settings, allogeneic NK cells were typically partially HLA matched, and most frequently derived from related donors (most often the same donor used for transplant). In the transplant setting, infusion of the allogeneic NK cells is provided to support disease suppression as the newly engrafted HSCT matures into a more fully functional immune system. While in general, adoptive transfer of NK cells in this setting has been well tolerated, some instances of GvHD

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have been observed, typically attributed to contaminating T cells in the NK cell infusion or resulting from the HSCT itself [21]. The methods used for production and purity of the NK product varied widely over these studies, and interpretation of both safety and efficacy results is complicated by the concomitant transplant.

In the non-transplant setting, over 300 patients have been treated with NK cells. Acute myelogenous leukemia (AML) and other hematological malignancies were the most frequently treated indications, but patients with various solid tumor diseases have also been included. No published studies have incorporated engineered cells. NK production methods in these studies have encompassed everything from overnight stimulation with IL-2 to several weeks' expansion in the presence of IL-21. Doses in these studies have ranged from under 1 x 10^6 NK cells/kg to over 1 x 10^8 cell/kg for primary NK cells, and up to 1 x 10^{10} cells/m^2 for the NK cell line NK-92 [22]. The wide variety of settings and product characteristics make systematic analysis difficult, but adoptively transferred NKs in the non-transplant setting are very well tolerated with no reported GvHD. Therapeutic responses have been variable in these studies; the largest such study, conducted by Bachanova and colleagues, reported a 30% overall complete response rate in a cohort of 57 patients with relapsed/refractory AML, with a 53.3% CR rate in a 42 patient cohort that received NK cells combined with a diptheria-toxin conjugated IL-2 for depletion of regulatory T cells [23]. A similar complete remission rate was achieved in a study by Romee and colleagues, where four complete responses were observed in nine treated patients with relapsed/refractory AML [24]; this study used a novel method for overnight stimulation of NK cells with IL-12, IL-15 and IL-18, a combination the authors demonstrated to result in differentiation of what the authors term cytokine-induced memory NK cell population (see below).

Immune suppression is required prior to either HSCT or adoptive transfer of allogeneic NK cells, and this has typically been conducted using a non-myeloablative conditioning regimen consisting of cyclophosphamide (Cy) and fludarabine (Flu); early studies showed that this chemotherapy conditioning was required for engraftment, and associated with transient elevation of IL-15 in patient plasma [25]. In addition to initial engraftment, key questions for allogeneic therapies surround the persistence of the NK cell product in the face of the host versus graft immune response, and the degree of persistence required to mediate a long-term benefit. Persistence can be measured by a variety of means, including flow cytometry using antibodies directed to HLA- or KIR-mismatched surface molecules, or more sensitively by qPCR to detect SNP or microsattelite chimerism. By either measurement, peak chimerism in peripheral blood usually occurs within 7 to 14 days following infusion [23,24,26,27]. Measurements at other anatomical sites, including bone marrow or lymph nodes, have been more sporadic; the general kinetic of peak chimerism seems to be similar [24,28]. The kinetics of NK cell engraftment correlate with suppression of the host immune system. While timing can vary,

recovery from lymphodepletion typically corresponds with loss of NK cell chimerism [23,27], as the transferred NK cells are presumably recognized and killed by the patient's immune system. In the study cited above, Bachanova *et al.* found that early persistence of NK cells at 7 days correlated well with AML clearance [23]. This finding is promising, especially for use of NK cells in hematological settings.

Overall, the safety and clinical responses rates achieved using allogeneic NK cells have encouraged further exploration of the therapeutic application of these cells. The methods used for production of the cells has been quite variable, often limiting the cell dose that could be administered, and perhaps the quality of the cell product delivered. The resultant variability in observed clinical outcomes has spurred research into optimizing techniques for both expansion and engineering of NK cells. As discussed below, these methods should permit delivery of multiple doses of cells engineered for greater activity and durability.

NK EXPANSION APPROACHES

At this point, several hundred cancer patients have been treated with adoptively transferred NK cells. A wide diversity of sources and methods have been used for derivation and expansion of NK cells for clinical use (Figure 2). While most methods start with allogeneic cells from a healthy adult donor, NK cells have also been expanded from cord blood [29], from differentiated induced pluripotent stem cells (iPSC) or hematopoietic stem cells (HSC) [26,30], or from autologous cells. NK-92, a cell line derived from a NK cell malignancy, has also been used in several studies [31].

Each of these sources introduces different opportunities and challenges to the manufacture and use of NK cell products. Cell lines like NK-92 are perhaps the most readily scaled starting material. Klingemann and colleagues pioneered the implementation of clinical scale GMP manufacturing processes for these cells [31]. Development has continued to advance, now incorporating both expansion and genetic engineering of the cell line, [32]. The ability to grow these cells in suspension to virtually any scale, using a well-characterized GMP master cell bank as starting material, represents an attractive option for development of an off-the-shelf product. Multiple trials using NK-92 cells engineered to express CARs are underway. As a transformed cell line, however, NK-92 must be irradiated prior to administration. In previous clinical studies, this has perhaps been responsible for the limited persistence observed for these cells; doses of up to 1 x 10^{10}/m^2 are detectable in patients' blood for only 48 hours [22]. Whether this will be sufficient to enable durable clinical responses remains an open question.

The use of stem cells as starting material also represents a powerful avenue towards development of scalable processes. Kaufman and colleagues demonstrated in 2005 that NK cells could be successfully differentiated from human embryonic stem cells [33]; his group later showed that NK cells can also be differentiated from iPSC in a multistep induction process [34] involving timed use of appropriate growth factors and cytokines. Clinical-scale

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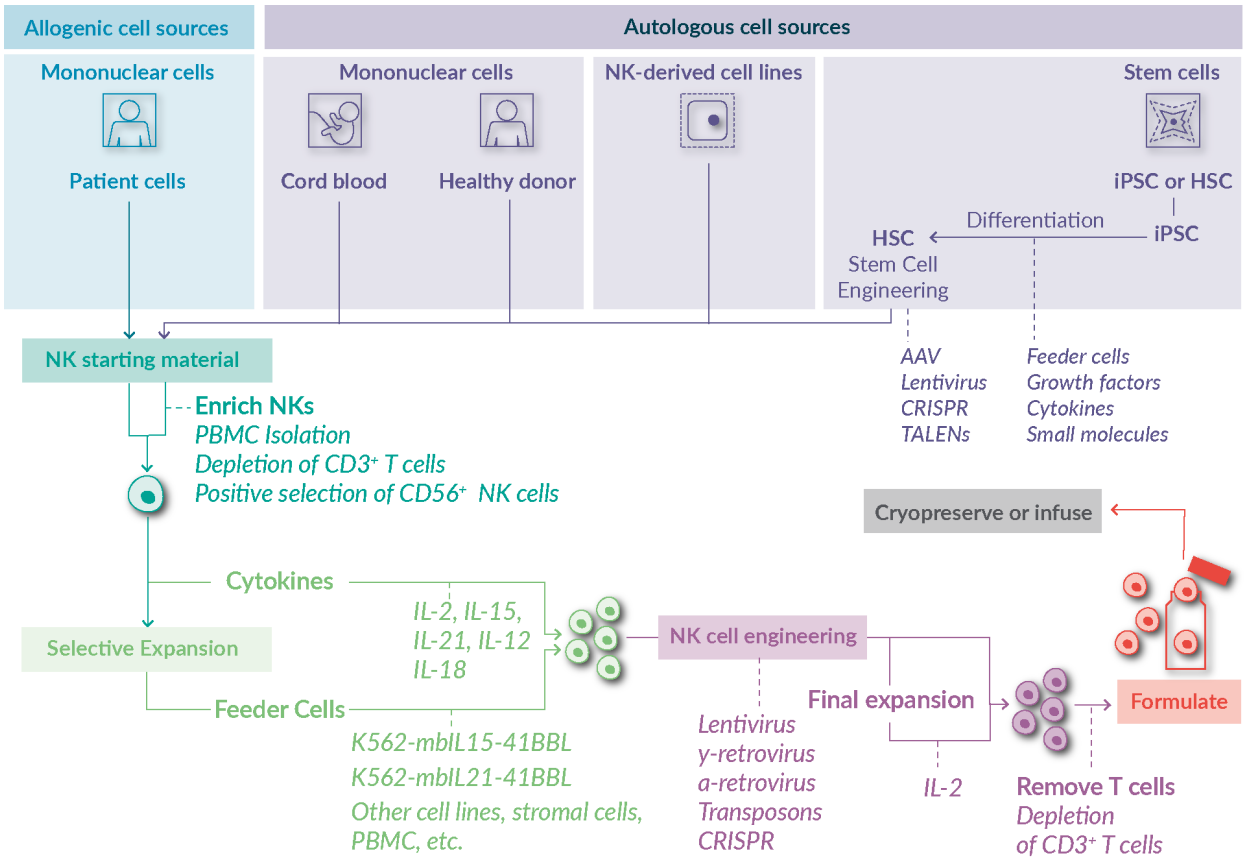
FIGURE 2

The divergent paths to CAR NK therapeutics.



NK cells are expanded and engineered using a wide range of processes; cells source, expansion methodologies, and engineering techniques have not been standardized.

methods for derivation of NK cells from iPSCs have been developed [35], as have methods for engineering iPSC prior to differentiation for production of CAR NK cells [36]. iPSC can be grown at scale in suspension with cytokine support, engineered with multiple modalities, and clonally isolated for GMP master cell bank production; they represent a powerful starting platform for production of engineered NK cell products [30]. It should be noted that, notwithstanding the evident scalability of iPSC production, NK cells must still be differentiated and expanded from the source material. The scalability and reproducibility of those process steps remains a challenge; further, prior to clinical assessment, the potential teratogenicity of residual iPSC in the NK final product will inevitably remain a concern. Initial clinical studies using iPSC-derived NK cells are now underway.

HSCs present many of the same opportunities as iPSCs. CD34+ human stem and progenitor cells (HSPCs) from umbilical cord blood have been successfully differentiated to NK cells and expanded in GMP-compliant clinical processes [26,37]. Stem cells are first expanded in culture using a mix of cytokine and growth factors; differentiation to NK cells can be initiated by addition of IL-2 and IL-15. The derived cells have phenotypic and functional characteristics typical of peripheral

blood NK cells [38]. To date, these cells have been clinically applied in HLA haplomatched patients [26]; scalability of the process has thus not been put to the test. Few reports have described engineering of HSCs prior to NK cell differentiation [39], and none to date have described clinical results.

Whether derived from allogeneic donors or autologously from patients, the earliest protocols for NK cell expansion used only IL-2 for cytokine stimulation in a suspension culture [40]. As an understanding of the basic biology of NK cells has grown, other cytokines have been added to either improve expansion or confer desirable biological properties on final product cells. form IL-15 has been used most frequently [41]. IL-15 is a key NK cell growth factor and is relatively selective in its support of NK cell expansion [42]. NK cells have been expanded in suspension using cytokine support alone [43], but since Campana and colleagues demonstrated robust NK cell expansion from peripheral blood mononuclear cells (PBMC) using a K562 cell line engineered to express 4-1BB ligand and a membrane-bound form of IL-15 [44], the use of stimulatory cell lines has become more common. Stimulatory support cells have either been sourced autologously [45], or more commonly are derived from engineered cell lines used in conjunction with soluble cytokines [44,46–48]. A membrane-bound form of IL-21 has frequently been substituted for IL-15 in engineered K562 cells [49]. Engineered K562 cells can be grown to large scale under GMP conditions and banked [50]; working cell banks are irradiated prior to use to ensure that no replicating stimulatory cells will be carried over to the patient. Alternately, engineered K562 cells expressing 4-1BBL and membrane-bound IL-21 have been converted to microparticles and used to successfully stimulate NK cell expansion from PBMC; use of the microparticles has been proposed both as a means to expand NK cells *ex vivo* and to maintain their expansion and persistence *in vivo* [51].

Several of the methods outlined above can be used to achieve expansion of NK cells by two logs or more in 7–9 days; 21-day expansion of NK cells of over 40,000-fold was reported by Denman and colleagues using stimulator cells expressing membrane-bound IL-21 [49], though expansion of this magnitude has not yet been reported at scale. Maturation of NK cell culture methods are bringing greater attention to the demands of developing scalable, GMP-compliant processes using closed systems and commercially available components, with the goal of producing an off-the-shelf cell product with the potential of longer term persistence *in vivo*. Several such methods have been reported; one closed and largely automated system for NK cell expansion from cord blood represents a promising avenue, though scalability remains to be determined [48]. Where scalable production of NKs has been reported, it has typically relied on initial expansion and differentiation of either hematopoietic [37] or more primitive pluripotent stem cells [35]. While these systems provide an avenue towards large scale NK production, they are inherently complex; mastering the control of such systems to limit lot-to-lot variability will continue to present challenges.

Final product cryopreservation will also be required to deliver a scalable, off-the-shelf NK cell product.

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NK cells are relatively large, granular cells, and have historically fared poorly following cryopreservation, with low recovery and hampered activity [52,53]; cellular function could be recovered following overnight re-stimulation with IL-2, suggesting that improved cryopreservation techniques might address the issues observed. More recent studies have reported more success through attention to freezing parameters [54] and cryopreservation media formulation [55], raising the promise that reproducible and reliable freezing methods may be achievable.

Past the ability to produce NK cells at large scale, questions have arisen around the selection or differentiation of the appropriate sub-set of NK cells for clinical application. Newer analytic methods such as CyTOF have shown that the diversity of phenotypically distinct NK cell sub-populations is complex, and at this point not well understood [56]. Moreover, many lines of research in both animal models [57,58] and in humans [59] have indicated the existence of NK cell populations with adaptive or memory properties. These cells are defined by their ability to respond more vigorously to repeat challenge with the appropriate antigen, or virus. Studies in mice showed that development of memory NK cells depended on key inflammatory cytokines, and that memory-like cells could be induced *in vitro* with appropriate cytokine stimulation [60], which included use of IL-15, IL-12 and IL-18. Culture with these cytokines also induces greater proliferation, persistence and IFNγ secretion in human NK cells; as described above, these memory-like cells could mediate impressive clinical responses [24]. It is safe to say that the phenomenon of NK cell memory is still incompletely understood in humans; various terms have been used to describe potentially related sub-sets of NK cells, including memory, adaptive, and memory-like NK cells, and phenotypic characterization of the cells has varied, though the maturation marker CD57 and the activating receptor NKG2C are frequently used for identification of cells with enhanced properties [61]. The attractive functional features of these cells has encouraged novel approaches to the selective expansion of NK cell sub-sets *ex vivo*. In addition to approaches using cytokines, small molecules may also be used to influence NK cell differentiation in culture. Cichocki and colleagues, for example, have shown that altering NK cell metabolism with inhibitors of Glycogen Synthase Kinase 3 allows expansion of NK cells with a more mature (CD57+) phenotype and improved anti-cancer activity [62]. Alternately, rather than biasing culture conditions to favor outgrowth of a given NK cell phenotype, cell isolation methodologies have also used at the outset of culture to select an NK population expressing low levels of KIR receptors to enhance NK cell function [63]. As the field is able to characterize the functional properties of these sub-populations more completely, it is inevitable that novel methods will continue to be developed to select for those cell types that possess desirable attributes.

Regardless of cell source, development of analytical methods to better characterize both source material and final product will also be essential for ensuring production of NK cells with consistent properties. Recent advances in mass cytometry have shown the complexity of the NK cell compartment [64];

multi-parametric analyses such as these may not be suitable for routine analytical purposes, but they may facilitate the identification of better-defined phenotypic attributes for donor selection, in-process analysis, and final release of NK cell products.

NK CELL ENGINEERING

As noted above the safety of allogeneic NK cells has been demonstrated in multiple clinical studies, durable complete response rates have been modest [14]. This has led to a more intense focus on engineering NK cells for increased potency, migration, or resistance to the tumor microenvironment. Viral transduction techniques using lentiviral or retroviral vectors developed for T cells initially proved of limited utility in NK cells, resulting in very low transduction efficiencies even at small scale [65]. Understanding has continued to advance of the determinants of efficient gene transfer to NK cells, including the importance of transfer vector and viral envelope, as well as the purity and ongoing proliferation of the NK cells. Under the correct conditions NK cells can be transduced using standard γ-retroviruses at efficiencies similar to CAR-T cells and recent studies have demonstrated transduction and transgene expression levels suitable for clinical use [66]. Engineering of NK cells at large scale remains a challenge for consistent manufacturing (as it does for T cells as well). Viral vectors are typically the most expensive raw material in the manufacturing process, and their production and performance can be inconsistent; moreover, the vectors used for gene deliver to NK cells have limited packaging capacity, limiting the complexity of genetic alterations that might be affected. New viral vector systems hold considerable promise for achieving more efficient and cost-effective genetic transgene delivery [67]. Successful transposon-based transgene delivery to NK cells has also been reported [68]; rapid advances in gene editing techniques will undoubtedly be rapidly applied to NK cells to expand the range of engineering possibilities for these cells.

NK cells were initially engineered over a decade ago with a first-generation chimeric antigen receptor [65]. Since then, multiple studies have shown that NK cell activity can be enhanced or redirected through expression of suitable transgenes, primarily chimeric antigen receptors [69]. Most of these transgenes have borrowed designs used in CARs designed for use in T cells, using the CD28 or 4-1BB costimulatory domains. While in some instances, costimulatory domains more characteristic of NK cell have been used, little systematic evaluation has been conducted to determine whether those domains that confer optimal function in T cells are identical to those that perform best in NK cells. One recent study by Li and colleagues compared the use of different transmembrane and costimulatory domains in NK cells derived from iPSCs [36], and showed that 'conventional' domains drawn from those used most frequently in T cells may not be best in NK cells. Undoubtedly there is a great deal of further exploration to be conducted before we fully understand how best to engineer chimeric receptors for use in NK cells.

In addition to conventional CARs, several groups have attempted to augment native signaling axes in NK cells by enhancing or altering the expression of the receptors

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that dominate NK cell responses. antibody-dependent cell-mediated cytotoxicity (ADCC) has been enhanced through forced expression of the naturally occurring low-affinity CD16 receptor, which binds IgG and triggers potent activity [70,71]. In the same vein, groups have focused on enhancing signaling through NKG2D, a receptor which binds 8 different ligands that are frequently upregulated in tumor cells [72,73]. Chimeric NKG2D receptors have been shown to broadly improve NK cells recognition of tumor cell lines; the low level of expression of ligands for this receptor in healthy tissues and their broad expression across both hematological and solid tumor malignancies makes this a promising avenue for NK cell clinical application.

Other aspects of NK biology have also been addressed through appropriate engineering. One of the greatest concerns in the clinical application of NK cells is their relatively short half-life when compared to T cells. While a population of adaptive or memory NK cells may exist, the bulk of NK cells have an effective half-life of 1–2 weeks *in vivo*. Limited persistence may have the beneficial effect of preventing long-term toxicities that have been sometimes associated with autologous T cell therapies, such as the prolonged B-cell aplasia observed in some patients treated with CD19-directed CAR-T cells [74]. There is, however, a concern that NKs may become exhausted too quickly during culture and after infusion, forestalling a consistent therapeutic benefit. To address this concern, several groups have engineered NK cells to express membrane-bound or secreted forms of IL-15 [75,76]. Expression of IL-15 extends the persistence of NK cells *in vitro* and *in vivo* and has been shown to improve the potency of the cells in animal models. Examples also exist of NK cells being engineered with dominant negative forms of the inhibitory TGFβ receptor to better resist tumor immune evasion [72,77]. Ongoing work in mouse models continues to reveal potential targets of genetic modulation to fortify NK tumor surveillance, including the suppressor of cytokine signaling 2 (SOCS2) [78] and the cytokine-induced SH2-containing (CIS) proteins [79].

PERSPECTIVES ON FUTURE DEVELOPMENT: PROMISE & CHALLENGES

Obstacles to the optimal development of NK cell therapies remain. Methods for expansion of NK cells are far more diverse than the methods that have been developed for T cells; while production of NK cells for early stage clinical study has become more tractable, it remains unproven that current methods will be suitable for late stage clinical development and eventual commercialization. Attaining truly consistent manufacturing processes and NK cell products will require continuous improvement in our understanding of the complexity of these cells and the dependence of final product potency and persistence on starting material and expansion methods. In particular we will need to further explore the potential for adaptive or 'memory-like' NK cells to control tumor growth and arrive at a more universal understanding of how best to select, characterize, and culture NK cell subsets with the greatest anti-tumor activity [24,63].

NK cells are well situated for allogeneic use. Like all allogeneic cell therapies, the efficacy of allogeneic

NK cells may depend on their ability to evade immune rejection by the patient's immune system. Most previous studies of allogeneic NK cells have been conducted in transplant settings, and have made use of haploidentical NK cells, typically derived from a close relative of the patient. While durable chimerism has been detected in some cases, more commonly NK cells achieve a peak of chimerism within 1–2 weeks following infusion and decline quickly thereafter [14]. Systematic study of the importance of the extent of HLA haplomatching to patient outcomes has not been conducted outside the transplant setting, but the enormous variety of HLA subtypes means that such matching will not be compatible with scaled manufacture of NK cells for off-the-shelf use. Nor is it feasible to lymphodeplete patients indefinitely without severe risk of infection and other serious adverse events. Thus, other approaches will be required to enhance NK cell persistence following infusion. Recent progress in masking T cells to patient immune responses may be applied to NK cells as well; genetic deletion of β-microglobulin, for instance, has been used to suppress HLA class I expression on the surface of embryonic stem cells, limiting one source of potential host versus graft responses [80]. As an alternate approach, molecules such as HLA-G have been overexpressed in to directly suppress immune response [81]. More extensive engineering of NK cells, which express both class 1 and class 2 HLA, may be required to limit immune rejection. Progress in this direction has recently been shown in an animal model of stem cell transplantation [82]; unquestionably the clinical application of allogeneic NK cells would also benefit by such approaches.

NK cells were discovered some 15 years after T cells, and for many years our understanding of the biology of these cells and their potential for therapeutic application has lagged behind that of T cells. That gap has begun to close however, as the pace of discovery in the NK field has steadily increased and several of the roadblocks to clinical development, including expansion, transduction, and effective engineering, have begun to fall. There is no doubt that the next decade of NK cell application will be bright.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The author is Chief Scientific Officer of Nkarta Therapeutics, which is engaged in the development and application of engineered Natural Killer Cells for the treatment of cancer. No writing assistance was utilized in the production of this manuscript.

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AFFILIATIONS

James B Trager, PhD

Chief Scientific Officer, Nkarta, Inc.

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CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

INTERVIEW

Invariant NKT cells: advances in transitioning to the clinic

ANASTASIOS KARADIMITRIS graduated in medicine at Aristotelion University, Thessaloniki, Greece. He undertook his postgraduate clinical training in the UK, first in general medicine and subsequently in haematology including in bone marrow transplantation at Hammersmith Hospital, London. He undertook his research training with Professor Lucio Luzzatto at Memorial Sloan Kettering Cancer Centre New York, USA, Professor Irene Roberts at Hammermsith Hospital, London and Professor Vincenzo Cerundolo at the Weatherall Institute for Molecular Medicine, Oxford. He is currently professor of haematology at the Centre for Haematology, department of medicine, Imperial College, London and honorary consultant haematologist at the department of haematology, Hammersmith Hospital, Imperial College Healthcare NHS Trust.

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Q Please can you summarize your current research activities for us?

AK: The immunology and immunotherapy programme in my lab focuses on the biology and therapeutic applications of invariant NKT cells (iNKT).

Over the years, we have demonstrated the ability of donor iNKT to modulate graft-versus-host disease. With forthcoming funding, we expect to be able to initiate first-in-human clinical trials in the context of allogeneic stem cell transplantation to see if donor iNKT cells, expanded *in vitro*, are safe to be used for ultimately preventing graft-versus-host disease.

Graft-versus-host disease is the main complication of hematopoietic stem cell transplantation. If you can get around this problem, you will have the possibility of offering this curative procedure to many more patients because at the moment, only younger patients are eligible for it. And a proportion of these patients die because of graft-versus-host disease, despite their leukemia or lymphoma being under control.

The second area of work for us relates to the question of whether introduction of a chimeric antigen receptor (CD19) to iNKT cells would be a worthwhile exercise – and if it were, how would this compare to more established CAR T cell therapy in CD19-expressing B-cell cancers, like B-cell lymphoma?

We demonstrated that CAR NKT cells fit the bill because they can be engineered with a CAR very efficiently. And importantly, despite the fact that they are very rare, they can be expanded to clinical scale and numbers, meaning they can be used for clinical immunotherapy.

Upon comparing their activity against different cancers head-to-head with same-donor CAR T cells, we found them to be more effective, *in vitro* and *in vivo*, against both primary lymphoma cells and chronic lymphocytic leukaemia cells, but also in animal models of lymphoma.

Very importantly, they demonstrated particular activity against brain-based lymphomas, which CAR T cells did not.

So this is the basis for us to now develop the NKT platform further, combined primarily with CARs against other blood cancers – we're working on multiple myeloma, for example. And we're exploring whether this can be used against solid cancers, too, although it is early days in that area.

Q What are the particular advantages of – and challenges in - utilizing iNKT cells in a therapeutic technology platform?

AK: The big challenge has been to make a clinical scale immunotherapeutic product from a very rare cell population. It took us some time to find the best conditions so we could select them to high purity, efficiently transduce them with CAR lentivirus, and then expand them. Although I must say that we expanded them with relative ease – they have an inherent ability to expand tremendously well compared to T cells: starting from something like 50,000-100,000 iNKT cells, you can expand to hundreds of millions of cells within a few weeks.

"The big challenge has been to make a clinical scale immunotherapeutic product from a very rare cell population."

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In terms of advantages, ourselves and others have repeatedly shown at the preclinical and also clinical observational level that iNKT cells protect from GvHD, which obviously means we can use iNKT cells sourced from healthy individuals as the basis for an off-the-shelf treatment.

This is a big focus for preclinical and clinical research right now in the CAR T field. But in order for CAR T cell immunotherapy to be suitable for off-the-shelf – i.e., utilizing cells sourced from healthy individuals – there's an additional genetic engineering step you need to carry out: as well as introducing the CAR, you have to remove the endogenous T-cell receptor of the CAR T cell by means of gene editing.

So you do two major genetic interventions with CAR T cells. However, with CAR NKT cells, you only have to introduce the CAR – you don't have to interfere with the endogenous T-cell receptor. In fact, the endogenous T-cell receptor is a valuable part of the CAR NKT cell because it adds to the anti-tumour activity when it finds its target (CD1d) and it also protects from GvHD. That's a big advantage, albeit a theoretical one at the moment because we have yet to see it in clinical practice – that would be the next step.

It would certainly solve a lot of logistical issues. For example, it might solve the issue in chronic lymphocytic leukaemia that was the subject of a recent paper from UPenn, which stated that only 20% of patients with chronic lymphocytic leukaemia achieve complete remission following CAR-T immunotherapy, simply because the autologous T cells are not very healthy. We do think there is really a lot of scope to develop CAR NKT immunotherapy in this regard.

There are other potential 'tricks' we can employ to selectively increase these cells' activity – pharmacological glycolipids, which are selectively targeting iNKT cells to make them more active, for instance – but these need to be tested further in preclinical models before they go into clinic.

Q Tell us more about the specific indications in which you have seen promise in the preclinical setting to date.

AK: We can cover the whole spectrum of mature B-cell malignancies, including multiple myeloma.

BCMA is a myeloma-specific target that investigators are looking at – there are several clinical trials with anti-BCMA CAR T cells. We're developing a preclinical programme around this idea, but we also have other targets that we will be developing CAR against including specific ones for both myeloma and lymphoma cells.

Q What is your approach to transitioning this platform to the clinic in terms of developing robust manufacturing protocols?

AK: That's our next challenge: moving outside the research lab, taking the research protocol and making it a clinically suitable one.

The current manufacturing platforms and protocols in this field are all focused around T cells, where you manipulate tens of millions of cells with lentivirus or other CAR transfer means. In our case, though, we start with a very small number of cells, and we take a 'scale-down scale-up' approach. That transition from scale-down to scale-up is where we will now be investing a lot of effort with our partners to see how we can make this into a seamless, streamlined manufacturing process. It is going to be quite different to what is currently out there and being applied in CAR T cell therapy manufacturing.

Q How and why did you come to work with Miltenyi and the Prodigy system?

AK: What we are doing with Miltenyi in the first instance is introducing their CAR T cell immunotherapy programme into our institution. So we will be hopefully using the Prodigy to make CAR T cell therapies in-house in our clinical cell therapy lab, using Miltenyi's own lentiviral CAR vector-what I would call CAR T cell manufacturing by the bedside, effectively.

Over the slightly longer term, the aim will be to try to adjust our own CAR NKT manufacturing to the Prodigy platform, because it's ideal as a closed manufacturing system.

But of course, the Prodigy system itself has been adjusted to the needs of CAR T cell therapy manufacturing, which as I mentioned involves tens of millions of cells. So alongside Miltenyi, we will be exploring how we can adjust our 'scale-down scale-up' approach accordingly to hopefully work on the Prodigy.

Q And what are, and will be, the particular advantages to this approach – in terms of impact on Cost of Goods, for example?

AK: First of all, the nature of CAR NKT immunotherapy will be such that the manufacturing process will use healthy individuals' cells, and can be done under controlled conditions without the

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time pressures one associates with autologous CAR T cell immunotherapy. That will immediately reduce the costs of manufacturing and logistics. Additionally, if the manufacturing is done on the Prodigy system, Milteny's initial assessments are that this will reduce the cost even of autologous CAR T cells by a significant margin.

Imagine having two aspects of manufacturing that will between them reduce both the costs of logistics and of the manufacturing process itself?

Hopefully, if the therapy is effective in the end (and we're all convinced it can and will be used early in the journey of the patient – much earlier than we're using them now in clinical trials) then there's the prospect of well-equipped academic cell therapy labs manufacturing CAR NKT products against different targets in their own time – only producing as many as they think they will need. They will then be able to immediately make these therapies available to patients as and when they are required.

Q Finally, where would you ideally see your work going over the next 3–5 years? What's on your list of aspirations?

AK: Well, it's hard to predict because of the very fluid field. And again, I think the Milteny approach is going to throw some spanners in the works of the established way of manufacturing and distributing CAR T cell immunotherapy.

My expectation is that we will be able to deliver effective immunotherapy at the lowest cost possible, which will be really affordable to all types of health systems in different geographies.

Certainly, given the way that manufacturing processes are developing, costs will become more sustainable. Combine this with what I believe is going to be a very effective and compelling treatment option – a curative one, in many cases – and I think this gives you a lot of advantages. But of course, if it is to be made available earlier in the patient journey, then it simply has to become affordable so that the larger patient population can access it – that's what I want to see.

"My expectation is that we will be able to deliver effective immunotherapy at the lowest cost possible..."

One possibility, as I've said, is that if we can get these new therapies to be manufactured on the Prodigy system, then facilities like ours can make their own products and have them ready to be delivered to patients.

Alternatively, I can see central, perhaps national, facilities like the blood transfusion service making this type of product and distributing it to hospitals in much the same way as blood is delivered today. I think the Prodigy can be adjusted for that purpose, too. This would mean all the commissioning, validation and quality control could be done centrally, which would only add to the potential to deliver these products to patients at a very affordable cost.

AFFILIATION

Anastasios Karadimitris
Professor of Haematology
Faculty of Medicine, Department of Medicine
Imperial College London,
UK

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CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

COMMENTARY

The advantage of allogenicity when using Inflammatory dendritic cells as antitumor immune primers

Sharon Longhurst, Juliana Kovacka, Emilia Heimann, Margareth Jorvid, Sijme Zeilemaker, Peter Suenaert & Alex Karlsson-Parra

For an allogeneic somatic cell therapy to become commercially successful in the field of oncology, where the patient population is significantly large, there needs to be an abundant supply of starting material, and the final product should be presented in a ready to use format that is available immediately, with no need to coordinate final stages of production with patient treatment schedules. Ideally the product should evade or modulate the immune system to avoid alloreactivity impacting on product efficacy, and the final cost of goods should be a low as is feasibly possible to ensure a cost-effective treatment. The allogeneic inflammatory dendritic cell product, ilixadencel, ticks a number of these boxes. The advantages of ilixadencel from a clinical and manufacturing perspective are discussed, as well as challenges that will need to be appropriately addressed to ensure future commercial success.

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INTRODUCTION

Our understanding of how the innate and adaptive immune system work together in response to alloantigens [1], and the experiences gained in organ transplant and hematopoietic stem cell transplants [2], tend to suggest that treating diseases using allogeneic cells is unlikely to be straightforward and may lead to serious adverse events. Unless the immune system in the recipient is suppressed [1,3] or tolerance/alloanergisation induced [4], or the therapy itself includes a mechanism to evade the adaptive immune responses that will be elicited [5,6] it may be difficult to demonstrate a favorable risk benefit profile and develop a commercially viable product. Indeed, most cell-based medicinal products gaining approval in the EU since the introduction of the ATMP regulation [7] have been autologous (Holoclar®, Strimvelis®, Chondroselect®, MACI®, Provenge®, Spherox®, Yescarta® and Kymriah®). The tide, however, is starting to turn, with the more recent approvals of Zalmoxis® [8] and Alofisel® [9] both of which are allogeneic cell therapies. Furthermore, following on from the success of autologous CAR-T therapies, considerable effort is now being invested in the development of allogeneic CAR-T cell products. The advantages allogeneic CAR-T products may have over their autologous counterparts and strategies used to disrupt the endogenous T-cell receptor (TCR) is reviewed by Graham *et al.* [10].

Immunicum AB is developing an immune primer as a strategy to elicit a *de novo* immune response to solid tumors following intra-tumoral injection of allogeneic inflammatory dendritic cells (DC) (ilixadencel, [INN]) [11]. Ilixadencel is a true 'off-the-shelf' product because one batch can be used to treat multiple patients, and it is manufactured in a ready-to-use presentation. Ilixadencel is being investigated clinically in combination with checkpoint inhibitors, which act to lessen the immunosuppression within the tumor microenvironment. In contrast to general dogma, that allogeneic cell-based therapies should evade or modulate the immune response, the allogeneic nature of the cells may be advantageous to the mechanism of action of ilixadencel. The advantages of the use of allogeneic DCs in this context will be discussed from a therapeutic, manufacturing and commercialization perspective.

MECHANISM OF ACTION OF ILIXADENCEL

Based on a plethora of data presented in the literature, which is reviewed in Karlsson-Para *et al.* [11], it is foreseen that the intra-tumoral injection of ilixadencel will have the potential to indirectly prime naïve CD8+ T cells *in vivo*, by acting as an immunogenic primer inducing recruitment and maturation of endogenous DCs, instead of acting as antigen presenting cells (APC).

The inflammatory DCs are expected to induce the recruitment of immune cells, including NK, endogenous 'bystander' DC and T cells to the site of injection, in response to the residual production of chemokines (including CCL4, CCL5 and CXCL10) by the injected cells. The injected cells also release cytokines such as IL-12p70, TNF-α and IL-1β and moreover express a number of co-stimulatory

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surface molecules (such as CD80 and CD86) which are important for immune cell activation [12].

Based on pre-clinical *in vitro* and *in vivo* findings [13,14] the proposed mode of action is as follows (see also Figure 1): the cross talk between ilixadencel and the recruited NK cells will induce NK cell activation, subsequently leading to local tumor cell killing and the release of cell-associated antigens, which will be taken up and processed by endogenous 'bystander' DCs recruited to the site. The activated NK cells secrete IFN-γ which, in concert with the TNF-α produced by both ilixadencel and the activated NK cells, will enhance the cross presentation of captured tumor antigens by the endogenous DCs. These antigen-loaded DCs will start to mature in response to pro-inflammatory factors such as TNF-α and IL-1β released by the injected ilixadencel and will migrate to the tumor-draining lymph node where they will activate tumor-specific T cells, including cytotoxic CD8+ T cells, thus generating a systemic immune response to the tumor. Furthermore, the production of IFN-γ from activated recruited NK and T cells will favor the differentiation of Th1 polarizing DCs and may inhibit immunosuppressive M2-macrophages [15] and drive T-reg fragility within the tumor [16].

In summary:

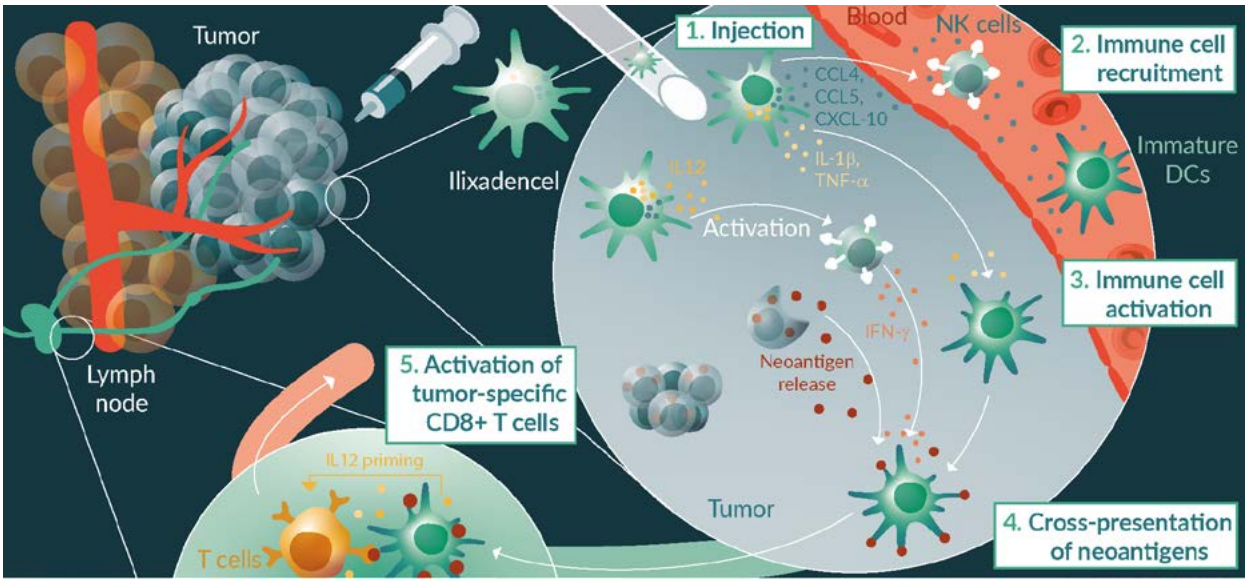
1. Intratumorally injected ilixadencel uniquely covers all major aspects of tumor specific immune priming:
   - Recruitment of immune cells including NK cells and DC into the tumor
   - Induction of local tumor cell death, leading to increased release of tumor-specific antigens
   - Maturation of antigen-loaded dendritic cells for subsequent migration to tumor-draining lymph nodes where the dendritic cells activate/prime tumor-specific T cells
2. The concept uses the patient's own tumor as the antigen source *in vivo* , which aims to ensure that the full set of neoantigens are used for activation of a tumor-specific immune response
3. Ilixadencel is applicable for all injectable solid tumors.

CLINICAL CONSIDERATIONS

In line with the proposed mechanism of action of ilixadencel, the product is administered intratumorally as its purpose is to initiate a Th1 and cytotoxic T-cell response against tumor-specific neoantigens released locally following NK cell activation and subsequent tumor cell death. However, it is well known that the tumor microenvironment (TME) is adapted to suppress T-cell responses by secreting immunosuppressive cytokines such as IL-10 and TGF-β, indoleamine 2,3-deoxygenase and nitric oxide and recruitment of immunosuppressive macrophages [15]. Furthermore, cells in the TME have up-regulated expression of inhibitory checkpoint molecules such as CTLA-4 and PD-L1 [17]. Given this hostile environment, ilixadencel may not be particularly efficacious as a monotherapy, however with the recent success in the treatment of solid tumors using checkpoint

FIGURE 1

Proposed mode of action for intratumorally injected ilixadencel.



After administration into the viable part of the tumor, ilixadencel DCs will release Th1-associated chemokines, including CCL4, 5 and CXCL10 that recruit immune cells, including NK cells and immature-DCs into the tumor. The interaction between recruited NK cells and ilixadencel DCs induce NK-cell-mediated killing of adjacent tumor cells, resulting in release of tumor-associated antigens, including neoantigens. The production of IFN-γ by activated NK cells and TNF-α/IL-1β released by ilixadencel DCs will induce maturation as well as increase cross-presentation of engulfed cell-associated tumor antigens by recruited endogenous 'bystander' DCs. Migration of these antigen-loaded and matured 'bystander' DCs to the tumor-draining lymph node will finally lead to Th1-polarized activation of tumor-specific T cells, including cytotoxic CD8+ T cells, by the release of IL-12.

inhibitors (CPI), which help to counteract these immunosuppressive mechanisms in the TME, it is considered likely that a combination therapy of CPI and ilixadencel may act to further improve response rates in solid tumors. Preclinical data demonstrate a synergistic effect of the combination treatment [18], and that a systemic adaptive immune memory may be induced [18]; clinical studies are currently on-going to test that combination in man. Continuous immune priming by re-administration of ilixadencel after the initial 1–3 cycles of treatment, is not foreseen once a robust response has been initially raised. The optimal dosing schedule is currently under investigation comparing the number of cells administered and the frequency of two versus three cycles. From a clinical perspective this treatment strategy is shorter, less expensive and ultimately less demanding for the patient, taking into consideration the IT route of administration.

From a clinical perspective there are clear advantages of allogeneic 'off-the-shelf' cell therapy products relative to autologous or personalised cell therapy products, these are summarized in Table 1. Most importantly, a true off-the-shelf product can be stockpiled so that they are available as soon as a patient is identified that may benefit from the treatment, whether it is a within the context of a clinical trial or commercial supply. Ilixadencel has currently a 36-month shelf life, as such can be manufactured and stored ready for immediate use, unlike autologous CAR-T cell where it is reported that manufacture can

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TABLE 1

Advantages and challenges of allogeneic inflammatory DCs from a clinical perspective.

Clinical	
Advantages	**Challenges**
▶ Off-the-shelf product; no delay in start of treatment	▶ Cryopreserved product; logistics of supply to (or storage at) pharmacy are challenging as LN2 is not always readily available at hospitals
▶ Patients with tumour types with a significant probability of rapid evolution towards progression prior to start of treatment can be included	▶ IT administration may not be routinely performed at all hospitals
▶ Short treatment duration with immune memory established; continual boosting of the immune response is not foreseen	▶ If tumour burden changes and new non-somatic mutations develop, re-treatment may be required to develop a robust T-cell response to the new neoantigens
▶ Ilixadencel does not need to become engrafted in the patient to elicit its therapeutic effect, as such rejection of the product for long-term efficacy is not a concern	
▶ Allogeneicity of ilixadencel may prove to be an advantage as a local reaction at the point of administration could potentially exacerbate the pro-inflammatory environment within the tumor	
▶ Positive tolerability including no evidence of clinical or serological auto-immunity observed to date	
▶ Ilixadencel has the potential to work in synergy with different product classes of immune modulators (and combinations thereof) so has the potential to remain a key therapeutic agent even as SOC changes	

IT: Intratumoral; LN2: Liquid nitrogen; SOC: Standard of care.

take up to 3 weeks after the patient has been identified [8]. As there is no delay in treatment with ilixadencel, the risk of disease progression occurring before the product is released for administration is reduced.

Biodistribution studies in rats indicate that injected rat DCs are not viable for more than 2–3 days [11] and no evidence of local or distant tissue damage or local accumulation of inflammatory cells are observed after intrarenal administration of inflammatory allogeneic rat DC in a rat model [11]. Moreover, adverse events seen in the clinic to date have mainly been mild to moderate fever and chills, which have been easily managed, and there is no evidence of clinical or serological auto-immunity being developed. Furthermore, because the cells not need to engraft in the patient for the therapeutic effect to develop, the clinical concerns related to rejection of the allogeneic cells and loss of efficacy is no longer relevant.

Given that the injected cells are allogeneic to the patient, about half of the evaluable patients developed donor-specific antibodies across two completed studies performed to date, which can also be viewed upon as a sign of systemic immune response [14,19]. Allogeneic responses could potentially involve both direct or indirect antigen recognition [1], dead or dying cells which can, via APC, activate the adaptive immune system [20,21], and/or the innate recognition of allogeneic non-self by endogenous monocytes, which results in the differentiation of host monocytes

into dendritic cells that, similar to ilixadencel, produce IL-12 and drive T-cell proliferation and IFN-γ production [22]. Whichever mechanism, or combination of mechanisms, that may occur within the TME, it is likely the allo-response will further exacerbate the pro-inflammatory environment within the site of injection and promote the activation of bystander DCs [23], which may be beneficial to the mechanism of action of ilixadencel, particularly following the second administration of ilixadencel as the same batch of ilixadencel is used in each cycle of treatment, and therefore manufactured from the same donor.

Finally, ilixadencel is not being developed in combination with a specific product, rather the approach is to show its' synergy with different product classes such as CPIs. This could be extended to other immune modulators, or new products as they become approved, or even triple combination therapies, as standard of care (SOC) regimens for different oncology indications will continue to change over time. As such, ilixadencel has the potential to become a key therapeutic agent in the future.

MANUFACTURING CONSIDERATIONS

Allogeneic products are likely to have quite disparate manufacturing processes given the potentially diverse types of products that may be approved for commercial supply, requiring different approaches with respect to CMC-regulatory strategies and supply logistics. The ilixadencel manufacturing process is described and contrasted to that of Alofisel® (an allogeneic mesenchymal stem cell (MSC) product [9]) and Yescarta® (an autologous CAR-T product [24]) in Table 2; and could be considered somewhat of a hybrid of the MSC and CAR-T processes, but without the cell expansion capacity, which limits the batch size. Unlike the majority of DC vaccines under clinical development, ilixadencel is not loaded with tumor specific antigens or neoantigens, as such there is no personalization of the product, resulting in a relatively straightforward and short manufacturing process. The advantages and challenges associated with the manufacture of ilixadencel are summarized in Table 3 and discussed in more detail below.

1. Starting material

Ilixadencel is manufactured from leukapheresis (LP) donations collected from healthy volunteers. The main advantages of using healthy donors rather than the patient themselves are that manufacturing failures due to the collection of insufficient numbers of cells will be unlikely, and the leukapheresis composition may be more consistent form donor to donor compared to patient to patient. In addition, because the donors are healthy the potential for collection of tumor cells that may be circulating is removed [25]. However, it would be misleading to suggest that manufacturing a clinical product from healthy donors removes donor variability, as Immunicum has observed between 20–40% variability in the LP cell composition (Table 4 [26]), which results in a similar level of variability in the resulting total

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TABLE 2

Comparison of allogenic (MSC and DC) manufacturing processes relative to an autologous CAR-T.

	MSC1	DC (ilixadencel)	CAR-T2	Comment
Starting material	Lipoaspirate from healthy living female donors	LP from healthy donors	Patient LP; Retroviral vector	For all processes' appropriate donation, procurement and donor screening in accordance with Directive 2004/23/EC, 2006/17/EC and traceability in accordance with Directive 2006/86/EC needs to be demonstrated For CAR-T viral vector requires its own complete module 3 defining manufacture and validation, control strategy (process and material) and shelf life
Process intermediates	MCB	None	None	
AS	Cell suspension of eASC	Cell suspension of pro-inflammatory DCs (arbitrary definition)	Not defined in EPAR	Ilixadencel and CAR-T: no formal release of AS
Process summary	Isolation of stromal vascular fraction from adipose tissue and selection of ASC	Monocyte enrichment from LP	Enrichment of T cells from LP	
	Expansion of ASC, harvest, filling and cryopreservation of MCB	Differentiation of monocytes to immature DC	T-cell activation	Alofisel: quality control of MCB intermediate is required and limits of population doubling defined (presumably to MCB and to active substance). Stability studies for shelf life of MCB also required Ilixadencel: no process intermediates defined, and cells do not expand during processing thus population doubling is not a pertinent quality attribute
	MCB thawing, expansion of ASC, harvest, vial filling and cryopreservation generating the active substance (eASC)	Stimulation of immature DCs generating a pro-inflammatory DC phenotype	Viral transduction and T-cell expansion	Alofisel: formal release of active substance and stability data to confirm shelf life (2 years a time of MAA) Ilixadencel: no formal release of active substance; processed immediately to final product CAR-T: no formal release of active substance; processed immediately to final product
	Final product manufacture requires the thawing and washing of active substance, cell recovery followed by formulation in DMEM/20% HSA, and filling (5 x 10^6 cells/ml; 30 x 10^6 cells/vial) in 9 ml type I glass vials Batch size not disclosed	Proinflammatory DCs formulated, vial filled and closed, then cyropreserved to generate the final product. 11.7 x 10^6 cells/vials	Formulated into cryopreservation medium, and cryopreserved ~68–200 x 10^6 cells per bag >1 bag is prepared but the exact number is not disclosed3	Alofisel: final dose consists of 4 vials/120 x 10^6 cells. Shelf life of final product is 48 hours. In process control testing is performed during the last steps of manufacture, immediately before and after final product formulation due to the short shelf life of the product. Product is shipped at 15–25°C Process validation included manufacture from 3 different donors and for 3 proposed batch sizes. Batch sizes are not disclosed Ilixadencel: cyropreserved final product with a 3-year shelf life currently, when stored at ≤-130°C CAR-T: container closure is ethylene vinyl acetate cryostorage bag. 12 months shelf life when stored at -150°C

[1]Alofisel EPAR [9].
[2]Yescarta EPAR [24].
[3]CMC review for Yescarta by FDA (BLA STN#125643/0).
AS: Active substance; eASC: expanded adipose stem cells;LP: Leukapheresis; MCB: Master Cell Bank.

DC yield at the end of the process (23.8% CV).

Key quality attributes of ilixadencel include the quantity of cytokines and chemokines secreted by the cells and cell surface markers common to DC, and again there is considerable variability observed from donor to donor. The clinical relevance of this variability and how the quality of raw materials used for differentiation and stimulation stages of the DCs influences this level variability needs to be evaluated as part of the clinical development and process characterization activities in order to justify acceptable release criteria. Nonetheless, the use of healthy volunteers clearly introduces variability from batch to batch when each batch is manufactured from a single donor; a means of minimizing this by the pooling of cells from >1 donor is discussed further in point 3 below.

2. Logistics of leukapheresis supply & manufacturing strategies

The challenges of orchestrating donor selection and consent, leukapheresis collection and manufacture when the leukapheresis must be processed within a few hours of collection (fresh) are manageable for Phase 1 and 2 studies as fewer batches are needed, and the manufacture can be scheduled in advance of use given the long shelf life of the final product, so any failures associated with batch manufacture may not necessarily impact on clinical supply. The strategy of manufacturing from 'fresh' LP is likely to give the best outcome in terms of product yield and possibly overall product quality and therefore a reasonable place to start. However, the logistics of supply become more complex when the number of batches required increases significantly or if there is more than one active study recruiting concurrently. For commercial supply manufacture from fresh leukapheresis may be achievable for an orphan indication with limited market demand, but for an indication such as cancer, where the patient population is 10s of 1000s per year, even if the products market share is small in comparison, it would be extremely complex and difficult to manage for the following reasons:

1. For each manufacturing slot pre-screening of at least two donors is highly recommended to mitigate screening failures or the donor falling ill prior to donation to avoid wasting manufacturing slots – rescheduling manufacture is not straightforward, particularly if a contract manufacturing organization is used.

2. To ensure commercial product supply multiple collection sites in all regions where the product is approved will be required. Each one will require a manufacturing facility to be located within a certain distance to ensure processing can start with the allotted window of time.

These two points act to increase the cost of goods (significant increase in resources, screening costs, facility operational costs), double the number of donors needed to be identified for each scheduled manufacturing slot and add to an ever-increasingly complicated comparability exercise as new sites come on board.

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TABLE 3

Advantages and challenges of allogeneic proinflammatory DCs from a manufacturing perspective.

Manufacturing	
Advantages	**Challenges**
Manufacturing process is straightforward, with no requirement to load the DCs with tumour antigens or neoantigens specifically identified from the patient (not a personalized product in any way)	▸ Cells do not expand during processing; – Maintaining viability and reducing cell losses during processing is critical to ensuring maximal yields
Healthy donor source material: ▸ More consistent product relative to autologous products; consistency might be improved further if manufactured from >1 donor ▸ No potential contamination of the source material with tumor cells ▸ Fewer manufacturing failures due to poor cell numbers in donation ▸ Potential to stockpile starting material from multiple collection sites if manufacture from frozen LP is feasible	▸ Commercial supply will require a consistent supply of donors in multiple regulatory jurisdictions – consider the possibility of setting up donor registries to ensure supply – aligning procurement, donation and testing procedures to meet all legal requirements in all regions may not be straightforward ▸ Commercialization of LP supply in the EU is not as advanced as in the US and could represent a bottleneck for manufacture of products derived from this starting material ▸ Processing of LP at blood collection sites needs to become more widespread, to permit immediate freezing of cells and extend the shelf life of the LP without significant impact on product yields or quality
Centralized manufacturing site could be feasible, if LP can be stored long term: ▸ Reduces the burden of process and analytical transfers to multiple sites ▸ Reduces the burden of demonstrating product comparability between different manufacturing sites	▸ If it is not possible to align procurement, donation and testing procedures between different jurisdictions regional (though still centralized) manufacturing sites i.e., USA/EU could be possible
Scale of the process is aligned with that of CAR-T, permitting use of (or modification of) closed, automated equipment already commercialised and permits a scale out approach to commercial supply	▸ A scale up approach could be possible if it is acceptable from a product quality, regulatory and safety perspective to pool starting material, enriched monocytes or final product from more than one donor
Final product has at least a 3-year shelf life and is stored cryopreserved. It can be stockpiled for immediate use	▸ Hospitals may not routinely have vapour phase storage vessels. Logistics of supply might involve: – Just-in-time shipments to site from a central depot – Sponsor/MAH provides storage vessels and supports maintenance in collaboration with pharmacy
Cost per dose is lower compared to autologous CAR-T costs	▸ Efficiencies of scale of production may not contribute to significantly lower cost of goods

LP: Leukapheresis.

In our opinion, the optimal approach for donor supply would be to manufacture from frozen LP, preferably LP that is frozen at the collection site, as the sooner it is processed the better the cell yield.

This would decouple the collection procedure from the manufacturing schedule, removing significant risk to supply. Such a supply strategy would be expected to reduce the costs of goods as a centralized manufacturing site could be used, if capacity permitted, which avoids lengthy product comparability evaluations. Though realistically, if commercially successful, regional manufacturing sites would be needed as a minimum.

The processing of LP at the collection sites will require a considerable investment by those facilities, with the construction of GMP processing suites and licensing for processing (albeit minimal manipulation). To the best of our knowledge, there are limited sites within the EU that could offer this commercially. To ensure the successful commercialization of allogenic products using LP as the starting material (whether fresh or frozen), it is critical that this potential bottleneck is addressed, which will require close collaboration between collection sites and product developers.

Another alternative strategy could be implemented - collection and shipping of fresh LP to a manufacturing site for freezing. Such a strategy would require shelf life of the LP to be prolonged, ideally for > 24–48 h, but this strategy still limits the distance the collection site can be from the production facility, and the viability of the cells also decrease over time, which will impact yield, even if final product quality is unaffected, and this will ultimately influence the final cost of goods calculation. This option, while better than processing within hours of collection, is still far from ideal; the location of regional manufacturing sites would have to be weighed against the number of collection sites needed for LP supply and the time needed to ship the LP to the manufacturer.

Another important point to note is when the clinical studies move to different regions restrictions may be made on the donors that can be used for manufacture. In our experience, the FDA has donor deferral criteria for cell-based products that essentially excludes the use of EU donors [27] for manufacture of products that are to be used under an IND in the USA. Interestingly, a draft guidance which amends FDA's current guidance on the preventative measures to reduce the transmission of (v)CJD by blood and blood products [28] was released in 2017 in which the donor deferral criteria for donors residing in the EU was eased; perhaps this will pave the way for a similar easing of restrictions for cell-based therapies $(HCT/Ps)_i$ in the future.

3. Scale-up vs scale-out and reducing the cost of goods

Based on the minimal cell numbers specified for a CAR-T production run (500 lymphocytes/µl; 150 CD3+/µl) [29], it is likely that similar process volumes are used during production with that used for ilixadencel, meaning that the closed, automated production platforms that are commercially available for CAR-T production, will almost certainly be adaptable for the ilixadencel production process. We consider that there are therefore three potential means of expanding production to meet late clinical phase and/or eventual commercial supply:

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TABLE 4

Variability in the composition of healthy donor leukapheresis.

Quality attribute	Patient (literature)[1]		Healthy donor (Immunicum)[2]		
	Ranges	Median	Range	Mean ± SD	%CV
Volume (ml)	129–173	153	207.6–320.4	258.9 ± 32.39	12.51
Viability (%; FACS)			98.6–99.7	99.29 ± 0.36	0.37
WBC/mL (x 107)			4.00–7.54	5.56 ± 1.02	18.42
WBC total (x109)	9.0–154.0	15.0	10.3–19.8	14.3 ± 2.53	17.74
Granulocytes (%)	0.10–2.28	0.38	0.12–4.12	1.34 ± 1.28	95.79
Monocytes (% CD14+)			11.67–28.22	14.73 ± 5.87	39.83
Monocytes (total number) ($x10^9$)	1.54–5.36	2.87	1.58–3.98	2.11 ± 0.89	41.98
Lymphocytes (%)			71.19–87.53	83.54 ± 5.48	6.56
Lymphocytes (total number) ($x\ 10^9$)	4.37–146.5	9.64	7.68–16.3	11.9 ± 2.39	20.04
T-cells (% CD3+)			55.47–75.53	65.62 (± 6.30)	9.60
B-cells (% CD19+)			4.45–9.09	6.70 (±20.06)	30.77
NK cells (% CD56+)			7.54–15.33	11.21 (±2.26)	20.17

[1]Svensson *et al.* 2005 [26]; n = 14.
[2]n = 12.
n/a: Not available.

1. Scale-out using pre-existing production platforms such as the CFC system (HCATS/Invetec) or Prodigy (Miltenyi)

2. Scale-up by pooling product from multiple donors and using a modular process for production such as larger volume platform bioreactors (i.e., GE HealthCare's' Xuri or similar) and automated but module cell washing equipment (i.e., Fresenius' LOVO or similar)

3. Use of mobilized LP (as a single batch or potentially pooled from multiple donors)

While ilixadencel is described as an off-the-shelf product, which indeed it is, the scale of production is still very small compared to the monoclonal antibody world where bioreactors of 10,000 L or more are used per batch. This means it is more difficult to reduce the costs of goods on the basis of efficiency of scale in the first instance. Given, as described above, there is pre-existing automated equipment already available, the ability to scale out and manufacture multiple batches from multiple donors in parallel is a potential means of meeting supply demands. This does not reduce in any way costs associated with donor screening, in-process control and release testing per batch; but facility/overhead costs per batch could be reduced using such a strategy. This approach, however, raises the issue of the need for high throughput release testing in order to 'keep-up' with the rate of production.

The scale-up approach using multiple donors is an interesting concept, which at first glance seems a logical approach, and

early experimental evidence suggests manufacture from pooled donors does not impact the biological activity of the resulting DCs [Unpublished Data]. However, on closer examination there are number of issues to consider, not least of which are the regulatory implications. The EMA take a pragmatic approach to pooling, but are concerned with the implications for viral safety; so, the questions to ask yourself as a product developer are how many donors do you want to pool and how can you justify this is not going to result in a safety risk to the patient? The FDA however, does not permit the pooling of source plasma from more than one donor (21CFR §640.69), it would therefore seem unlikely that licensure would be feasible in the US using cells pooled from multiple donors for production purposes.

Furthermore, consideration needs to be given to where in the process pooling takes place – LP, enriched MO or final product? LP may not be ideal due to the presence of other immune cells such as T cells as these could become activated due to the HLA mis-match between donors, which may activate the monocytes and influence product quality. There may also be limitations on the volume and number of cells that can be processed for monocyte enrichments which would limit the number of donors that could be pooled.

Pooling of monocytes may be feasible, but there would need to be consecutive processing to limit the hold time of monocytes from each donor before pooling as this could influence overall product quality and yield. Pooling at the point of final product is likely the most problematic, as each donor would have to be processed in parallel (perhaps not an issue if using automated platforms), and there is a limited time from harvest to freezing to ensure maximal product viability. This latter point also impacts on the maximum number of cells that could be formulated and filled/frozen in a reasonably short period time and would require ideally fully automated filling processes. The level of testing performed on each 'pre-batch' versus that of the final pooled product would also need careful thought and discussion with the regulatory authorities. The approach of pooling material from several donors would require a new clinical development plan, as the resulting product is unlikely considered to be comparable to a batch generated from a single donor.

Finally, there is the possibility to use mobilized LP, which increases the number of PBMCs in the donation and has therefore, the potential to increase the number of monocytes enriched from the same volume of LP. However, consideration needs to be given to the impact this may have on the number of donors that could be identified, given the additional treatment required prior to donation. Secondly, the mobilization treatment may influence the activation status of the monocytes, which could impact on their differentiation to DC, and could ultimately influence the final product characteristics. Such a change to the starting material would be considered a major process change requiring extensive evaluation to confirm its feasibility.

The cost of goods calculations for autologous vs allogeneic stem cell products has been reviewed elsewhere [30] and will not be repeated for the ilixadencel process here.

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However, it is anticipated that while the scale of manufacture is not that dissimilar to the CAR-T process, the fact that each batch of ilixadencel can treat between 20 and 50 patients, depending on the optimal dose, the cost of treatment would be expected to be significantly lower than autologous products (Yescarta is reported to be priced at $373,000 per patient [31]). If scale-out, or eventually scale-up of the process proves to be feasible, the cost per dose could potentially be reduced even further.

CONCLUSIONS

The authors are of the opinion that it is not possible to generalize on which product class is better – allogeneic or autologous; each may well have their own clinical niche in the long term and their niche will likely depend on the indication to be treated and potentially the mechanism of action of the product. Of importance will be whether the treatment is intended to persist in the recipient for a prolonged time, as such a therapeutic approach is likely to be more challenging for allogeneic products due to the potential for cell rejection and graft-versus-host disease. Furthermore, if allogeneic products are to become commercially viable, the development of true 'off-the-shelf' products that are available for immediate use is preferable. Having to align the manufacture of a product (or even just the final stages of manufacture) with the date of administration at a hospital adds to the complexity of supply. There is also the expectation that allogeneic products will be less expensive as more than one patient can be treated per batch, and this has already been seen in the cost of Alofisel® which is reported to be priced at GBP £54,000 [32] per treatment compared to £285,600 (US $373,000) for Yescarta®.

Ilixadencel ticks a number of these boxes: it can be stockpiled in a ready to use presentation, for immediate use; it does not persist in the patient and, uniquely for an allogeneic product, the allogenicity may be advantageous to its effectiveness *in vivo*. Furthermore, there has been no measurable evidence of auto-reactivity in any of the patients treated and evaluated to date, which may suggest the allogeneic nature of the product may not be detrimental to patient safety. Given the relatively early stage of development the cost of a course of treatment of ilixadencel has yet to be calculated but given between 20 and 50 patients could potentially be treated from one batch, a cost saving relative to an autologous product would be expected.

However, there is one factor that represents a key challenge to the manufacture of ilixadencel and this is the starting material; not because it may not be possible to identify sufficient numbers of healthy donors for manufacture, but more due to the logistics of supply of that starting material to the manufacturing site, and the fact that one LP equates to one batch of product which limits the batch size and therefore will significantly influence the cost of goods, as efficiencies of scale cannot be brought into the calculation.

As discussed, these are not necessarily insurmountable hurdles, but they will require careful consideration during product development in readiness for commercial

supply and will ultimately require close collaboration with an extensive network of blood collection sites across multiple regulatory jurisdictions. Furthermore, an expansion of blood collection sites that can perform LP processing, at least minimal manipulations such as freezing of the cells, would, in our opinion, pave the way for successful commercialization of allogeneic products derived from donor LP.

Clearly there are other potential avenues to investigate in relation to the possibility of scaling up manufacture; this could include the use of mobilized LP or pooling from multiple donors. Although, as discussed, these strategies may impact on product characteristics, requiring independent clinical development plans.

Despite the challenges that still need to be addressed, we consider ilixadencel to have significant clinical and manufacturing advantages that improve the likelihood of commercial success, and we expect that allogeneic cell products will have a promising future in the emerging world of cell-based therapeutics.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

Alex Karlsson-Parra, Juliana Kovacka, Emilia Heimann, Sijme Zeilemaker, Sharon Longhurst and Peter Suenaert report ownership of stocks in Immunicum AB and are all Immunicum employees. Margareth Jorvid report ownership of stocks in Immunicum AB and is a consultant to Immunicum AB. No writing assistance was utilized in the production of this manuscript.

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AFFILIATIONS

Sharon Longhurst,
Immunicum AB, Östermalmstorg 5, 114 42 Stockholm, Sweden

Juliana Kovacka,
Immunicum AB, Östermalmstorg 5, 114 42 Stockholm, Sweden

Emilia Heimann,
Immunicum AB, Östermalmstorg 5, 114 42 Stockholm, Sweden

Margareth Jorvid,
Immunicum AB, Östermalmstorg 5, 114 42 Stockholm, Sweden

Sijme Zeilemaker,
Immunicum AB, Östermalmstorg 5, 114 42 Stockholm, Sweden

Peter Suenaert
Immunicum AB, Östermalmstorg 5, 114 42 Stockholm, Sweden

Alex Karlsson-Parra
Immunicum AB, Östermalmstorg 5, 114 42 Stockholm, Sweden

and;

Department of Immunology, Genetics and Pathology, Uppsala University, Rudbeck Laboratory, Dag Hammarskjölds Väg 20, 752 37 Uppsala, Sweden

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CELL & GENE THERAPY INSIGHTS

CELLULAR IMMUNO-ONCOLOGY 4.0

SPOTLIGHT

EDITORIAL

The immunosuppressive tumor microenvironment: more than just immune checkpoints

Mark W Lowdell

"If the second decade of the 21st century represents the 'coming of age' of cancer immunotherapy, we can only hope that the next decade delivers a maturity of understanding that comes with added years and we are able to combine CPIs with targeted breakdown of the TME and appropriate adoptive immunotherapies to create truly patient-specific treatment packages."

In this issue we have review articles covering a wide spectrum of tumor immunotherapy options; embracing T cells and NK cells as immune effectors and dendritic cells as immune potentiators. These approaches include genetic manipulation to add function via chimeric receptors and control inhibition by targeted gene editing. The one thing they have in common is that the clinical outcome will be determined to a greater or lesser degree by the microenvironment in which the effector cells encounter tumor. In this second decade of the 21st century, oncologists have become true believers in the role of the immune response in the treatment and cure of cancer but it wasn't always so. This sea change in belief has been driven by the substantial successes of the use of monoclonal antibodies which

block immunoregulatory signals delivered by tumor cells to the patient's cytotoxic T cells; so called 'immune checkpoint inhibitors', or CPIs. The first of these was an antibody to CTLA-4 and this was followed by others targeting PD-1. These off-the-shelf monoclonal antibody drugs have had significant success in a wide range of solid cancers including melanoma, non-small cell lung cancer, pancreatic cancer and prostate cancer. Many more are still in clinical trials and alternative therapies using the same regulatory axes are in development – anti-PD-L1, anti-CD39 and anti-CD73 to name but three. Despite these successes, it is fair to say that the majority of cancer patients are not cured by any of the current CPIs, even when used in combination. Nonetheless, the intense interest they have generated has raised awareness of the highly immunosuppressive nature of the tumor microenvironment which has led to a greater understanding of the complex interactions within a tumor and is leading to broader immunopotentiation strategies and the development of drugs to target those.

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Cancer immunotherapy has a very long and somewhat torrid history, from the use of the eponymous Coley's toxin in the 19th century to the adoptive cell therapies of today. The discovery in the 1980s and 90s that the cures achieved by allogeneic bone marrow transplantation in chronic myeloid leukemia (CML) were due to the immune response of the donor lymphocytes gave the first indication of the power of adoptive immunotherapy and it was proven by the first trials of allogeneic donor lymphocyte infusions which eradicated minimal residual disease in CML patients relapsing post-transplant. Despite this and some notable successes with tumor-infiltrating lymphocytes and autologous NK cell therapies, the widespread acceptance of the importance of the immune system in the treatment and eradication or control of cancer was something that the oncology community largely ignored. That changed in the first decade of the 21st century as Phase 1 clinical trials of monoclonal antibodies which blocked key immunoregulatory molecules on T cells began to be reported. The first regulatory molecule to be targeted was CTLA-4 [1] and subsequent trials with anti-PD-1 led to the development of licensed medicines Ipilimumab and Nivolumab/Prembrolizumab respectively, and the 'era of CPIs'.

The significant but still limited success of these CPIs, as single agents or in combination, has driven the academic and commercial interest in the complexity of the tumor microenvironment to seek more, suitable targets for clinical trial. The current CPIs work by blocking the immunosuppressive signals delivered to the tumor-resident T cells. T lymphocytes are central to the adaptive immune response to infection and cancer but their proliferation and function need to be tightly controlled

to prevent autoimmunity. This regulation is called 'peripheral tolerance' and is a normal physiological process throughout the body. However, many cancers evolve in vivo to switch on the genes for the expression of the PD-1 ligand which leads to aberrant blockade of T cell function and inhibits the anti-tumor cytotoxicity. Blockade of the PD-1:PD-L1 interaction by antibodies to one or either partner can overcome this immune suppression and allow the tumor-resident T cells to target the cancer cells.

Whilst these pathways are important immune evasion strategies for tumors – indeed, these discoveries led to the award of the Nobel Prize in Physiology/Medicine to James Allison and Tasuko Honju in 2018 – they are a small part of the whole immunosuppressive milieu of the tumor.

Most solid cancers are a heterogeneous mix of cells and blood vessels of which the tumor cells are only a part. Like all 3-D structures, cancers require a scaffold of interstitial and stromal cells with a rich blood supply. Within this 3-D structure it is common to find regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM) and tumor-resident fibroblasts, often called 'cancer associated fibroblasts' or CAF. Each of these cell types is able to participate in the suppression of anti-tumor immunity and many work in tandem.

The MDSC is central to the creation and maintenance of the immunosuppressive milieu of the tumor microenvironment (TME). Indeed, the number of MDSC in the peripheral blood or tumor biopsy of cancer patients is a negative prognostic marker for overall survival and predictive of increased resistance to current CPIs [2–4]. The impact of raised MDSCs is due to their multiple roles in creating the TME. MDSCs drive the differentiation of naïve CD4+ T cells to Tregs through secretion of TGF-b [5] which are able to inhibit T-cell proliferation and cytotoxicity [6]. Once they have migrated from the circulation into the TME, MDSCs can differentiate into TAMs and promote the differentiation of resident fibroblasts into CAFs.

> "The next logical step in enhancing endogenous immunity to cancer is to target the breakdown of the rest of the suppressive mechanisms in the TME..."

MDSCs are characterized into two distinct subpopulations called 'monocytic' (M-MDSCs) or 'granulocytic' (PMN-MDSCs) and it is believed that these differentiate from macrophage and granulocyte precursors alongside conventional macrophages and granulocytes but preferentially expand under chronic inflammation such as that experienced in cancer [7]. M-MDSC can be identified in low numbers in the peripheral blood of healthy donors as CD11b+/CD14+/CD15-ve/CD33+ with low expression of HLA-DR. It is this latter characteristic which distinguishes the M-MDSC from conventional monocytes. PMN-MDSCs are phenotypically identical to normal granulocytes except for expression of LOX-1 and a morphology which is more lymphoid [8].

The predominant immunosuppressive activity resides within the M-MDSC and it appears to be mediated by secretion of prostaglandin E2, ARG1 and nitrous oxide, but their ability to differentiate rapidly into TAMs when they invade the tumor microenvironment is probably their most significant role [9,10].

TAMs have multiple direct immunosuppressive actions within the TME including release of IL-6 and IL-11, which drive tumor growth though activation of intra-tumoral STAT3 and secretion of CCL18 which is pro-metastatic. TAMs further contribute to tumor survival through direct and indirect suppression of tumor-infiltrating T cells and, possibly, NK cells. This is via secretion of IL-10 which directly inhibits cytotoxic T-cell activation but also though secretion of TGFβ which induces differentiation of naïve CD4 cells into Treg cells and thus, indirect suppression of cytotoxic cells. Finally, TAMs are unable to synthesize IL-12 and since the TAMs displace conventional macrophages within the TME, the result is a deficiency of local IL-12 and indirect reduction of NK and T cell activation.

TRANSLATION INSIGHT

This brief snapshot of the principal cellular constituents of the TME highlights the plethora of complex interactions determining a patient's ability to mount a curative immune response to their cancer. It is hardly surprising that single agent approaches with CPIs have a high degree of treatment failure; in fact, it is remarkable that CPIs have achieved the notable successes they have.

The current approach of combining CPIs to maximize clinical effect will remain limited since it fails to address the cells in the TME which directly and indirectly suppress the tumor infiltrating T cells which are the target of current CPIs. The next logical step in enhancing endogenous immunity to cancer is to target the breakdown of the rest of the suppressive mechanisms in the TME but doing so risks side effects of systemic dysregulation of control of autoimmunity. One approach may be to selectively target the MDSC which should reduce TAM and Treg within the tumor but is unlikely to affect systemic peripheral tolerance since MDSC are an ultra-rare population in healthy individuals. Immune targeting of MDSC is challenging since no MDSC-specific surface antigen has been found to date. However, MDSC differentiation in the patient bone marrow is heavily dependent upon soluble TNF-α and selective blockade may be a solution. Trials of a molecular mimic of TNF-α which disrupts sTNF-α but spares the membrane-bound form are underway and we look forward to the outcome.

If the second decade of the 21st century represents the 'coming of age' of cancer immunotherapy we can only hope that the next decade delivers a maturity of understanding that comes with added years and we are able to combine CPIs with targeted breakdown of the TME and appropriate adoptive immunotherapies to create truly patient-specific treatment packages.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

Mark Lowdell is co-founder of INmune Bio Inc., an innate immunotherapy company developing NK-related and MDSC targeting therapies in clinical

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trials. He is also co-founder of Achilles Therapeutics Ltd, developing neo-antigen specific, autologous TIL therapies, and of Autonomous Ltd, a company creating software solutions to cope with the challenges of commercially delivering autologous immunotherapy products to thousands of patients. No writing assistance was utilized in the production of this manuscript.

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AFFILIATION

Mark Lowdell
CSO and Founder, InMuneBio, UK, Director of Cellular Therapy at the Royal Free London NHS Foundation Trust, London, UK