EXPERT ROUNDTABLE

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

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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.

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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.

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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.

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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 hypoxic environments such as the TEM.

Persistence is something we must consider, too: what are the types of mechanism 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 point of 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 factors involved in TME such as hypoxia and 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.

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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 feeling 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 target in solid tumors – it's a far more challenging scenario than for hematological malignancies, where CD19 B cells are the tissue involved – they can be targeted more or less indiscriminately, because they are not essential to life. With solid tumors, many of these targets are also present in healthy tissues in the heart, the brain and other vital organs. A lot more work needs to be put into identifying antigens that seem to be highly specific, but there are already some great approaches targeting them.

For instance, we have experimented with affinity-tuning CARs, modifying scFvs, trying to introduce switches to turn things 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 – 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 do you require for the CAR-Ts to 'see' the right target? We've experimented with tumor-associated antigens, which are highly associated with the tumor, but they're also present in normal tissue – so what's the ratio there? What specifically do you need to be very sensitive about? Where are the cut-offs?

Tuning 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 bid to decrease off-target activity. There are many very clever approaches to overcoming this issue, but it remains a central question – one we're going to have to focus on as we move forward.

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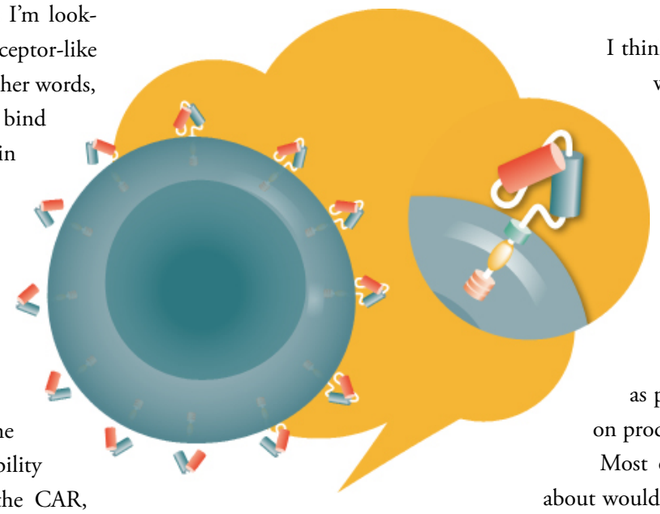
JK: I agree. Identifying a unique target is very important, but so too is fine-tuning the specificity or affinity. There have been many different approaches to this – of late, people have trended towards humanised CARs, which come with a slew of both advantages and disadvantages. The signalling part 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 tumour cells, although that will be less of an issue with solid tumours, 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 which cytokine do we want to use? People have used IL-2, IL-7, IL-15, IL-21, and combinations of these.

That brings is to transduction, which I think is one of the key bottlenecks in manufacturing, especially when 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 a specific 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 lentiviral vector (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 player in this field. We've focused a lot on creating this idea of an 'off-the-shelf' approach, which is 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, so you go back to the source. 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. So now you're looking at specific approaches that fit a particular patient population of interest, which might not be the same route you would use for PDMCs.

An off-the-shelf source would allow you to standardise characterisation. We modify these cells. let's not forget that – we

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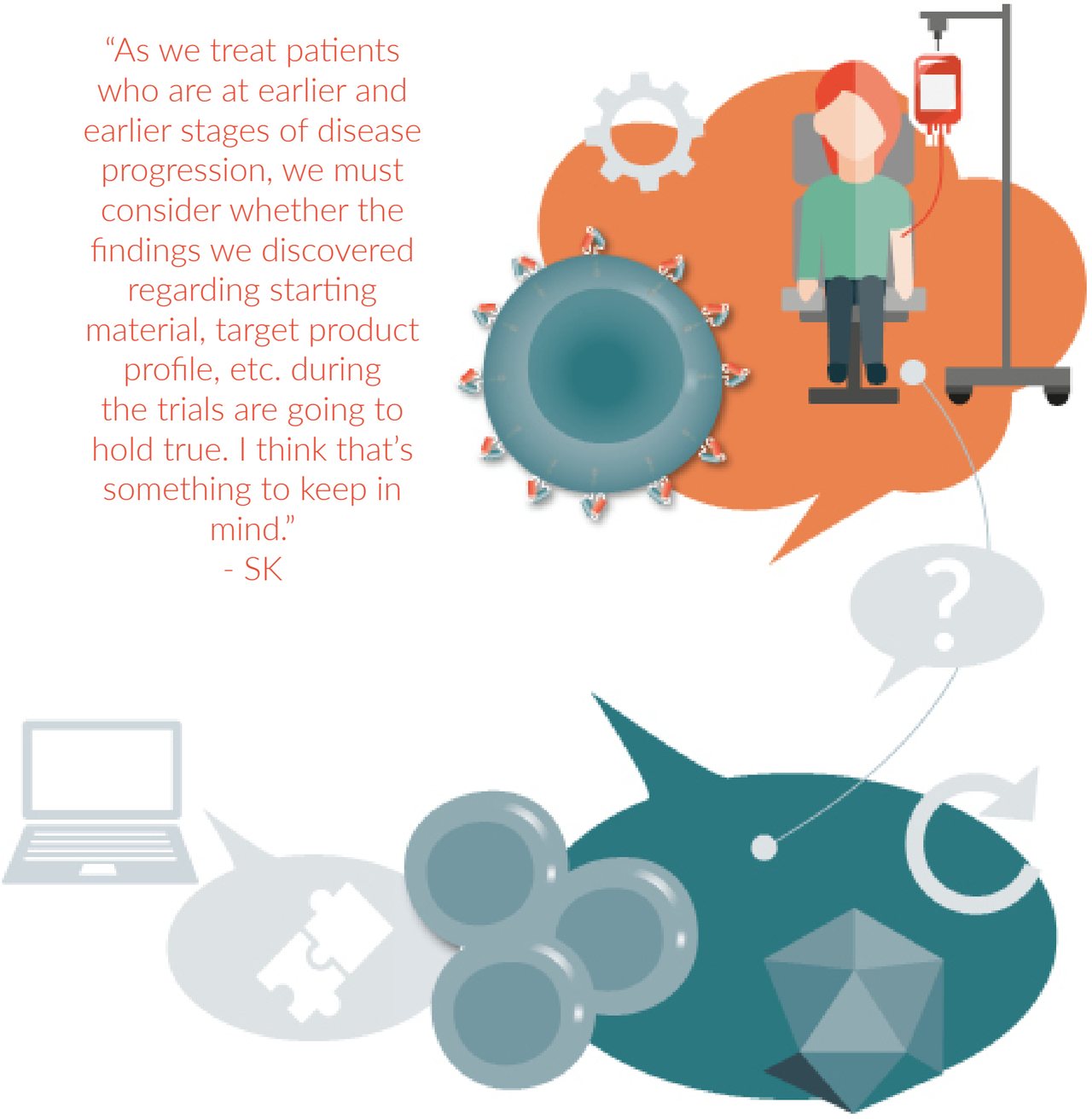

modify them with CRISPR or TALEN or LVV, or whatever the choice of tool may be – and it's conceivable that it's within our obligation to also investigate whether the desired modifications are are the only modifications that happen in the genome. This kind of question can be better addressed with a source that can be fully characterised then frozen to be used 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 co-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 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 TET2 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?

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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 create a lot of 'noise', making it difficult to distinguish what is relevant from what is 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 are relevant changes, 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, despite the challenges, it's headed in a good direction, overall, and we're learning from a different angle.

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 tumours. 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 are using PDX models, which uses patient-derived tumour cells that have been grown *in vitro*, or passaged in mouse in order to make them grow better.

However, there is discrepancy between the in vitro and *in vivo* assays. The antigen expression could differ in a 3D versus a 2D culture. Sometimes you see excellent CART activity in an in vitro 2D culture, but once you go *in vivo* with the same cell line you don't see any antigen expression. That's probably because when the tumour cells are growing in 3D, the antigen expression is either lost or decreased. There's certainly the possibility of it changing.

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

Regarding other *in vivo* models for predicting tumour microenvironment, we mostly use immune-deficient mice, such as NSGs, to test the CART activity. However, these mice are actually deficient in IL-6- and IL-1-producing macrophages, so they don't really represent a typical tumour microenvironment 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 group in New York that used immunotolerant beige mice, but they infused CARTs through the IP route only in those mice with high tumour 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's not just generating the data, but how can you analyse it in real time fashion to really make actionable decisions?"
> - SK

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

TL: I think another thing we often consider, but which is incredibly difficult to model *in vivo*, is that patients come to immunotherapy having undergone 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 variables exist, we just can't necessarily replicate them accurately in our models.

Having said that, I do think many of our models give us good understanding of the challenges we're dealing with. For instance, the TME characteristics. We know that different solid tumors originate in different organs, different cell types. They evolve differently, they create different internal structures the immune cells will encounter. 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 immune 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 until we move to in-human trials.

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> - JO'R

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

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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.

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 extensive modifications and characterisations.

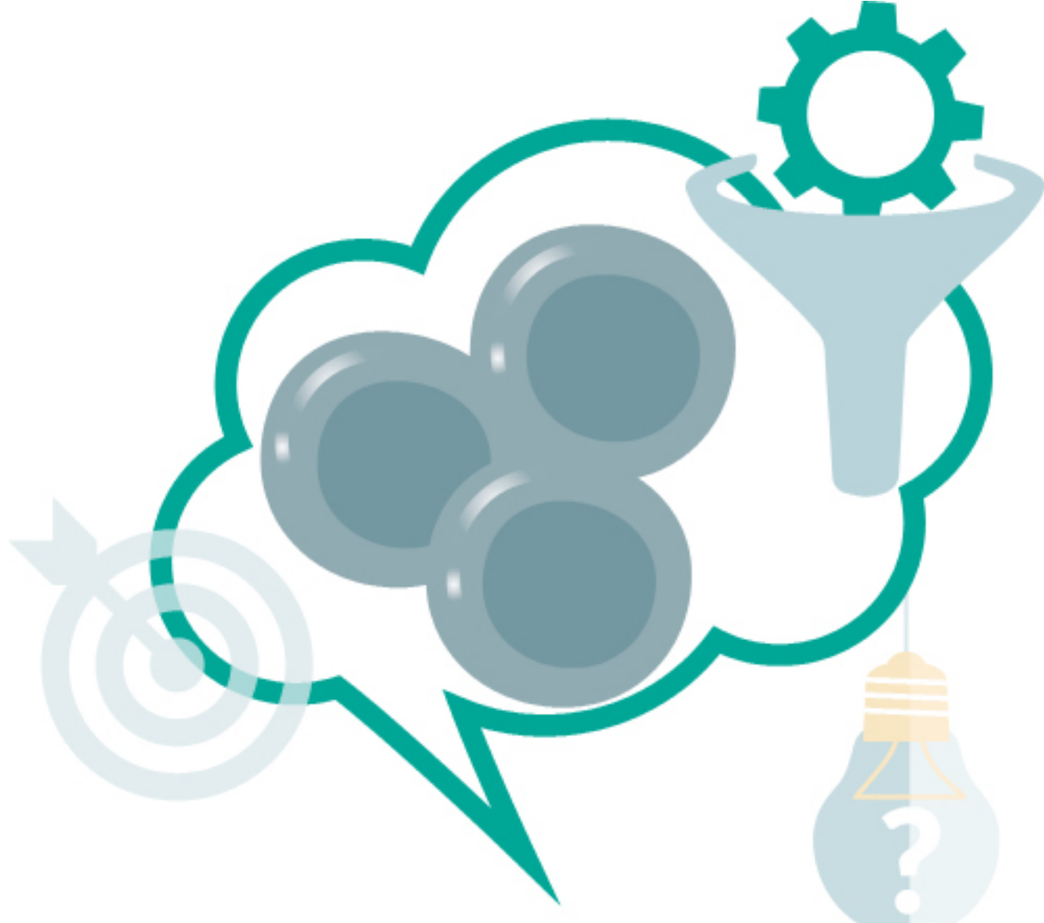
I think once these elements are figured out, then time from bench to bedside will be reduced, along with cost.

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, and 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 the direction of creating reliable, time- and cost-effective analysis pipelines.



"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

"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"
- SK

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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-1 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

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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.

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 identify the 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 BCMA CAR for multiple myeloma. The idea being that BCMA 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

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