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IMMUNO-ONCOLOGY INSIGHTS

SPOTLIGHT ON:

Modality/platform development trends

Guest Editor: David Dilillo, Senior Director, Immuno-Oncology

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"These are truly exciting times in the immuno-oncology research field, as seemingly innumerable novel platforms and modalities emerge and enter the clinic for crucial proof-of-concept studies."

FOREWORD

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For decades, cancer treatment was largely defined by non-specific therapies, such as radiation and chemotherapy, that were associated with significant side-effects. However, with the more recent advent of immunotherapy, we're witnessing an unprecedented explosion of novel technologies and investigational agents that have been designed to harness the power of the immune system to specifically attack malignant cells, leading to promising responses and more manageable side effects. In this issue of *Immuno-Oncology Insights*, our contributing authors share their perspectives and expertise on a number of emerging platforms for engaging the immune system to target tumors.

Cell therapies have become a leading platform for re-targeting immune cells to recognize and kill tumor cells. In the chimeric antigen receptor T (CAR-T) cell space, multiple autologous T-cell therapies have been approved, including anti-BCMA CAR-T cells (Abecma®) for the treatment of multiple myeloma developed by the team at 2seventy Bio. In this issue, Steve Shamah, head of oncology research at 2seventy, discusses the innovative technologies that his team is developing to tackle challenges associated with targeting solid tumors. He stresses the importance of understanding the tumor indication that they are trying to cure in order to define the key problems, which may be solvable using 2seventy's sophisticated cell-engineering toolbox. This toolbox includes 'flip-receptors', dual-targeting strategies, gene editing, and combinations with biologics being developed with their collaborators at Regeneron.

Blair Madison, Chief Scientific Officer at Poseida Therapeutics, shares a Viewpoint on key aspects of developing gene-edited, off-the-shelf cellular therapies designed to evade detection by the host immune system to enhance persistence. He defines key criteria that should be considered for maximal safety and efficacy of a CAR-T product, and suggests that Poseida's site-specific transposition technology meets these criteria.

Continuing with the adoptive T-cell therapy theme, Frank Borriello and James Lederer of Alloplex Biotherapeutics discuss their unique SUPLEXA cell platform, which is a non-engineered, multifaceted cellular approach that provides a robust anti-tumor response with natural immunostimulatory mechanisms. SUPLEXA cells are an autologous mixture of NK cells, $CD8^+$ and $CD4^+$ T cells, TCR γδ T cells, and NK T cells that have been 'trained' on tumor cells engineered to express a combination of immunomodulatory ligands. The authors discuss the potential therapeutic advantages of Alloplex's mixture of non-engineered immune cells and highlight the strong safety profile that has been demonstrated in patients to date.

Switching gears to protein-based biologic therapies, Rony Dahan, Principal Investigator at the Weizmann Institute of Science, discusses how antibodies can be engineered to enhance both their anti-tumor activities as well as for increased safety. He speculates that the next-generation of antibody-based therapies will achieve both of these goals through more complex structures, including multi-specifics, Fc-engineered molecules, and antibodies conjugated to various payloads. For example, Dr Dahan's work has demonstrated that agonistic anti-CD40 antibodies can be Fc-engineered for enhanced anti-tumor activity, or can be engineered to deliver CD40 agonism to specific cell types to reduce undesired toxicities associated with non-specific CD40 agonism.

These are truly exciting times in the immuno-oncology research field, as seemingly innumerable novel platforms and modalities emerge and enter the clinic for crucial proof-of-concept studies. We look forward to learning from these important studies that will, in turn, inform the next generation of immunotherapeutic platforms designed to treat patients with malignant disease.

BIOGRAPHY

DAVID J DILILLO is a Senior Director in the Immuno-Oncology department at Regeneron Pharmaceuticals in New York, where he leads a team developing novel immunotherapies to treat cancer. His team is responsible for target discovery and the preclinical development of immune cell-engaging multi-specific antibodies to treat liquid and solid tumors. His team also develops cell-based therapies and works to understand detailed mechanisms-of-action of immunotherapies in order to advance next-generation therapeutics. Dr. DiLillo holds a Ph.D. in Immunology from Duke University, where he studied non-classical B cell effector functions, and he completed his post-doctoral studies in Fc-receptor biology at the Rockefeller University.

AFFILIATION

David J DiLillo
Senior Director, Immuno-Oncology,
Regeneron Pharmaceuticals

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FASTFACTS

AI pathology in immuno-oncology research

Marija Pezer, Senior Scientific Project Manager, Aignostics

The tumor microenvironment is an important area of research in immuno-oncology for elucidating the complexities of cancer and potentially discovering patient populations that are more likely to respond to existing and/or novel therapies. Here we present a proprietary method called 'label extraction' to extract immunohistochemistry or multiplex immunofluorescence-based labels which are used as 'ground truth' to train models which predict cell status in hematoxylin and eosin stains alone. The resulting models can be deployed to detect and classify different cell types in the tumor microenvironment of clinical sample cohorts, the data for which is used in downstream spatial biology analyses for correlation to clinical outcomes and hypothesis generation.

ADDRESSING THE COMPLEXITY OF THE TUMOR MICROENVIRONMENT WITH AI

Aignostics have developed a platform to allow researchers to view and measure features and biomarkers of the tumor microenvironment (TME). Using this platform, disparate data sources such as immunohistochemistry (IHC) and multiplex immunofluorescence (mIF) images can be overlayed with the same-section hematoxylin and eosin (H&E) image to create more precise algorithms which generate reproducible results in an efficient, scalable and contextual manner. These methods can provide key information about the spatial heterogeneity within the TME by combining image analysis with spatial feature statistics in H&E-stained tissue images **(Figure 1)**.

Figure 1. Viewing platform hosting H&E, IHC and mIF images to provide context and display the output of AI algorithms at scale.

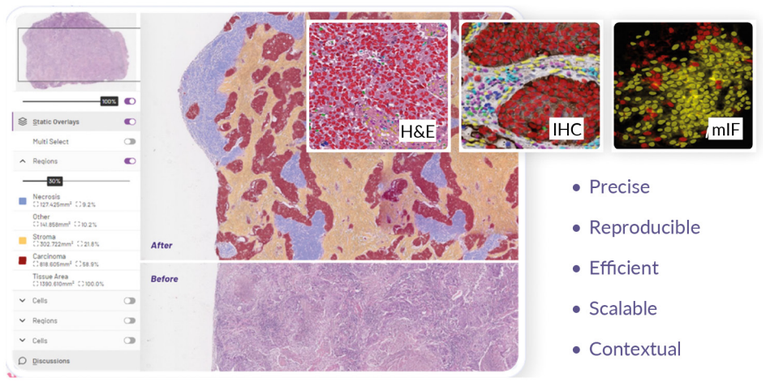
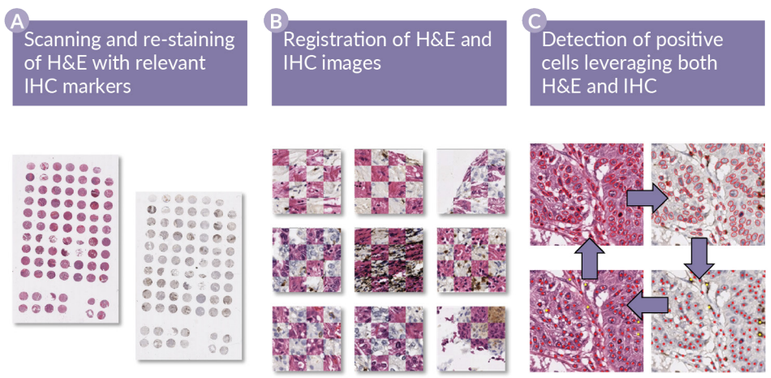


Figure 2. Aignostics' proprietary 'label extraction' for AI training.



'LABEL EXTRACTION' APPROACH

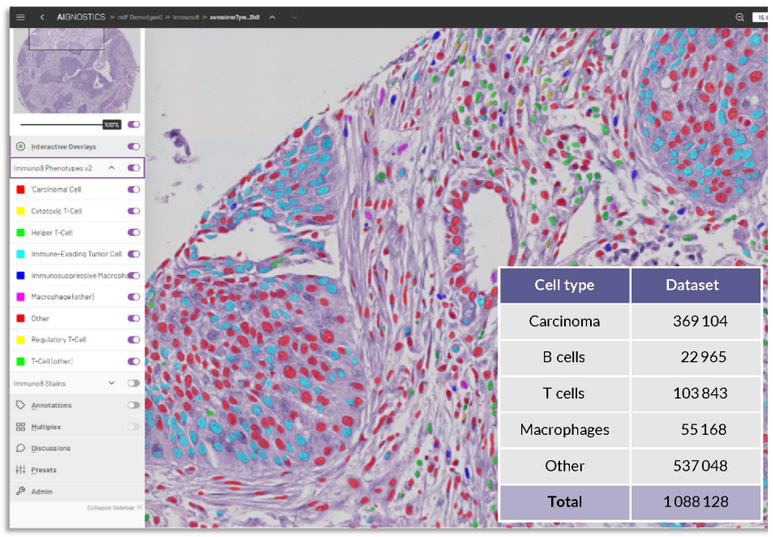
In 'Label Extraction', signals generated from cell specific biomarkers in IHC or mIF stained images are used to establish ground truth labels to train H&E models. In this way, the resulting models have a higher degree of accuracy than labels which are generated via manual pathology annotations made directly from the H&E. As shown in **Figure 2**, H&E stains are bleached before re-staining the same section with a cell specific IHC assay. The positive signal from the IHC is then digitally transferred (extracted) to the original H&E image and these labels are then used to train the H&E model. The performance of the final model is dependent on four main criteria:

1. IHC optimization
2. Same section (not consecutive) H&E & IHC stains
3. Precise overlay of the two images and
4. Machine learning model optimization

H&E-ONLY MODEL TRAINING WITH mIF-INFORMED 'EXTRACTED LABELS'

The image in **Figure 3** is a representation of mIF-informed labels which were generated from the mIF image at scale. These can also be used to train cell classification models, which predict TME status in H&E images alone. The table highlights the scale of data collection from this tissue microarray core where every data point on the slide is collected and exported – for example, into a .csv file – to perform spatial and correlation analysis with drug effects.

Figure 3. Extraction of labels from mIF images can also be used to train models to better discriminate different types of immune cells.



Cell type	Dataset
Carcinoma	369 104
B cells	22 965
T cells	103 843
Macrophages	55 168
Other	537 048
Total	1 088 128

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In partnership with: aignostics

INTERVIEW

Discovering cellular immunotherapy approaches in solid tumors & beyond

Roisin McGuigan, Editor, *Immuno-Oncology Insights,* speaks to (pictured) **Steven M Shamah,** Senior Vice President, 2seventy bio, about his work driving an early discovery engine to explore novel cellular immunotherapy approaches in I–O.

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Q Can you tell me a bit about your current role, and your key areas of focus?

SS: I head up the Oncology Research group at 2seventy bio, a group that drives the company's early discovery engine. There is a tremendous amount of innovation happening within my group and across the company as we build unique and impactful products, especially across the cellular engineering, assay development, and process development innovation areas. We are integral to bringing new targets and programs into the company and driving

those programs forward through to the clinic. We don't do this alone, and there is a tremendous amount of cross-functional interaction, but we are at the beginning of it all.

Q What differentiates 2seventy bio's approach to cellular immunotherapy from others in the space?

SS: Our fundamental philosophy is that we believe that T cells can cure cancer. It is our mandate to unleash that power. We believe that autologous cell immunotherapy is here to stay; it has been proven to be successful and is already benefiting thousands of patients.

We take an approach that forces us to understand the biology of cancer. We spend a lot of time understanding the indication that we are trying to cure in order to define the problems to solve. We also have a sophisticated toolbox to generate the products that will ultimately solve that problem. Although our company is relatively new and we take a startup mentality, our sophisticated toolbox is one aspect that differentiates us. Alongside our toolbox, we have the downstream functions and the built-in infrastructure to then bring those products forward into the clinic.

What makes my team so energized about what we do is that there are not many places in the field of cell therapy where you can focus on innovative early-stage work, build unique and impactful products, and know there is a full-scale development path forward – including in-house drug product manufacturing – to get these products to patients in need. We learn from our preclinical data sets all the time, but we are also in a privileged position to learn from the data coming out of our clinical programs. Those invaluable learnings help to feedback and cross-pollinate into our earlier stage programs.

Q What do you see as the key benefits and drawbacks when considering autologous versus allogeneic cell therapies for I–O applications?

SS: The advantage of autologous therapies is that we know those therapies are working across multiple indications and are bringing incredible benefit to patients and their families. We are excited about 'off-the-shelf products' just as everyone else is, but there is still a considerable amount of work to be done. Autologous therapies are working in the here and now, and we believe they can work beyond the indications in which they are currently working.

However, there are clear challenges to autologous cell therapy and we're seeing them play out in real time. Manufacturing bottlenecks exist, and the demand is outpacing supply. Our company name, 2seventy, comes from the speed of human thought – neurons transmit signals at 270mph. Time is so important to us and to our patients. If patients are on a waitlist, we have to solve this as a field and we are all motivated to do this as quickly and effectively as possible.

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For this reason, off-the-shelf therapies could be incredibly disruptive to the field in a very positive way, as they would lessen many manufacturing challenges. Allogeneic therapies also have the advantage of utilizing cells that have not been through a battery of treatment regiments that the patients we typically treat have gone through. In terms of the disadvantages of allogeneic therapies, there are two major issues. First, we must be able to exemplify and enable the platform, keeping in mind that it took a long time for us to figure out how to get autologous T cells to work as a viable cell therapy platform. A central challenge for any allogeneic platform is in enabling engineered cells to evade the host immune system. In addition, the platform still must solve the same biological challenges imparted by the targeted indication that autologous therapies have to solve in order to bring impactful benefit to patients. As a result, the challenges to enable allogeneic cell therapies are compounded.

We are particularly interested in *in vivo* delivery, which is a different way to think about off-the-shelf therapies in which it's the vector that is stocked and available on the shelf rather than a cell product. While the mode of delivery changes from *ex vivo* manipulation of a given cell type (e.g. CD8+ T cells) to transduction of cells *in vivo*, the hope is that the engineering cargo, whether it be a chimeric antigen receptor (CAR) or some other vector-encoded enhancement, remains constant to unleash the full potential of the cell therapy. Given the bespoke complexities that each cell chassis presents, the biological problems that we are solving for with autologous therapies might slot more seamlessly into an *in vivo* delivery approach than an allogeneic approach. Clearly, identifying ways to deliver vector with a high-fidelity approach that drives target cell type selectivity is the ultimate challenge for *in vivo* delivery.

Q Looking at the early discovery/pipeline development aspect, what would you name as the most important considerations for success?

SS: First off, it is critical to dedicate the time and effort to understand the key problems to solve for in any given indication. That's true for any modality. In oncology, we know that every malignancy is a little different and the challenges and problems to solve will follow that.

An approach we take in my team is to consider ourselves to be drug hunters. With small molecules and biologics, drug discovery has historically taken a high throughput screening approach that starts with enormously diverse compound libraries and utilizes sophisticated screening platforms and biorelevant assay readouts to identify hits. More recently, display technologies have been developed that utilize selective pressures to drive highly diverse libraries to individual hits that possess specific attributes. The underlying premise from these approaches is that there may be many molecules that drive desired activity, and so casting a wide net by implementing libraries of potential solutions make sense as long as there is a way to identify 'the winner'. Our approach to cell therapy is no different, albeit on a smaller scale. In the case of CAR-Ts, for example, we are building constructs into a cell that encode antibody fragments tethered to activation and co-stimulatory domains through regions that embed in the plasma membrane. This creates a large number of combinations as we consider

"When it comes to understanding the problems to solve and building a product to match that, a good example lies in lymphoma, where CD19 CAR-T cell therapies are proving successful and have changed lives in a positive way."

stitching together these various domains. To find the optimal design, we adopt a drug hunter mentality implementing high throughput screening approaches to find that diamond in the rough. The readout for this type of screening approach has to be biorelevant, so the screening platforms that we've implemented involve primary T cells and miniaturized assays that allow us to measure and identify optimal T cell activation upon target engagement.

Q Can you tell us more about your approach to translating projects from an initial idea to a development candidate and beyond?

SS: When it comes to understanding the problems to solve and building a product to match that, a good example lies in lymphoma, where CD19 CAR-T cell therapies are proving successful and have changed lives in a positive way. Despite this success, we know that a significant fraction of those patients will relapse. They relapse for different reasons, including antigen escape when tumors stop expressing CD19. We know that there are durability and persistence issues as well, and internal mechanisms that are pumping the brakes on T cell activity.

In response to these challenges, we strategically identified ways to create a product that will help those patients who have either relapsed or are refractory to CD19 CAR-T. We developed our bbT369 program that is currently in Phase 1 in the clinic to address those problems. It encompasses dual targeting orthogonal to CD19, but like CD19, both targets are highly expressed in non-Hodgkin's lymphoma. Even if patients have relapsed because they have lost CD19 expression in those malignancies, bbT369 allows for engagement and activation of T cells by two distinct cell surface targets. In addition, bbT369 incorporates synergistic and enhanced signaling, pairing a CAR plus chimeric costimulatory receptor (CCR) which drives both 4-1BB and CD28 costimulatory signaling upon target engagement in an OR-gate configuration. This structure achieves a more robust peak expansion than CD19 CAR-Ts which we expect will drive a more durable T-cell activation phenotype. If we drive peak expansion further and harder upfront, patients are more likely to see benefits for longer periods of time. Finally, we also have a unique gene editing technology in our toolbox which we've used to edit out a target gene called CBL-B, an intracellular mediator of T cell exhaustion, thus potentially driving a more durable functioning therapy. Taken together, the bbT369 product is designed to overcome limitations of existing CD19 CAR-T therapy – a

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great example of the approach that we take in the early stage to understand the problems to solve and address those by engineering new solutions into our pipeline products.

Q What are the biggest challenges still to be addressed when applying cell therapy engineering approaches to solid tumors?

SS: There are a number of challenges, so it is certainly going to take longer for us as a field to solve solid tumors. However, it is not fair to label solid tumors unilaterally, as every one of those is likely to be a bit different. As a field, we cannot try to solve all the problems all at once, but it is still important to understand them.

Working from the outside in, stromal barriers exist external to the tumors themselves which often make it difficult for T cells to even reach the target. Once through the stromal barrier, cytotoxic T cells have to be able to identify the majority of tumor cells via specific cell surface antigens. In solid tumors target heterogeneity is a major challenge, as targets that are homogenously expressed throughout the tumor are scarce and quite distinct from the expression profiles of CD19 and B-cell maturation antigen (BCMA) in certain hematological malignancies. Beyond targeting, any number of suppressive factors exist in the TME which serve as barriers to cytotoxic T cell activity and also need to be confronted. We know that driving a robust expansion of T cells once administered is key to driving clinical responses. In B cell malignancies, for example, the levels of CD19 antigen in the bloodstream are significant and helps to drive that early expansion. We do not expect to be the beneficiaries of that in the solid tumor space given the reduced access that T cells have to target, so we need to think about other ways to help drive their expansion.

A good example of our approach to treating solid tumors is in our MAGE-A4 program, wherein we are engineering T cells with a highly selective TCR against a MAGE-A4 peptide presented on the surface of numerous solid tumors in the context of HLA 02:01. Other groups are also focused on this target due to its attractive on-tumor versus off-tumor expression profile. As we studied the target indications we wanted to pursue and thought about the other challenges to solve for, we leveraged the knowledge that transforming growth factor beta (TGFβ) is expressed with high prevalence in those indications and is an important immunosuppressive factor. Therefore, we have engineered a 'flip' receptor into T cells co-expressing the MAGE-A4 TCR that allows us to turn the tables on TGFβ, converting what would otherwise be a suppressive signal into potent T cell activation. This is a Regeneron-partnered program that will be accelerated into the clinic in China through a subsequent partnership with JW Therapeutics. We anticipate learning much from this initial clinical proof of concept for the flip receptor technology and look forward to bringing those learnings back from the clinic into Research where we can identify other programs where it might be impactful. This kind of reverse translational 'bedside-to-bench' approach is going to be critical for us to learn about where we're having success against solid tumors, and where we need to continue to push to address remaining obstacles. There is no question that it is a conglomerate of issues that we and others are dealing with when trying to develop impactful

approaches to treat these types of malignancies, but I do believe that in time, cell therapy will be highly impactful for patients with solid tumors.

Q For cell immunotherapy, how can we make cells that address the known immunologic barriers posed by the TME?

SS: As discussed above there are a multitude of barriers that exist in the TME and for immunotherapy to be successful, we are likely to require a multitude of solutions, including novel targeting approaches, boosting intracellular signaling pathways, and engineering T cells to produce and secrete factors such as interleukins that can be impactful in neutralizing and/or counteract the TME. However, one needs to be careful about the uncontrolled secretion of some of these factors due to the risk of severe toxicities. Thus, devising ways to control or regulate the release of these factors is going to be important.

We are developing on a number of flip receptors that can be engineered into T cells to convert inhibitory signals into ones that activate. As mentioned above, one that we have created is CTBR12, a chimeric receptor that consists of the extracellular ligand binding domain of the TGFβ receptor coupled to the intracellular signaling domain of the IL-12 receptor. Upon binding to TGFβ, the CTBR12 flip receptor induces a potent intracellular IL-12 signaling cascade which drives cytotoxic T cell activity. This lets us 'flip the script' on the otherwise suppressive activity ordinarily imposed by TGFβ by connecting it to IL-12 signaling, which promotes T cell activity. We're also able to reap the T cell intrinsic benefits of IL-12 without releasing it into the circulation where it has proven to elicit toxicities in the clinic that have been challenging to manage. There are numerous other negative factors that exist in the TME that can be converted into positive signals by taking similar approaches.

Gene editing is another important tool that can be used to address immunologic barriers – and we have a proprietary approach in our MegaTAL technology that allows us to selectively knock out genes that would otherwise convey inhibitory signals to cytotoxic T cells. It is likely that approaches to step on the T cell accelerator while simultaneously pumping the brakes on the suppressive TME will be essential to yielding impactful therapies for patients.

Q Turning to combination therapies, what progress is being made in developing and rationalizing combination approaches that include cellular therapy?

SS: The immune-oncology space is flooded with a multitude of combination approaches that, in many cases, have been executed on simply because they have been available to the field. Combinations need to be hypothesis-driven and we are excited about the potential to achieve targeted and impactful products by combining cell therapy products with biologics.

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> " The early pipeline work is focused on beginning to explore and understand what it takes to make an engineered T cell work against solid tumors in an impactful way and to bring that benefit to patients."

With our partners at Regeneron, our work revolves around the belief that these types of combinations can bring great benefit to patients, in particular in the treatment of solid tumors. We have recently expanded the 2seventy/Regeneron partnership built on the belief that biologics can orthogonally engage and enhance engineered T cells. Regeneron is one of the leaders in the biologics space, with a repertoire of bispecific antibodies and checkpoint inhibitors that can complement and, in some cases, synergize with cytotoxic T cells to drive more robust anti-tumor responses. We are working with Regeneron to understand the specific areas of biology that each of these combinations can address and incorporating them into product concepts across our partnered portfolio.

The initiation of our new 270-mph internal drug product manufacturing facility this year will be integral to our approach to understanding which biologic and cell therapy combinations can have the most clinical impact. The facility will accelerate product development by deepening process and product understanding and correlating patient profile and outcome with process data. The result is the operational realization of a unique 'ask-answer' engine for 2seventy bio and a significant value proposition for our Regeneron partnership: an opportunity to sample and test out hypotheses regarding drug product manufacturing processes and modality combinations *ad libitum* in the clinic. We believe that this approach will enable a level of ideation and iteration in the clinic that will ultimately yield the optimal drug product and drug combination for our patients.

Q What are your predictions for the future of autologous cell therapy? What progress do you hope to see in the next 5-10 years?

SS: We believe autologous cell therapy has already become a major modality of cancer treatment, and we believe that the modality is here to stay. My first hope is that in 5–10 years we will have learned enough from our own and our peers' work to be able to cure patients in the solid tumor arena. Recently published data demonstrating a 63% overall response rate (including nine complete responses) from GD2 CAR-T therapy treating pediatric neuroblastoma patients provides the type of encouragement to suggest that we're on the right path [1]. With enough collective shots on goal, we are all learning about what it takes. This drives me and keeps me motivated. The early pipeline work is focused on beginning to

explore and understand what it takes to make an engineered T cell work against solid tumors in an impactful way and to bring that benefit to patients.

Another aspect to consider as we work to get these therapies to all patients in need is that, over time, these therapies are going to be approved into earlier lines of treatment. For example, we recently published results in the New England Journal of Medicine regarding our KarMMa-3 trial revealing promising data using Abecma, our BCMA CAR-T, in earlier lines of treatment for multiple myeloma patients [2]. Continued success in this space bodes as a bit of a double-edged sword: most importantly it translates to greater benefit for patients, but clearly will also increase the pressures on solving for current manufacturing bottlenecks. Once we achieve our goal, the goalposts move – but we're excited to accept that challenge in order to help more patients. It is my expectation that in 5-10 years the field will have made great strides toward solutions for these significant manufacturing challenges.

BIOGRAPHY

STEVE SHAMAH is head of oncology research at 2seventy bio and held the same position previously at bluebird bio. Steve has over 20 years of experience driving innovative science in biotech settings including Obsidian Therapeutics where he led the Research team working on next generation, regulated CAR T cell-based products, and at X-Body Biosciences, building a fully human antibody discovery company later acquired by Juno Therapeutics. Prior to X-Body, Steve made significant contributions to the scientific progress at various startups in the Boston/Cambridge area, including Archemix Corp., Hydra Biosciences, and Phylos, Inc. Steve earned his Ph.D. in biological chemistry and molecular pharmacology from Harvard Medical School and his B.S. in neuroscience from the University of Rochester.

AFFILIATION

Steve Shamah, PhD
Senior Vice President,
2seventy bio

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Advancing best-in-class allogeneic CAR-T therapies

Blair B Madison
Chief Scientific Officer, Gene Therapy,
Poseida Therapeutics, Inc.

"Typically, the targeted inactivation (knockout) of key genes is the go-to approach to remove mediators of alloreactivity."

VIEWPOINT

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For allogeneic chimeric antigen receptor (CAR)-T cell therapies interactions between the host immune system and engrafted T cells must be significantly minimized for safety and better durability. Typically, the targeted inactivation (knockout) of key genes is the go-to approach to remove mediators of alloreactivity. Targeted knockouts are often achieved with a site-specific nuclease (SSN). Site-specific RNA-guided nucleases (RGNs), like Cas9 [1,2], are revolutionizing therapeutic gene editing in the cell and gene therapy sectors of

modern medicine. Approaches for gene edited CAR-T therapies are rapidly advancing since the first Cas9-engineered clinical trial in treatment-refractory cancer [3], especially with the development of base editing [4], prime editing [5], Cas9 variants [6,7], and the high-fidelity Cas-CLOVER nuclease developed at Poseida Therapeutics, Inc. [8]. Nucleases that create double-stranded breaks (DSBs) are repaired through the nonhomologous end-joining (NHEJ) pathway, while nicks generated by base (or prime) editors are repaired though the template-directed single-strand break repair (SSBR) pathway. Either approach can provide multiplex editing for CAR-T engineering [9], but safety concerns arise as nucleases can yield high rates of translocation and high rates of off-target editing [10–12], while base editors can yield off-target mutations in both RNA and DNA [13,14].

Such unintended mutations can increase the risk of cellular transformation or cause cellular senescence and poor efficacy. These risks necessitate precaution, careful design, and diligent safety evaluations. The fidelity of an SSN is certainly a central consideration; however, several other key criteria should also be considered for maximal safety and efficacy of a CAR-T product. These include:

1. The cell cycle status at the time of gene editing;
2. The efficiency of CAR transgene delivery and gene editing, and;
3. The proportion of early memory CAR-T, especially stem cell memory T cells (T_{SCM}), in the final product.

These key criteria, elaborated below, are each integral to the performance and success of our platforms and processes used at Poseida to engineer allogeneic CAR-T therapies.

GENE EDITING IN RESTING NON-DIVIDING T CELLS

Cell cycle status of target cells is highly relevant for minimizing unintended genotoxicity, regardless of the fidelity of the genetic engineering technology. Although NHEJ repair will be functional in both dividing and non-dividing T cells, there are advantages to performing gene editing in quiescent T cells. This is because DSBs created by SSNs in dividing cells are frequently not repaired prior to chromosomal segregation (especially if the DSBs occur just after DNA replication or just before cytokinesis) [15]. In turn, such DSBs can cause the loss of a chromosomal arm that lacks a centromere, leading to micronuclei formation and chromosomal instability. Even with a hypothetical nuclease with perfect fidelity, this remains to be a risk in dividing T cells. When using the high-fidelity Cas-CLOVER nuclease in non-dividing cells we observe an incredibly low rate of chromosomal translocation and excellent maintenance of genome integrity [8]. This underscores the need to perform precise gene editing in resting non-dividing T cells.

EFFICIENT CARGO DELIVERY & GENE EDITING

While RGNs have been developed for robust cutting and ease of programming, cargo delivery at DSBs is generally inefficient, especially using non-viral approaches. Non-viral approaches with donor repair templates typically yield only 4−12% efficiency [16], although some ssDNA approaches can yield 30−40% CAR knock-in efficiency [17]. Current Super piggyBac (SPB) transposase variants developed at Poseida yield highly efficient transposition into the genome (more than 50% average efficiency to date for P-BCMA-ALLO1, an allogeneic CAR-T product candidate partnered with Roche being developed to treat patients with relapsed/refractory multiple myeloma). The piggyBac® DNA Modification System generates no DSBs, has a large cargo capacity (10 kb and higher) [18,19] and a favorable insertion profile, with integration preferentially in intergenic regions [20–22], and a less intragenic profile compared to lentiviral vectors [21,23]. Such a profile, coupled with the low vector copy number (average of 2–4), mitigates

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the risk of insertional mutagenesis and cellular transformation. The large cargo capacity enables the addition of a selection cassette (yielding more than 99% transposed cells) and an inducible safety switch for the rapid elimination of cells, if needed [8]. This occurs in the context of highly efficient Cas-CLOVER-mediated editing, with 90% or higher editing at *TRBC1*, *TRBC2*, and *B2M* [8]. For Poseida allogeneic CAR-T therapies, the manufacturing process is completely non-viral, using electroporation to deliver the nucleic acids encoding Cas-CLOVER and the SPB transposase, and a plasmid bearing the transposon cargo, all into non-dividing resting T cells.

T_{SCM} PRESERVATION & ENRICHMENT

These key criteria (low genotoxicity and highly efficient engineering in non-dividing cells) lay the foundation for preservation and enrichment of the favorable T_{SCM} subset in Poseida CAR-T products. In addition to driving more robust activity, the T_{SCM} subset possesses greater self-renewal and long-term engraftment potential [24–27]. T_{SCM} cells also have high telomerase activity [28–33], which will mitigate the potential for telomere shortening and replicative senescence and boost replicative potential. Thus, it is no surprise that multiple efforts have been described to preserve or enrich for this valuable T cell subset [28–31]. One additional benefit of the piggyBac DNA Modification System is that transposition occurs preferentially in naïve and T_{SCM} subsets relative to more differentiated T cells. This feature, combined with efficient engineering of resting T cells, drives the favorable phenotypic composition of Poseida's final CAR-T product, which currently consists of 40–60% T_{SCM} cells [8].

Ultimately, the next advance in allogeneic CAR-T therapies may involve simultaneous gene editing and CAR delivery via targeted knock-ins. This can be achieved by integration into the TRAC locus via homology directed repair, as demonstrated using Cas9, Cas12a, or homing endonucleases [34–36]. However, reliance on homology-directed repair for knock-ins will require the manipulation of dividing cells, with a high risk of chromosomal translocations. Thus, we need to maintain the development of new technologies beyond nucleases. A site-specific integrase or transposase could conceptually capture all the benefits of site-specificity, without the downsides of a nuclease. Such technologies, like a site-specific transposition technology in development at Poseida, would be the obvious choice for gene edited therapies, especially given the inherent risks of DSBs generated by a site-specific nuclease, even with 100% fidelity.

BIOGRAPHY

BLAIR MADISON is the Chief Scientific Officer of Gene Therapy at Poseida Therapeutics and holds a BA in Biology from Washington University and a PhD in Cell and Molecular Biology from the University of Michigan where he studied how developmental pathways control epithelial proliferation and morphogenesis. After continuing investigations into mechanisms regulating epithelial-mesenchymal crosstalk in his postdoc at the University of Pennsylvania, he transitioned to lead R&D efforts at Transposagen Biopharmaceuticals under Dr Eric Ostertag, the founder of Poseida Therapeutics. Dr Madison led the development of early enhancements of the piggyBac DNA transposon for functional genomics applications and in related efforts also studied the transcriptional regulation of the LINE1 retrotransposon. Following work on the role of Let-7 microRNAs in carcinogenesis pathways Dr Madison pursued academic research as an NIH-funded principal investigator at Washington University. There Dr Madison studied how onco-fetal microRNA pathways regulate stem cell specification and control of epithelial carcinogenesis using organoid, CRISPR, GEMM, and piggyBac transposon models. Dr Madison joined Poseida Therapeutics in November, 2019 and has helped advance allogeneic CAR-T programs and has led the development of key platforms, including the Cas-CLOVER nuclease, and site-specific super piggyBac for applications in gene and cell therapies.

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AFFILIATION

Blair B Madison, PhD
Chief Scientific Officer,
Gene Therapy,
Poseida Therapeutics, Inc.

AUTHORSHIP & CONFLICT OF INTEREST

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INTERVIEW

Creating the next generation of antibodies for I–O

Roisin McGuigan, Editor, *Immuno–Oncology Insights*, speaks to **Rony Dahan,** Principal Investigator at the Weizmann Institute of Science

DR RONY DAHAN is an assistant professor at the Department of Systems Immunology, the Weizmann Institute of science. He completed his BA in Molecular Biology at the Technion-Israel Institute of Technology in 2004. Dr Dahan completed his PhD in Molecular Immunology at the Technion in 2010. He served as a Cancer Research Institute Irvington Postdoctoral fellow in Cancer Immunology at the Laboratory of Prof Jeffrey Ravetch at Rockefeller University in New York from 2013 until joining the faculty of the Weizmann Institute of Science in August 2017. Dr Dahan has been awarded several U.S. patents for antibody-based immunotherapies. His translational work in the immunotherapy field includes licensing of technologies from his lab to pharmaceutical companies, collaborations and consulting positions in pharma and biotech companies. His prizes include the Technion's Presidential Excellency Award (2004), the Pollack Prize for Academic Excellence (2007), the Fulbright Foundation Doctoral Dissertation Fellowship, the Sanford Kaplan Prize for Creative Management in 21st Century High Technology (2011), the Immune Therapies Training Award of the Juvenile Diabetes Research Foundation (2011), the Hershel Rich Technion Innovation Award (2011), the Israel Cancer Research Fund (ICRF) Career Development Award (2018), the Harry J Lloyd Charitable Trust (HJLCT) Career Development Award (2019), and the Melanoma Research Alliance (MRA) Young Investigator Award (2019). Dr Dahan is the incumbent of the Rina Gudinski Career Development Chair.

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Antibodies such as PD-1/PDL-1 checkpoint inhibitors represented a huge breakthrough for cancer immunotherapy – but what's next for antibody-mediated immune therapies in cancer, and which approaches hold the potential to move the needle towards improved safety and efficacy in the clinic? We spoke to Rony Dahan, Principal Investigator at the Weizmann Institute of Science, about his lab's work on the next generation of engineered antibodies, and his predictions for the future of the space.

Q What is your lab currently focused on?

RD: In my lab, we aim to better understand the factors that limit an effective anti-tumor response in general, and those that contribute to an effective anti-tumor response in response to immunotherapies. We study the mechanism of action of immunotherapies that are already approved, such as programmed death (PD)-1 inhibitors, and also potential targets of immunotherapy that show preclinical promise but have yet to be translated to humans.

One of our approaches is to look at the detailed mode of action driving some of the most promising antibodies. This includes exploring how the antibodies act at the tumor microenvironment (TME), and what critical factors for response we are missing in terms of secondary events, interactions between immune cells, and the identity of different participating cell types and cell states. Achieving such a new understanding and mechanistic insight into these factors gives us new knowledge and the opportunity to optimize these antibodies. We then utilize antibody and protein engineering techniques to design such next-generation therapies.

For example, one major effort in my lab is to attain better understanding of the role of the Fc domain in checkpoint antibodies, and how these antibodies interact with Fc receptor pathways. Once we better understand those pathways, it is possible to harness them and engineer the Fc domain to exhibit more precise properties, resulting in significant improvements in tumor reactivity.

Another major issue, which is related mostly to agonistic antibodies, is the issue of dose-limiting toxicities that limit clinical use. These toxicities limit the maximum tolerated dose to a very low level, far below the optimal biological dose, and before they can induce effective anti-tumor immunity. By understanding cellular pathways that lead to anti-tumor activity and efficacy, we are able to use antibody engineering to produce bispecific antibodies and give them enhanced selectivity for the cell types required for efficacy without toxicity, thereby increasing the therapeutic window.

To summarize, our aims are to attain new mechanistic insights into how antibodies used in immunotherapy work, how efficacy can be enhanced while limiting toxicity, and then find solutions from the antibody engineering world to generate antibodies with enhanced potency and improved therapeutic window.

Q What for you are the most promising approaches that have the potential to move the needle for antibody-based cancer immunotherapy?

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"...the next wave of immunotherapies will be a new generation of antibody-based drugs that have a more complex structure: bi- and multi-specific antibodies that have a more complicated mode of action, thanks to their multiple specificities."

RD: I predict that the next wave of immunotherapies will be a new generation of antibody-based drugs that have a more complex structure: bi- and multi-specific antibodies that have a more complicated mode of action, thanks to their multiple specificities.

Fc-engineered checkpoint antibodies that harness Fc-mediated activity on top of their Fab-mediated activity result in improved efficacy in preclinical models and are another promising approach evaluated currently in clinical trials. In addition, chimeric antibody-based molecules, such as cytokine-conjugated antibodies (immunocytokines) and ligand-conjugated antibodies, that work through induction of multiple beneficial pathways, are emerging as promising approaches for inducing potent antitumor immunity.

Q What is that current state of play for the development of myeloid-targeted immunotherapies?

RD: Myeloid cells are an extremely heterogenous population of cells with different functions, and they can have significant effects on tumor growth and the immune response against tumors.

One relevant and important function of myeloid cells, especially dendritic cells, is their role as antigen presenting cells; priming and activating the T cell response against the tumor. These activities of dendritic cells can be leveraged either by antibody-mediated therapies or cellular therapies, to potentiate the T cell response against the tumors.

On the other hand, there are many myeloid cell populations in the TME that promote the disease progression and limit the repose to immunotherapies. They either directly promote the growth of the tumor, or support the immunosuppressive microenvironment, and thereby suppress the immune response against the tumors. As our knowledge continues to increase, myeloid cells represent very promising targets for different therapeutic approaches targeting suppressive cells in the TME. However, this has not yet been achieved. To date, there are more failures than successes in terms of clinical translation – but we're still at an early stages, and hopefully new clinical data will emerge from different approaches to help us better understand the potential of drugs that enhance the infiltration of myeloid cells, that promote their proliferation and differentiation, or that enhance their anti-tumor immune-stimulating effect or make them less suppressive.

Q What about obstacles facing the development of safer and more effective CD40 agonistic antibodies?

RD: CD40 is a great example of an important target for dendritic cell activation and myeloid targeting, but generating the right drugs that can fully harness the potential of this target has proved challenging.

Looking back at the last 20 or 30 years of research and at more than a decade of clinical trials, I believe we can highlight two major challenges. The first is efficacy; many of the antibodies that were introduced to clinical trials were just not efficacious enough. They were poor agonists, and were not able to activate the full potential of CD40 signaling. A linked issue is related to Fc interactions and the IgG scaffold. This is due to the role of the Fc receptor in cross-linking the CD40 agonists in order to cluster CD40, which is a requirement for intercellular signaling of CD40. We did a lot of work during my post-doc with Jeffrey Ravetch at Rockefeller University to better understand the human Fc receptor requirements for anti-CD40 antibodies, and how we can engineer their Fc domain. This resulted in second-generation Fc-engineered human CD40 agonists that have enhanced ability to cluster cell surface CD40, and that are therefore very potent agonists.

There are also Fc receptor-independent approaches to increase the agonism of anti-CD40 antibodies. These are alternative antibody engineering approaches to achieve the key issue here of generating super agonists that have enhanced capacity to cluster CD40 to achieve maximum efficacy.

This leads us into the second challenge, which is currently more challenging and significant: the matter of dose-limiting toxicity. Even if once we have available super agonist or a very potent Fc-engineered antibody, treatment is still limited by this toxicity. You will achieve doses that result in toxicity long before reaching the optimal biological doses that are needed for an effective anti-tumor response. The field must address the challenge of dose-limiting toxicity, and several solutions that have been proposed are in preclinical and early clinical testing.

One approach is intertumoral administration, i.e. using low and safe doses that are injected directly into the tumor. This approach can avoid systemic toxicity, but can still attain very potent local activation of dendritic cells that results in systemic and abscopal effects. This is very promising for some indications – for example in bladder cancer you can use intravesical injection, via a catheter, for local administration. For other clinical indications, intertumoral administration is more challenging.

Another approach is to generate bispecific reagents that couple CD40 agonism with targeting of tumor antigens. Such reagents can be injected systemically, but will be directed by the anti-tumor arm so it can be mainly located in in the TME so safely activate dendritic cells.

A study from our lab suggested using a bispecific antibody not to target a selective tissue such as the tumor, but rather to deliver the antibody to selected cell types. This is based on our mechanistic studies showing that type 1 conventional dendritic cells (cDC1) must be engaged by CD40 agonists in order to achieve anti-tumor activity in different tumor models. We also demonstrated that several other myeloid cell populations that express CD40 and are engaged by these antibodies result in toxicities. By understanding that different cellular

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"We will see improved clinical results from new and additional combinations of existing antibodies that work together to result in better efficacy."

pathways lead to efficacy versus toxicity, we engineered bispecific antibodies that only engage CD40 signaling in cDC1, the cell type that lead to anti-tumor efficacy. We recently demonstrated in a mouse model that such cDC1-selective CD40 bispecific antibodies increase the therapeutic window compared to the traditional approach of using CD40 agonistic monoclonal antibody. This bispecific approach also allows the antibody to be administered systemically, which is an advantage and more practical for clinical translation.

Q If the current challenges can be overcome, what potential advantages can CD40 agonist approaches offer?

RD: The interaction of CD40 and CD40 ligand is an important central pathway in the immune synapse during the early priming of the T cell response by the dendritic cells. Unlike checkpoint inhibition that affects the exhausted T cells, manipulating CD40 signaling can prime the early response. This can be very beneficial in relatively 'cold' tumors that exhibit low infiltration of T cells. An agonistic antibody can prime T cell activation in the context of a tumor antigen, and thereby increase their infiltration to such tumors. You also have great synergy with, for example, PD-1, and additional inhibitory checkpoints that act at later timepoints to engage exhausted T cells. I see this as is a great opportunity for controlling very hard to treat tumor types, and patients who are not responding to currently available immunotherapies.

Q What are be your predictions for the antibody-based immunotherapy space as a whole in the next 5–10 years?

RD: We will see improved clinical results from new and additional combinations of existing antibodies that work together to result in better efficacy – this is something we will probably see in the outcomes from clinical trials that are currently ongoing.

What I think is most promising and most exciting is this new wave of engineered antibodies including multi-specific, bispecific and protein-conjugated antibodies, that bring novel biological mechanisms to the table.

Q In that same timeframe, what will you be focusing on?

RD: Based on our mechanistic insights we suggested some ideas for engineering antibodies to achieve improved responses, for example the cell-selective bispecific CD40 antibody, which we are now working to translate into a fully human, optimized, cell-selective agonist. Looking forward, we hope to see it enter clinical trials, and determine how well our preclinical models were able to predict an increased therapeutic window.

In addition, I hope to see the next wave of Fc-engineered antibodies harnessing Fc receptor pathways translated to clinical trials. Seeing how all of these ideas and proofs-of-concept in preclinical models from the lab translate to clinical efficacy will be very exciting.

AFFILIATION

Rony Dahan, PhD,
Principal Investigator,
Weizmann Institute of Science

AUTHORSHIP & CONFLICT OF INTEREST

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IMMUNO-ONCOLOGY INSIGHTS

MODALITY/PLATFORM DEVELOPMENT TRENDS

SPOTLIGHT

COMMENTARY

Alloplex Biotherapeutic's SUPLEXA cells represent a new type of autologous adoptive cellular therapy for cancer

Frank Borriello & James A Lederer

SUPLEXA immunotherapeutic cells are derived by activation, differentiation, and expansion of cancer patient peripheral blood mononuclear cells (PBMCs) by an 'training' melanoma tumor cell line that has been engineered to express multiple immunomodulatory factors. SUPLEXA cells generated by our manufacturing process develop into potent and broadly cancer reactive cells that do not damage normal cells or tissues. Our SUPLEXA cellular therapeutic approach is currently being tested in a Phase 1 metastatic cancer clinical trial in Australia. The majority of the first 20 metastatic cancer patients, which had progressive disease (PD) have shown disease stabilization without any drug related adverse events. Furthermore, several patients have reported improved quality of life. In addition to tumor size measurements, we performed comprehensive, longitudinal single-cell PBMC profiling and plasma cytokine measurements of enrolled patients as a measure of changes in immune health over time. Patients with stable disease (SD) showed marked changes in specific immune cell type abundances and altered circulating cytokines that are indicative of improved immune health. These laboratory observations serve as pharmacodynamic markers of SUPLEXA activity, which will be used clinically to optimize the dosing schedule and select the target cancer patient population most likely to benefit. The lack of negative adverse event observations will facilitate the developmental path for SUPLEXA cellular therapy with feasibility to explore combinations with other cancer therapies without concern about compounding side effects. Our basic insights into the biology of SUPLEXA cells strongly suggest that our SUPLEXA cellular therapy approach is a novel and multivalent personalized cellular therapy with potential for treating multiple types of cancers.

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The cellular therapy space has witnessed an explosion of innovation beginning with CD19 targeted CAR-T cells for the treatment of leukemias and has been extensively reviewed [1–4]. These first-in-class autologous therapies involved the transduction of T cells obtained from a patient's peripheral blood with a lentiviral vector encoding a chimeric antigen receptor (CAR). These first-generation CARs encode a type I transmembrane proteins comprised of an extracellular scFv domain with antibody-like specificity to the CD19 antigen linked to a transmembrane segment and an intracellular signaling domain which activates T cells following CAR engagement with its cognate ligand. While the CD19 target protein is broadly expressed on acute leukemia cells, it is also found on normal B cells, which means that all B cells are also eliminated upon treatment with CAR-T therapeutic cells. Fortunately, it has been established that long term B cell depleting therapeutics such as the anti-CD19 mAb Rituxan® is well tolerated. These early efforts have led to the commercialization of three therapies in the CAR-T class, including Novartis/University of Pennsylvania (Kymriah®), Bristol Meyers Squibb (BMS)/Juno Therapeutics (Breyanzi®), Gilead/Kite (Yescarta®) and has initiated many follow-on CAR-T programs among various biotechnology companies using updated CAR design elements and technologies [3,5].

While these early CAR efforts focused on cells of the adaptive immune system, more recent efforts, have explored leveraging the arguably superior inherent features of innate immune cells to counter tumors by CAR engineering strategies in NK, iNKT, γδ T cells, macrophages and B cells [6–9]. In addition, induced pluripotent stem cells (iPSC) have presented an unparalleled opportunity which has been exploited to accommodate significantly more complex multi-step engineering approaches that extend beyond a single CAR [10–12]. For example, knocking out β-2 microglobulin to reduce HLA Class I expression to reduce host versus graft rejection of allogeneic immune cells [13,14]. iPSCs have the virtue of being infinitely expandable and able to support multiple genetic manipulations leading to the development of stable cell lines that can then be differentiated to the desired immune cell type just before administration to a patient. While it remains to be proven that iPSC derived immune cells are functionally equivalent to their naturally occurring counterparts, especially those mechanisms involving immune memory, they clearly can mediate anti-tumor activity. Cells of the innate immune system also avoid the requirement for HLA matching, which makes them better candidates for allogeneic cellular therapies.

Despite a demonstrable level of anti-tumor activity by cells produced by these varied approaches, solid tumors remain substantially recalcitrant to cytolysis via these approaches. One major hypothesis to account for this *in vivo* resistance is that the tumor microenvironment (TME) of various cancers is a major impediment to cellular therapeutics gaining access to the tumor [15–17]. Major efforts have been made to address modifying the tumor microenvironment to make it more susceptible to immunotherapy [18,19]. Furthermore, the TME is enriched by immunosuppressive cells such as Tregs, MDSC and M2 macrophages, all of which produce immunosuppressive cytokines like TGF-β and IL-10 that would suppress most immune cells even if they did gain access to the tumor. The problem is further compounded by the possibility that different solid tumor types may have different immunosuppressive mechanisms of action in their TME and elsewhere [20], which may impede a single, one cell type cellular therapy approach as well. Beyond the choice of effector immune cells to be deployed against cancer, the most limiting feature for any CAR based approach is the need for a specific target protein that distinguishes tumor cells from normal tissues. CD19 is an example of a tumor target that is shared with a dispensable normal cell type, but most tumor target antigens are expressed to some level on normal tissue and are responsible for some of the CAR-based therapeutic side effects.

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In contrast to the various CAR approaches listed above, tumor infiltrating lymphocytes (TILs) have offered a non-engineered autologous approach with the advantageous feature that they are derived from cells that have already demonstrated the capacity to enter and survive in the TME [21,22]. One must however question the hypothesis behind expanding a TIL population that was not able to control the tumor in the first instance. It has been demonstrated that such cells can be functionally impaired [23]. Beyond this theoretical consideration, perhaps the most limiting factor in TIL cell therapy development has been the manufacturing process. For example, since TILs are tumor derived, it is essential to demonstrate no tumor cells remain in the expanded final product since that would be tantamount to providing metastatic cells to the patient. In addition, Lovance, an early leader in the TIL field, has been considerably delayed with issues related to establishing a potency assay acceptable to the FDA and more recently Instil has had to suspend enrollment in its first clinical trial at great reputational cost because of inability to consistently make their cellular product. These difficulties have resulted in TILs having yet to yield a commercial product despite preceding CAR technology by several decades [21].

HISTORICAL CONTEXT

The extraordinary number of approaches being pursued by both academic and commercial groups have spanned the range of cell types (e.g., NK, iNK-T, T cells, macrophages) and sources (e.g., PBMC, cord blood, iPSC) (Table 1). What they have in common however is a focus on a single cell type with a specific engineering concept, be it CAR, genetic insert/deletion, or a combination. Alloplex Biotherapeutics Inc., hereafter referred to as Alloplex, reasoned that picking a single immune cell type or engineering approach was biased by limiting the cellular therapy to a specific antitumor response. Furthermore, we felt that a multifaceted cellular approach against tumor cells would provide a more robust antitumor response and that natural immunostimulatory mechanisms could generate tumor killing cell subsets without genetically engineered enhancements.

Accordingly, Alloplex has focused its efforts on a differentiated approach that was inspired by an earlier generation of cellular vaccine developers. Specifically, GVAX developed by Cell Genesys over 25 years ago utilizing a prostate tumor cell line (PC3) genetically engineered to express GM-CSF (GVAX), an immunomodulatory cytokine that is known to stimulate the maturation and function of dendritic cells (DCs), a type of professional antigen presenting cell [24,25]. The hypothesis driving this approach was that GVAX would be able to release cross-reactive prostate tumor antigens to DCs while simultaneously activating their function and in so doing, lead to a more productive anti-tumor immune response. Early-stage clinical trials demonstrated that GVAX could be used to yield clinical responses in prostate cancer patients. These early data were promising [26] but the limited durability was an impediment to further development, which led to perhaps the most consequential development decision made in the pivotal registration trials; namely to combine GVAX vaccination with chemotherapy [27]. Unexpectedly, the pivotal trials, VITAL-1 and VITAL-2, showed that patients treated with the combination of cellular vaccination and chemotherapy performed considerably worse than patients treated with chemotherapy alone with a shorter progression free survival (PFS) and overall survival OS. Unfortunately, no vaccination-only arm was included in these trials. The outcomes of the VITAL registration trials were deemed a failure with devastating consequences not only for the GVAX developers but also for the field of cellular vaccination approaches for immunotherapy leading to reluctancy in investor support for second generation efforts.

At Alloplex, we interpreted the data for the VITAL trials in a different and more favorable way. We reasoned that if vaccination was not having an effect, then there should

TABLE 1

Representative biotechnology companies pursuing diverse cell therapy strategies.

Therapeutic cell type	Company
Non-engineered trained immune cells	Alloplex Biotherapeutics
CAR-T cells (autologous and allogeneic)	Allogene
	Sana
	Poseida
	Precision Biosciences
	Adaptimmune
TILs (autologous)	Iovance
	Instil
iPSC (allogeneic)	FATE
	Century Therapeutics
	Cytovia Therapeutics
	Shoreline Biosciences
	BrightPath Biotherapeutics
NK cells (autologous and allogeneic)	Nkarta (allogeneic)
	NKGEN
iNKT (autologous and allogeneic)	MINK (allogeneic)
γδ T cells (autologous and allogeneic)	Adicet (allogeneic)
Macrophages (autologous)	Carisma Therapeutics
	Myeloid Therapeutics
B cells (autologous)	Be BioPharma

be no difference in PFS and OS between the two study arms. Instead, we suspect that the GVAX cellular vaccine is inducing an immune response in patients that is being negated by the combined chemotherapeutic treatment specifically eliminated key effector immune cells that were activated to proliferate in response to the vaccine. The elimination of these vaccine activated immune cells specifically hobbled the emerging anti-tumor response. Alloplex used this realization to conclude that a GVAX cellular vaccine approach may indeed induce a beneficial anti-tumor response. This led us to further speculate that if engineered expression of one immunomodulator on tumor cells could provide beneficial immune effects then perhaps it may be justified to test additional immunomodulators both individually and in combination to further augment this tumor vaccine strategy.

SUPLEXA ORIGIN STORY

Alloplex expanded on the seminal GVAX concept by exploring the combinatorial space of a highly curated list of immunomodulatory proteins selected for their ability role in activating complementary immune cell types. Higher order combinations were achieved by a reiterative process whereby multiple rounds of viral transductions were used to introduce immunomodulators into a tumor cell line with *in vitro* testing after each cycle. Using PBMC as the starting material (Figure 1), highly engineered tumor cells called ENLIST (engineered lymphocyte stimulator) cells were tested using *in vitro* mixed lymphocyte tumor reaction (MLTR) assays. The MLTR allowed us to precisely evaluate the biological effects of ENLIST cells on PBMCs by measuring immune cell activation, differentiation, proliferation, cytokine production, and most importantly, cytolytic activity.

We observed that sequential addition of immunomodulators in the ENLIST cells greatly affected PBMC proliferation, cytokine release, and tumor cytolytic activity of the expanded PBMC populations. Our engineering efforts were halted only when we reached the maximum value in the dynamic range of the assays employed. One

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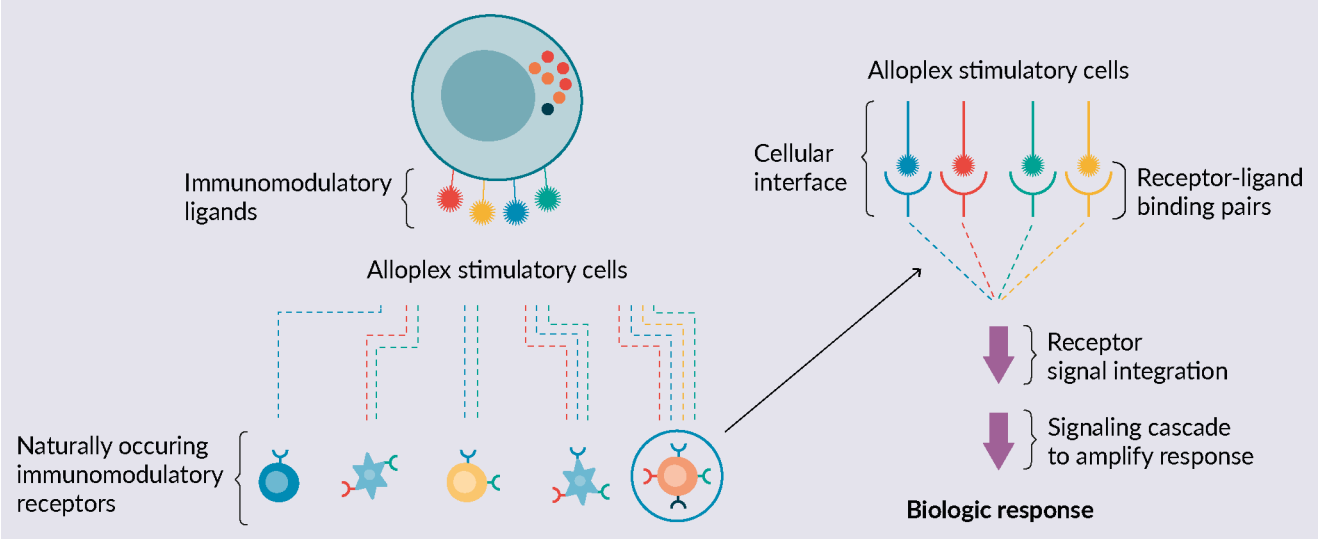
observation of particular concern was a dramatic increase in the inflammatory cytokines released during the MLTR, which we felt might lead to serious adverse events in a cellular vaccine setting. However, we were fortunate that the MLTR approach showed us that *ex vivo* cellular expansion was possible and could be used as basis for a manufacturing process leading to an autologous or allogeneic adoptive cellular therapy for cancer. We called these *ex vivo* expanded cells SUPLEXA therapeutic cells – alluding to the multiple immunomodulators used to activate complementary immune cell types. Incidentally, the name SUPLEXA derives from the word 'suplex', which is a technical term in wrestling to describe an offensive maneuver intended to control the opponent.

During the construction of ENLIST cells, numerous combinations were tested but one set of immunomodulators proved to demonstrate a remarkable and unexpected synergistic activity and is the subject of an issued US patent (US10731128B2). This core set is comprised of a CD28 ligands (CD80 or CD86), OX-40 ligand and CD27 ligand. Each of these ligands showed little individual enhancement of PBMC activity in the MLTR, but when used together, a striking 300-fold induction in the number of CD8 positive, cytotoxic T cells occurs. Indeed, this unexpected synergy demonstrates that this approach for analyzing the combinatorial space of immunomodulators can be utilized to efficiently identify previously unappreciated functionally intersecting or synergistic immune pathways. Using this initial core set as a starting point, Alloplex significantly expanded and refined immunomodulators and moved into a higher order of combinatorial variations. The final combination of immunomodulators used in ENLIST cells and specific cell manufacturing know-how are foundational to Alloplex initiatives in cellular therapy and are proprietary trade secrets. We now refer to ENLIST cells as immune training cells because they have the capacity to train PBMCs to develop into immune cells with potent anti-tumor effector function and phenotype.

Most impressively, SUPLEXA cells demonstrated broad cytolytic activity against all tumor cell lines tested, irrespective of HLA matching or tumor type. This HLA

FIGURE 1

The Alloplex approach.



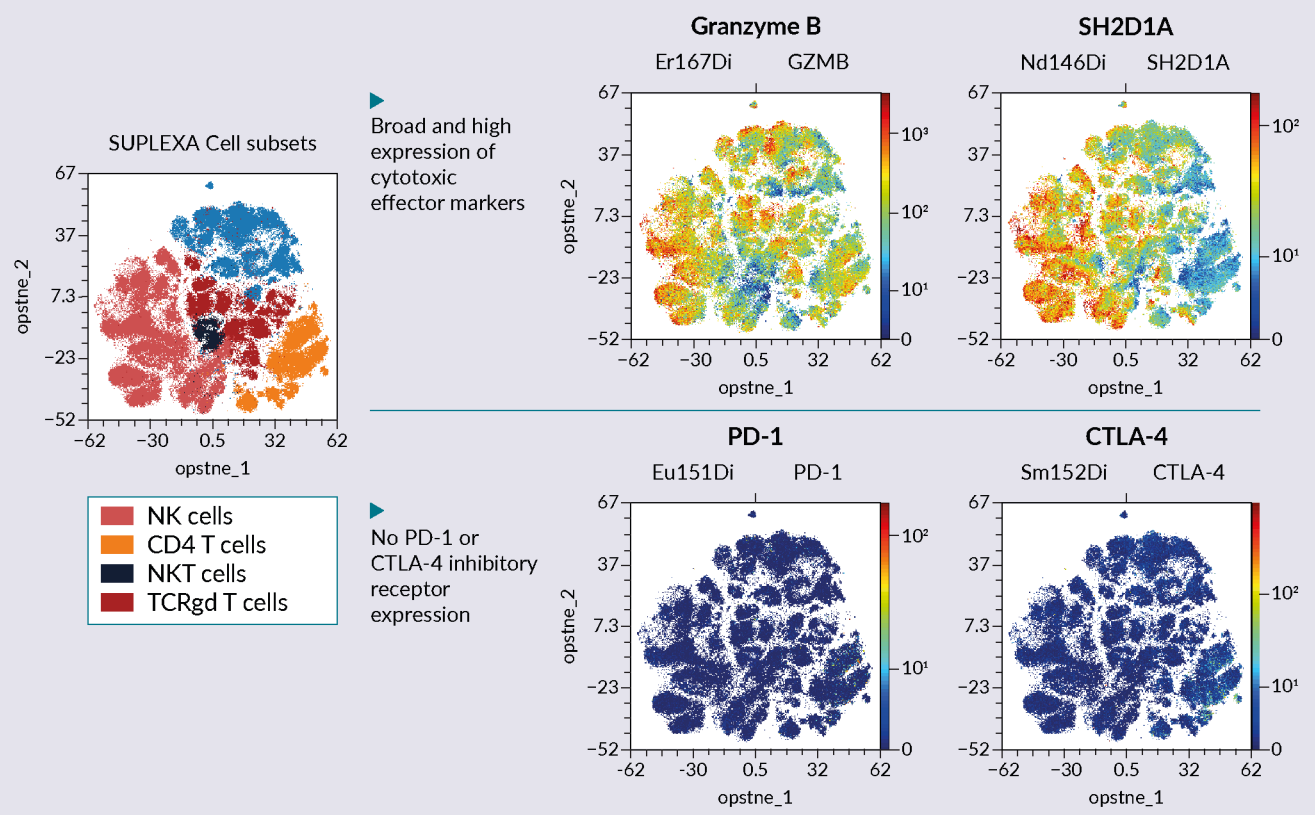
Engineered lymphocyte stimulator (ENLIST) training cells are engineered to express a combination of immunomodulatory ligands designed to stimulate a set of complementary immune cells to acquire tumor killing capacity. These activated immune cells are cytolytic against a broad array of tumor cells but without causing harm to normal cells. It is hypothesized that activation through multiple signaling pathways is integrated and amplified to yield a biologic response such as differentiation, proliferation, and cytokine production.

independence is perhaps not surprising given the large percentage of innate immune cell types such as NK, NK T and γδ T cells that express the NKG2D activation marker, which is known to bind stress signals typically found on the surface of cells undergoing metabolic stress due to infection, cancerous transformation, or senescence. Comprehensive phenotyping by mass cytometry (CyTOF) identified the cellular composition of SUPLEXA cells as a mixture of NK cells, $CD8^+$ and $CD4^+$ T cells, TCR γδ T cells, and NK T cells. Phenotyping by CyTOF showed that SUPLEXA cells express high levels of tumor cytolytic markers like granzyme B and SH2D1A, but not inhibitory checkpoint inhibitors like PD-1 or CTLA-4 (Figure 2). We have performed iRepertoire (Huntsville, AL, USA) T cell receptor (TCR) sequencing analysis of αβ and γδ TCRs in SUPLEXA cells and identified a significant increase in αβ and γδ TCR clonality to suggesting that there may be antigen-specific T cell activation and expansion occurring during SUPLEXA manufacturing. Future work will seek to identify antigen specific mechanisms involved in T cell training by ENLIST cells.

While genetic engineering of the SUPLEXA therapeutic cells was also considered, we found that the broad anti-tumor activity of SUPLEXA cells we reasoned that further genetic engineer of SUPLEXA cells was not necessary. Avoiding genetic engineering allows for a more efficient and less expensive manufacturing process than CAR-T processes. A first-generation SUPLEXA manufacturing process has already been developed for our Phase 1 clinical trial (Figure 3). SUPLEXA cells are autologous and therefore have lower risk for adverse events or clinical complications like graft-versus-host (GVH) disease or host-versus-graft (HVG) rejection

FIGURE 2

CyTOF analysis of SUPLEXA cells.



A composite CyTOF analysis of SUPLEXA therapeutic cells from the first three patients at the end of the expansion process reveals high expression of the cytotoxic effector markers, Granzyme B and SH2D1A and no expression of checkpoint receptors PD-1 and CTLA4.

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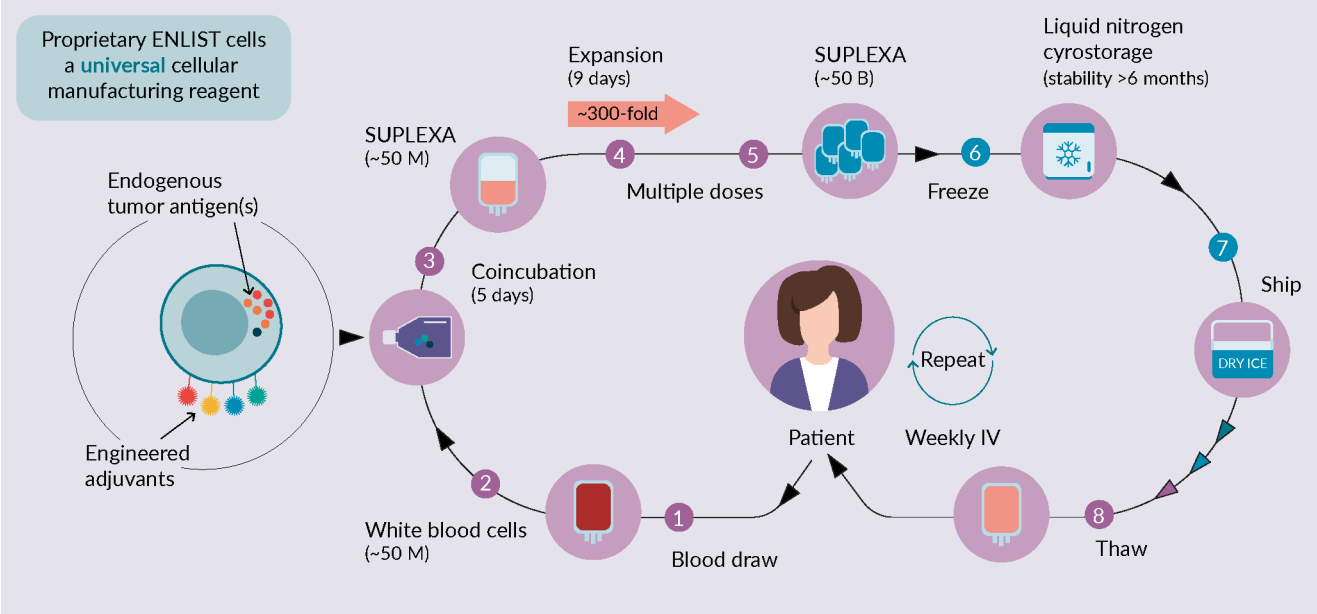
FIGURE 3

The SUPLEXA manufacturing process.



The multi-step manufacturing process begins with 1) a blood draw of about 50 mL, followed by 2) isolation of PBMC using Ficoll density centrifugation, 3) coincubation of PBMC with ENLIST training cells to induce SUPLEXA cells, 4) expansion of SUPLEXA cells with cytokine support by approximately 300-fold, 5) aliquots of SUPLEXA cells into individual IV dosage bags, 6) controlled freezing followed by long-term cryogenic storage in liquid nitrogen, 7) shipping to the clinical site on dry ice and 8) controlled thawing followed by IV administration back to the patient.

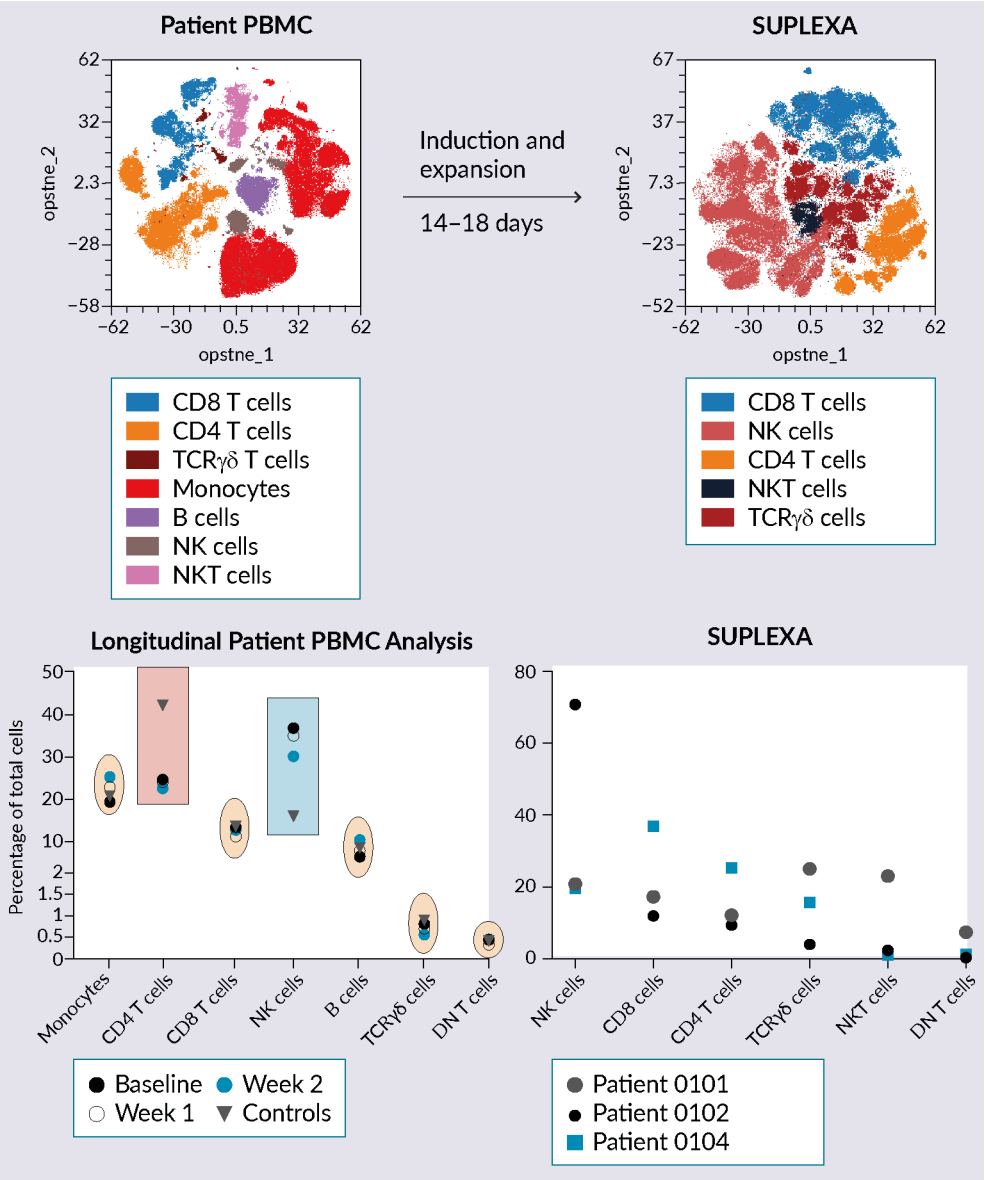
of SUPLEXA cells as compared to allogeneic cellular therapeutics.

Furthermore, by virtue of not being engineered, SUPLEXA cells do not carry the theoretical risks associated with possible tumorigenesis resulting from the lentiviral transformation. SUPLEXA cells develop from the patients' PBMCs by activating naturally occurring receptors functioning at regulated normal physiologic levels. We posit that their anti-tumor activities will also be naturally physiologic when given as a therapeutic cell. By contrast, CAR proteins deliver supraphysiologic signals that can lead to the clinical toxicities such as cytokine release and tumor lysis syndromes. The SUPLEXA therapeutic cells currently in clinical testing are a mixture of cells comprised of NK, NKT and T cells but are notably devoid of Tregs, myeloid and B cells. The exact composition of SUPLEXA therapeutic cells varies among individuals owing to the autologous and personalized approach and the nature of PBMCs from cancer patients that have received diverse prior therapies (Figure 4). Despite a significant level of inter-patient variability in percentage immune cell types, SUPLEXA cells consistently show similar activation morphology, functional phenotypic marker expression, and consistent cytolytic potency profiles. These phenotypes of SUPLEXA are used as measures for our quality control release assay (Figure 5).

By contrasting SUPLEXA cells against CAR-T cells highlights several additional key differences (Figure 6) with implications for clinical trial design and use as a broadly active cancer therapy. It was anticipated for instance that unlike CAR-T cells, SUPLEXA would not induce cytokine storms or work so vigorously as to induce tumor lysis syndrome. Implementation of SUPLEXA in a clinical setting would also prove very different from that of CAR engineered cells because chemotherapeutic preconditioning and systemic IL-2 treatments are often used to foster *in vivo* expansion of a single dose of about 250 million CAR engineered cells [28]. Multiple lines of evidence support the rationale for the use of chemo preconditioning [19,29] but some evidence is

FIGURE 4

CyTOF analysis of PBMC and SUPLEXA therapeutic cells from the first three patients.



DN T cells: Double-negative T cell; NK: Natural killer cells; NKT: Natural killer T cell; PMBC: peripheral blood mononuclear cell;: TCRγδ: T cell receptor gamma delta.
Compared to normal controls, the PBMC analysis of the first 3 cancer patients revealed a lower percentage of CD4⁺ cells both at baseline and over the initial 2 weeks of SUPLEXA cell therapy but an increase in the percentage of NK cells. SUPLEXA cells showed considerable variability in cell composition between patients as anticipated due to individual heterogeneity in patients and the personalized therapeutic approach.

also emerging, which suggests that it may not be necessary [30]. This issue is critical because the chemo preconditioning used with CAR engineered cells comes with significant toxicities, including profound cytopenia. Immune system recovery after cell ablation is a dynamic process that can span years and often does not recover to pre-treatment cellular composition [31]. Since SUPLEXA cells can be expanded to large numbers *ex vivo* without loss of activity and may not require *in vivo* expansion, they can be administered to the patient in ten-fold larger cellular doses than CAR cell therapies. Hence the patient will not experience preconditioning induced neutropenia, lymphopenia, thrombocytopenia. Furthermore, the ability to

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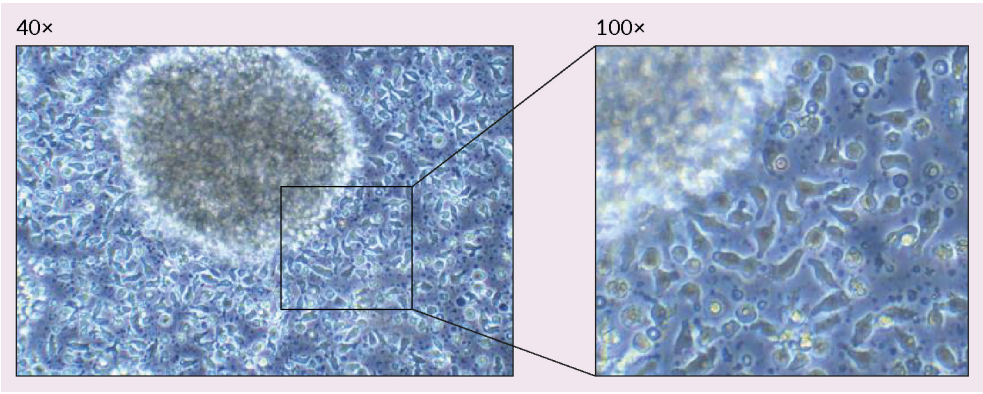
FIGURE 5

Photomicrographs of SUPLEXA cells.



SUPLEXA cells grow in dense clusters and develop elongated forms as shown in the low and high magnified photomicrographs of SUPLEXA cells in culture at 2 weeks after induction and expansion. The morphology of SUPLEXA cells is typical of activated lymphocytes with large oblong polarized cells.

manufacture unlimited numbers of SUPLEXA therapeutic cells allows for the ability to administer multiple doses, something that would not be possible if chemotherapeutic preconditioning were required prior to every dose.

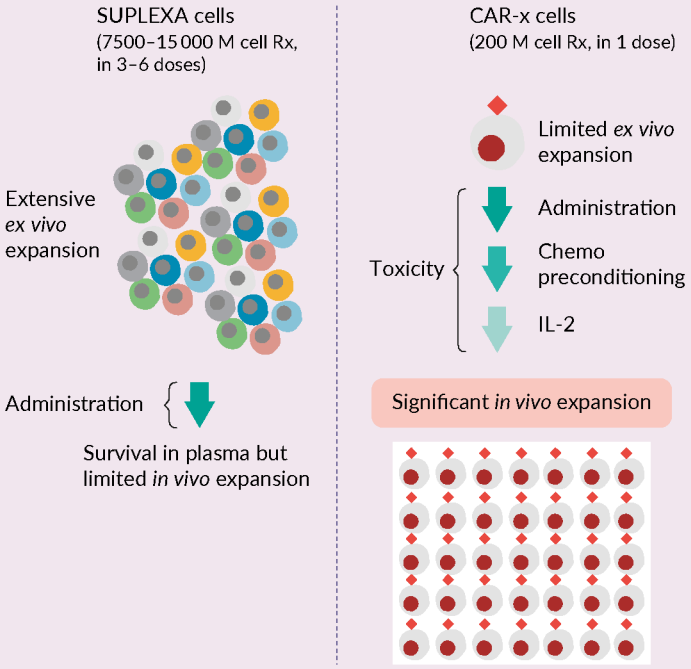
For our initial clinical trial, we adopted a once weekly dosing regimen of 2.5 billion cells for a minimum of 3 weekly doses and this dosing schedule is limited only by the manufacturing yield of the patient's specific

FIGURE 6

Differentiation of SUPLEXA cell from CAR-x cell therapies.

SUPLEXA versus CAR

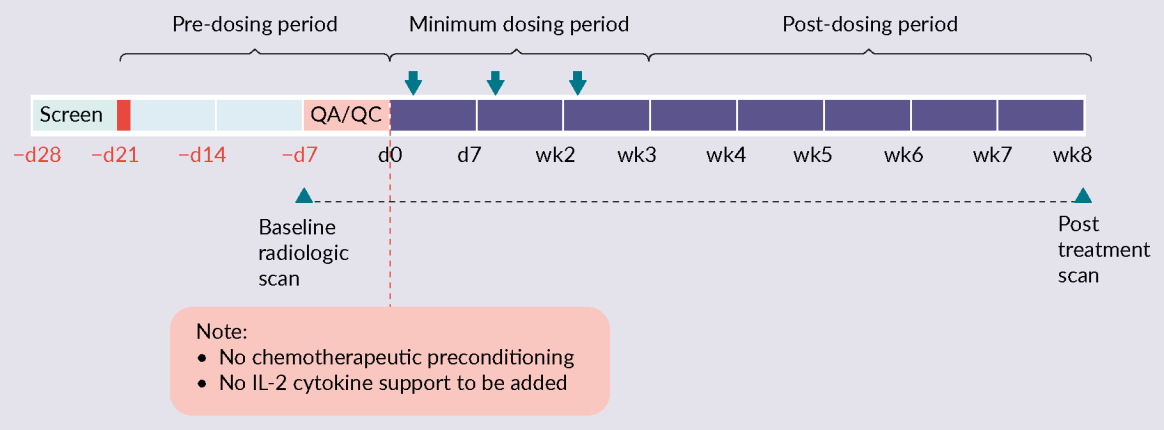
Feature	SUPLEXA	CAR-x
Overall	Non-engineered Hetreogeneity high	Engineered Hetreogeneity limited
Spectrum of activity	Broad pan-tumor	Tumor-type specific
Growth factors needed for expansion	Exogenous only	Exogenous and intrinsic due to CAR signaling domains
Expansion potential	High	High
Administered dose	7500–15 000 M cells	200 M cells max. (Yescarta)
Chemo pre-conditioning	NO No need for additional survival factors or cell expansion	YES Induce homeostatic cytokines and reduce cytokine sinks for greater therapeutic cell expansion



SUPLEXA therapeutic cells are differentiated from CAR modified (CAR-x) cell therapies as listed in the table. Unlike CAR-x cells which are administered in a single dose of 250 M cells, SUPLEXA are administered as multiple weekly doses of 2.5 B cells or 30–60 times as many cells. As such *in vivo* expansion is not essential for SUPLEXA cells and thus does not require chemotherapeutic preconditioning to foster such expansion.

FIGURE 7

SUPLEXA-101 study design.



This is a single agent basket trial designed to enroll metastatic solid tumor patients or those with hematologic malignancy. A screening period 21–28 days prior to first dose confirms patients meet enrollment criteria. Blood is then drawn for SUPLEXA manufacturing approximately 3 weeks prior to the first SUPLEXA dose. The 2-week manufacturing period is shown in blue followed by about 1 week of quality control prior to product release. A minimum of three weekly SUPLEXA doses comprised of 2.5 billion cells is shown although more is possible depending on the manufacturing yield. Importantly, no chemo preconditioning or IL-2 cytokine support is used which spares the patient significant toxicity. Scans are performed at baseline and approximately every 8 weeks afterwards. The focus of this Phase 1 study is safety but has been used to demonstrate single agent activity and for exploratory analyses.

SUPLEXA batch. Having observed no drug related adverse events in the first 20 patients, we are now in the position to explore more intensive multiple dosing regimens and the utility of combining SUPLEXA treatments with already approved anti-tumor drugs such as Rituxan, Herceptin® and checkpoint inhibitor antibodies.

MANUFACTURING PROCESS

The first generation SUPLEXA manufacturing process currently requires approximately 2 weeks of laboratory expansion followed by 1 week each for QA/QC and logistical coordination of delivering the first dose back to the patient. The 4 weeks that patients must wait for their individualized therapy

TABLE 2

Early clinical findings on the first 11 patients.

Patient	Cancer type	RECIST	
0101	Anal canal squamous cell carcinoma	SD	
0102	Ovarian (serous papillary)	SD	
0104	Ovarian		PD
0105	Endometrioid carcinoma		PD
0106	Cervical SCC	SD	
0107	Pancreatic	SD	
0201	Ureteric transitional cell carcinoma		PD
0202	Endometrioid carcinoma		PD
0203	Endometrioid carcinoma of the ovary	SD	
0204	High-grade serous carcinoma of ovary		PD
0205	Bladder transitional cell carcinoma	SD	

The first 11 patients in the SUPLEXA phase 1 study had progressive metastatic disease from diverse tumor types upon enrollment. Each patient received a minimum of 3 weekly SUPLEXA doses comprised of 2.5 billion cells per dose without any reported drug related adverse events. RECIST analysis resulting from imaging approximately 8 weeks after first SUPLEXA dose revealed disease stabilization in a major of the patients (6/11) with the 5 patients showing progressive disease comprised of ovarian or uterine disease, all of which had ascites at the time of enrollment.

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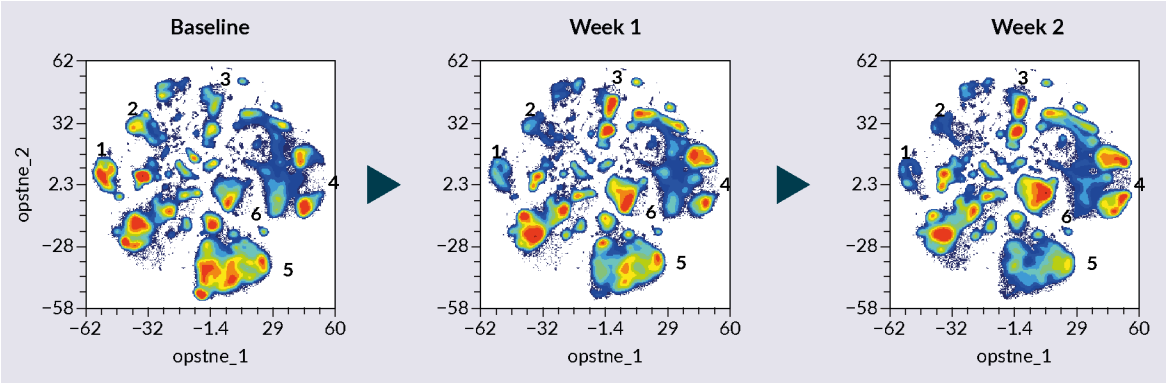
FIGURE 8

CyTOF analysis of the PBMC from the first three patients (0101, 0102 and 0104) over the first 2 weeks of therapy demonstrated favorable changes in the immune cell profile.



Several examples of undesirable cell populations decreasing over time are indicated by number 1 (exhausted CD4⁺ T cells), 2 (exhausted CD8⁺ T cells) and 5, CD16⁺/Arginase-1+ suppressive monocytes. Several examples of desirable cell populations increasing over time are indicated by number 3 (cytotoxic NK-T cells), 4 (activated proliferating monocytes) and 6 (B cells).

is perhaps the biggest current liability of the approach; and one which can lead to anxiety for the patient. In the future, we hope to bridge this waiting period for the patient by using a single dose of allogeneic SUPLEXA cells immediately after drawing blood from the patient.

SUPLEXA cells exhibit individual batch-to-batch variability in immune cell lineages (e.g., NK versus T cell ratio). However, they consistently express an activation signature that is a composite of cytolytic cells (Granzyme A, B, Perforin and Granulysin) and cells that have uniquely acquired an antigen presenting cell like phenotype (HLA Class II, IL-3R, CD28 ligands). Given this observation, we suspect that SUPLEXA cells may be able to present released tumor antigen after the initial cytolysis of a tumor target and thereby activate and amplify the response of host immune cells against the tumor. However, this is the subject of ongoing research at Alloplex that is addressing the biology of SUPLEXA cells. Since these are unique and consistent findings, these activation markers comprise the foundation for a phenotypic release assay conducted on each SUPLEXA batch. Complementary to the phenotypic analysis, each SUPLEXA batch undergoes assessment in a cytolysis assay in which a tumor cell line is employed as a reference target to ensure a minimum level of anti-tumor activity is reached in each SUPLEXA batch.

FIRST SUPLEXA CLINICAL TRIAL

SUPLEXA cells are currently being tested in a Phase I basket trial designed to enroll patients with solid tumors and hematologic malignancies (clinicaltrials.gov, NCT05237206). The trial is enrolling highly pre-treated patients who have progressed through numerous prior therapies and therefore have no standard of care treatment options left. The trail design is shown in (Figure 7). This trial makes use of a first-generation manufacturing open process that begins with about 50 mL of whole blood. PBMC are isolated using standard density centrifugation isolation in a regional manufacturing facility, are activated to differentiate by proliferation attenuated ENLIST cells, and then expanded to yield a minimum of 7.5 billion SUPLEXA cells for administration in 3 or more IV doses of 2.5 billion cells each. The numbers of SUPLEXA doses have varied among enrolled patients (ranging from 3 to 15 doses), which was anticipated given the autologous nature of the starting PBMC material. Despite this

FIGURE 9

Plasma from the first three patients were screened for cytokine levels by 40 cytokine Luminex assays.

The 40 cytokine Luminex panel used to measure plasma cytokine levels included Luminex bead regions to detect the following cytokines: IL-2,TNFα,IL-4,IL-18,IL-1α,IL-1β,IL-1RA,IL-5,IL-10,IL-33,IL-23,IL-22,IL-6,IL-21,IL-8,Tweak,IFNβ,MCP-1,G-CSF,MIP-1α,IFNγ,ST2,GM-CSF,IL-13,Trem-1,MIP-3α,GROα,Rantes,IL-17A,ENA-78,P-DGF-AA,PDGF-BB,MCP-3,MIG,MDC,FLT3L,IL-15,IP-10,TGFβ1, and MCP-2. Inflammatory and immune suppressed phenotypes were detected in these patients and SUPLEXA treatments had effects on systemic cytokine levels in each patient as illustrated in these radar plots. Patient 0101 had high levels of cytokines, while patients 0102 and 0104 had low levels of cytokines. All patients showed increases in IL-8, Rantes, and FLT3L.

intrinsic variability, SUPLEXA cells reproducibly demonstrate phenotypic and cytolytic release assays within acceptable ranges to be used in patients.

The emerging data from the first 11 patients receiving a minimum of 3 doses demonstrates disease stabilization in most patients as determined by the first post treatment imaging time point taken at ~8 weeks post first SUPLEXA infusion (Table 2). Remarkably, this was achieved with no reported drug related adverse events, not even infusion site reactions. The only feature on which patients have remarked is a garlic or sweet corn odor of limited duration, which is likely due to the DMSO in the cryopreservation media. In addition to these early safety and efficacy findings, we are highly encouraged by anecdotal accounts from the nursing staff and patient reports that suggest an overall improved quality of life with instances of increased energy and reduced pain and narcotic use. The trial remains open to enrollment and patients continue to be monitored.

As part of our exploratory studies, patient blood samples were collected over the course of the study and assessed for cellular composition (Figure 8) and plasma inflammatory markers (Figure 9). Surprisingly the first several patients showed an improvement in 'immune health' based on a comprehensive CyTOF phenotypic analysis of longitudinal PBMCs and Luminex cytokine profiling of plasma samples. Interestingly, we found that a patient with high levels of systemic cytokines showed a progressive reduction in circulating cytokine levels, which suggests that SUPLEXA therapy may have significant impact on the pro-inflammatory nature of certain types of cancer (Figure 10). These pharmacodynamic observations provide an unexpectedly important tool for optimizing the SUPLEXA cell dosing regimen and target population. Moreover, these pharmacodynamic measures on overall immune health are especially important for autologous SUPLEXA cell treatment since pharmacokinetic measures are not possible owing to the difficulty of distinguishing SUPLEXA cells

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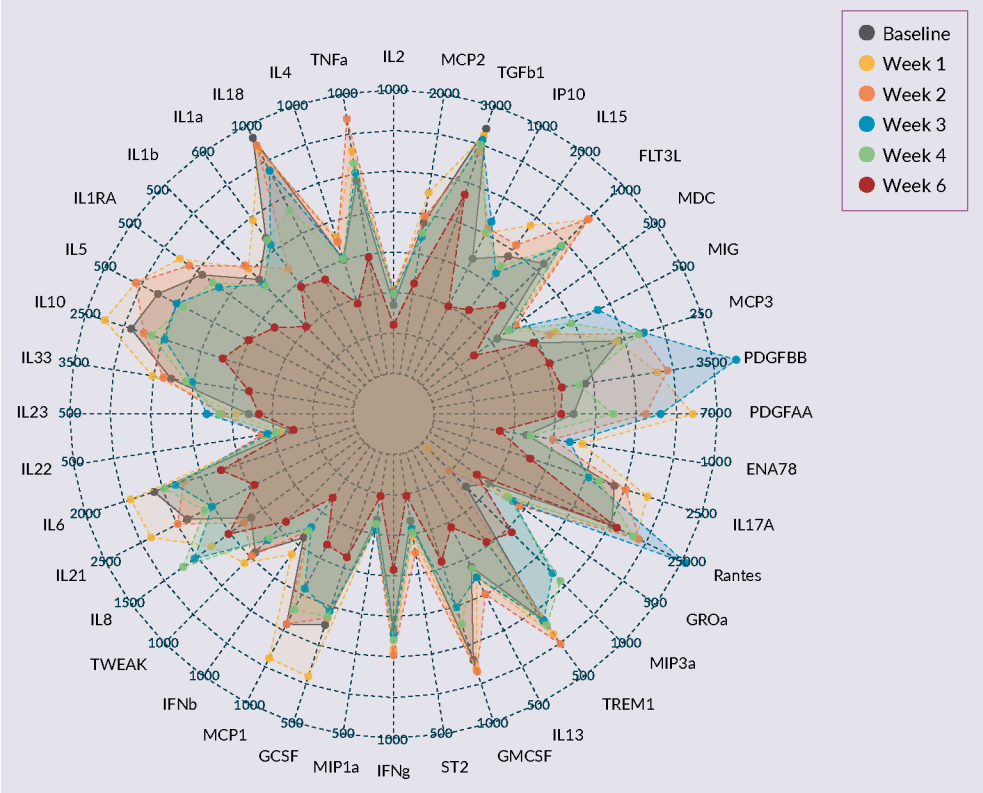
FIGURE 10

In a patient with high baseline levels of inflammatory cytokines, SUPLEXA cell therapy reduced circulating cytokine levels.



Patient 0101, 56- year-old male with metastatic squamous cell carcinoma presented at baseline with uniformly elevated cytokine levels as depicted on this radar plot. This patient received 5 weekly doses of SUPLEXA cell therapy of 2.5 B cells. Shown are progressively decreased inflammatory cytokines at week 1, 2, 3, 4 and 6 weeks following the first dose. Reductions in TNF, IL-6, and IL-1/IL-18 levels are consistent with improved immune health.

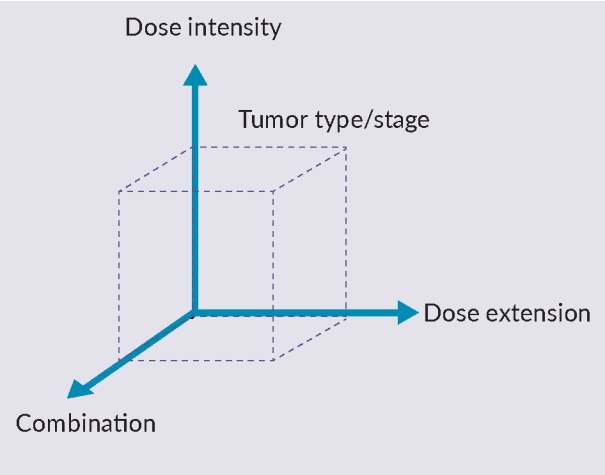
from normal activated cells endogenous to the host. Fortunately, the pristine safety profile exhibited by SUPLEXA so far creates an unprecedented opportunity to consider combination therapies with tumor targeting antibodies as well checkpoint inhibitors/engagers to enhance the efficacy of this autologous cellular therapy.

SUPLEXA cells are a developmental work in progress with many potential applications both in oncology, autoimmunity, infectious diseases, and senescence that will play out over the next 5–10 years (Figure 11). However, near term goals for the ongoing SUPLEXA trial include:

1. Optimizing the clinical SUPLEXA dosing regimen;
2. Exploring a second PBMC-derived SUPLEXA batch for responding patients;
3. Determining which tumor types are most likely to respond;
4. Correlating clinical responses with the pharmacodynamic assessments of immune health;
5. Incorporating validated quality of life measures into our clinical trials, which can capture clinical activity beyond what is apparent in scans and other laboratory assessments;
6. Integrating a single allogeneic SUPLEXA dose to cover the period in which the autologous SUPLEXA cells are being prepared.

FIGURE 11

Diagram of future development for SUPLEXA.



Future therapeutic decision for SUPLEXA will focus on several variables including tumor indication, dose schedule (number and intensity), potential for combination with other therapies, and industrializing the manufacturing process.

CONCLUSION

The Alloplex approach of activating and expanding immune cells by using a highly engineered training cell line represents a novel cellular therapeutic development platform approach with extraordinary promise fueled by our early emerging clinical safety and efficacy data. While the first generation SUPLEXA cells used an unbiased approach resulting in a final product comprised of multiple cell types, we have already demonstrated that the ENLIST immune training platform can be used to specifically enhance B cells, γδ T cells, and Treg cells. In the future, cells enriched for these specific cell types using this approach may have additional specific applications in infectious diseases and autoimmune indications. The pristine safety profile exhibited so far will undoubtedly facilitate the development of SUPLEXA cells either as a stand-alone therapeutic option or in combination with various biologic cancer therapeutic agents such as tumor targeting antibodies, checkpoint inhibitors and cell engagers. While initial SUPLEXA development has focused on an autologous approach, the integration of allogeneic approaches may also be possible in settings where shorter term bridging therapies are required until the patient's own cells can be prepared [32,33]. The enhanced second-generation manufacturing methods will also greatly facilitate future developments by increasing process efficiencies and exploiting economies of scale, critical features for making such therapies more accessible to patients.

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AFFILIATIONS

Frank Borriello
Author for correspondence
Alloplex Biotherapeutics, Inc.,
300 Trade Center, Suite 6580
Woburn, MA 01801, USA

James A Lederer
Alloplex Biotherapeutics, Inc.
300 Trade Center, Suite 6580
Woburn, MA 01801, USA
and
Brigham and Women's Hospital,
Harvard Medical School, USA

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given his approval for this version to be published.

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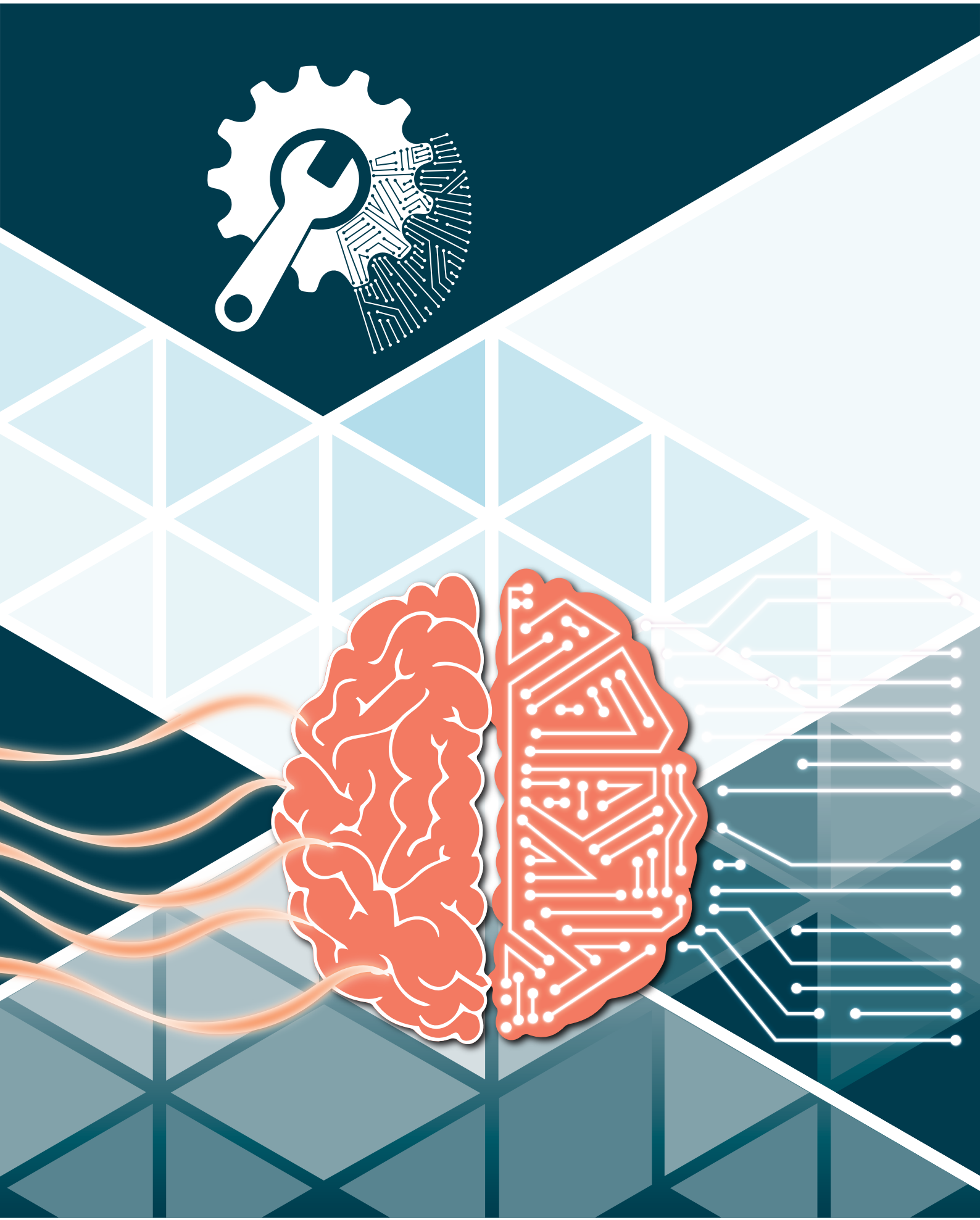
IMMUNO-ONCOLOGY INSIGHTS



IMMUNO-ONCOLOGY INSIGHTS

TOOLS & TECHNOLOGIES CHANNEL

April 2023
Volume 4, Issue 3

FASTFACTS

AI-driven image analysis for cancer cell biology

Gillian Lovell

FASTFACTS

AI-driven image analysis for cancer cell biology

Gillian Lovell, Senior Scientist, Sartorius

Live-cell image analysis is an important tool for gathering cell growth data in the field of cancer cell biology. This poster describes how Incucyte® AI Confluence analysis can be used to enable non-perturbing measurement of cell proliferation using a trained convolutional neural network.

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The field of cancer cell biology is rapidly moving towards the use of more complex biological models, as well as more sensitive and precious cell types including primary and stem cells. Label-free live-cell imaging and analysis is therefore a useful method for acquiring data on cell growth and behavior without perturbance.

INCUCYTE AI CONFLUENCE ANALYSIS

The use of artificial intelligence (AI) has enabled highly accurate, robust, and unbiased image quantification. Incucyte AI Confluence analysis uses an expert-trained convolutional neural network (CNN) to accurately identify a wide range of cells including tumor cells, fibroblasts, and stem cells.

AI Confluence enables straightforward cell segmentation with a user-friendly workflow **(Figure 1)**. After images are acquired, they are automatically analyzed using the trained CNN. Cells in the images are segmented, providing a percentage confluence measurement that can be quantified over time as cells proliferate. AI Confluence requires minimal user input, providing unbiased and accurate segmentation to a wide variety of cell types. Robust and reproducible data can be acquired in up to 384-well throughput.

Figure 1. Incucyte AI confluence image analysis workflow.

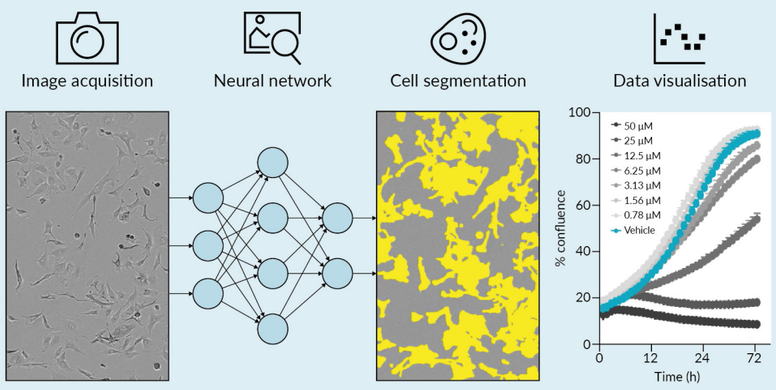
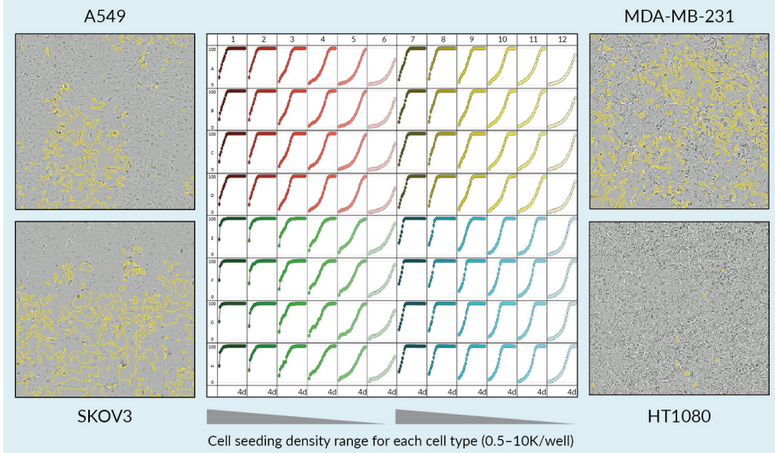


Figure 2. Robust quantification of cell proliferation.



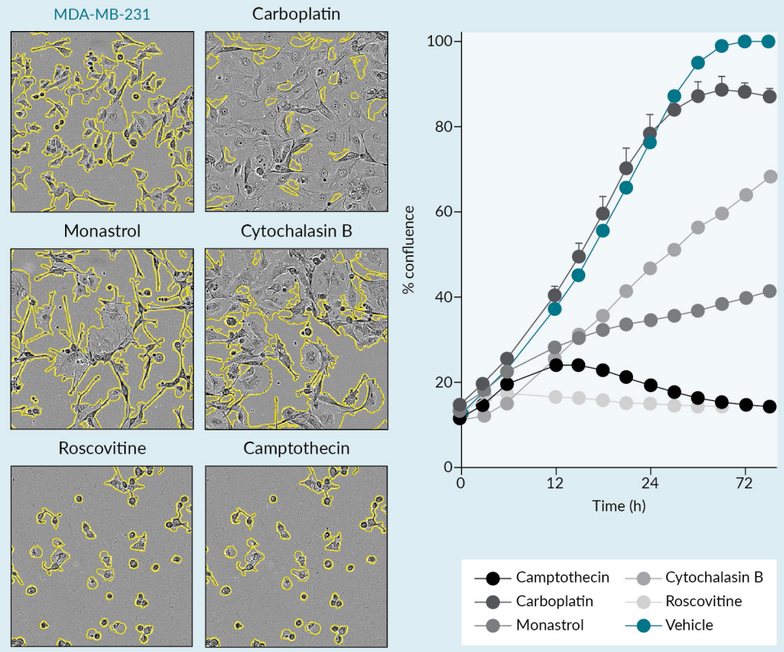
QUANTIFYING CELL PROLIFERATION

The CNN is trained to identify a wide variety of cell morphologies, and provides quantification that can be directly compared across cell types. **Figure 2** shows example cell growth data from four different cell types seeded at a range of densities into a 96-well microplate. The same analysis adapts well to all four morphologies as well as across the increasing confluence as cells proliferate.

QUANTIFICATION OF COMPOUND EFFECTS

In addition to cell growth, AI Confluence has been used to quantify compound effects. In the example shown in **Figure 3**, triple-negative MDA-MB-231 cells have been treated with compounds with different mechanisms of action. Each of these compounds induces a unique morphology and the cell segmentation has adapted accordingly in all cases.

Figure 3. Accurate analysis of compound effects.



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IMMUNO-ONCOLOGY INSIGHTS

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INNOVATOR INSIGHT

Patient-derived organoids: an emerging platform to de-risk immunotherapy development

Sylvia Boj & Andrea Bisso

Recent advances in cancer immunotherapy have had a positive impact on the life expectancy of patients with liquid cancers, whereas solid tumors remain an open challenge for immunotherapeutic development. The lack of clinically predictive biomarkers coupled with the poor translatability from conventional 2D cancer models represent major hurdles for preclinical development. Patient-derived organoids (PDOs) generated from healthy and malignant tissues recapitulate complex characteristics of the original parental tissue, including molecular heterogeneity and morphological and functional traits. Importantly, they preserve tumor-specific antigens that are conventionally lost in standard *in vitro* models, therefore representing an excellent system to investigate efficacy, target engagement, and mechanism of action, and to stratify a patient population based on tumor molecular features. In this article, the development of PDO and immune cell biobanks relevant for testing immuno-oncology agents, and co-culture assays to evaluate different products, are described.

Immuno-Oncology Insights 2023; 4(3), 143–154

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AN INTRODUCTION TO PATIENT-DERIVED ORGANOIDS

The current drug development paradigm has proven over the years to be an inefficient, unpredictable, and expensive process, with a high attrition rate of new compounds in the clinic. This is partly due to a lack of clinically relevant preclinical models that can be used as patient avatars to test treatment responses before entering clinical trials. Drug development can be improved by replacing standard preclinical models with patient-derived organoids (PDOs) that hold a high predictive value of patient response in the clinic. PDOs' predictive value of patient response in the clinic has been extensively demonstrated by multiple independent clinical validation studies

published in high-impact peer-reviewed papers [1–3], and led in 2015 to treating the first cystic fibrosis patient [4] with ultra-rare mutations based on organoid data. More recently, a proof-of-concept study [5] was published demonstrating the feasibility of progressing a new oncology clinical candidate to clinical trials using organoid screening.

PDOs – or HUB Organoids® – are adult stem cell-derived organoids that capture patient and tumor heterogeneity and mimic patient response to treatment. These organoid models are physiologically relevant and genetically and phenotypically stable. They are expandable for large-scale screens, suitable for genetic manipulation, and have a high establishment efficiency.

The technology that allows the development of organoids directly from patient tissue biopsies or resections is a proprietary technology based on extensive knowledge of stem cell biology and organ development. HUB Organoids (HUB), which holds the patent for organoid technology, was founded in 2013 to refine, upscale, and commercialize adult stem cell-derived organoids and their applications.

HUB Organoid Technology allows the development of PDO models from virtually any epithelial organ. Living biobanks that represent various human diseases can be generated, which undergo strict quality control and characterization to ensure they preserve original patient features over multiple passages and after cryopreservation. This allows us to recapitulate in the lab the diversity of the patient population, and to conduct preclinical studies that enable patient stratification and the identification of biomarkers of response – something which is still lacking for most immunotherapies.

Currently approved cell therapies which have shown success in hematological malignancies were developed to target linear-specific markers. However, this approach has failed in solid tumors due to the lack of tumor-associated antigens (TAAs) that can be targeted while sparing normal tissue. Where TAAs have been identified in patient tumors, standard preclinical models have failed to recapitulate TAA expression, thus limiting therapeutic development. Interestingly to immuno-oncology (I–O) applications, PDOs preserve patient heterogeneity and specific TAAs, which is key for developing cell therapies for solid tumors.

Co-cultures of organoids can be set up with various immune cell types, either in an allogenic or autologous setting to investigate immunotherapeutics targeting the tumor, the immune microenvironment, or both. Thanks to HUB Organoid Technology's unique culturing conditions, PDOs can be established from both normal epithelia and cancer lesions, in some cases from the same patient, thus allowing testing for off-target/off-tumor effects by comparing normal and tumor responses.

To adapt to the growing demand for patient-relevant models for immuno-oncology, HUB has focused on developing I–O biobanks, with protocols to isolate and expand not only tumor cells from resected tissue but also other cell types such as fibroblasts and T cells to establish autologous and non-autologous systems. These complex biobanks will enable us to dissect the role of different components of the tumor microenvironment in treatment response. Different readouts have been established and more are under validation to allow the collection of the most relevant data from co-cultures to understand the mechanism of action, efficacy, and activity of test compounds.

PDOs IN I–O: CASE STUDIES

Figure 1 shows morphology read out and T cell activation following allogenic co-culture of engineered T cells and organoids. Tumor and normal organoids were placed in screening wells in combination with engineered T cells designed to identify and kill tumor organoids based on antigen recognition. After co-culture for 3 days, bright field images confirmed tumor organoid killing, whereas no significant morphological changes were detected in the normal organoid culture.

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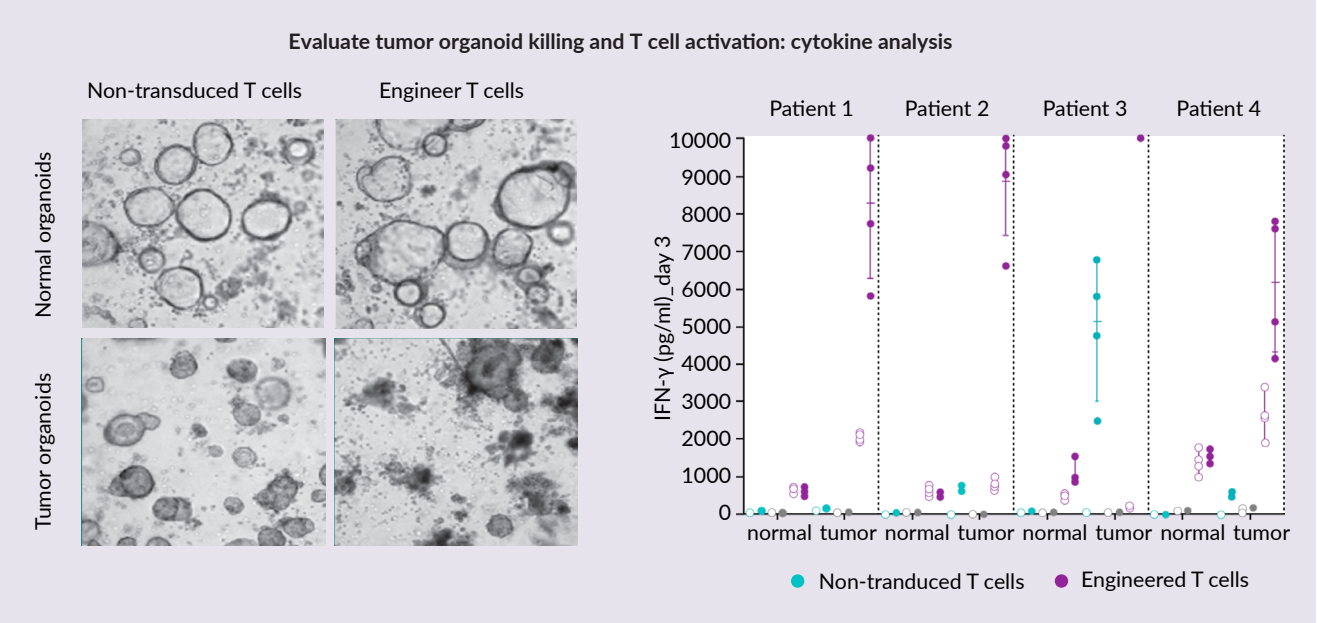
FIGURE 1

PDO and engineered T cell co-cultures.

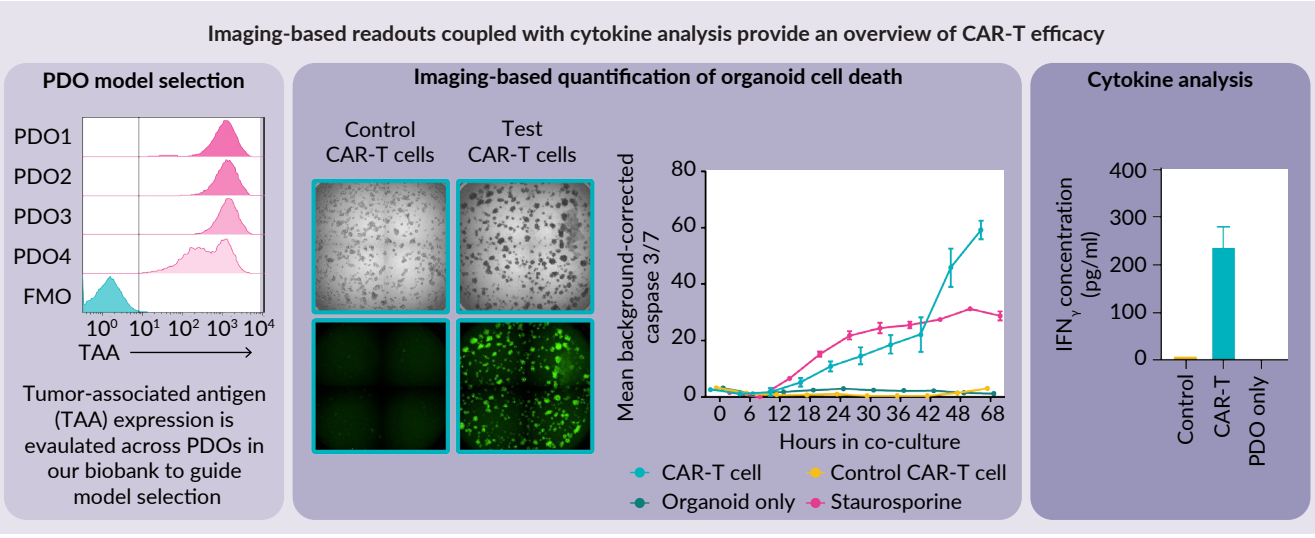


IFNγ measurement confirmed engineered T cells activation in the presence of tumor organoids but not normal organoids.

Additional readouts were developed to offer alternative options to explore the activity of these cellular products. A typical chimeric antigen receptor (CAR)-T testing workflow (Figure 2) first involves organoid model selection based on CAR-T target antigen expression by tumor cells. Subsequently, a co-culture is established, and CAR-T cell activation is detected by ELISA IFNγ measurement. In this example, an imaging-based readout (caspase 3/7 signal) is used to measure tumor organoid killing over time by CAR-T cells. Alternative readouts are also under development to measure a larger spectrum of activities depending on the compound mode of action.

FIGURE 2

CAR-T cell testing workflow.



Autologous PDO and T cell co-cultures have been adopted in this sample study to assess the efficacy and tumor specificity of bispecific antibodies (Figure 3). In this example, T cells were isolated and expanded from patient peripheral blood mononuclear cells (PBMCs), and PDOs were selected based on target tumor antigen expression, with tumor PDOs expressing high levels of target antigen and matched normal PDOs expressing lower antigen levels, similarly to corresponding patients. No killing of normal organoids was detected by caspase 3/7 signal, even in the presence of high-dose T cell bispecific antibody, whereas dose-response curves display a correlation between increasing therapeutic dose and tumor PDO killing.

EXPLOITING γδTCRs TO TARGET HEMATOLOGICAL TUMORS

γδ-T cells are a subpopulation of T cells, either tissue-resident or circulating, with unique features that allow them to recognize stress-related molecules on cells that experience some level of alteration, including malignant transformation. Recent publications have shown that the presence of γδ-T cells within tumors is associated with a better patient prognosis, suggesting their general role in counteracting tumor progression. At the molecular level, γδ-T cells are able to target cancer cells by recognizing surface antigens using γδ-T cell receptors (γδTCRs) in a human leukocyte antigen (HLA)-independent manner. This offers the possibility of using γδTCRs in a broad spectrum of patients.

Gadeta, a biotechnology company founded in 2015 and based in Utrecht, is focused on harnessing the unique capacity of γδTCR to target tumors for the development of first-in-class cell therapies.

Gadeta´s innovative technology led to the development of GDT002, a first-in-class cell therapy targeting CD277, a tumor cell antigen presented on the surface of cancer cells with altered bisphosphonates metabolisms (Figure 4).

GDT002 has broad tumor reactivity but does not cause cross-activity to healthy tissue from different organs. It has now been tested in a multicenter Phase 1/2 clinical

FIGURE 3

PDO and PBMC-derived T cells co-cultures to assess the efficacy of bispecific antibodies.

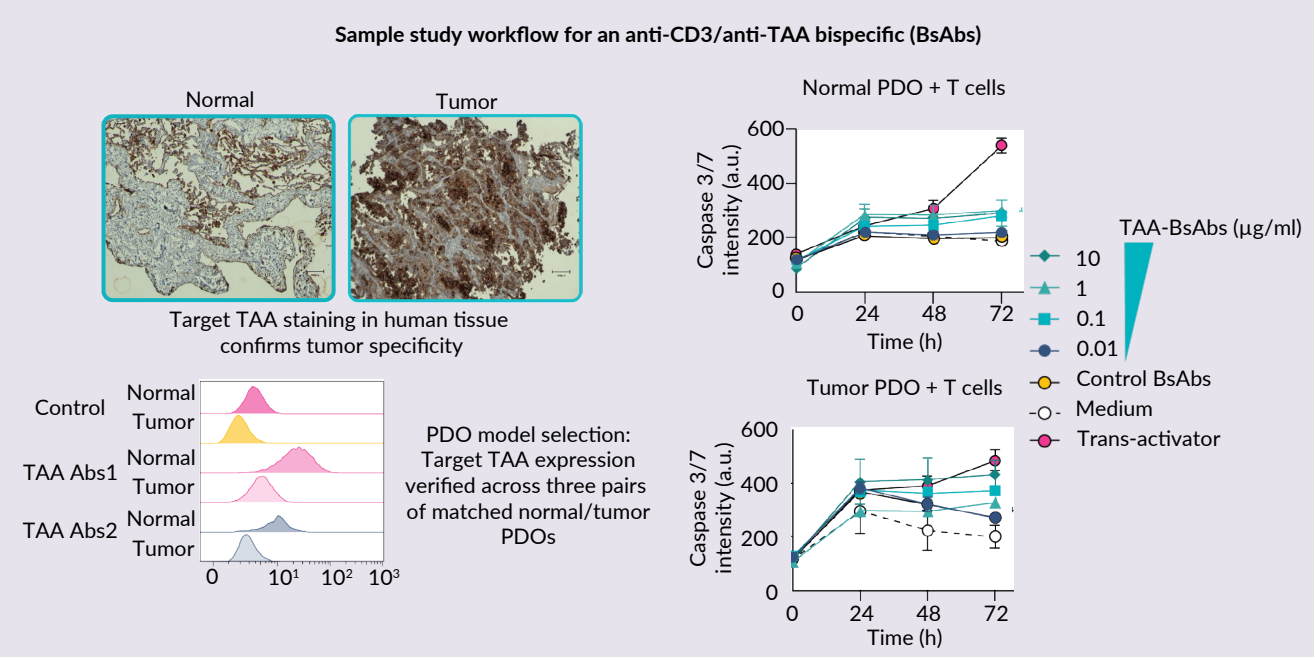


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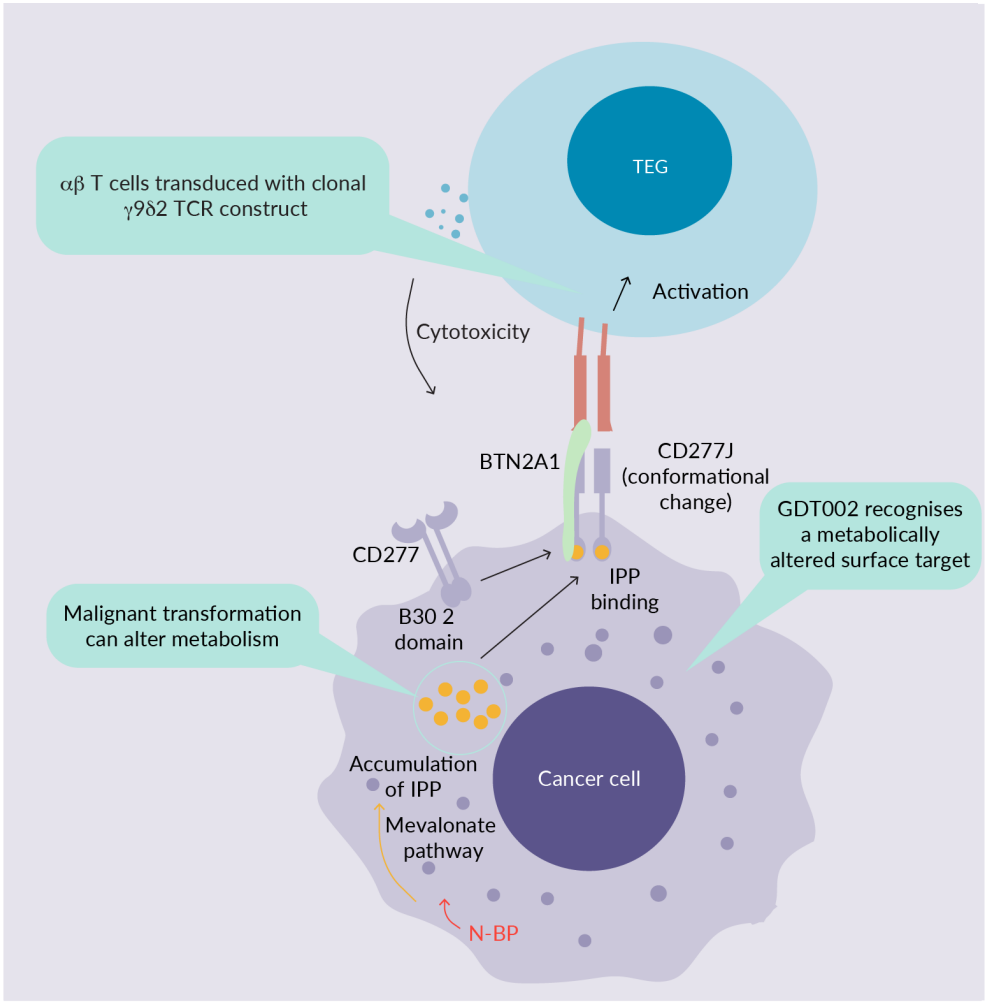
FIGURE 4

GDT002 Mechanism of action.



trial evaluating safety, tolerability, and preliminary efficacy in patients with relapsed or refractory multiple myeloma.

HARNESSING PDO SCREENING TO EXPAND GDT002 INDICATIONS TO SOLID TUMORS

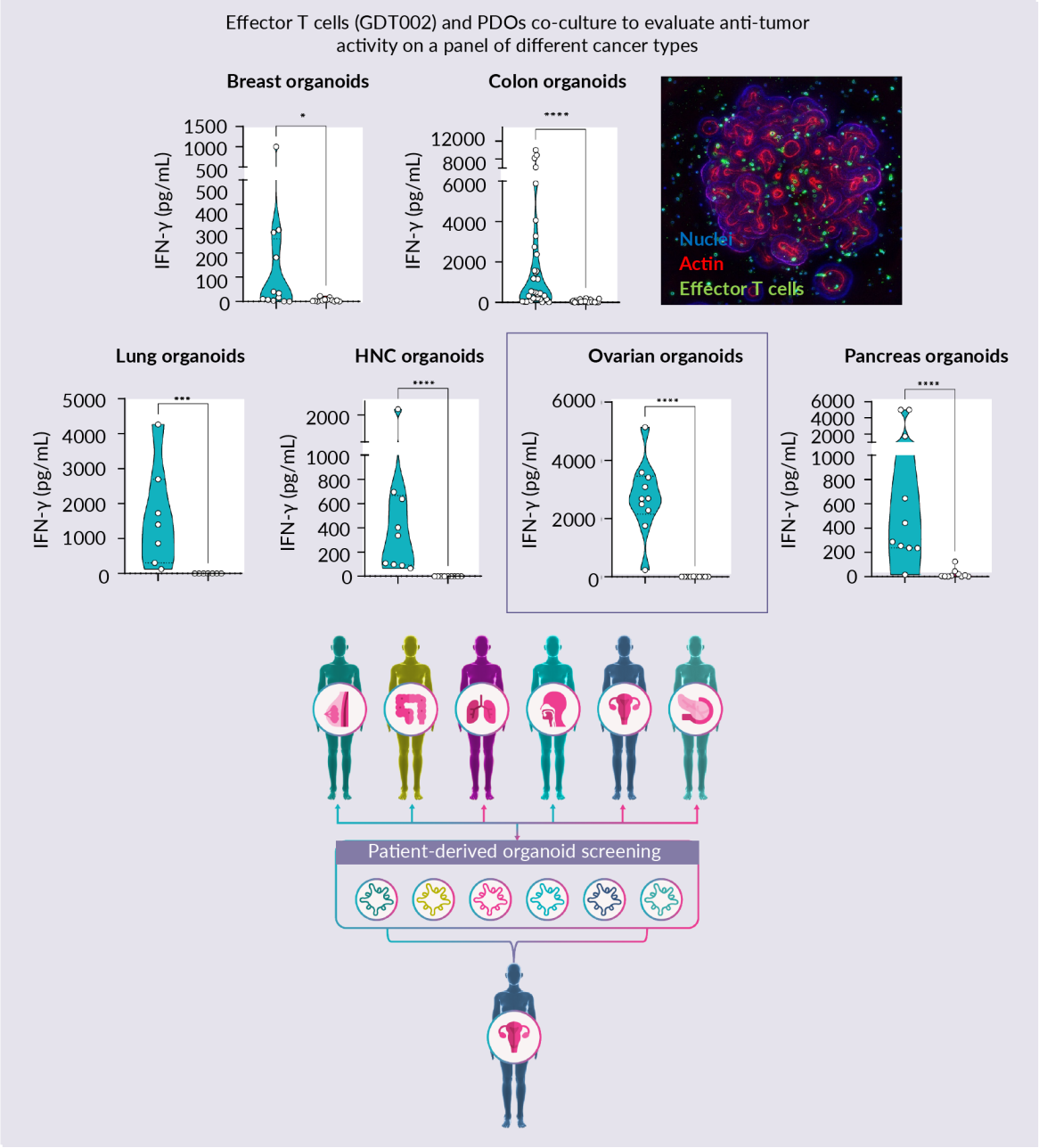
Given the positive results obtained with GDT002 in hematological cancer, Gadeta was interested in expanding the application of the drug to solid tumors and selected a PDOs basket trial as a patient-relevant platform to obtain preclinical data for their IND package submission. GDT002 showed a broad reactivity against tumor PDOs, with a remarkable 90% reactivity against ovarian PDOs (Figure 5). Therefore, ovarian cancer was prioritized as the chosen indication for the first clinical trial to assess the safety of GDT002 in solid tumors.

TRANSLATION INSIGHT

Autologous PDO and immune cell biobanks can be co-cultured for testing a variety of T cell targeting therapies. Building these patient-relevant biobanks supports immunotherapeutic development by providing a scalable and physiologically relevant system that recapitulates patient heterogeneity and preserves key TAA to investigate immune cell

FIGURE 5

GDT002 shows broad reactivity against tumor PDOs.



activation and tumor cell killing downstream of target engagement. PDOs co-cultures with autologous or allogenic immune cells allow to test the efficacy and off-target toxicities in parallel, thus providing a comprehensive profile of the therapeutic efficiency of a new agent before moving with confidence to patient trials.

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ASK THE EXPERTS

Roisin McGuigan, Editor, BioInsights speaks to (pictured left to right) **Sylvia Boj,** CSO, HUB Organoids and **Andrea Bisso,** Director Pharmacology, Gadeta

Q How large are your I–O biobanks?

SB: Our immuno-biobanks began recently. In the context of colorectal (CRC) models, we have a dozen models from which we have isolated tumor-infiltrating lymphocytes (TILs), and in non-small cell lung cancer, we have around five models. In a subset of these CRC immuno-oncology (I–O) models, we have isolated cancer-associated fibroblasts (CAFs). We are also interested in indications like bladder and head and neck cancers, where we see a lot of interest in I–O. Patient-derived biobanks are not limited to particular indications – we can develop specific biobanks depending on need.

Q How do you identify the ligands of γδTCRs?

AB: Our approach to identifying new tumor-reactive γδTCRs is based on functional assays: initially, we want to ensure that these γδTCRs are tumor-specific and not recognizing any healthy cells. Then, it is important to identify the ligand on tumor cells. We normally do this through two approaches. One is functional genetic screening. The second is based on a more classic biochemical approach. The identification of γδTCR ligands has been and still is a challenge in the field: we think that a combination of these two approaches is the right way to tackle this aspect.

Q How can I work with HUB to develop I–O compounds?

SB: We have different service offerings. I would advise anyone interested in our services to contact our business development (BD) team and explain the mechanism of action and the area in which your compound is developed. Our team can offer different solutions and approaches, either from existing assets or ones that require further development. Our strong scientific team can address any challenge and offer a solution.

Q Is there an option to validate target expression in both the patient material and in the organoids to control for differential expression between *in vivo* and *ex vivo* situations?

SB: One challenge we face is that when we receive the tissue to establish the organoid, we know that there are interests for different tumor antigens but performing a complete analysis on the tissue to make comparisons is difficult. At a basic level, we must use immunohistochemistry, which is not quantifiable but can confirm that the tissue expresses a specific antigen. Then, we can confirm it is also expressed on organoids with flow analysis. If there is a particular application in which this is an important question, we could run a project in which we get new tissue for PDO generation. While establishing the organoids, we can characterize the original tissue using flow analysis. It is outside our standard activities, but we can address this.

Q How do you expand the tumor PDOs without losing the original phenotype and genotype, and how many passages can be maintained?

SB: The culture conditions for expanding organoids were first established by culturing healthy cells to ensure we could expand stem cells, proliferative cells, and other cell types. We use the same principles to establish the tumor model. We believe that because there is no high selection pressure, we can maintain the original tumor heterogeneity in organoids.

When the first tumor biobanks were generated, original tissue and derived organoids were sequenced. In most cases, there was more than 80% overlap between driver mutations detected in original tissue and organoids. Of course, tumors are, by definition, genetically unstable, but we know that culture conditions do not drive this genetic instability. When working with tumor-derived organoids, we recommend expanding them for a maximum of 5–6 passages, as an average and depending on the model. We do not recommend expanding a tumor model for a year because it can vary too much from the original tumor. However, 2–3 months of expansion and cryopreservation can allow us to preserve the genetic landscape that the original tumor contained.

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Q Based on the results of assessing your two treatments in organoid models, do you have plans to use organoids for future projects?

AB: To characterize γδTCR tumor reactivity we proceed with a step-by-step approach. We start with tumor cell lines *in vitro* for the first layer of investigation. As soon as possible, we move selected γδTCRs into more clinically relevant models. For this, our collaboration with HUB is beneficial. Most of our γδTCRs can recognize broadly different tumor types, so we want to narrow down the tumor types that can be selected to be used in an initial clinical trial. We also would like to understand whether there is a genetic setting that is preferentially targeted. For both of these aspects the use of organoid models provides valuable information and will certainly be included in our strategy.

Q How do you know the reactivity is tumor-specific?

AB: Our first layer of characterization of γδ TCRs is a screen for lack of cross-reactivity against a large set of healthy primary cells to ensure there is not recognition of healthy vital tissues. Then, we test a set of tumor models, including organoids, to show that tumor cells are killed. In the next steps, we use mixed toxicology and pharmacology models with primary material from patients, to show specific killing of tumor cells, sparing the normal tissues in the samples.

Q In the allogeneic setting, could this T cell reactivity be due to HLA mismatch?

AB: It is true that in most of the models there is a potential allogeneic reaction due to human leukocyte antigen mismatch. To control for this, we use either untransduced T cells, or our recently developed set of control TCRs that are engineered to not recognize tumor tissues. These two controls can be used for measuring the background level of possible allogenic activity.

Q Is there an imaging platform that can be used for organoids derived from patients and grown without dissociation into single cells, but as small tissue chunks?

SB: When we perform our screens, the organoids are not single cells but structures. The average size of these organoids in our screening assays is ~50–70 µm in diameter. We use confocal microscopy in a high throughput format for imaging. As we are developing

the technology, we are also working on selecting the best imaging platform for our data. There are several publications from different labs that can achieve good quality imaging data from screens performed on organoids. We do not see imaging as a limitation.

Q For co-culturing methods such as organoids with T cells, can I use non-activated T cells?

SB: Yes, this is possible. In our co-culture assays, we have a control of activated T cells, but we have set up co-cultures where, for example, we wanted to evaluate tumor reactivity on TILs isolated from tumors without pre-activating the cells. We have seen a response with this, so it is possible to see cell activity.

Q What do you see as the most exciting application for organoids, now and in the future?

SB: The developments we are working on in I–O and inflammatory diseases by combining organoids with other cell types is an exciting area. Our organization is also putting effort into validating the predictive value of organoids to show that organoids can predict patient response. A solution in the industry could be to run 'avatar' clinical trials with organoids before moving into patients to identify the patient populations likely to succeed in clinical studies. We believe that our technology will significantly contribute to this, and we are putting great effort into this.

BIOGRAPHIES

SYLVIA F BOJ received her PhD in 2006 at the University of Barcelona, Spain for her work at the Hospital Clinic in the laboratory of Prof. Jorge Ferrer, where she conducted functional genetic analysis to understand the transcriptional role of MODY genes in pancreatic beta cells. With a long term EMBO fellowship, she subsequently joined the HUB Organoids (Utrecht, the Netherlands) as a postdoctoral fellow. In the laboratory of Prof Hans Clevers she first studied the role of TCF7L2 in regulating metabolism. Then, she established human pancreas organoids from tumor resections in collaboration with the Surgery and Pathology departments from the UMC and the laboratory of Prof DA Tuveson. In 2014, she joined HUB as one of the founding scientists and worked as a Group Leader for the cystic fibrosis and pancreatic cancer organoid programs. In 2016, she was appointed Scientific Director at HUB and in her new role she was responsible for leading both the contract service and research programs. Under Sylvia´s leadership the Organoid Technology evolved from a highly innovative basic research tool to a industry leading drug development platform. Since 2020 she holds the position of Chief Scientific Officer.

ANDREA BISSO is the Director Pharmacology at Gadeta BV. There he oversees the investigation of the efficacy, safety and mechanism of action of the new proprietary candidate cellular products, by leading the Preclinical Pharmacology team and managing the collaborations with external partners and CROs. He joined Gadeta at the end of 2020, after a nearly 15 years career in academia, during which he gained extensive experience in the cellular and molecular

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mechanisms at the basis of cancer. Working as Scientist at the European Institute of Oncology (Milan, Italy), he contributed to the understanding of the role of the MYC, WNT and Hippo pathways in tumorigenesis, by performing functional genetic screenings and by developing new preclinical mouse models of B cell lymphomas and liver tumors. Dr Bisso received his PhD in Molecular Medicine from the University of Trieste (Italy), focusing on the role of microRNAs regulating the activity of the p53 pathway and on novel potential therapeutic approaches to block the oncogenic functions of p53 tumor-associated mutants. He holds a patent covering the application of peptides and aptamers as specific modulators of mutant p53.

AFFILIATIONS

Sylvia Boj, PhD
CSO,
HUB Organoids

Andrea Bisso, PhD
Director Pharmacology,
Gadeta

AUTHORSHIP & CONFLICT OF INTEREST

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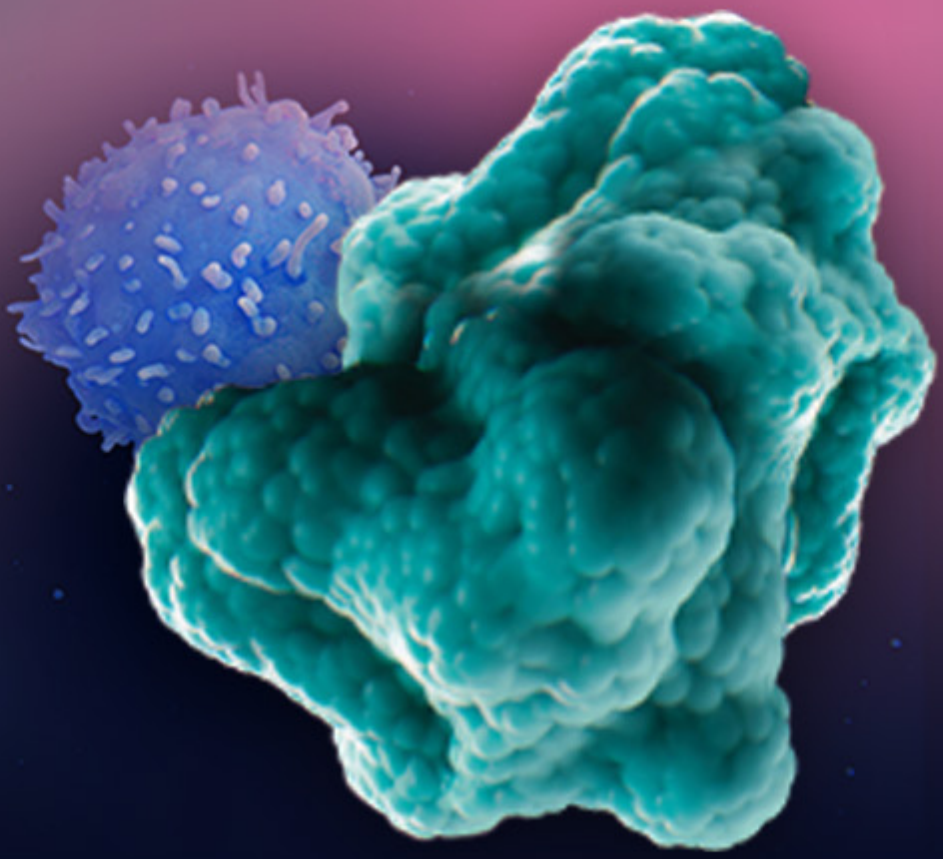
HUB ORGANOIDS

Recapitulate tumor-immune system interaction with patient-derived organoid co-cultures



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