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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:
New horizons in cellular immunotherapy: next-gen platforms & modalities

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SPOTLIGHT

COMMENTARY

Developing harmonized immune platforms: a must-have for realizing personalized therapies in solid tumors

David Morrow, Jan Langermans, Anton Ussi, Antonio L Andreu & Lucia Gabriele

The future of healthcare lies deeply in personalized medicine which we are all aware places the patient at the center of the therapies we develop. Advanced therapies are at the forefront of this strategy, but we are still not harnessing the true power of the data in immunology at our disposal that allow us to understand how our complex 'individual' biological systems react to these novel therapies. The goal of CAR-T expansion into solid tumors remains elusive to our scientists with meta-analysis of solid tumor CAR-Ts tested in these tumors revealing only a mere 4.2% complete response, for example [1]. Understanding the interplay between a cell therapy, immune checkpoint inhibition, tumor environment, and host that has critical impact on immunotherapies is paramount for the developer and clinician. It demands an integrated, harmonized characterization strategy where innovative partnerships among clinical centers, academic institutions and research infrastructures represent a key strategy. Through the development of harmonized immune platforms comprising these inter-disciplinary teams for personalized therapies in solid tumours, we can provide the optimum innovation strategy and ensure reduction in the present failure rates. Done right, this can further advance the development of these must-have therapies to the clinic for the right patients at the right time and at the right dose.

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THE LONG ROAD TO SUCCESS IN SOLID TUMORS

The impressive effectiveness of T-cells in oncology seen in patients is well established in the field [2–5]. Gene engineered T-cells are now non-exhaustively investigated for various cancer types, with the greatest success still falling in hematological cancers [3]. There are currently two gene modified T-cell therapies for B-cell malignancies - Kymriah and Yeskarta [4–6] - and one pending market approval. Of note, in 2019 there were over 800 clinical trials ongoing using CAR-T technology alone [7]. However, despite the promise and excitement of these therapies, little success has been seen in the last 3 years for these types of therapy in solid tumors. The road to success for CAR-Ts in solid tumors falls now into well-established research areas. These include amongst others:

1. CAR design with the type of co-stimulatory domain being central to the creation of next generation therapies,
2. Target antigen selection,
3. Delivery approaches,
4. Tumor microenvironment (TME),
5. Co-therapies including immunomodulatory reagents,
6. Innovations in CAR-T trials [8–9].

This commentary piece however is not trying to highlight or discuss these well-established research questions. Instead, we aim here to discuss the pivotal need to develop platforms that harness the right technical expertise in immunomonitoring. Such platforms must be suitable to correlating response to CAR T therapy with TME or systemic immunologic dysfunctions determined by several variables, including immune cell balance, immune signature, and tumor lymphocyte function. Certainly, it is now well established that the responsiveness of patients to cell therapies and immune checkpoint inhibition relies on the immune status of the TME, which needs to be evaluated through immunoprofiling and immune signature. These both show great potential to be independent prognostic and predictive biomarkers. However, although they provide crucial insights into immune cell behavior, how they correlate with clinical outcome still requires a lot more investigation. Peripheral blood immune profiling, for example, has been investigated as a non-invasive method to predict response to immunotherapies [10]. In view of this, improving the capability to integrate and harmonize complementary sets of immune parameters across different tumors from different patients will allow us to stratify patients into responders versus non-responders, thereby having the effect of achieving greater success from a more personalized approach. This will result in the increased success of clinical trials where the failure rate currently stands at 80%, in addition to reducing relapse rates post treatment [7]. Furthermore, this can also help decrease the enormous, almost unaffordable price tags for these therapies due to the high clinical trial failure rate because of the unresolved technological challenges that such platforms could remedy.

WHAT SHOULD A PLATFORM CONSIST OF?

Nowadays, immuno-technologies for Precision and Personalized Medicine (PPM) are ever more present in research programs and have begun to be adopted in clinical medicine [11–12]. Their applications however need to be enhanced, implemented, and integrated with other platforms to have a real impact on human health, and to spur policy and economic actions. Technology platforms furthering a novel scientific agenda for diagnostic and prognostic screenings as well as for Prevention Medicine (PM) and/or PPM interventions is a priority. The right platform should have the necessary technologies and expertise in place that can discover

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and validate immune correlates to predict response and guide therapeutic decision-making for the individual patient. Central to this is standardized methodologies of measuring all immune parameters. Such platforms should bring together leading expertise and innovative technological approaches, thus meeting the needs of biotechnology companies, the pharmaceutical industry, and the academic research community. This is pivotal to the successful development of their novel cell therapies and immunotherapies including vaccines. Such an integrated and harmonized approach to immune correlation and prediction of therapeutic response and clinical outcome will improve the output of high utility, validated clinical tools.

The acceleration of the research required to implement such processes for immunomonitoring of patients requires a high level of knowledge and coordination, as well as the application of standards and quality to reduce uncertainty and ascertain the immunotherapy pipeline for solid tumours [13–14]. This can be made possible through effective interaction between private-public networks. In Europe, this collaboration is facilitated by research infrastructures such as EATRIS, which is the European research infrastructure for translational medicine [15–17]. EATRIS has developed a dedicated platform that consists of a dynamic flow of knowledge and resources among 110 world class Institutions across 13 EU countries that integrate and harmonize innovative technologies to monitor immune parameters across different solid tumour types. This enables the identification of patients that can benefit from novel cell therapies and immunological strategies for the treatment of cancer.

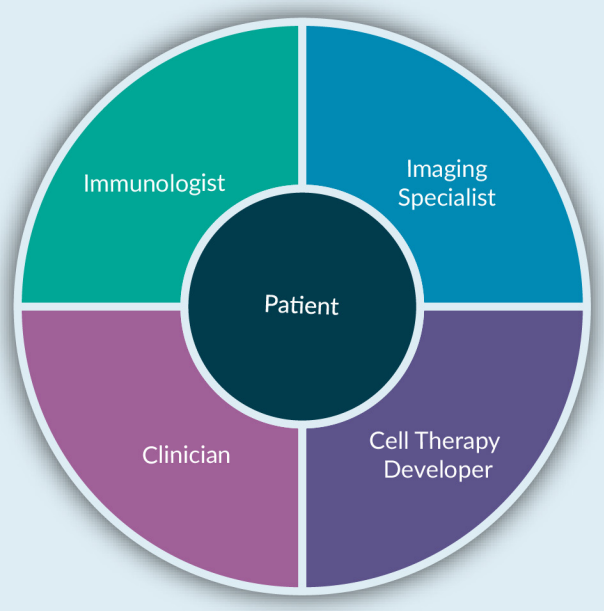
High-end, validated analytical technologies are central to a deep and appropriate immune monitoring for pre-clinical and clinical studies aimed at a comprehensive elucidation of the underlying bases of immune responses following the administration of a given therapeutic. These innovative tools range from high-throughput analyses of the transcriptomes and proteomes of single immune cells and integrated mass cytometry immunophenotyping, to imaging immune strategies and standardized immune functional tests, pioneering the use of non-invasive technologies to explore and track the dynamics of the immune system. For example, clinical use of the innovative imaging technology constitutes an unprecedented advance beyond the state of the art, allowing the tracking of the fate of cells after its use in cell therapy in human beings [18–20]. Multimodal imaging approaches in its right form can complement the immunomonitoring tests of any such platform by acting potentially as an early prognostic indicator of therapy success by evaluating proliferation of T-cells, localization and subsequent change in tumor sizes. It is noteworthy that with many new therapies being assessed on the initial response of the patient, robust, standardized, and validated capabilities to monitor immune responses are essential for the correct evaluation of the shaping interactions between immune cells in tumours leading to achieve better outcomes in preclinical projects, translational projects, and clinical trials.

Building such a platform with standardized analytical procedures across different institutions is paramount towards producing new scientific knowledge in the field. The EATRIS Vaccine, Inflammation and Immunomonitoring Platform is one example of a research resource that offers a complete array of expertise and innovative tools allowing high-quality translational research capacity in the immunology field to the developers of immunotherapies.

The key to success of such innovative technological offerings relies primarily in the ability to bring different expertise together in the same workflow. Immunotherapy developers, researchers, immunologists, multimodal imaging specialists, clinicians, regulators, and patients must all be part of such harmonised platforms to address the multifactorial research questions and hurdles in answering them [Figure 1]. Only with a concerted approach, utilizing the right technologies, can the long road to achieving the success of cell

FIGURE 1

Key stakeholders of harmonized immune platforms for personalized cell and immunotherapy development for solid tumors



therapies and different immunotherapies in solid tumours be a clinical reality.

CONCLUSION

Despite the initial success of CAR T cell therapy in hematologic cancers, realizing the same degree of benefit in solid tumors still seems a very long way away. Similarly, although revolutionary therapeutic benefit for immune checkpoint inhibitors was predicted and hoped for, response rates range are still only between 10% to 40% [21]. Both therapeutic strategies still come with significant toxicity and high costs for healthcare systems across the globe, with many countries simply not able to make these therapies available. Hence, there exists an urgent and unmet medical need for reliable predictors of response for patient selection. In this regard, the research landscape lacks faithful models and strategies to standardize methods of analysis and there is still a lack of coordination between all stakeholders in the field of developing new innovative therapies for solid tumors. This commentary piece calls for the utilization of innovative platforms such as the EATRIS Vaccine, Inflammation and Immunomonitoring Platform and similar standardized technology platforms that overcome these bottlenecks through harmonized approaches. These platforms must be validated across European and global laboratories, and proficient in integrating immune correlates that predict patient outcome and therapeutic decisions. Achieving this goal will facilitate faster and more efficient cell and immunotherapy development and as a result, lower health costs making these therapies a reality for patients.

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY: NEXT-GEN PLATFORMS & MODALITIES

SPOTLIGHT

EXPERT INSIGHT

Engineering precision cell therapy for immune-mediated disease

Michael C Milone & Gwendolyn Binder

Autoimmune disease (AID) is a major medical concern for a significant part of the world population. AID comprises a heterogenous group of medical conditions characterized by a shared etiology of the adaptive immune system targeting normal tissue antigens in concert with a breakdown in immune tolerance mechanisms. Until recently, treatment for AID has fallen primarily to systemic therapies including metabolic inhibitors (methotrexate and mycophenolate mofetil), immune suppressants (cyclosporine and corticosteroids), and cytotoxic therapies such as cyclophosphamide for severe manifestations. Although the emergence of novel therapeutics have increased the therapeutic options for patients, the mechanism of action for each of these newer approaches are generally immunosuppressive without specificity towards the aberrant autoimmunity and most are unlikely to restore specific immune tolerance or precisely remove autoreactive cells to permanently cure disease. New approaches to treating AID are still needed, and engineered cell therapy offers a new therapeutic paradigm for addressing pathogenic autoimmunity with the potential to restore immunologic tolerance. Here, we summarize the state of development for precision cell therapy in AID, focusing on redirected T cells to eradicate pathogenic immunity, and antigen specific or engineered regulatory T cells for specific suppression of pathogenic immune responses and restoration of immune tolerance.

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INTRODUCTION

This review has been designed to provide the reader with a line of sight to the emerging field of precision cell therapy for immune-mediated disease. Focus is given to why new therapies are needed, rationale behind various precision cell therapeutic approaches for various immune-mediated diseases, and to the current state of development of these therapies. Where possible, references to deeper technical reviews for individual approaches has been provided.

Autoimmune disease (AID) is a major medical concern for a significant part of the world population. AID comprises a heterogenous group of medical conditions characterized by a shared etiology of the adaptive immune system targeting normal tissue antigens in concert with a breakdown in immune tolerance mechanisms. This can result in organ specific damage (e.g. autoimmune hepatitis or Hashimoto's autoimmune thyroiditis) and systemic manifestations (e.g. systemic lupus erythematosus [SLE] or rheumatoid arthritis [RA]). The ability to identify and diagnose AIDs has evolved significantly over the last quarter century leading to an increase in those identified from 30 to over 100 today [1–3]. US based disease prevalence ranges widely from extremely rare (e.g. fewer than 200 cases) to common (e.g. >2.5 million cases) [4]. An estimated 4.5% of the worldwide population and approximately 14 million people in the US are living with AID [4, 5]. A significant economic burden associated with chronic treatment and associated comorbidities that accompanies AID has been estimated at greater than $100 billion, based on the data acquired from the seven most common diseases [6]. The incidence of several AIDs appears be increasing, possibly resulting from environmental factors, and also due to changes in reporting from improved epidemiologic surveillance and more accurate clinical diagnoses [7,8]. Thus, AID represents a major global unmet medical need for which new therapies, and in particular curative therapies, are badly needed.

Although the etiology of AID is incompletely understood, it is well accepted that a breakdown in immune tolerance is a hallmark of the disease. Despite the effectiveness of central tolerance mechanisms in deleting or anergizing T and B cells that express receptors with affinity to self-proteins, self-reactive antibodies and T lymphocytes are now understood to be common in healthy people and are thought to play a role in systemic homeostasis [9–13]. These self-reactive cells are prevented from becoming pathogenic through a series of peripheral tolerance mechanisms, including regulation of co-stimulation, low avidity between self-reactive immune cells and their cognate antigen, and regulatory T cell-mediated immune suppression. It is not yet possible to predict the likelihood or timing of AID onset, and while factors associated with future disease have been identified, including the presence of antibodies against specific proteins, family history, genetic profile, and lifestyle and environmental exposures, initiation of AID initiation remains a stochastic process [14,15]. This is exemplified by the low concordance of several different AIDs amongst identical twins [16]. Initiation of pathogenic autoimmune responses requires a breakdown of immune tolerance, combined with expression of sufficient self-antigen to activate a dormant adaptive autoreactive immune response. This can be instigated though several mechanisms thought to include: infection which provides an inflammatory environment for activation thus increasing avidity of autoreactive cells, molecular mimicry induced by infection (where foreign proteins are expressed by the immune system but look like self-antigens), and environmental changes that induce a breakdown of normal immune regulation [14]. Therefore, the ideal way to treat AID would be to restore immune tolerance through specific eradication of autoreactive cells, restoration of robust peripheral tolerance mechanisms, or a combination of both.

Until recently, treatment for AID has fallen primarily to systemic therapies including metabolic inhibitors (methotrexate and mycophenolate mofetil), immune suppressants (cyclosporine and corticosteroids), and cytotoxic therapies such as cyclophosphamide for severe

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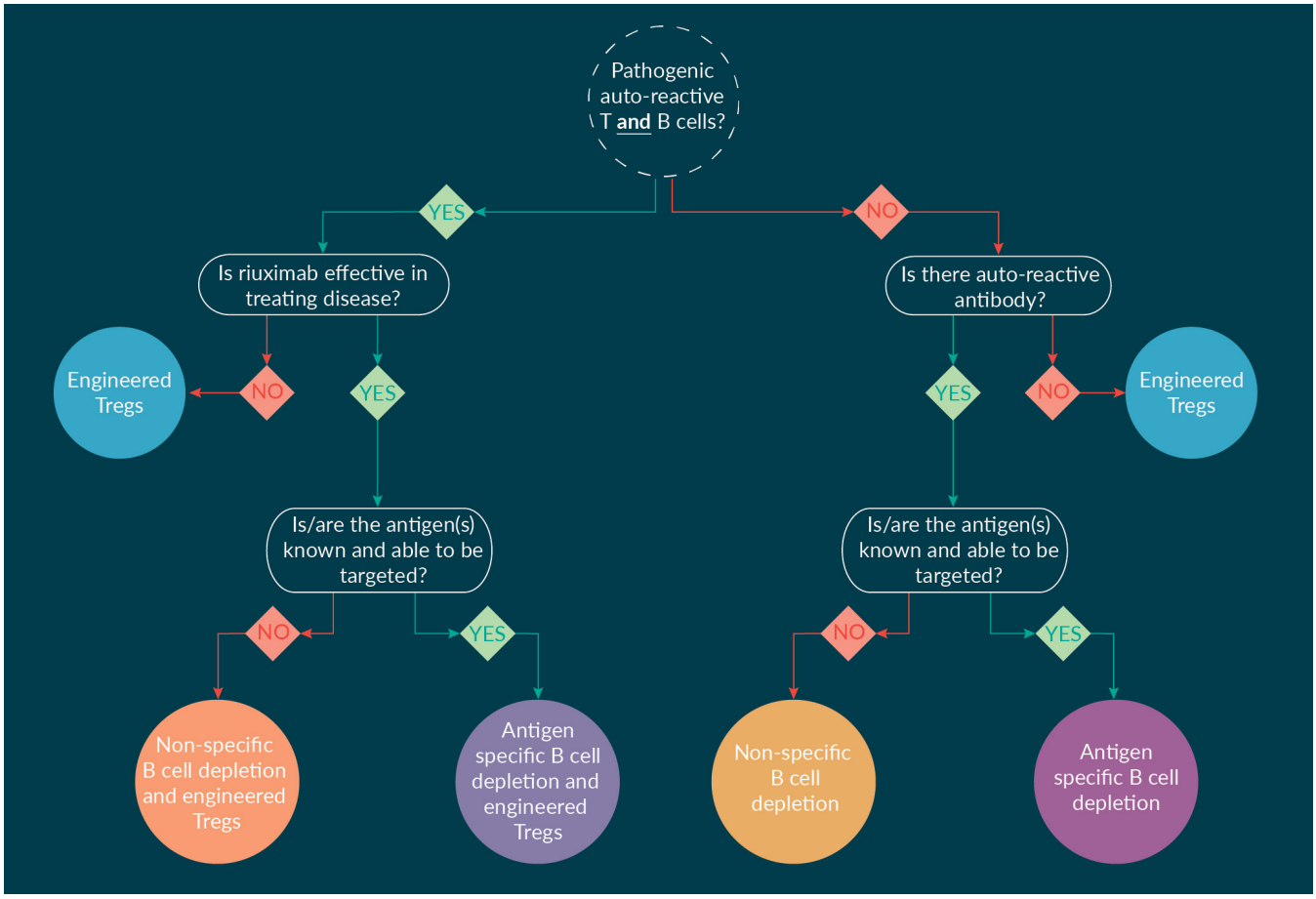
manifestations. These therapies are a cornerstone for AID management, however broad immune suppression comes with associated risks of infection and increased malignancy risk, as well as other non-immune related side effects. For patients with severe disease and treatment side effects, hematopoietic stem cell transplant has been seriously explored as a method to achieve medication free remissions [17–19]; mesenchymal stem cell transfer has also been explored with less promising clinical outcomes [20]. Recently, the utilization of the B cell depleting reagents, such as rituximab, have offered meaningful improvement in acute disease outcomes and reduced comorbidities in certain AIDs [21]. Although data continues to emerge, rituximab therapy of antibody-mediated AID primarily remains non-curative despite prolonged administration over time due, in part, to incomplete B cell depletion in the lymphoid compartments [22–24]. Furthermore, non-specific B cell depletion has the potential to worsen disease through the promotion of long lived plasma cells resulting from excess BAFF/Blys (B cell maturation cytokines which promotes long lived plasma cells), and the reduction in B regulatory cells [25–27]. A series of additional novel approaches to AID treatment have been developed and are continually emerging, and which are aimed at blocking pro inflammatory and B cell maturation cytokines (anti-IL-1α, -IL-1β, -IL-6, -IL-17, -IL-23, -TNF-α, -IL-12, -BAFF and -APRIL), B and T cell signaling (BTK and JAK inhibitors), cell trafficking (integrin inhibitors) or promoting depletion of B cells (next generation B cell depletion reagents including bi-specific antibodies) or plasma cells (anti-BCMA or bortezomib) [28]. Although the emergence of these novel therapeutics have increased the therapeutic options for patients, the mechanism of action for each of these newer approaches are generally immunosuppressive without specificity towards the aberrant autoimmunity and most are unlikely to restore specific immune tolerance or precisely remove autoreactive cells to permanently cure disease. New approaches to treating AID are still needed.

Engineered cell therapy offers a new therapeutic paradigm for addressing pathogenic autoimmunity with the potential to restore immunologic tolerance. In cases where the autoantigen(s) are well defined and are few to one, antigen-specific precision cell therapy is one possible solution that is being applied in an attempt to suppress antigen-specific autoimmunity while minimizing the generalized immunosuppression, thus reducing overall longitudinal risks especially of serious infection. Approximately half of all AID have been associated with well-defined target antigens [4]. The underlying immunopathology of AID is disease specific and can be primarily antibody mediated, primarily T cell mediated, or B and T cells may both play a role. In antibody-mediated AID such as pemphigus vulgaris, thrombocytopenic purpura and myasthenia gravis, antibody binding to self-antigens can be pathogenic via several mechanisms including disruption of normal membrane protein function [29,30], inhibition of serum protein function [31,32], and induction of target cell death through complement mediated mechanisms or antibody-dependent cellular cytotoxicity (ADCC) [33–35]. There is increasing evidence that B cells play a pathogenic role in AIDs classically recognized as being T cell-mediated such as type 1 diabetes (T1D), multiple sclerosis (MS), and rheumatoid arthritis (RA) based upon their responsiveness to anti-CD20 therapy especially in early phases of disease [36–38]. Although it cannot be entirely excluded that the responsiveness of anti-CD20 therapy derives from depletion of $CD20^{+}$ CD8 T cells [39], the role of these cells in AID pathogenesis is far from clear [40]. B cells may contribute to T cell-mediated autoimmunity through both their antigen presenting function as well as regulatory roles in maintaining immune tolerance [41]. Therefore, when considering precision cell therapy for a given AID, it is helpful to evaluate the role of antibodies and B cells when considering effector approaches (Figure 1). As an alternative approach, precision tolerogenic therapy has the potential to work across all AIDs in a manner that similarly does not induce the

FIGURE 1

Algorithm for selecting a precision engineered cell therapy approach for immune-mediated disease.



While immune-mediated diseases typically have some level of involvement of both T and B cells, this dendogram is intended to illustrate the decision tree for selection of T-cell based therapeutic approaches based upon whether B cells, T cells, or both are the primary drivers of pathology in AID. The role of B cells in disease can be significant, even in conditions recognized to be dominantly T cell-mediated, and the impact of B cells can be determined by measuring the impact of rituximab, a B cell depleting therapy, in disease outcomes. For diseases where B cells contribute to disease pathogenesis, cell therapy for B cell depletion may be considered. In disease where T cell mechanisms are dominant, suppressive cell therapy approaches may be considered.

broad immunosuppression seen with current therapies. Here, we summarize the state of development for precision cell therapy in AID, focusing on redirected T cells to eradicate pathogenic immunity, and antigen specific or engineered regulatory T cells for specific suppression of pathogenic immune responses and restoration of immune tolerance.

PRECISION REMOVAL OF PATHOGENIC AUTOREACTIVE B CELLS

After birth, B cells are continuously formed in the bone marrow as pre-B cells which become immature B cells upon the rearrangement of its immunoglobulin loci for expression of IgM on the cell surface. Like T cells in the thymus, B cells are subject to central tolerance mechanisms and will be positively or negatively selected based upon IgM binding affinity in the marrow. B cells migrate from the bone marrow to the spleen where peripheral tolerance mechanisms further remove autoreactive B cells, and expression of BAFF/BLyS cytokine is critical for survival of the remaining B cell repertoire [42]. Despite this selection rigor, autoreactive antibodies, or "natural" autoantibodies (for example anti-nuclear antibodies [ANA] that tend to increase with age), remain which are thought to play an important role

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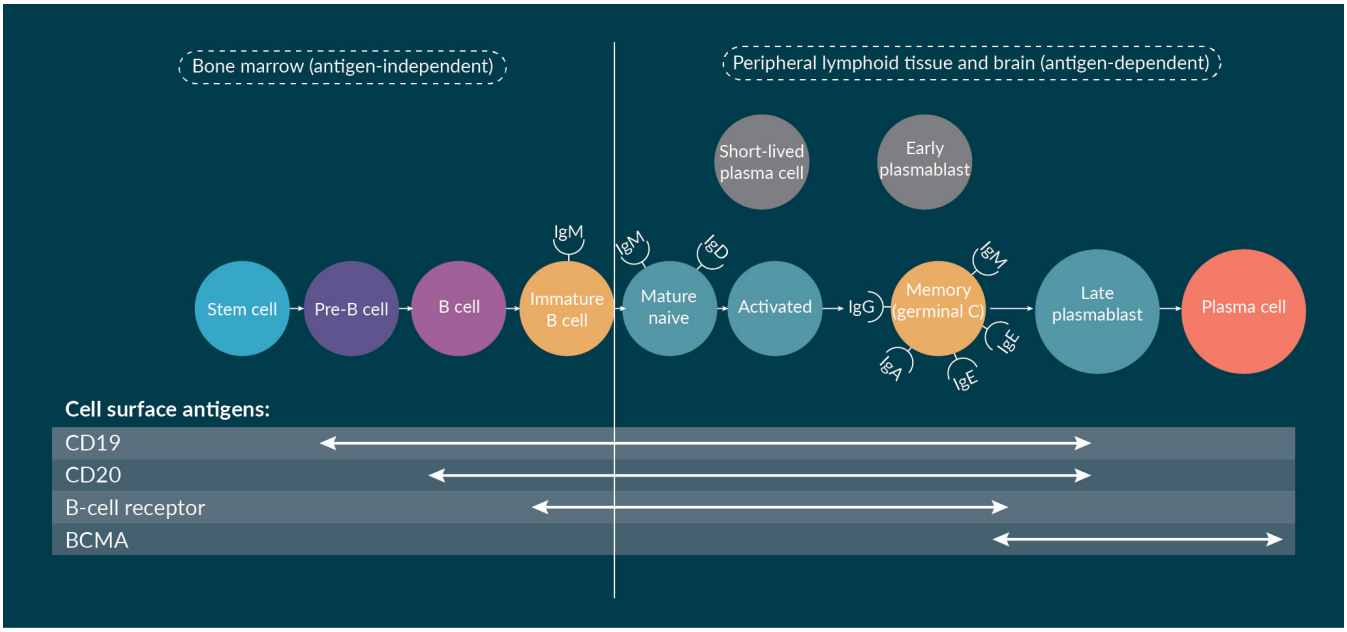
in systemic homeostasis [10,11]. As B cells mature, they will move to secondary lymphoid organs where they can become activated by exposure to antigen and undergo affinity maturation, and become professional antibody producing cells (plasma cells) or memory B cells. Targetable markers on B cells vary at these different stages, and there is a notable loss of B cell markers such as CD19, CD20 and the B cell receptor (BCR; the BCR has identical specificity to the soluble antibody produced by plasma cells) with plasmacytic differentiation [Figure 2]). Importantly, plasma cells can be short lived or long lived; short lived plasma cells survive in the body only a few days while long lived plasma cells can survive for years and antibodies produced from these cells are not eradicated by memory B cell depletion [43,44]. The mechanisms by which long lived versus short lived plasma cells develop is not well understood and is likely dependent upon the strength of signal given to developing B cells and the availability of key cytokines such as BAFF/Blys [25,43]. In general, autoantibodies tend to be produced by short lived plasma cells, which can be empirically tested by observing the impact of B cell depleting therapy on autoantibody titer. Nevertheless, occasional reports of conversion from short lived to long lived autoantibody producing plasma cells have been made [27]. Thus, targeting and eradicating autoreactive pathogenic memory B cells offers a potentially optimal approach to overcome antibody-mediated AID driven by largely short-lived plasma cells. However, the depth and durability of response to antibody-mediated B cell depletion with anti-CD20 antibody leaves room for therapeutic improvement [21,22].

In 2017, in an historic step, the first two engineered T cell therapies (tisagenlecleucel and axicabtagene ciloleucel [axi-cell]) achieved regulatory approval in the United States for the treatment of B cell malignancy. These approvals came nearly 30 years after the first proof of concept report of a chimeric antigen receptor (initially termed a "T-body") [45]. During that time, several discoveries around enabling

FIGURE 2

B cell and plasma cell markers throughout development which may be used for precision cell therapy in immune mediated conditions.

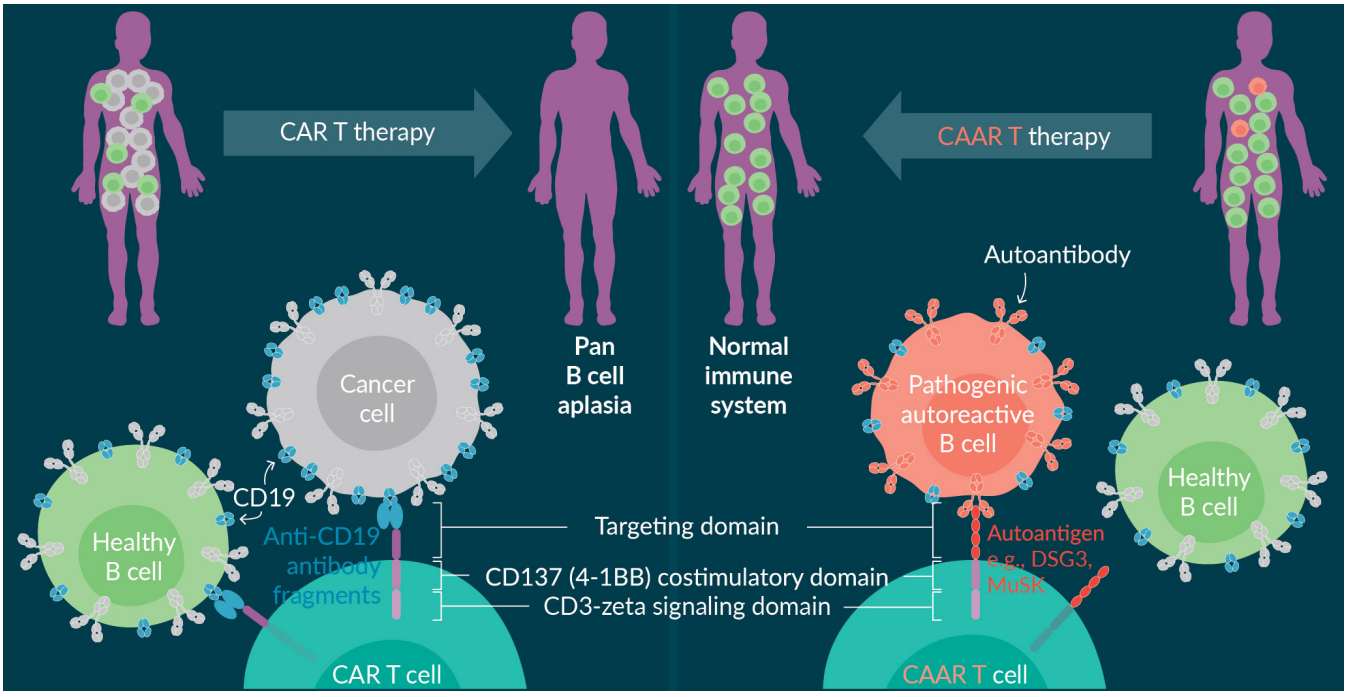


Targeting B cell surface antigens (e.g. CD19 or the B cell receptor) is an effective strategy for reducing antibodies produced by short-lived plasma cells. For antibodies produced by long-lived plasma cells, B cell depleting strategies will not provide an effective therapeutic strategy, but plasma cell depleting strategies may. The markers shown in this figure illustrate how different markers can be used to target subsets of B cells and plasma cells. Additional markers not shown include B220, CD27, CD38, and CD24.

and optimizing gene transfer, improving CAR design, and optimizing T cell expansion and function were made in parallel to support the advancement of this novel therapeutic modality. These continue to be optimized in the oncology setting [46,47]. As reviewed previously in this journal, building on this success, CAR technology evolved to enable specific targeting of autoantigen specific B cells, while ignoring non-pathogenic B cells, by utilizing the T cell expansion and CAR signaling mechanisms proven to be effective in tisagenlecleucel. This was achieved by expressing all or part of the autoantigen on the chimeric receptor (rather than an scFv) targeted by the autoantibodies (Figure 3) [48,49]. This new type of receptor for effector T cells has been named a Chimeric AutoAntibody Receptor or "double-A" CAAR T cell approach (CAART) for B cell-mediated AID. A similar receptor design has been developed for regulatory T cell suppression of specific B cell populations, and has been named B-cell Antigen Receptor (BAR) approach. In this review, we use the term CAAR when referring to effector T cells specific for antigen specific B cells. Initial designs were developed for pemphigus vulgaris (PV), which is the prototype antibody-mediated AID, with anti-DSG3 antibodies being 98–100% sensitive and specific for the mucosal-dominant form of the disease [29]. Proof-of-concept data were published in 2016 [50]. In this published study, an optimized design was developed, which demonstrated specific elimination of anti-DSG3 expressing cells while sparing normal cells *in vitro* and *in vivo* even in the presence of soluble antibody to DSG3 that is expected to neutralize the CAAR interaction with the surface immunoglobulin on antigen-specific B cells. A series of IND-enabling studies, including CAAR specificity testing against a human membrane proteome array, manufacturing qualification, and *in vivo* safety assessments in murine models of PV disease, led to the opening of a clinical trial which is now recruiting patients [51,52]. This

FIGURE 3

Comparison of the CAR T cell versus the CAAR T cell approach for immune mediated disease.



Shown on the left is the FDA approved CD19 chimeric antigen receptor T cell (CAR T) approach, which eliminates all CD19 expressing B cells (healthy and pathologic). On the right is the chimeric autoantibody receptor T cell (CAAR T) approach which specifically targets pathogenic B cells only, while leaving non-pathogenic B cells in-tact. This is done by expressing the targeted autoantigen on the surface of the chimeric receptor (Figure provided courtesy of Cabaletta Bio).

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Phase 1 study has been designed with an initial dose escalation phase comprising 4 total doses which covers the range of doses shown to be effective in oncology studies. In order to mitigate any unexpected toxicity (such as CAART activation in response to soluble antibody in the bloodstream), while providing each patient with the optimal opportunity to receive a potentially therapeutic dose, each total dose is split into 4 escalating dose fractions of 1%, 5%, 25% and 69% which are administered over a week. The learnings from this trial (e.g. optimal dose, safety profile, durability of response/dosing frequency, and any preconditioning requirements) will be instructive to support development of CAAR for a host of follow on antibody-mediated conditions, including for MuSK myasthenia gravis, and factor VIII inhibitors associated with hemophilia A for which CAARs are currently under development [53–55]. Of note, despite the promising data utilizing bispecific technology in oncology that demonstrates deeper responses than with antibody approaches, CAART cells may have a significant advantage in autoimmune disease due to their ability to recycle receptor onto the cell surface and remain functional the presence of soluble antibody [50].

Effective CAAR T cell technology is restricted to AIDs where the autoantigen is well-defined. In antibody-mediated AID where multiple antigens are implicated, such as with systemic lupus erythematosus (SLE), pan-B cell depletion will likely be needed, unless a tolerance approach is taken such as with engineered regulatory T cells (covered later in this review). The pan-B cell depletion approach has been modelled recently in a murine model of SLE through targeting of CD19 using a tisagenlecleucel-like (CAR) approach [56–58]. Alternate approaches to depletion of B cells might include targeting CD20, CD22, BAFF, APRIL, or BCMA [59–61]. CD19 CAR T cell therapy has demonstrated efficacy in patients with B cell cancers, where treatment with the B cell depleting agent, rituximab, has failed [62]. Thus, such an approach may similarly prove more curative than rituximab in AID. Of note, unlike with autoantibody specific approaches discussed earlier, bi-specifics may be competitive to B cell specific CAR therapy since there is no soluble antibody against anti pan-B cell antigens that might block the bi-specific agents activity (notably there is soluble BCMA which has been shown to interfere with anti-BCMA bi-specific function [63]). The relative safety and efficacy of bi-specific antibodies to CAR T cells is being tested in oncology studies and will need to be tested in AID. One potential advantage to bi-specifics in this setting could be the ability to terminate dosing once disease has resolved, whereas the CAR T cells may induce long-lasting B cell depletion in patients with persisting CAR T cells that is undesirable due to the risks of serious infection [64,65]. Notably however, depletion of long-lived plasma cells does not occur following CD19 CAR T cell depletion, and therefore some protective humoral immunity may nevertheless persist during the period of B cell aplasia [44,57]. Furthermore, several approaches may be considered to limit the duration of B cell aplasia, including allogeneic products which are rapidly rejected by the recipient's immune system [66], and autologous products utilizing RNA mediated gene transfer or a suicide gene [67–69].

RESTORING IMMUNE TOLERANCE: ENGINEERING REGULATORY T CELL THERAPY

Many AIDs are characterized by a regulatory T cell imbalance or ineffectiveness, contributing to loss of peripheral tolerance [70–72]. Regulatory T cell defects in AID have several potential etiologies including defects in the IL2 signaling pathway, inflammation-mediated destabilization of regulatory T cells due to cytokine effects, and acquired resistance of effector T cells to regulatory T cell effects [73]. The critical importance of this CD4 T cell subset in maintaining systemic and immunologic homeostasis is illustrated in patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) who have defective regulatory T cells resulting

from a mutation in the master regulatory T cell gene FOXP3 [74]. These patients experience extreme dysregulation of the immune system resulting in autoimmune polyendocrinopathy and enteropathy, and the disorder has universally poor outcomes with hematopoietic stem cell transplant and immune suppression resulting in an estimated 73% (median follow-up 2.7 years) and 65% (median follow-up 4 years) overall survival rate, respectively [75]. Based on the known mechanism of action for regulatory T cells in AID pathogenesis and data from animal models of AID, restoration of effective regulatory T cell function in patients with AID is expected to improve patient outcomes.

Regulatory T cells (Treg) are a subset of CD4 T cells characterized by their suppressive activity on multiple cells of the immune system including effector T and B lymphocytes, mature dendritic cells, activated monocytes, and granulocytes [76]. Tregs exert their suppressive effect through multiple mechanisms including competition for the T cell growth cytokine IL-2 (acting as a "cytokine sink"), production of immunosuppressive cytokines like IL-10 and TGF-β, and blocking costimulatory effects of antigen presenting cells and thus blunting the adaptive immune response [77]. Tregs are difficult to definitively distinguish from other CD4 T cell subtypes, but are characterized by a high level of the IL-2α receptor on their surface, low to no levels of the IL-7 receptor CD127, and active expression of the *FOXP3* gene [78]. Definitive identification of Tregs comes from evaluation of demethylation at the FOXP3 promoter and suppressive function *in vitro* against effector T cells. Tregs are classified broadly into two groups. The first is natural Tregs or thymic Tregs (tTReg) which are typically quite stable due to demethylation at the FOXP3 control locus, and these may be enriched for specificity against self-antigens [79]. The second is induced Tregs (iTReg), which include a subset of iTreg characterized by IL10 production. Induced Treg are typically more plastic, responding to local inflammatory mediated cues resulting in changes in FOXP3 expression levels, and are at a higher risk of conversion into effector T cell, although like tTReg, iTreg also serve a critical role in the maintenance of peripheral tolerance [80]. The history of Treg discovery, ontology, and characterization and function have been excellently reviewed by others elsewhere [79–81].

Manufacturing regulatory T cells proves to be more complex than for effector T cells. Due to the plastic nature of Treg, lack of definitive markers for purification, and competition for growth *ex vivo* by effector T cells, the source, purification and expansion methods for Treg are critical to yield an effective product at a dose useful for therapeutic purposes. Purification methods are tailored to the target regulatory T cell population ($CD4^+FOXP3$ tTreg, or induced Treg cells have both been manufactured for clinical trials, and have different markers). Tregs are typically sourced from peripheral blood, but allogenic sources of thymus and cord blood have also been utilized due to the high purity of the starting material. In addition, multiple rounds of stimulation are required to reach clinical doses. Despite the challenges, the ability to manufacture large numbers of Treg in a manner compliant with clinical trial requirements is now well established [82–85]. Methods for purification and expansion of Treg have been reviewed in detail elsewhere [76,80,86]. Minimal standards for the characterization and release of Treg cells have been proposed [87].

More than 40 clinical trials utilizing Treg as a therapeutic intervention have been carried out, according to a clinicaltrials.gov search for "regulatory T cell" and "cell therapy". These include studies to evaluate the effect of Treg on organ transplantation, allogeneic stem cell transplant, and autoimmunity. Initial clinical studies utilized polyclonal Treg, which are not antigen specific, and all studies have demonstrated safety. Indications of therapeutic effect have been observed in allogeneic stem cell transplant studies; this setting may be ideal for Treg proof of concept since alloreactive Tregs are essentially mediating an antigen-specific suppressor response as a result of alloreactivity to the graft [88]. Other studies

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using polyclonal Treg have not yet yielded clear efficacy, although studies in T1D have demonstrated long term engraftment and stability out to one year post infusion, thus indicating the potential for durability of this approach [89]. Further improvements in Treg product potency may be required to routinely achieve efficacy in non-allogeneic settings.

One way to improve the specific potency of Treg is to target them to the affected organ, or to the auto- or allo- antigen that is driving immune-mediated disease. Several animal models of AID have demonstrated the superiority of antigen-specific, or retargeted, Treg therapy, including in models of colitis, T1D, allogeneic transplantation, and EAE (the animal model serving as an analogue for multiple sclerosis in humans) [90–94]. Methods for engineering in specificity to Tregs, including through CAR, BAR, or TCR, has been previously reviewed [95]. Notably, the T1D model clearly shows that antigen specificity enables a significantly lower dose for efficacy, and the EAE model demonstrated efficacy with a novel intranasal delivery route of administration. Methods for generating antigen specific Treg include antigen-dependent Treg expansion *ex vivo*, *in vivo* generation of antigen specific Treg using tolerizing dendritic cells, and engineering antigenic specificity into Treg utilizing through cloned T cell receptors (TCRs) or CARs. *Ex vivo* antigen stimulation and tolerizing dendritic cell therapies are potentially promising; each have technical challenges which are outside of the scope of this review and are summarized elsewhere [96–98]. TCRs have been shown to be exquisitely sensitive sensors of target antigen, with fewer than 10 target antigens capable of triggering cytokine production and cytotoxicity, and are capable of targeting intracellular antigens which greatly improves tissue specificity, and would enable targeting of any autoimmune antigen [99]. This is far more sensitive than current generation CARs, several of which have been shown to have a threshold activation limit of thousands of targets [100], although the sensitivity is likely to differ based on the antibody affinity and signaling mechanism. The costimulatory domain of the CAR also plays a significant role in Treg function, with the CD28 domain showing marked superiority over other costimulatory domains in two recent studies [101,102]. Furthermore, CARs are restricted to cell surface antigens; only about 30% of the human proteome contains transmembrane regions, and only a subset of those proteins would contain extracellular domains capable of targeting by antibodies [103]; this limitation restricts tissue specific targeting of Treg, which is a limitation for CAR-redirected Treg. Although on tissue toxicity is an unlikely concern, targeting an antigen too broadly may serve as a "tissue sink" that reduces the efficacy of the therapy or produce more broad and undesirable immunosuppression. A safety concern specific to engineered Tregs is their stability, as Treg are known to be responsive to inflammatory conditions and may lose their suppressive phenotype. If this were to occur to an engineered Treg, it could then become a pathogenic T cell and exacerbate disease. To address this, approaches to improve stability such as through constitutive expression of FOXP3, or expression of a suicide gene, may be required to ensure safety. Additional discussion pertaining to the engineering of Tregs has been presented elsewhere [86,104,105].

SUMMARY & FUTURE PERSPECTIVES

The era of engineered T cell therapy for AID is here and is sorely needed where current treatments fall short in order to provide potentially curative therapy (or even good clinical responses without significant chronic side effects) for a large unmet global medical need. Characterization and diagnosis of AID continues to improve. Understanding of the immunopathogenesis of AIDs is rapidly increasing which is driving a new era of possible therapeutic interventions. In particular, the role of B cells in AID pathogenesis is greater than previously understood, a learning that in large part comes from the increasing use of anti-B cell depleting therapy

in AIDs, including those that are thought to be classically T cell mediated such as RA, and T1D. We are on the cusp of understanding how precision B cell depleting effector T cell therapy can be utilized in such indications, with data expected from the first CAART study now open and recruiting in patients with pemphigus vulgaris. Coming up quickly behind are engineered Treg therapies, which have seen major advances in the past decade and should yield interesting clinical data in the coming few years. Although the cost benefit of cellular therapies is The future is looking brighter for patients with AID.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY: NEXT-GEN PLATFORMS & MODALITIES

SPOTLIGHT

INNOVATOR INSIGHT

Evolving the role of gene editing in cell therapy

Jonathan Frampton, Torsten Meissner & Tristan Thwaites

The potential of cell therapy has captured the interest and imagination of many – but all cell therapies are not created equal. Autologous approaches offer significant drawbacks relating to cost and economy of scale, and as a result, interest is also growing in the idea of an off-the-shelf 'universal' cell that can be produced at large scales and be given to many patients. However, a significant barrier to this goal has yet to be fully addressed: the immune response.

Gene editing could offer a solution to a range of issues facing the cell therapy field, and could prove critical for delivering the next generation of cell therapies. This article will provide an overview of gene editing options for cell therapies, a discussion of the practical considerations and tools available, and the personal account of an immunologist working to overcome the issues of the immune system via gene editing in order to enable safer and more practical cell therapies.

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Gene editing in cell therapy

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Autologous versus allogeneic therapy: cost & speed

The main benefit of an autologous approach to CAR T cell therapy is that there is little risk of graft-versus-host disease (GvHD). The cells are taken from and returned to the same patient, and therefore an immune response from the procedure is unlikely. There are also several challenges, as some cancer patients will not have sufficient T cells for isolation. If they do, the quality of the cells isolated may not be sufficient for the manufacturing process. There is currently a 10–15% failure rate in manufacture when isolating cells from patients with the aim of turning them into CAR T cells.

Additionally, there is no economy of scale. The process provides one treatment per person, which leads to it being very costly – in the region of US$300,000 to US$500,000 for each treatment cycle. Because the patient's own cells are being utilized, there is also a relatively complex supply chain as collection and the transport is required to the facility where the transfer and modification of the cells occurs. Facilities must be close to each other to ensure this can be done effectively and efficiently. Depending on the logistics, this results in a timeframe of 2–4 weeks.

On the other hand, there is the allogeneic or 'universal cell' approach. Utilizing a healthy donor allows more control, as a donor who can provide high quality and fully characterized cells can be chosen. There is a very attractive economy of scale, as one donor could potentially be utilized for thousands of patients. This could potentially bring down the cost per dose to the region of US$7,500 to US$10,000 – a 50-fold decrease compared to autologous therapy.

The supply chain remains relatively complicated for allogeneic therapy but is considerably simplified compared to autologous, as most of the allogeneic cell manufacturing process can be done anywhere in the world. This allows for biobanks that are spread out around countries and regions, driving down the timeframe and allowing for cells to be shipped to the required hospital within one or two days.

Of course, the allogeneic approach does not provide a perfect solution, and runs the risk of creating an immune response. The therapeutic cells come from a foreign body, and the T cell used must be modified to decrease the risk of GvHD. Despite this challenge, the advantages described above have resulted in a significant drive in both academia and industry to produce allogeneic cells.

Utilizing gene editing for improved cell therapies

Gene editing can play a role in decreasing GvHD risk in cell therapies – and can bring several other benefits.

Looking to the current autologous therapies on the market, there has been encouraging early therapeutic successes, particularly for liquid tumors such as leukemias. As yet,

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successful transfer to solid tumors has not been achieved, and remission is not always guaranteed. As a result, one area of particular focus in the field is to find ways to generate T cells that have increased persistence and proliferation, which will allow them to survive within toxic tumor microenvironments. Precise gene editing of the T cell may prove critical for delivering the next generation of cell therapies.

The design of the CAR for identifying and targeting a cancer cell is very important – but the modulation of the T cell is just as crucial. Combining CAR T therapy with methods to increase the persistence, proliferation, and survival of a CAR T cell in a solid tumor will result in a very strong therapy.

To achieve enhanced persistence and effector response, there are a number of known genes which can be targeted. For example, knocking down the *CBLB* or *SHP1* genes may enhance proliferation. For improving T-cell response within a microenvironment, *TSC1* may be a good option, and for improving allogeneic responses, *TRAC*, *B2M* or *PD1* are all potential approaches.

Next, the gene editing techniques which could be adopted to achieve these changes must be considered – and there is a considerable portfolio to choose from. There are the more historical approaches such as zinc finger nucleases, rAAV or TALENs editing systems. And of course, there is CRISPR, which has made a considerable impact in the last few years and which might currently be considered the leading gene editing technology. Another breakthrough technology seeing more use in recent years is base editing.

Gene editing of cell therapies: the challenges

Irrespective of which technology is being utilized to modify the T cell for cell therapy, there are a number of issues to bear in mind. When considering intellectual property, it is important to know how you are going to get to market, and to perform freedom-to-operate analyses, as these areas are still relatively murky for this field. There are also technical challenges – understanding the editing efficiency for a particular cell line, and whether it is high enough to deliver what is required, are crucial steps.

The supply chain is another issue that can be overlooked. Once a good process for delivering gene editing is in place, it must be ensured that all of the equipment and reagents can be sourced or accessed at a GMP grade, so they will stand up to regulatory requirements.

Last, but very much not least, there is the safety profile of the technologies and the potential long-term impact on the patient. If a modified T cell targets a tumor, will there be off-tumor effects as well? And does the gene editing technology being adopted have off-target effects? The majority of the approaches discussed here create a double strand break as part of their mechanism, which brings the risk of unintended translocations, insertions or deletions. Base editing is an exception, as it creates a nick on one strand only, and therefore removes these risks.

Gene editing workflow & tools

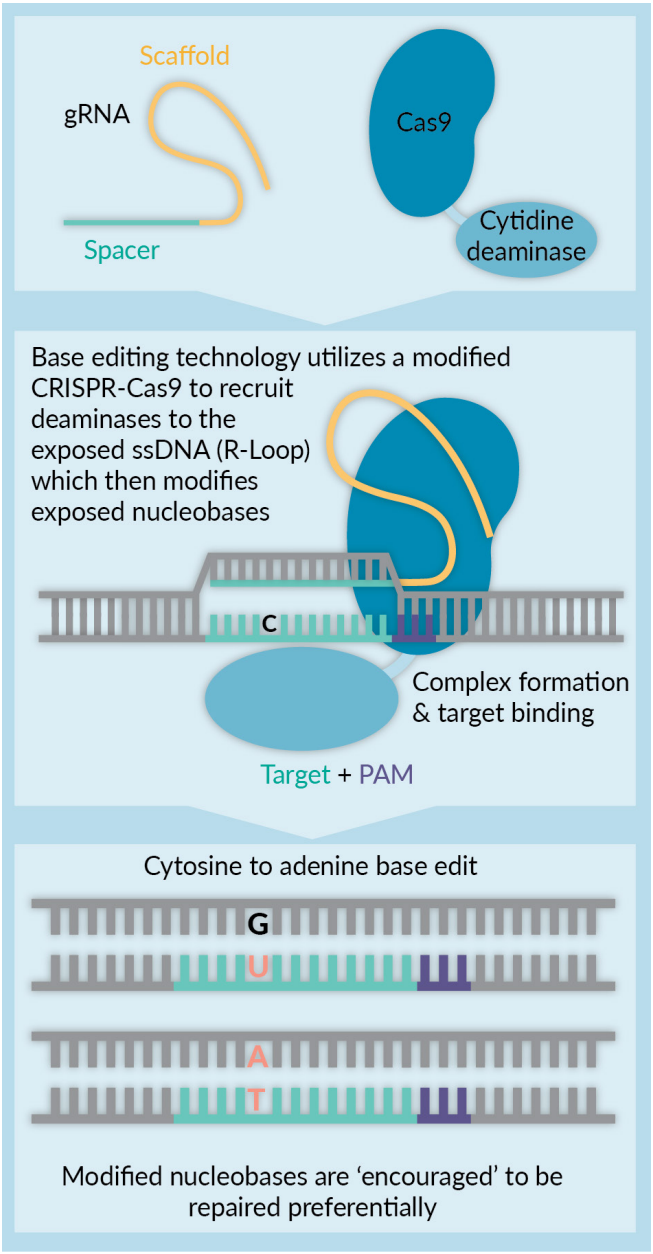
For primary cell gene editing, once the primary cells have been isolated, a level of preparation is required for both the cells and the reagents, whether these are guide RNAs or recombinant Cas9 protein.

Once the cells are prepared, the next step is delivery, which could involve a viral or non-viral approach. Next there is the readout, to determine which cells have been edited. Once the cells have been screened, various phenotypical analyses can be performed.

Base editing, which utilizes a modified Cas9, creates no double strand breaks. As can be seen in Figure 1, it works in a similar way to CRISPR, where a guide RNA will direct the Cas9 to the targeted site. As no double strand break is introduced, it is possible to introduce a stop codon at a given gene, and

FIGURE 1

Base editing mechanics



The attraction of base editing is; it can gene edit without generating a double-strand break.

for example, knock out PD1. This approach lends itself to multiplex editing where multiple genes can be targeted at once, with a good safety profile and limited or no off-target effects. For applications where the aim is to knock out upwards of 10 genes in order to generate the desired allogeneic cell, base editing has the potential to the gene editor of choice.

Finally, a key aspect of the process is being able to identify the cells which have been modified. With any gene editing approach, there is never a perfect correlation between a knock out or knock down and a phenotype exchange. The Sartorius iQue3 Advanced Flow Cytometry Platform (Sartorius) has proven to be a powerful and cost-effective tool to confirm direct quantitative phenotypic data at single cell resolution, with a number of simple assays available. In situations where clonal expansion is not feasible, single cell resolution techniques are the only way to determine complex, multiplex gene knockout and advanced flow cytometry offers a fast and versatile approach which can assist with a high-throughput approach to gene editing.

Conclusion

Gene editing can offer solutions to a range of challenges facing the development of cell therapy, including increasing the persistence and proliferation of therapeutic cells, and reducing the risk of immune response when introducing foreign cells to the body and paving the way for 'universal' stem cells. However, off-target editing effects present a significant risk which must be addressed in order for gene edited cell therapies to reach their potential.

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Gene editing in cell therapy: experience & perspectives from an immunologist

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The immune response: the ultimate barrier to cell therapy?

Many people in the cell therapy field, including in industry, share the opinion that personalized medicine is simply too expensive in its current state. An autologous approach to cell therapy will not be feasible for many applications, and this has generated a lot of interest in a 'universal stem cell' – an off-the-shelf, quality-controlled product that is compatible with any patient and can be produced in large quantities. However, for this to become a reality, a significant hurdle must be faced: the issue of the immune response.

Organ compatibility, or incompatibility, is determined by human leukocyte antigens (HLAs), a group of highly polymorphic molecules encoded by the major histocompatibility complex (MHC) gene complex. They come in two main 'flavors': class I and class II; they are surface antigens that communicate with T cells by showing them what is essentially a passport displaying the identity of the cell. Ideally for cell therapy applications, these HLA molecules need to be removed. For HLA class I molecules there is already a relatively simple approach: if you delete the B2M gene, class I molecules cannot traffic to the surface of the cell. For HLA class II, the story is not so simple.

My own work in this area has provided a lot of food for thought. Gene editing truly gained traction in 2013 with the advent of the CRISPR/Cas system, and its application to mammalian cells – what previously took months with the TALEN system could now be achieved within weeks.

In 2013, with the help of a graduate student, Leonardo Ferreira, we discovered one guide RNA catchily named the B2M bulldozer. In a HEK 293T cell line, the gene targeting activity was mind blowing. In $CD4^+$ T cells, CRISPR/Cas9 gene targeting was less efficient. However, all the guides that we tested showed some activity, which we were able to improve upon by using a dual guide strategy [1].

Delivery presented a significant challenge, as primary immune cells do not like DNA. There are different ways of getting around this using viral and non-viral systems (Figure 2).

One option is lentiviral delivery targeting the T cell receptor. With lentivirus and antibiotic selection, you can greatly facilitate and improve on efficacy. However, whether this will be accepted by regulatory agencies remains to be seen.

In my view, the real game changers are ribonucleoproteins (RNPs). In this approach, recombinant Cas9 is complexed with synthetic guide RNA, and this provides extremely low toxicity coupled with high targeting efficacy. If we fast forward to 2020, we now find RNPs in the clinic. One example is a famous trial from Carl June's lab at the University of Pennsylvania [2].

Why stem cells?

Stem cells are ideal for cell therapy due to two key features: they rapidly divide, so you can make many building blocks of genome edited stem cells; and they are pluripotent and can therefore differentiate into many different cell types.

As discussed earlier, a potential strategy to overcome the immune barrier is to create genome-edited universal stem cells that can be differentiated into various therapeutic cells. For us, this project gained traction upon Xiao Han joining our laboratory. She deleted the entire locus of the polymorphic HLAs. HLA-A, and HLA-B and -C are the most polymorphic, and hence drive allorejection. We then performed a series of studies to check on-target activity and demonstrated that these knockout cell lines were indeed prevented from expressing HLA [3].

Next, we wanted to test whether the function of the cells was maintained, and if there was a therapeutic effect. In this case, this would be immune evasion of the transplanted cells – and we demonstrated that the HLA-deficient cell lines evaded $CD8^+$ T cell activation and killing. We also needed to protect the cell lines from natural killer (NK) cell responses. To this end, we inserted immunoregulatory molecules which will be very familiar to anyone working in the cancer space – the checkpoint inhibitor PD-L1, CD47 (a macrophage 'don't eat me' signal) and HLA-G. Using these methods, we saw a drop in NK degranulation and toxicity.

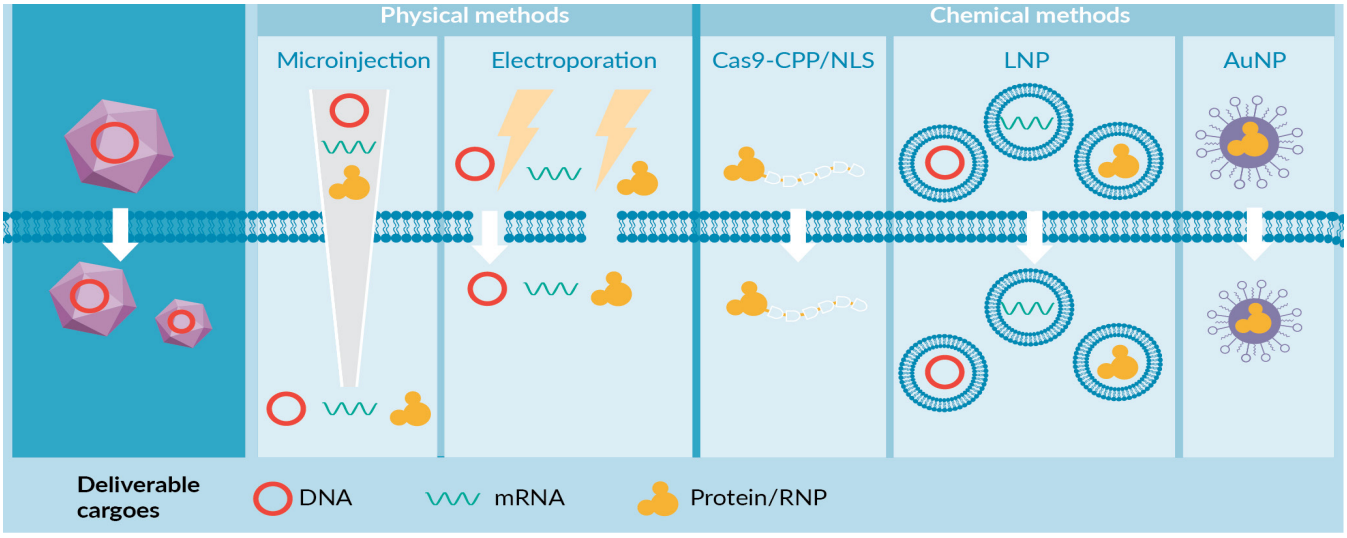
Lastly, we extended this work to an *in vivo* assay in a humanized mouse model, and demonstrated that compared to wild type cells, in these knockout and knock in cell lines lower levels of T cell infiltration and cytotoxicity were observed. Of course, this does not provide a complete answer on how well these cells are immunoprotected – but we have shown that the CRISPR/Cas9 system can be used to ablate HLA expression in human pluripotent stem cells. Crucially, the cells remain pluripotent and retain a normal karyotype. We observed minimal off-target activity, and these cells evaded T cell responses and were protected from NK and macrophage responses both *in vitro* and *in vivo* [3].

Gene edited cell therapies: the future?

Engineering cells through gene editing technology has the potential to impact on a number of different fields: in regenerative medicine for cell replacement therapy across the allobarrier, in cancer immunotherapy, and potentially to replace cells that are lost due to autoimmunity, such as in type I diabetes or multiple sclerosis. By using modified cells that fly under the immune system's radar, true cellular cures for a variety of conditions may be possible.

FIGURE 2

The delivery challenge. Adapted from [4] .



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Q & A

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Q Let's begin with a discussion of the off-target effects of gene editing in the cellular immunotherapy context – what impact can they potentially have on the therapy?

JF: There are a few effects it could have in the short term. For example, if you have edited a T cell, this could impact either the proliferation or the persistence of the cell, and as a result the therapy may not be as effective as it could be. The impact of editing could also be off-target but simply not do anything. There are lots of non-coding regions in the genome, and it could just fall where there is no impact.

On the more extreme side, if an indel or translocation occurred in a tumor suppressor gene, that could potentially lead to an oncogenic event. The cell therapy you are treating a patient with could deal with the particular cancer they are suffering from, but in the medium or longer term, having those cells within the body could then lead to a secondary cancer. There needs to be strict regulation on monitoring and understanding what off-target effect there could be with a cell therapy.

TM: I agree – and in my view it depends also on the timespan that the cells will be in the patient's body. For example, with cancer immunotherapy, it is transient, and if the approach is non-autologous, the cells will actually be rejected after a certain amount of time. In these situations, off-target effects may not play such an important role, as opposed to cells that are meant to be in the body for a much longer time, for example in cell replacement therapies for diabetes or Parkinson's. In these cases, you need to make sure the cellular function is not compromised. And if you begin with stem cells as a starting population, you want to ensure that you do not have any residual stem cells, and they do not drive any transformation due to off-target effects. This is an important safety concern for regenerative medicine.

TT: There are two questions that I tend to consider here. The first is that we know gene editing is specific, but nevertheless can cut DNA in unintended locations. The extent

to which this happens is still up for debate, and we need better tools to assess the magnitude and effect of off-target editing.

The second point is that there are still questions around the longer-term effects of gene editing. Are these genetic manipulations stable, and are there unexpected or unintended consequences of these manipulations?

The initial trials we are seeing at the moment are going to be crucial for addressing these questions, as will be the new tools we see coming in.

Q Looking more broadly at the safety challenges with current autologous and allogeneic cell therapies, how can gene editing either add to or help alleviate these concerns?

TT: Elaborating on autologous CAR T therapy, we know there are many well-defined issues here. These include time for manufacturing, wide variation in terms of quantity and quality of T cells, and difficulty in obtaining enough cells for re-dosing. This is really driving the interest in off-the-shelf allogeneic CAR T cells, which can be pre-manufactured from third-party donors and theoretically provide solutions to these different problems.

Allogeneic T cells possess foreign immunological identities, and this can lead to histocompatibility considerations such as GvHD, as discussed earlier. This is an exciting opportunity for gene editing. Indeed, some groups have already demonstrated high efficiency targeting of endogenous targets such as the T cell receptor (TCR) and HLA molecules. Our own platform at the Catapult is able to achieve greater than 99% knockdown of TCR expression in primary T cells. I certainly believe that there is therapeutic potential with this approach.

The challenge I see here is enrichment efficiency. No approach is going to give you 100% removal of residual TCR/HLA-positive cells, and this is a really important consideration to reduce the risk of contaminating cells potentially inducing GvHD.

At the Catapult we have programs looking at the feasibility of other cell sources such as NK cells, which do not express T cell receptors and, therefore, present a significantly lower risk for GvHD. The challenge here is how to enhance their persistence *in vivo*, and again, this is where gene editing has a lot of potential.

In the longer term I expect we will see greater adoption of induced pluripotent stem cell technology, and there are already companies leading the way, such as Fate Therapeutics with their CAR NK cell candidate. The real benefit is that you can use single-cell engineered clones as your starting material. The challenge will be the implementation of quality control measures that allow you to ensure genotypic stability and efficient cell differentiation.

Q Looking at multiplexing and gene editing, what will the impact on off-target effects be?

TM: To first bring everyone on to the same page, multiplexing is when you use several guide RNAs to target multiple genes in one round of transfection.

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For example, in CAR T cells, you want to prevent allorejection, hit the T cell locus by targeting either the alpha or beta chain of the T cell receptor, and you also want to increase persistence. You don't want the cells to get exhausted, so there are checkpoint inhibitory receptors such as PD-1 that you can target.

It's important to note, you can only deliver so many RNPs or nucleic acids to one cell. If you perform multiplexing you dilute out each individual guide RNA, and reduce the on-target efficacy. With regards to the off-target effects, that is actually a good thing, as you will also see reduced activity on those off-targets.

However, at least for the Cas9-based system, in the lab we see a higher degree of toxicity with multiplexing in most cases. You reduce the off-target intensity, but the overall number of off-targets may increase when you multiplex, and cell viability is ultimately linked to double-strand breaks. Cells have a way of keeping track and counting mounting genotoxic stress and are then driven towards senescence and cell death. It is not only the off-target effects you should keep in mind, but also the cell viability.

This can be overcome by using either pharmacological inhibition, or an alternative is the base editing approach, as the base editor does not rely on double stranded breaks. We observed less genotoxicity and hence increased viability with the base editor.

JF: Building on what Torsten mentioned, it is definitely something that needs a lot of attention as people start to perform more and more gene edits. If you are going after five genes, and you are creating double strand breaks for each of those genes, you have now got at least ten strands of DNA which will need to be joined. The non-homologous end joining machinery needs to be able to cope with that level of genomic instability.

Then let's say the Cas9 endonuclease is also creating one off-target edit per gene editing event. You could then end up with 10 double strand breaks, and now you have got 20 strands. This is a question I asked my biostatisticians about a few times. You have to make a lot of assumptions to try and figure the impact of this. It is a very tricky thing to answer but theoretically, the less double stranded breaks, the better. This is why we are focusing a lot of our efforts on base editing in order to avoid working with double strand breaks.

On the flipside, if cell therapy is moving away from T cells or NK cells and going down the induced pluripotent stem cell route, you could do multiplexing then perform mass screening for cells that do not have any off-target effects. Even if 90% or higher have quite catastrophic off-target effects, as long as you can identify the cells that have been edited in a way you want without off-target edits, then you could tackle it at the screening level.

It is a big challenge, but there are multiple paths we could take to overcome it.

Q What are the challenges facing *ex vivo* and *in vivo* gene editing approaches in the cellular immunotherapy field, and what you see as the relative advantages of each? Do you expect a 'best approach' to eventually emerge?

TT: The commonality between the two approaches is that safe and effective delivery of the CRISPR/Cas component into the nucleus is required for effective

therapeutic gene editing. The gene editing machinery can be delivered in several formats, such as plasmid DNA, viral vectors or ribonucleoprotein complexes.

In the ideal case, a delivery system should address the current limitations of CRISPR gene editing: the lack of targeting to specific tissues and cells, the inability to enter cells, activation of the immune system, and off-target events, as we have already covered.

To circumvent most of these problems, therapeutic applications of CRISPR/Cas9 are usually performed *ex vivo*. Typically, they use technology such as electroporation, but there are concerns around the impact of electroporation on cell quality. At the Catapult we have an *ex vivo* delivery program looking at what we call non-viral technologies, including electroporation alternatives such as lipid nanoparticles and chemical transfection. These technologies offer advantages in that they do not subject the cells to any sort of electrical or mechanical stresses, but they still have some way to go to reach the GMP readiness and scales that current electroporation platforms offer.

The other issue with *ex vivo* is the fact that most commercially available reagents cannot transfect T cells, and this is because uptake is a major barrier for gene delivery. Endosomal acidification, for instance, is slower and not as robust in T cells compared to the HeLa cell line that is typically used to evaluate cationic polymers. You can see how this can start to impact the kinetics of your gene editing experiments. If we want to move away from electroporation *ex vivo*, we need delivery systems that can be tailored specifically to the biological traits of the material that we are working with.

For *in vivo*, the gold standard is to use adeno-associated virus (AAV). It has established itself as the vehicle of choice for gene therapy, and many *in vivo* CRISPR efforts are following suit. However, these systems suffer from problems relating to packaging constraints, immunogenicity, and longevity of Cas expression, which can favor off-target events.

Finally, the other issue is that for HDR-based therapies, most *in vivo* strategies currently involve dual AAV vector delivery systems, one for the nuclease and a second for the donor. Whilst this shows preclinical promise, it may face regulatory, manufacturing, and clinical delivery challenges.

Q Torsten, what would you expect to see as the approach that might eventually emerge victorious?

TM: This depends on the application and the cell type. For cancer immunotherapy, *ex vivo* seems safer because you can perform a quality control screening and assessment of whether you really knocked out the genes you wanted by sorting and enriching them.

In vivo would be fantastic for the treatment of inborn diseases if we could do that without any side effects. Again, we have the delivery problem, not only to cells but to reach the organ that you intend to target to correct an inherited disease. The liver is the easiest because lipid nanoparticles, or whatever you decide to inject into the blood, end up in the liver or in the lungs. The lung you could also target using aerosols.

I am particularly interested in immune therapies, and there you would have to ultimately reach the bone marrow to cure a congenital disease. How can you deliver your modality into a particular cell type in the bone marrow niche? This can only be accomplished if we come up with better, more targeted delivery systems.

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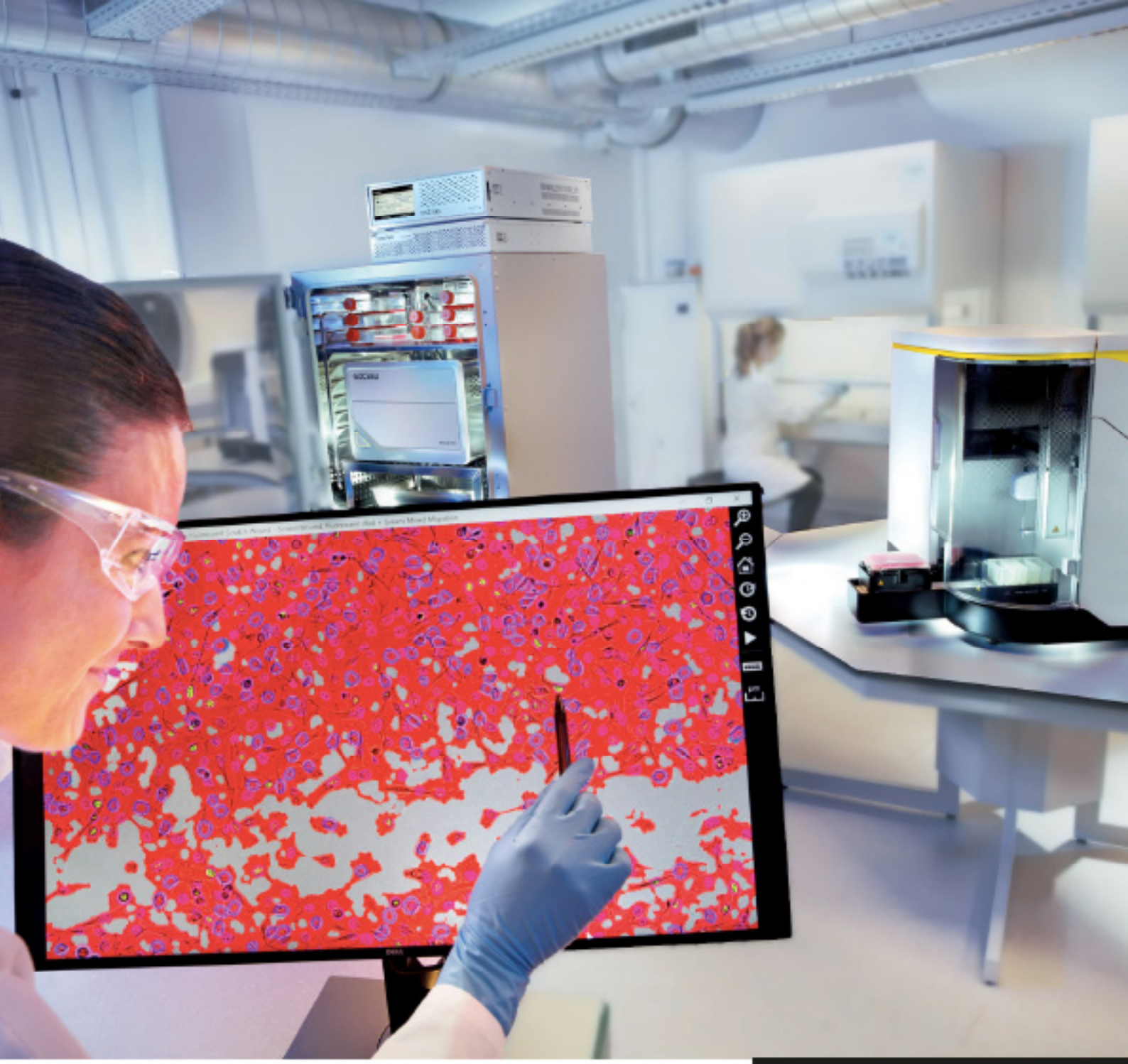

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Promising non-viral vector for efficient and versatile delivery of mRNA for antigen-specific immunotherapy

Rebuma Firdessa-Fite, Jorge Postigo-Fernandez, Valérie Toussaint-Moreau, Fabrice Stock, Alengo Nyamay'antu, Patrick Erbacher & Rémi J Creusot

Cellular immunotherapy involves the modification of immune cells *in vivo* or *ex vivo* to elicit, modulate or suppress immune responses. Modification by mRNA has become an attractive alternative to DNA vectors as a non-viral delivery approach, due to high transfection efficiency including in non-dividing cells. However, widespread adoption of this strategy is currently limited by the lack of commercially available ready-to-use reagents for *in vivo* delivery of mRNA. In this study, we evaluate the newly launched *in vivo*-jetRNA® for the delivery of mRNA-encoded antigens into nanoparticles and the resulting $CD4^+$ T cell responses to one of the expressed epitopes. Four routes of administration were compared to determine the *in vivo* biodistribution of the nanoparticles based on evidence of antigen-specific T cell responses in various lymphoid tissues. Systemic routes achieved efficient delivery with most antigen-specific T cells stimulated in all sites tested. In the case of local routes, responses were confined to draining lymph nodes. Some of the activation markers (CD25, PD-1) were only induced in specific sites using specific routes, suggesting a role for the local dose of nanoparticles and the nature of antigen-presenting cells present involved in different sites. When applied to splenocytes from different mouse strains *in vitro*, the mRNA nanoparticles had marginal effect on the maturation of antigen-presenting cells, did not negatively affect viability and did not induce proinflammatory cytokines. We conclude that *in vivo*-jetRNA® is a promising mRNA formulation for efficient delivery of genes and antigens *in vivo*.

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Cellular immunotherapy aims to harness the adaptive immune system in order to develop a targeted and curative response for unmet medical needs in the effective treatment of cancer, chronic infections or autoimmune diseases. The adaptive immune system is a specific immune response mainly driven by highly specialized and specific cells: lymphocytes (B and T). B lymphocytes, once exposed to an antigen in a peripheral lymphoid organ, can be activated and differentiate into plasma cells that secrete soluble or membrane-bound antigen-specific antibodies. T lymphocytes are subdivided into helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) whose role is to help activation of other immune cells (B cells, $CD8^+$ T cells, macrophages, etc.) and to kill infected target cells, respectively. Successful immunogenic vaccines and therapies rests on activation of as many antigen-specific T cells as possible in different lymphoid tissues to create a robust immune response. Conversely, in the case of autoimmune diseases, effective immunotherapy relies on elimination or inactivation of self-reactive T cells. When nucleic acid vectors are used to express the antigen of interest or antigenic subparts (epitopes), they should be efficiently delivered to antigen-presenting cells (APCs) who will in turn present the encoded antigens to T cells in different lymphoid tissues. Thus, regardless of the desired outcome, there is a need for efficient and safe delivery modalities for successful antigen-specific and cellular immunotherapy.

mRNA delivery on the rise

In vivo delivery of plasmid DNA to APCs was demonstrated to result in the induction of primary adaptive immune response more than two decades ago [1]. Since then, there has been a keen interest in DNA-based vaccines compared to protein/peptide-based vaccines; they are easier to produce, more cost-efficient and their delivery can be enhanced using transfection methods. Still, there are challenges with the use of plasmid DNA, mainly attributed to the insufficient immune response level elicited. This low immunogenicity of plasmid DNA is dependent on the delivery efficiency to target cells [2]. To improve delivery of plasmid DNA into tissues, several delivery systems have been developed, such as cationic polymers, to condense and protect DNA from degradation while facilitating its delivery into APCs via endocytosis. As previously discussed [3], we developed the leading cationic polymer-based transfection reagent *in vivo*-jetPEI® that is currently used in several ongoing nucleic acid-based drug development programs, with a majority of clinical trials for cancer treatment. As an alternative to DNA, mRNA-based immunotherapy is a promising tool due the fact that it retains many of DNA's advantages including delivery of multiple antigens with one immunization while allowing a higher transfection efficiency, avoiding promoter-dependent inhibition of expression and genome integration [4,5]. The higher transfection efficiency stems from the fact that mRNA does not need to reach the cell nucleus for expression nor require cell division for efficient gene expression. This is particularly interesting to improve the efficiency of delivery into slow-dividing and quiescent cells *in vivo*. The main drawback of mRNA is that it is not a stable molecule, hence the need for a transfection reagent that can efficiently protect mRNA during its *in vivo* delivery to cells and can easily be administered through systemic and local administration routes. The ideal transfection reagent should efficiently condense and protect mRNA from degradation by nucleases, as well as facilitate endosomal escape [6].

in vivo-jetRNA®, A PROMISING VECTOR FOR mRNA DELIVERY

Delivery of mRNA into cells, and even more so *in vivo* is challenging because it depends on efficient condensation of mRNA molecules to prevent degradation by extracellular nucleases. Although significant progress has been made on efficient intracellular delivery of mRNA, thanks to extensive optimization efforts in the field to make mRNA-based therapeutic strategies viable for immunotherapy, there is still no such therapy that

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is approved and commercialized. Strategies for non-viral delivery of therapeutic mRNA include *ex vivo* transfection of isolated vehicle cells (e.g. dendritic cells [DCs] as APCs), direct injection of naked mRNA, and nanoparticles. Nanoparticles carrying mRNA molecules have the potential to significantly improve the efficiency of mRNA delivery to cells non-invasively and with improved safety. These mRNA-containing nanoparticle formulations are typically made in-house and require significant experience and, in some cases, specialized equipment, for successful production. However, the lack of commercially available options to produce such nanoparticles that are validated and accessible to the average researcher constitutes a barrier to the widespread evaluation of this therapeutic strategy. Based on our expertise in developing non-viral based transfection reagents for *in vivo* applications, we engineered a ready-to-use transfection reagent, *in vivo*-jetRNA®, specifically to ensure delivery of intact mRNA molecules to various tissues and cell types, the nature of which depending in part on the route of administration.

CASE STUDY: BIODISTRIBUTION, AMPLITUDE & QUALITY OF A T CELL RESPONSE INDUCED BY AN mRNA-ENCODED EPITOPE DELIVERED BY *in vivo*-jetRNA®/mRNA NANOPARTICLES

Delivery of antigen-encoding mRNA to exogenous DCs versus endogenous APCs

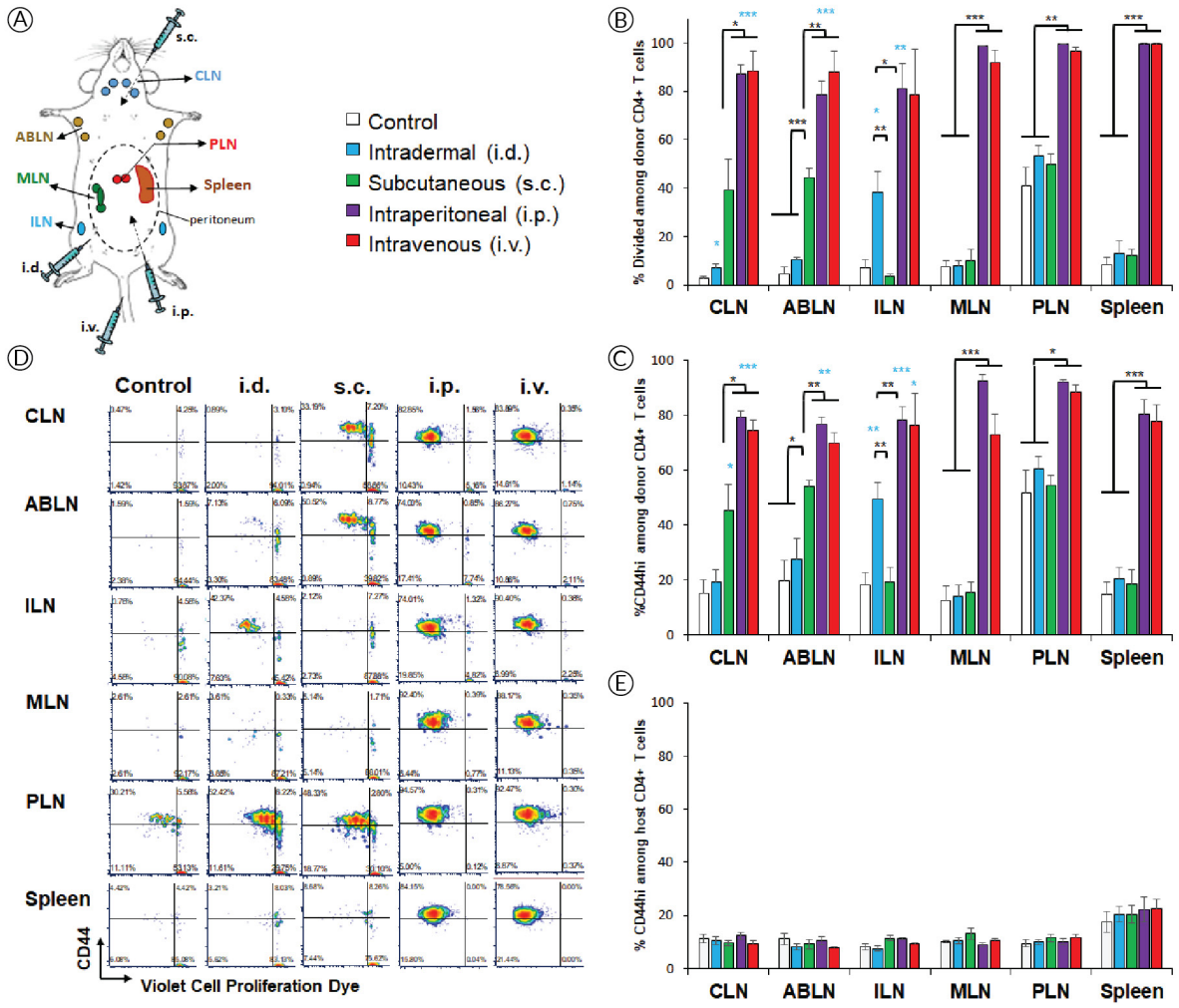
We have recently compared delivery of mRNA-encoded epitopes by *ex vivo* mRNA-electroporated DCs to nanoparticle delivery as means to elicit effective antigen-specific T cell responses in immunotherapy [7]. We reported that nanoparticle-mediated mRNA delivery to professional (bone marrow-derived DCs) and non-professional (stromal) types of APCs *in vitro* using jetMESSENGER® was gentle and most efficient in stromal cells. While not designed for *in vivo* delivery, these mRNA-carrying nanoparticles achieved transfection of various hematopoietic APCs (conventional and plasmacytoid DCs and other myeloid cells; 1–7% of cells) and endothelial cells (~14% of cells) within lymph nodes. Using antigen-encoding mRNA, this level of transfection was adequate to induce robust $CD4^+$ and $CD8^+$ T cell responses, which showed a broader biodistribution than those induced by *ex vivo* mRNA-electroporated DCs injected by the same route (intraperitoneal). When administered intravenously, DCs were primarily retained in lungs, while the nanoparticles efficiently targeted lymphoid tissues such as the spleen. These studies took advantage of a new platform (Endotope) that enables optimal engagement of $CD4^+$ and $CD8^+$ T cells with multiple antigenic peptides encoded by DNA or mRNA [8].

Systemic injection of *in vivo*-jetRNA®/mRNA nanoparticles induces robust & widespread antigen-specific T-cell responses

In this study, we evaluated how *in vivo*-jetRNA®, a specifically developed *in vivo* transfection reagent, performs in delivering mRNA-encoded epitopes to different lymphoid tissues based on route of administration. To this end, we assessed the amplitude and phenotype of antigen-specific T-cell responses elicited by *in vivo*-jetRNA®/mRNA nanoparticles in various lymphoid tissues as readout. Two systemic routes (intravenous [i.v.] and intraperitoneal [i.p.]) and two local routes (intradermal [i.d.] and subcutaneous [s.c.]) were compared (Figure 1A). We adoptively transferred T cell receptor-transgenic $CD4^+$ T cells specific to one of the expressed epitopes followed by *in vivo*-jetRNA® nanoparticles containing mRNA-encoded epitopes. A wide variety of lymph nodes (Figure 1A) as well as the spleen were collected 3 days later (before activated T cells can redistribute to other sites) for a comprehensive assessment of the biodistribution of nanoparticles and where antigen presentation takes place.

FIGURE 1

Activation of antigen-specific T cells by an mRNA-encoded epitope in different lymphoid tissues after delivery via different routes using *in vivo*-jetRNA®.



(A) Schematic of the sites of injections (see 'Methods' section) and lymphoid tissues analyzed. **(B–D)** Response of antigen-specific T cells: percentage of T cells that have divided at least once, based on proliferation dye dilution **(B)**, percentage of $CD44^{high}$ cells **(C)** and representative plots for the different routes and locations **(D)**. In this system, the PLN serves as an internal positive control because BDC2.5 T cells also react to a self-antigen naturally and uniquely presented in these lymph nodes, hence the higher background. **(E)** Response of recipient polyclonal $CD4^+$ T cells (mostly nonantigen-specific) based on CD44 upregulation. Data are mean ± SEM (n=4–6 mice per group). Statistical significance measured by two-way ANOVA: * p<0.05, ** p<0.01, *** p<0.001. Stars in black (*) are for comparisons indicated by the lines. Stars in blue (*) are for comparison with the control group.
Lymphoid tissues analyzed: ABLN: Axillary and brachial lymph nodes; CLN: Cervical lymph nodes; ILN: Inguinal lymph nodes; MLN: Mesenteric lymph nodes; PLN: Pancreatic lymph nodes, spleen.

Based on proliferation and CD44 upregulation (indicators of antigen recognition), T cell responses were maximal (>80%) in all lymphoid tissues evaluated following systemic administration of only 5 µg of mRNA per mouse (Figure 1B–D), indicating a broad distribution of mRNA and T cells responses with these routes. In contrast, more modest albeit significant responses, which were limited to the draining lymph nodes (inguinal lymph nodes for i.d., axillary, brachial and cervical lymph nodes for s.c.), were seen with local delivery routes. We found no evidence of visible non-specific stimulation of nonantigen-specific (polyclonal) $CD4^+$ T cells based on CD44 expression (Figure 1E).

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Local injection of *in vivo*-jetRNA® nanoparticles carrying antigen-encoding mRNA induce a T cell phenotype distinct from systemic administration

The high affinity interleukin-2 (IL-2) receptor chain CD25 can be induced upon activation and is important for T cell proliferation supported by IL-2 as the major T cell growth factor. However, it is interesting that, despite evidence of extensive proliferation (Figure 1), $CD4^+$ T cells did not induce CD25 in response to systemic delivery (Figure 2A & B). On the contrary, CD25 was uniquely upregulated in draining lymph nodes in the case of i.d. and s.c. routes, and marginally in peritoneal lymph nodes (MLN and PLN) in the case of the i.p. route (Figure 2A & B), but not changed on polyclonal $CD4^+$ T cells in all lymph nodes (Figure 2C).

PD-1 is another activation marker that serves as negative regulator of the T cell response. PD-1 is usually more highly expressed on exhausted T cells following repeated antigen exposure. Interestingly, PD-1 upregulation was restricted to lymphoid tissues within the peritoneal cavity after i.p. injection (Figure 2B & D), contrasting with proliferation and CD44 upregulation (Figure 1B & C), which appear more sensitive. PD-1 was also prominently induced in the spleen after i.v. injection, which is where mRNA/nanoparticles primarily accumulate when using this delivery route [7]. Again, the treatment did not affect PD-1 expression on other T cells (Figure 2E). Small but significant increases in $CD25^+$ and $PD\text{-}1^+$ polyclonal T cells were observed in spleen after systemic delivery.

The results of this study revealed interesting differences in the regulation of CD25 and PD-1 induction, which were uncoupled from simple TCR engagement that was evidenced by CD44 upregulation and followed by proliferation. Although more experimental work is needed for confirmation, our interpretation of those data is as follow:

1. CD44 upregulation and proliferation in antigen-specific $CD4^+$ T cells are achieved wherever there is sufficient antigen presentation. These responses are lower when antigen presentation is only contributed by migratory APCs (e.g. in draining lymph nodes after local delivery) and higher when resident APCs are directly transfected (e.g. systemic delivery). These responses are maximal when the local dose is higher (e.g. in peritoneal lymphoid tissues compared to skin-draining tissues after i.p. delivery).

2. CD25 may be most readily induced by migratory APCs. In the case of i.d. and s.c. deliveries, these APCs may be dermal DCs and/or Langerhans cells from the skin. In the case of i.p. delivery, they may be APCs from the omentum, which serves as the port of entry for cells and particles introduced into the peritoneal cavity [9,10].

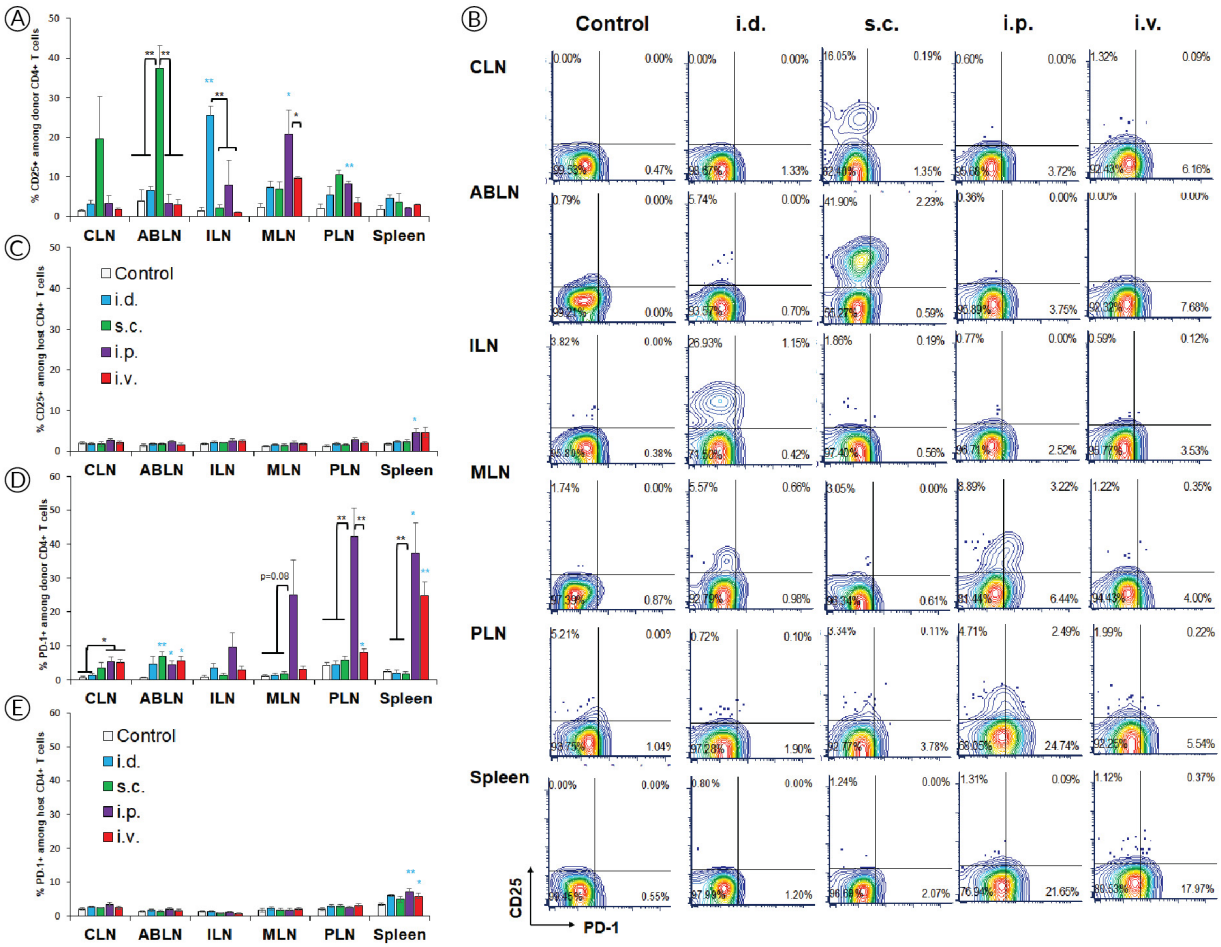
3. In contrast, PD-1 may be more readily induced by resident APCs, but at a higher local dose than is required for CD44 upregulation and proliferation, thus is not observed in distal lymph nodes. Although these responses are prominent in all lymphoid tissues after i.p. injection for example, we find that they are nonetheless significantly lower ($p<0.03$) in ABLN and CLN (non-peritoneal) than in MLN and PLN (peritoneal). The same may apply for the spleen as it is within the peritoneal cavity. Nanoparticles that are injected i.v. disperse easily and can reach all lymphoid tissues with a dose sufficient for CD44 upregulation and T cell proliferation, but not necessarily for PD-1 upregulation. However, the spleen is by far the most vascularized of the tissues evaluated, and as such, it is expected to have a higher local dose of nanoparticles [7], thereby enabling PD-1 upregulation. The small effect on CD25 and PD-1 seen on splenic polyclonal $CD4^+$ T cells may be attributed to this higher accumulation of nanoparticles

combined with cumulated T cell responses to other epitopes, besides p79, expressed by the mRNA.

The differences seen between routes may reflect both local antigen doses and the nature of APCs transfected and/or presenting antigens, keeping in mind that transfected cells incapable of presenting antigens may nonetheless transfer encoded antigens to professional APCs. The environment of each lymphoid tissue may also influence the immune responses. Both MLN and PLN drain the intestinal mucosa [10], and their APC content and microenvironment milieu is expected to differ dramatically from skin-draining lymph nodes (ABLN, CLN, ILN). Substantial heterogeneity in immune responses is even observed within gut-draining lymph nodes depending on the sections of the gut they drain [11]. Thus, it is important to understand the biodistribution of T cell responses and how it affects the profile of the responding T cells, and select the route accordingly.

FIGURE 2

Expression of CD25 and PD-1 by antigen-specific T cells are dependent on the route antigen mRNA/*in vivo*-jetRNA® nanoparticle injection.



(A–C) Percentage of CD25⁺ cells among donor antigen-specific CD4⁺ T cells **(A & B)** and recipient polyclonal CD4⁺ T cells **(C)**. **(B & D & E)** Percentage of PD-1+ cells among donor antigen-specific CD4⁺ T cells **(B & D)** and recipient polyclonal CD4⁺ T cells **(E)**. Panel B: representative contour plots for the different routes and locations. Response to the natural self-antigen in the PLN (control group) does not involve CD25 or PD-1 up-regulation. Data are mean ± SEM (n=4–6 mice per group). Statistical significance measured by two-way ANOVA: * p<0.05, ** p<0.01. Stars in black are for comparisons indicated by the lines. Stars in blue are for comparison with the control group.

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in vivo-jetRNA® nanoparticles do not exhibit toxicity & overstimulation of proinflammatory cytokines on immune cells *in vitro*

Since *in vivo*-jetRNA® is a recently launched formulation for mRNA delivery, we addressed whether mRNA formulated with this reagent had deleterious effects on immune cells, including an excessive non-specific release of proinflammatory cytokines by overstimulation of Toll-like receptors (TLR). To gain insight of these possible effects on immune cells across genetically diverse individuals, we cultured splenocytes from three strains of mice for 24h in the presence of 'naked' mRNA, mRNA/*in vivo*-jetRNA® nanoparticles or a variety of TLR ligands. We used a relatively high dose of mRNA (0.5 µg per $2x10^5$ cells, same mRNA as *in vivo* studies), which is only ten times lower than the dose injected *in vivo*, and we used comparable amounts of TLR ligands. The mRNA nanoparticles had no to marginal effects in increasing the expression of MHC and costimulatory molecules on the surface of $CD11c^+$ cells (Figure 3A) and $B220^+$ B cells (Figure 3B), and these effects were minimal compared to most TLR ligands tested. Furthermore, the mRNA nanoparticles did not negatively affect the viability of the cells (Figure 3C). Importantly, the mRNA nanoparticles did not induce the expression of proinflammatory cytokines IFN-β, IFN-γ, TNF-α and IL-6, or the regulatory cytokine IL-10, unlike most TLR ligands tested (Figure 3D), and none of the treatments induced IL-1α, IL-1β, IL-12p70, IL-17A, IL-23, IL-27, MCP-1 and GM-CSF (data not shown). Thus, this formulation appears safe, and given the relatively low dose used to achieve robust responses *in vivo* and the high dispersion of the nanoparticles after systemic delivery supported by our biodistribution data, we do not anticipate toxicity or cytokine storm to occur. These data are in line with the lack of unspecific/bystander effects on polyclonal $CD4^+$ T cells *in vivo*. However, it remains to be assessed whether delivery resulting in high local concentrations of mRNA nanoparticles, for example in the intradermal space, can result in localized toxicity and/or inflammation.

in vivo-jetRNA® constitutes a versatile delivery vehicle for mRNA-encoded antigens

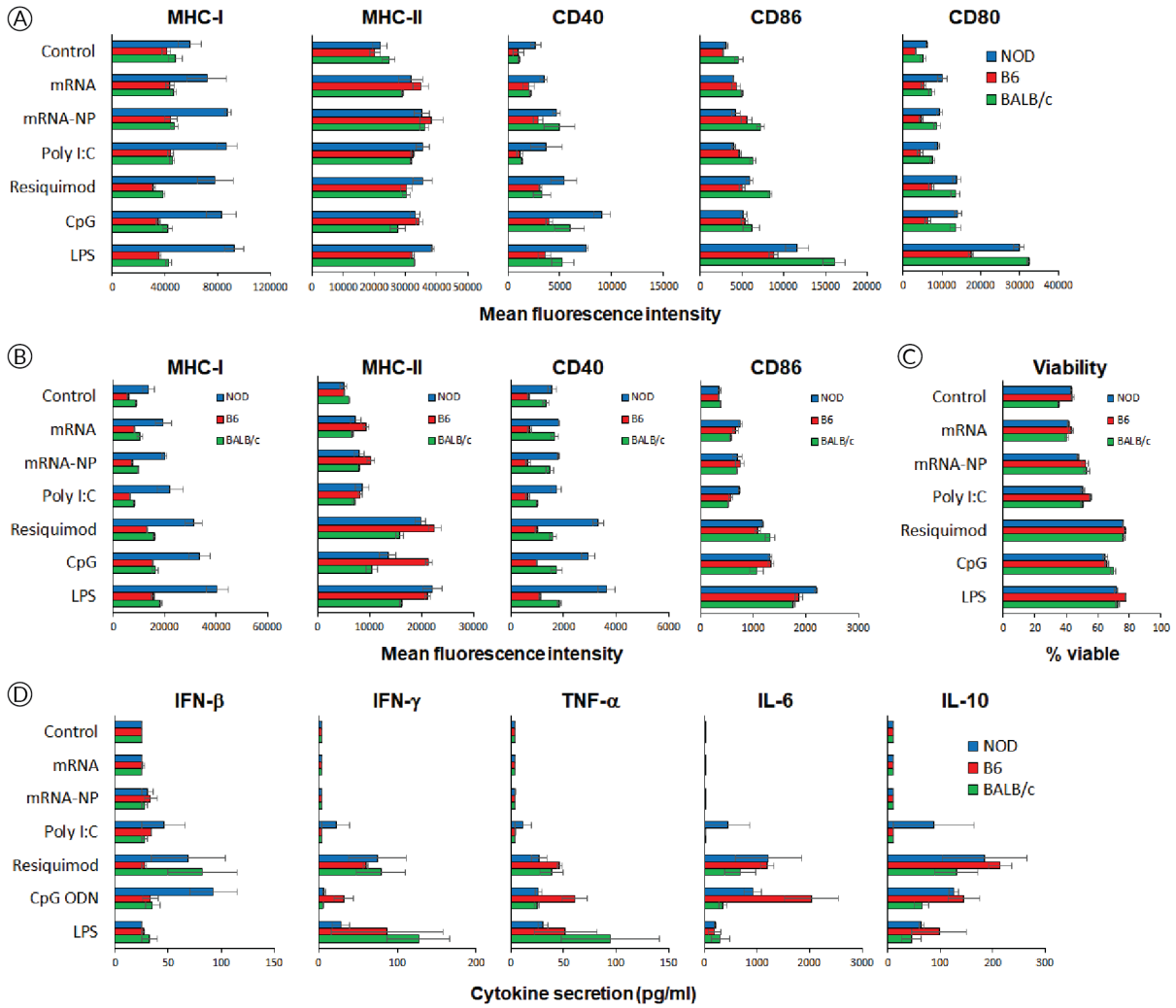
Irrespective of the administration route used, systemic or local, this data set shows that *in vivo*-jetRNA® is a promising non-viral delivery modality for effective *in vivo* delivery of therapeutic mRNA. Our previous studies demonstrated that the delivery of mRNA using nanoparticles can successfully target antigens to various types of APCs in several lymphoid tissues [7], a winning combo to elicit a robust immune response. The phenotype and expansion of the stimulated T cells can be modulated based on the route of delivery, suggesting that this approach is versatile. The relative contribution of different transfected APCs to the overall immune response and how route of vector delivery, microenvironment of the target lymphoid tissue and dose, as parameters, modulate the quality of the T cell response remain to be further explored using models in which the outcome of the T cell response can be tested (viral clearance, tumor rejection, etc.).

Methods

NOD mice (strain #001976, females, 8 weeks of age), used as recipients, and T cell receptor-transgenic congenic (CD45.2) BDC2.5 mice (cross of strains #004460 and #014149, females, 8–16 weeks of age), used as donors, were obtained from The Jackson Laboratory, the latter bred in our barrier facility. All procedures were performed following protocols approved by the Columbia University Institutional Animal Care and Use Committee. *In vitro*-transcribed mRNAs, expressing multiple epitopes including the p79 mimotope recognized by BDC2.5 T cells [7,8], was

FIGURE 3

***In vivo*-jetRNA®/mRNA nanoparticles have minimal effect of antigen-presenting cell maturation, and lack toxicity and the ability to induce non-specific release of proinflammatory cytokines *in vitro*.**



(A & B) Effect of *in vivo*-jetRNA®/mRNA nanoparticles (mRNA-NP) compared to medium alone, non-complexed mRNA and Toll-like receptor (TLR) ligands on the expression of MHC and costimulatory molecules on splenic CD11c$^+$ cells **(A)** and B220$^+$ B cells (B) after 24h in vitro. B cells did not express measurable amounts of CD80 (data not shown). **(C)** Viability of splenocytes after 24h of treatment based on propidium iodide exclusion. Data are mean ± SD (n=3 technical replicates per strain). **(D)** Secretion of cytokines by splenocytes after 24h *in vitro* stimulation. Eight other cytokines tested (IL-1α, IL-1β, IL-12, IL-17A, IL-23, GM-CSF, MCP-1) were not detected in any condition. Data are mean ± SEM (n=3 biological replicates per strain). Culture conditions: Medium only (Control), mRNA ± *in vivo*-jetRNA® formulation (500 ng mRNA), poly I:C (TLR3 ligand; 50 ng), Resiquimod (TLR7 ligand, 200 ng), CpG oligonucleotides (TLR9 ligand, 200 ng) or lipopolysaccharide (LPS, TLR4 ligand, 500 ng). All doses are per 2x10^5 splenocytes per well.

produced by TriLink Biotechnologies. The mRNA was produced with modifications (Anti-Reverse Cap Analog as well as 5-methyl-cytosine and pseudouridine substitutions) to increase stability and reduce immunogenicity. For isolation of antigen-specific CD4$^+$ T cells, spleen and pooled lymph nodes were collected from donor CD45.2$^+$ BDC2.5 mice and CD4$^+$ CD25- T cells were purified using the MoJo™ Mouse CD4 T Cell Isolation Kit (BioLegend) supplemented with biotinylated anti-CD25. Cells were then labelled with Violet Cell Proliferation Dye (eBioscience) and 0.5–1x10^6 T cells were injected i.v. into recipient NOD (CD45.1$^+$) mice. The formulation of mRNA into *in vivo*-jetRNA®/mRNA nanoparticles was done according to manufacturer's instructions.

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Following adoptive transfer, recipient mice were injected with nanoparticles containing 5 µg mRNA per mouse either i.v. (tail vein), i.p., i.d. (shaved abdominal area) or s.c. (neck fold) (Figure 1A). Control mice received T cells but no nanoparticle treatment. After 3 days, lymphoid tissues (as shown in Figure 1A) were collected separately and processed for single-cell suspensions. The cells were stained with antibodies to CD4, CD45.2, CD25, CD44 and CD279 (PD-1), all from BioLegend, and analyzed on a BD Fortessa™ flow cytometer. Splenocytes from NOD, C57BL/6 (B6) and BALB/c mice were cultured *in vitro* for 24h in the presence of mRNA with or without formulation with *in vivo*-jetRNA® or with various TLR ligands known to stimulate antigen-presenting cell maturation and cytokine secretion. Supernatant from these cultures were separately analyzed for 13 cytokines using the LegendPlex™ Mouse Inflammation Panel (BioLegend). The cells from these cultures were analyzed on a BD Fortessa™ flow cytometer after staining for B220 (B cells), CD11c (primarily dendritic cells), MHC class I (H2-K^d for NOD and BALB/c, H2-K^b for B6), MHC class II (I-A/I-E for B6 and BALB/c, I-A^k for NOD), CD40, CD80, CD86 as well as propidium iodide (all reagents from BioLegend and BD Bioscience). Flow cytometry data were analyzed with FCS Express 7.

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Contributions: *Rebuma Firdessa-Fite made all mRNA nanoparticle preparations, performed* in vivo *experiments and multiplex cytokine assays, Jorge Postigo-Fernandez set up the* in vitro *treatment of splenocytes and performed their flow cytometric analysis, Valérie Toussaint-Moreau, Fabrice Stock and Patrick Erbacher worked on the development of in vivo-jetRNA®, Patrick Erbacher and Rémi J Creusot designed and directed the study, Alengo Nyamay'antu and Rémi J Creusot wrote the manuscript.*

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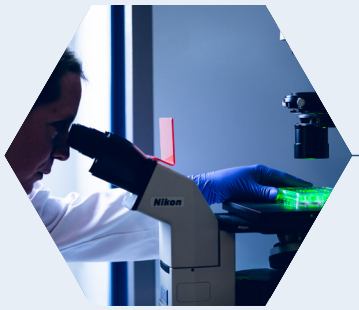

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SPOTLIGHT

REGULATORY PERSPECTIVE

FDA perspective on the preclinical development of cell-based immunotherapies

Alyssa Kosmides Galaro & Christopher Saeui

Preclinical studies are conducted to support administration of investigational products in clinical trials. Assessment of the safety profile and product activity for an investigational product are important to inform the clinical trial design and characterize the benefit–risk profile related to the product and target clinical population. However, identifying appropriate animal models and designing informative preclinical studies can be challenging for cell-based immunotherapies. This review discusses the general expectations for preclinical assessment of cell-based immunotherapies, including resources to support transition from bench to bedside.

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INTRODUCTION TO CELL-BASED IMMUNOTHERAPIES

Cell-based immunotherapies constitute a diverse array of products designed to harness the immune system to treat individuals with medical conditions that can range from cancer to autoimmune disease. These products may be isolated and expanded *ex vivo* without additional modifications, such as adoptively transferred autologous or allogeneic T cells, or may have significant genetic modifications, such as viral transduction to express chimeric antigen receptors and knock-down of immunomodulatory molecules.

There are several aspects of cell-based immunotherapies that can present challenges for a preclinical development program. The frequencies of immune cell subsets, expression of signaling molecules, and antigen recognition domains differ between humans and many traditional laboratory animal species. Thus, it can be difficult to identify an appropriate animal species or establish an animal model that is biologically responsive to the investigational product of interest. Patient-specific products, such as those targeting cancer neoantigens, can present further challenges related to the design of comprehensive preclinical studies to evaluate the safety of the investigational product. An informative preclinical development program for cell-based immunotherapies is thus product-specific and will often include a range of *in vivo*, *in vitro*, and *in silico* studies, each evaluating targeted questions regarding safety and activity.

In the United States, cell-based immunotherapies are regulated by the Office of Tissues and Advanced Therapies (OTAT) in the Center for Biologics Evaluation and Research (CBER) of the US Food and Drug Administration (FDA). This review highlights the scientific and regulatory challenges associated with the preclinical development of cell-based immunotherapies. In the context of this review, cell-based immunotherapies denote products that utilize cells to modify the immune response for the treatment or prevention of disease.

OVERVIEW OF PRODUCT TYPES

The diversity of immunotherapies being developed for treatment of various medical conditions is considerable. Clinical indications range from those of recent product approvals in the field of cancer immunotherapy to those in ongoing development in fields such as autoimmune and infectious diseases. This section will first discuss the various cell sources that are used for cell-based immunotherapies, followed by examples of the different types of genetic modifications that can be incorporated in these products.

Cell source

Cell-based immunotherapies can be derived from isolating immune cells from patients, donors for adoptive cell transfer, or bacterial sources. Non-genetically modified immune cells made up approximately one third of cell-based immunotherapy IND submissions in 2019 (Figure 1). Manufacturing often includes *ex vivo* enrichment and expansion of specific immune cell subsets prior to infusion. Examples include tumor infiltrating lymphocytes activated *ex vivo* with tumor antigens, dendritic cells pulsed with tumor or infectious disease antigens, and regulatory T (Treg) cells stimulated against an autoimmune antigen.

Immune cells can also be derived from a pluripotent stem cell source, such as induced pluripotent stem cells (iPSCs). Stem cell products may be differentiated into a desired cell type, such as NK cells or other immune cell subsets. *Ex vivo* differentiation may be preferred over isolation of specific immune cell subsets from peripheral blood or bone marrow in cases where an allogeneic off-the-shelf product obtained from healthy individuals is desired or for an autologous product obtained from a clinical population that has a dysfunctional immune subset compartment.

Genetic modifications

Genetic modifications can be introduced into the different cell types to redirect their antigen specificity, express immunomodulatory molecules, prevent alloreactivity, introduce a safety mechanism, and/or further tailor cell activity. Common examples of *ex vivo* modified cells include chimeric antigen receptor (CAR) T cells, T cell receptor (TCR)-engineered T cells, and engineered bacteria for immunomodulatory applications. This field is rapidly expanding, enabled by advances such as genome editing technology and development of modern manufacturing tools that facilitate genetic engineering.

CAR T cells are a common genetically modified cell-based immunotherapy product

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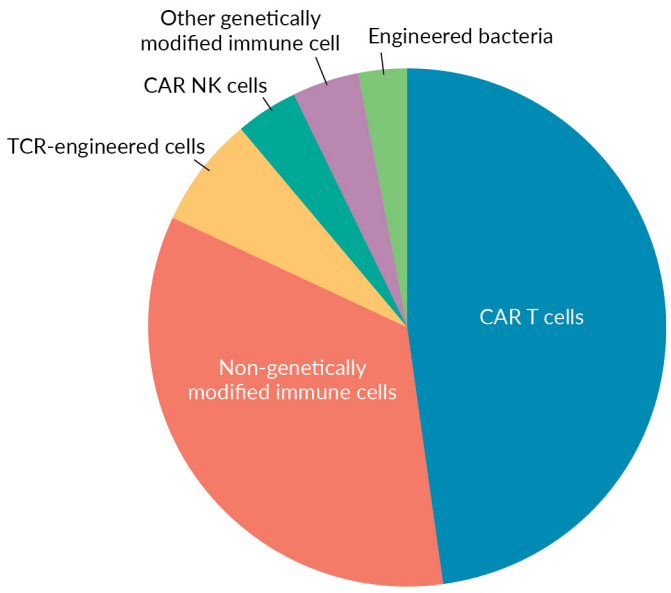
for the treatment of cancers comprising nearly 50% of all cell-based immunotherapy IND submissions to FDA in 2019 (Figure 1). CAR T cells are a type of adoptive cell-based immunotherapy in which introduction of a transgene construct redirects the T cells to recognize antigens expressed on the surface of tumor cells (tumor-associated antigens; TAAs) or other endogenous cell targets. A typical CAR construct consists of an extracellular antigen binding domain, such as a single-chain variable fragment (scFv) specific to a TAA or an endogenous cell target, fused to cytoplasmic signaling domains by hinge and transmembrane segments. The scFv allows recognition of TAA-expressing cells in a human leukocyte antigen (HLA)-independent manner, while the cytoplasmic signaling domains drive T cell effector functions (such as proliferation, cytokine production, and cytotoxicity) upon scFv engagement. As a result, CAR T cells are highly potent "living drugs" engineered to recognize and kill target cells and have the potential to expand and persist long-term following administration. CAR T cells can be further modified to express, for example, additional receptors, cytokines, or suicide genes, or to reduce expression of immunomodulatory signaling receptors. The CAR can also be introduced in natural killer (NK) cells to provide additional antigen-dependent effector function. CAR NK cells comprised nearly 5% of cell-based immunotherapy IND submissions to FDA in 2019 (Figure 1).

Similar in principle to CAR T cells, TCR-engineered T cells are transduced to redirect their antigen-specificity. This product type constituted approximately 5% of cell-based immunotherapy IND submissions to FDA in 2019 (Figure 1). TCR-engineered T cells are human T cells modified *ex vivo* with a transgene construct encoding a desired TCR. In contrast to CAR T cells, this product type recognizes peptides in an HLA-restricted manner. Examples include TCR-engineered T cells that recognize a tumor neoantigen or a TAA. Cell-based immunotherapies with other genetic modifications, such as transduction to induce expression of immunomodulatory molecules, constituted less than 5% of cell-based immunotherapy IND submissions to FDA in 2019 (Figure 1).

Bacteria can also be genetically modified to secrete immunomodulatory molecules.

FIGURE 1

Distribution of product types for cell-based immunotherapy IND submissions to the FDA in 2019.



Examples include bacteria modified to secrete peptides intended to induce tolerance in an autoimmune disease or secrete tumor-associated peptides intended to induce a pro-inflammatory response against tumor cells. Genetically modified bacteria consisted of less than 5% of cell-based immunotherapy IND submissions to FDA in 2019 (Figure 1).

In addition to the products described above, mesenchymal stem cells (MSCs) are another group of cell therapy products being evaluated for various indications, such as graft versus host disease, autoimmune diseases, and trauma, due to their purported immunomodulatory properties. As their mechanism of immunomodulatory action is still undetermined, MSC products are not discussed further in the context of this review.

Trends in IND submissions & marketing approvals

The field of immunotherapy is growing rapidly. In 2019, FDA received 99 IND submissions for cell-based immunotherapy products, in contrast to 40 IND submissions in 2009 (per internal agency numbers). More than 75% of the 2019 submissions were for products being developed for the treatment of hematological malignancies or solid tumors, and are intended to enhance an anti-cancer immune response (Figure 2). Similar approaches are being evaluated for cell-based immunotherapies in the setting of infectious disease, making up approximately 5% of cell-based immunotherapy IND submissions to FDA in 2019. A smaller fraction of products is under investigation as immunomodulators for the treatment of autoimmune diseases and other medical conditions.

To-date, three CAR T cell products, KYMRIAH® (tisagenlecleucel), and YESCARTA® (axicabtagene ciloleucel), TECARTUS™ (brexucabtagene autoleucel), and one *ex vivo* activated peripheral blood mononuclear cell product, PROVENGE® (sipuleucel-T), are cell-based immunotherapies that have received FDA approval. KYMRIAH® is approved for the treatment of patients with B-cell precursor acute lymphoblastic leukemia or relapsed or refractory diffuse large B-cell lymphoma, YESCARTA® for relapsed or refractory large B-cell lymphoma, TECARTUS™ for relapsed/refractory mantle cell lymphoma, and PROVENGE® for metastatic castrate resistant prostate cancer.

Nonetheless, challenges with clinical translation of cell-based immunotherapy products remain, and science-based approaches are needed to establish an optimal preclinical development program, often utilizing innovative testing strategies and assays tailored to answer targeted questions. Thus, product evaluation can vary based on case-by-case considerations. Several communication modalities with FDA/CBER are available to obtain guidance on preclinical development programs for these complex product types, with a shared goal of facilitating product development and achieving a successful IND submission.

THE REGULATORY PROCESS: EARLY COMMUNICATIONS WITH FDA/CBER & THE IND

In order to initiate a clinical trial in the US, an IND application is first submitted to the FDA. There are numerous opportunities to obtain FDA feedback during the development of an investigational product [1]. Early communication with the FDA to obtain feedback prior to submission of an IND is possible primarily through a pre-IND meeting. For innovative products with unique challenges in preclinical development, earlier feedback from the FDA can be obtained in the context of an Initial Targeted Engagement for Regulatory Advice on CBER products (INTERACT) meeting (Figure 3). While these communications are not required prior to an IND submission, they are encouraged due to the challenges with the preclinical development of cell-based immunotherapies (further discussed in Section 4 of this review).

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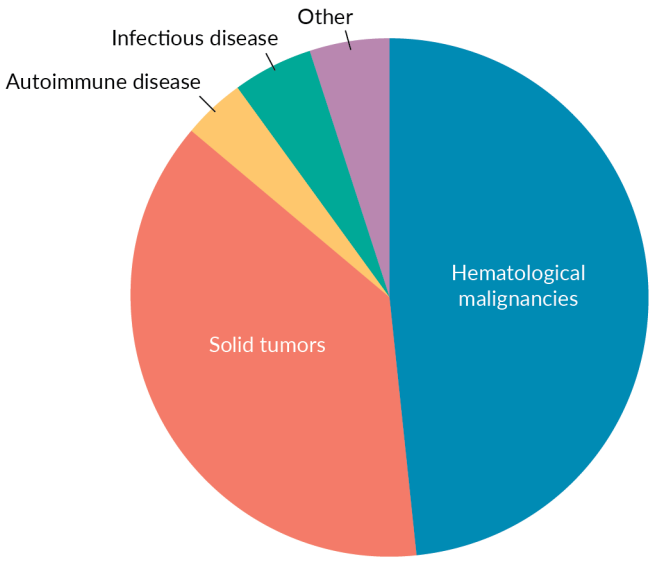
FIGURE 2

Distribution of product indications for cell and gene immunotherapy IND submissions to FDA in 2019.



INTERACT meetings

The INTERACT meeting provides a forum to discuss novel investigational products at an early stage of product development [2]. An INTERACT meeting, which is held prior to a pre-IND meeting, is a non-binding, informal discussion with key FDA/CBER personnel, generally consisting of experts in the areas of:

1. Chemistry, manufacturing, and controls (CMC);
2. Pharmacology/toxicology (P/T); and
3. Clinical trial design.

This interaction serves as an important opportunity to obtain guidance from regulators for products with unique challenges, complex manufacturing methods, innovative devices, and novel testing methods. The information provided on the FDA website [2] includes detailed instructions on how to submit a request for an INTERACT meeting, the expected contents of an INTERACT meeting package, and the logistics of these meetings.

Pre-IND meetings

A pre-IND meeting consists of a non-binding, formal discussion with key FDA/CBER personnel (CMC, P/T, clinical, and other experts). These meetings are an opportunity to obtain FDA feedback and discuss concerns related to manufacturing, preclinical program development, and first-in-human (FIH) clinical protocols. Pre-IND meetings often occur prior to the initiation of definitive preclinical safety studies such that FDA input can be obtained and incorporated into the study designs. At the pre-IND stage, product development programs are typically further along in comparison to INTERACT submissions, and more extensive preclinical data are available to facilitate a productive pre-IND discussion.

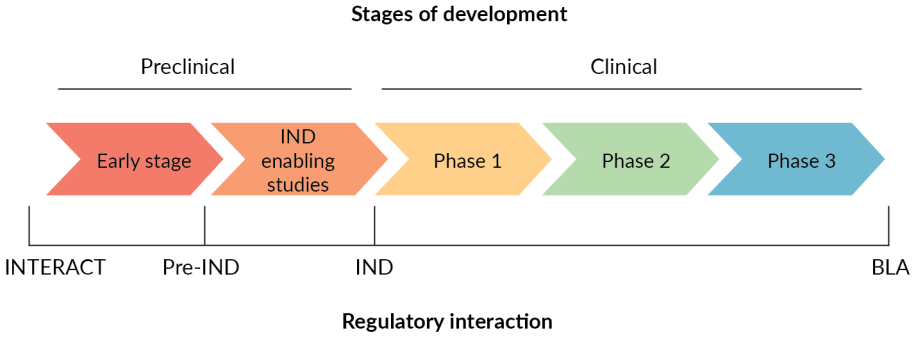
The IND submission

The content and format of an IND submission to the FDA should adhere to 21CFR 312.23 and should contain:

1. CMC data to characterize the composition, manufacture, and control of the investigational product;

FIGURE 3

During the preclinical stage of the product development lifecycle, several opportunities exist for engagement with the FDA.



INTERACT meetings are a mechanism to obtain feedback at an early stage of preclinical development once preliminary pharmacology data are obtained. As product development progresses and more extensive proof-of-concept (POC) results are obtained and the pharmacological properties are better characterized, the opportunity exists for a pre-IND discussion with FDA for guidance on the design of the definitive safety studies. Following completion of the preclinical pharmacology and toxicology studies, an IND requesting initiation of an early phase clinical trial can be submitted. The goal of early interaction with the FDA is to guide sponsors towards a successful IND.

2. P/T data to support the safety and feasibility of the proposed clinical trial; and

3. A detailed clinical protocol.

Considerations for P/T studies to support an IND submission are discussed further below; CMC data and clinical protocol(s) submitted to an IND are outside of the scope of this review.

The objective of a preclinical program is to demonstrate that an investigational product is reasonably safe to administer in a clinical trial (21CFR 312.23(a)(8)). Preclinical data can also provide proof-of-concept (POC) data to justify product administration in a first-in-human clinical trial and inform trial design (e.g. initial safe starting dose level, dose-escalation scheme, dosing schedule, and clinical monitoring). Data can originate from:

1. GLP-compliant toxicology studies conducted by a qualified testing facility;

2. Well-controlled studies conducted in-house;

3. *In silico* analyses; and

4. Published data in peer-reviewed journals.

Clinical trials evaluating product(s) that are similar to the investigational product of interest may also provide supporting information. Pharmacology/Toxicology studies to support an IND submission for a cell-based immunotherapy product are further discussed in Section 4 of this review.

Other resources

In addition to meetings with the FDA, published guidance documents communicate FDA's current thinking on certain topics. The document, Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products [3] is a resource that can be used for cell-based immunotherapies. Additional guidance for preclinical evaluation of gene therapy products, which include genetically modified cells, is presented in the Guidance for Industry: Long Term Follow-Up After Administration of Human Gene Therapy Products [4], and guidance regarding the preclinical evaluation and clinical considerations for therapeutic cancer vaccines is found in the Guidance for Industry: Clinical Considerations for Therapeutic Cancer Vaccines [5]. The recommendations

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conveyed in these and other guidance documents provide a high level framework that may be used when approaching preclinical assessment of cell-based immunotherapy products. However, the diversity and inherent biological properties of cell-based immunotherapies often necessitates a product-specific testing strategy.

PRECLINICAL CONSIDERATIONS FOR ASSESSMENT OF CELL-BASED IMMUNOTHERAPIES

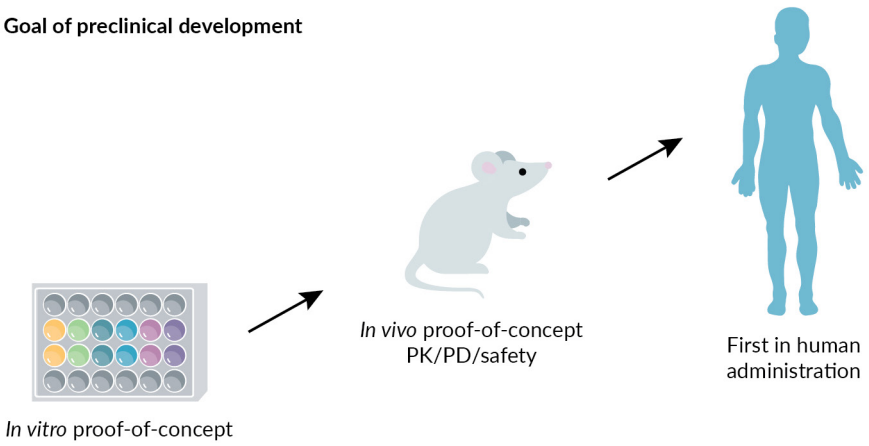
Preclinical testing of an investigational product provides data that contribute to the decision to initiate testing in humans. Such data can also provide scientific support for the selected dose levels and planned dosing regimen in humans. The scientific rationale for administering a cell-based immunotherapy in a clinical trial is based on data generated in POC studies, which accompany safety testing. A weight-of-evidence approach is thus used to assess the benefit/risk profile, and to determine whether the preclinical data support administration of the investigational product in a clinical trial (Figure 4). Preclinical review of cell-based immunotherapies focuses on several key considerations to guide this decision-making process:

- Product activity: Understanding the biological activity of the product and the desired therapeutic effect provides context when approaching the design of preclinical safety studies. *In vitro* and *in vivo* data are important to characterize product activity and to provide evidence for the purported mechanism of action for a given cell-based immunotherapy.

- Product safety: Incorporation of appropriate methods to assess product safety is an important consideration. Traditional *in vivo* toxicology studies may be challenging or limited in their ability to fully assess product safety for cell-based immunotherapy products. For example, model species commonly selected for preclinical testing are often not biologically responsive to cell-based immunotherapy products. Using *in vitro* or *in vivo* testing methods that are sensitive, reproducible, and specific for a human target antigen are important for identifying risks for this product type.

FIGURE 4

A preclinical development program for cell-based immunotherapies can determine administration of the investigational product in a FIH clinical trial.



Preclinical assessments for safety and activity can consist of *in silico*, *in vitro*, and *in vivo* based studies.

- Biological relevance of animal models: Animal models should be biologically responsive to the investigational product and relevant to the target clinical population. Considerations include disease pathogenesis, anatomical similarities and differences between each animal species and humans, and the timing of product administration relative to disease status. Additionally, understanding the limitations of the selected animal model(s) is important for interpretation of the preclinical data. For example, human tumor-bearing rodent models are typically immunocompromised, limiting the ability to assess any immune-related effects. Development of xenogeneic graft-versus-host-disease can also occur with administration of an investigational human product.

- Sample size and strength of the *in vivo* data: *In vivo* studies designed according to the three 'R' principles of replacement, reduction, and refinement are encouraged and should be balanced with the need for sufficient numbers of animals (i.e., sample size) that enable interpretation of results. Various factors, such as animal model relevancy and the specific preclinical safety and activity questions being addressed may affect this consideration.

- Dose level rationale: Integration of data obtained from preclinical POC and safety studies, as well as existing clinical data for similar product types, is important when identifying a starting clinical dose level and dosing regimen for FIH clinical trial.

A more comprehensive discussion of preclinical testing considerations along with product-specific examples for cell-based immunotherapies are provided below.

Proof-of-concept studies

Preclinical studies are important to support the rationale for a given clinical trial. For example, *in vitro* testing of CAR T cells is often used to demonstrate that the engineered CAR can recognize a specific antigen. Activity may be demonstrated by measuring the induction of various cytokines (i.e., cytokine release) and cytotoxicity against cancer cells that express the target antigen. An immunocompromised rodent, such as the NOD scid gamma (NSG) mouse, bearing a clinically relevant tumor type (i.e., xenograft model), is permissive to a human CAR T cell administration and can enable *in vivo* testing. POC studies can be performed in this type of model or in a syngeneic tumor-bearing model using a surrogate animal product to assess anti-tumor activity, with determination of tumor burden and overall survival. *In vivo* studies can also be helpful for understanding differences in the kinetics of CAR T cell trafficking, expansion, and persistence.

When additional cell surface ligands, secreted cytokines, or genetic modifications are incorporated in a cell-based immunotherapy product, *in vivo* studies can provide insight on the functionality and kinetics of these components. The combination of cell-based immunotherapies with other investigational agents is also becoming increasingly common. In addition to demonstrating activity of a proposed combination immunotherapy, *in vivo* studies can provide insight regarding appropriate dosing regimens.

Other cell-based immunotherapies, such as microbial vectors engineered from live bacteria, are often attenuated and are designed to have limited growth potential. Thus, POC studies in animal models of disease evaluating repeat dosing regimens are often performed to characterize the effect of multiple administrations on achieving a durable response outcome.

Safety studies

While POC studies can provide a rationale for a proposed clinical trial, preclinical evaluation of potential toxicities is important to

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determine that a clinical trial is reasonably safe to proceed. Toxicology studies can assist in identifying adverse local and/or systemic effects to inform clinical monitoring and may guide patient eligibility criteria. Key safety concerns for cell-based immunotherapy products often include product specificity, aberrant cell proliferation or differentiation, cell transformation, and toxicities related to the pharmacologic activity of the product.

Assessing the safety profile for products such as CAR T cells is challenging because the scFv element of the CAR T cell construct often does not cross-react with the corresponding antigen in species commonly selected for preclinical testing. Evaluation of potential off-target effects or off-tumor/on-target reactivity can also be accomplished with cross reactivity studies employing panels of human tissues, membrane protein arrays, and co-culture studies with various normal human cells. Assessment of antigen expression profiles, combined with publicly available clinical trial results or published literature providing information for other therapeutic products (e.g., monoclonal antibodies) targeting the same antigen, may support identification of potential target organs of toxicity of the investigational CAR T cell product of interest.

For products that incorporate genome editing, preclinical studies should also be conducted to evaluate potential off-target genome editing events and genomic integrity. Additionally, *in vitro* studies can be performed to assess the presence of cytokine independent growth for T cell or NK cell-based products. When biologically responsive animal models are available, *in vivo* studies may provide insight regarding the safety of expressed transgenes, such as cell surface ligands or secreted antibodies.

For microbial vectors, safety evaluations of host innate and adaptive immune response, induction of proinflammatory cytokines and chemokines, and the potential for local, acute, systemic, or chronic toxicity can inform clinical trial design. Although the route of administration may be local (e.g. intratumoral), systemic safety studies can be useful to evaluate a hypothetical 'worst case' type scenario should systemic exposure occur. Demonstration that a microbial vector under investigation is sensitive to available antibiotics is also important. Because microbial vector administration often occurs via routes that differ from natural routes of exposure, assessment of the biodistribution profile to characterize bacterial colonization in off-target tissues, is typically conducted. Additionally, preclinical evaluation of shedding can assess the potential for transmission of the intended clinical product from treated to untreated individuals (Guidance for Industry: Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Product [6]). For further discussion regarding the preclinical assessment of this product type, refer to the Guidance for Industry: Recommendations for Microbial Vectors used for Gene Therapy [7].

CONCLUSION

A product-specific approach to preclinical testing for a new cell-based immunotherapy product can support transition to FIH clinical testing, in accordance with the Code of Federal Regulations (CFR) Title 21, Part 312. Progress towards this important objective may be facilitated through early interaction with FDA to discuss preclinical aspects of a product development program. Due to the complexity of cell-based immunotherapies, designing preclinical programs to comprehensively assess product safety and activity is often challenging. However, becoming familiar with the regulatory considerations and opportunities for engagement with the FDA may help facilitate successful transition from bench to bedside.

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY: NEXT-GEN PLATFORMS & MODALITIES

SPOTLIGHT

INNOVATOR INSIGHT

The progression & delivery of adoptive cellular immune therapies: leveraging single-cell resolution & novel algorithms to overcome the challenges associated with allogeneic & autologous immune cell therapies

Vicki Moignard, Alessandra De Riva & Raul Elgueta

The success and progression of chimeric antigen receptor (CAR) T cells in adoptive cellular therapies for B cell malignancies can be attributed to the effective engraftment, efficient expansion, and the persistence of the cells after transplant. However, that success has yet to be translated into solid tumors, which present their own set of distinct challenges. As the knowledge in the field grows and more data reach the public domain, it has become clear that both intrinsic and extrinsic mechanisms of tumor resistance need to be addressed in order to improve immune-based adoptive cell therapies (ACT). Computational approaches and novel algorithms are well placed to identify new genes needed to overcome resistance and enhance efficacy of the current and future ACT. This review discusses the current challenges for autologous and allogeneic ACT and how big dataset analysis is opening paths to overcome resistance and enhance the efficacy of ACT.

Cell & Gene Therapy Insights 2020; 6(9), 1411–1430

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INTRODUCTION

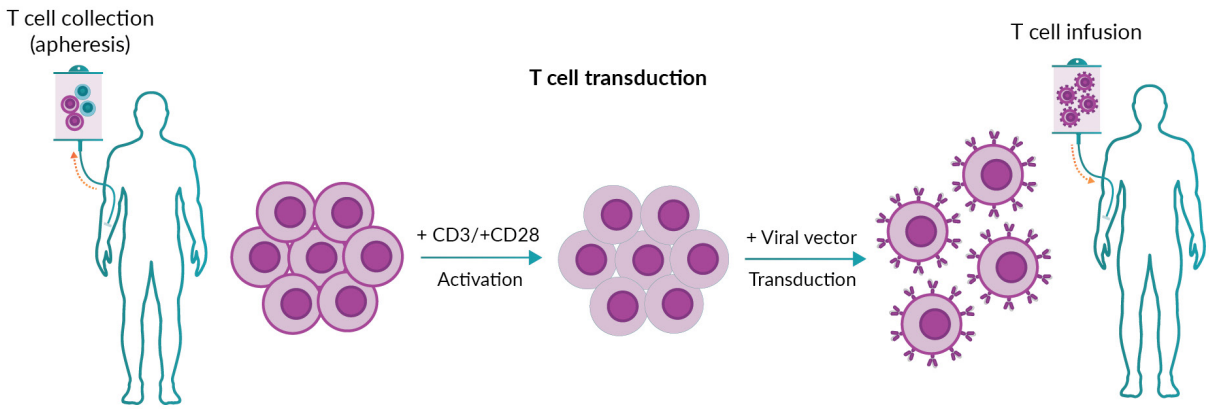
The idea that immune ACT could play a central role in the fight against cancer was developed in 1964 by Alexander and Delorme [1]. Their work demonstrated that sarcomas in rats can be treated by ACT of lymphocytes from immunized syngeneic animals. So far, only three immune ACT, using CAR T cells, are available on the market for the treatment of hematological blood malignancies, but the field is rapidly expanding. Currently, several clinical trials are running or approved, for both autologous and allogeneic immune ACT. This is due to incremental knowledge acquired in several fields, including stem cell transplantation, monoclonal antibody, and HIV research, together with the technical advances achieved in molecular biology. However, alongside the excitement of developing new therapies, the industry is faced with new challenges at the scientific, manufacturing, and regulatory level.

CAR T cell therapy uses autologous T cells isolated from patients, which are genetically modified to insert the CAR construct (Figure 1). These CAR T cells are then expanded and reinfused into the patient, where the recognition of the cognate tumor antigen triggers activation and the anti-tumoral immune response [2]. The first-generation of CAR construct was designed by merging the single chain of a monoclonal antibody (scFv), with a transmembrane domain and intracellular domain of CD3ζ (Figure 2). The lack of costimulatory signals in the first generation of CAR T cells resulted in low efficacy in their activation [3]. The second generation of CAR T cells was therefore designed to introduce the intracellular domain of costimulatory signals such as CD28, 4-1BB, or OX-40. Since the primary and costimulatory signals are activated, the second generation of CAR T cells provide an efficient anti-tumoral response in patients with diffuse large B cell lymphoma (DLBCL) [4,5]. A third generation of CAR, current under clinical trial, combines the intracellular domain of the CD3ζ and the intracellular domain of both costimulatory signals CD28 and 4-1BB [6].

Despite these successes, almost 30% of patients with DLBCL are not able to receive autologous CAR T cell therapy due to low product quality during the processing and manufacturing of the CAR T cells [4,5]. This is mainly due to a reduction in T cell numbers or T cell exhaustion (lack of functionality) because of the severity of the disease and/or treatment [7]. An allogeneic ACT could potentially address these issues, by providing an 'off the shelf' and reproducible alternative. An allogeneic product could be immediately available to

FIGURE 1

Autologous cell therapy, T cells are collected from a cancer patient by apheresis



Those T cells are then activated with antibodies and exposed to a viral or other vector encoding the CAR molecule. CAR T cells are allowed to expand before reinfusion into the patient.

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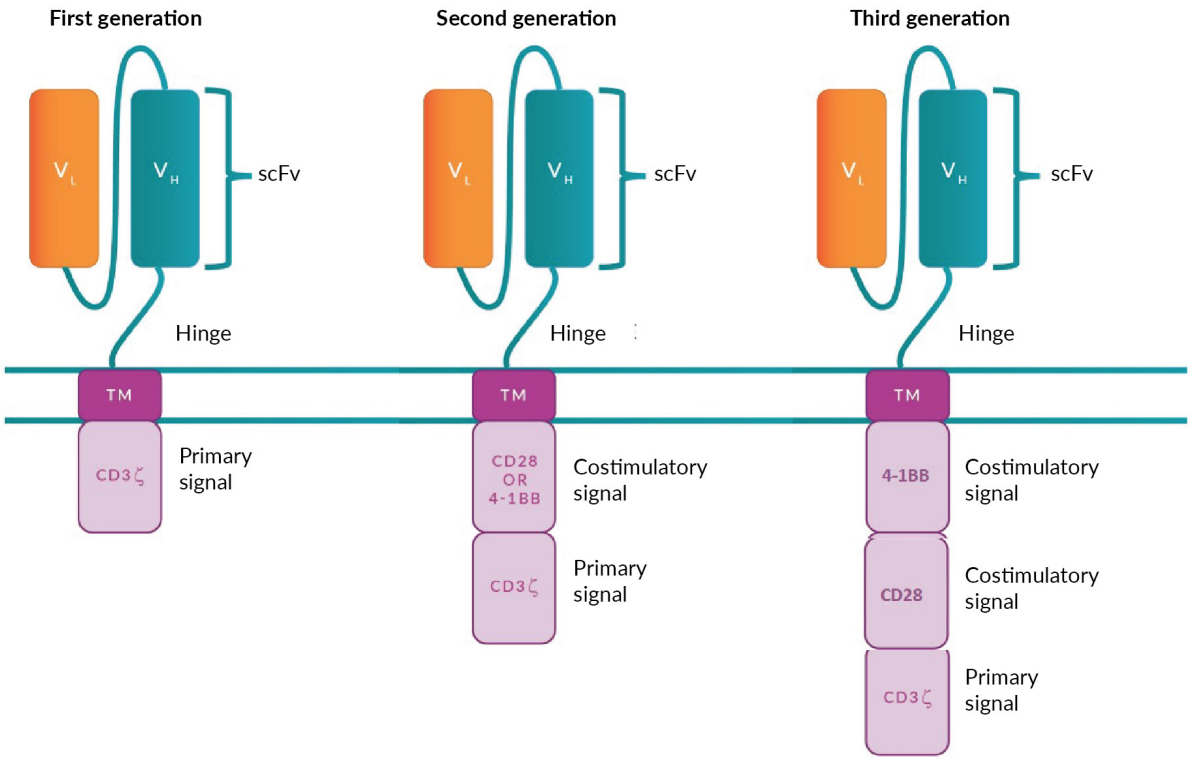
FIGURE 2

Different generations of CAR structures utilized in current and past clinical trials.



CARs that contain only the CD3-ζ intracellular domain are identified as first-generation CARs, whereas those that contain one costimulatory domain (such as CD28 or 4-1BB) are known as second-generation CARs and lastly, those that contain two or more endodomain of costimulation are known as third-generation CARs.

the patient, reducing treatment lead time, and ensuring availability if redosing is necessary. Despite the promise, allogeneic ACT will face two major barriers to success; graft versus host disease (GvHD, whereby donor T cells attack the recipient cells), and rejection. Nonetheless, there are several approaches that different groups have developed to tackle these issues (reviewed in Depil *et al.* [8]).

Next-generation sequencing and high throughput data approaches will play a key role in the identification of gene regulators or small molecules that can prolong the effectiveness of CAR T cells. For example, datasets such as the FANTOM5 consortium data have been employed in new approaches by Mogrify® to identify the optimal combination of transcription factors (TFs) required to directly convert any human cell type into any other human cell type [9]. In addition, the same dataset has made it possible to identify enhancers and promoters that are important in T cell and macrophage differentiation by profiling human T cells [10] and monocytes [11]. This type of large-scale data could identify regulatory molecules needed to overcome resistance and enhance efficacy in CAR T-cell therapies.

In this review, we look at the status of immune ACT, discussing some of the challenges and solutions that the current therapies are facing. We will describe how single-cell technologies and analysis of large-scale data could provide some answers to those issues and how Mogrify®, using its proprietary direct cell conversion technology, is able to tackle some of the issues associated with immune ACT.

TRANSLATION INSIGHT

Three cell therapy products based on CAR T cells have so far reached the immuno-oncology

market: YESCARTA® (axicabtagene ciloleucel) and TECARTUS™ (brexucabtagene autoleucel) from Kite Pharma and Gilead Sciences; and KYMRIAH® (tisagenlecleucel) from Novartis. KYMRIAH®, TECARTUS™ and YESCARTA® target CD19 which is expressed on malignant, as well as normal, B cells in hematological cancers (e.g. DLBCL and lymphoblastic leukemia [ALL]). Generally employed after two or more lines of systemic therapy, these therapies have elicited complete and lasting tumor regression in up to 40% of patients [4,5,12]. These are autologous therapies, meaning that the patient is both the donor and the recipient of the product. Although this approach has the advantage of avoiding GvHD, there are safety considerations related to unwanted toxicities that may develop following CAR T-cell infusion, such as cytokine release syndrome (CRS), neurological toxicities, 'on-target/off-tumor' recognition, and anaphylaxis. CRS occurs in most patients receiving CAR T-cell therapy and based on accumulated experience through many clinical trials, clinical risk management protocols have been put in place so that toxicity is graded according to clinical symptoms and managed pharmacologically [13,14]. Scientists are already working towards a new solution to this problem such as the introduction of 'suicide genes' into the CAR construct, so that CAR T cells can be selectively depleted [15] or turned off [16] if neurotoxicity and CRS are observed in the patient. The use of safety mechanisms may become particularly relevant for allogeneic therapies, where the donor and the recipient (the patient) are two different individuals. In these conditions, GvHD is an unwanted complication likely to occur depending on the degree of Human Antigen Leukocyte (HLA)-mismatch between the donor and the recipient [17].

The success of KYMRIAH®, TECARTUS™ and YESCARTA® CD19 CAR T-cell therapies has led to exceptional growth in the number of other CAR T-cell therapies targeting this same antigen. Figure 3 shows that among the 312 CAR clinical trials currently active or recruiting, the most popular target is CD19, with 61% of therapies directed against this antigen. The landscape is dominated by CARs directed against hematological cancers, with 84% of the therapies targeting CD20, CD22 and B Cell Maturation Antigen (BCMA), in addition to CD19. While this reflects the success of CARs and points to the potential for the development of new CARs directed to cancer antigens in addition to CD19 and B cells, it also highlights the fact that this therapeutic approach has yet to demonstrate efficacy in indications other than hematological malignancies. There is the hope that CARs will be successful to treat solid tumors too.

Solid tumors represent a highly challenging environment, as they involve many different cell types that promote, sustain, and protect the growth of the tumor mass via several mechanisms. Myeloid-derived suppressor cells, tumor-associated macrophages, and regulatory T cells generate a suppressive microenvironment by releasing cytokines like IL-10 and upregulating surface markers that inhibit T cell activation. The cells that manage to infiltrate the tumor microenvironment are put on idle by these coercive actions, but often T cells are simply spatially excluded from the tumor. Several mechanisms acting at once may have to be put in place to subvert the tumor microenvironment. Many preclinical studies have shown that CAR T cells genetically modified to secret cytokines (e.g. IL-12, IL-15 and IL-8) could enhance T cell proliferation and anti-tumor activity [18,19]. Moreover, the genetic insertion of chemokine receptors into CAR T cells could lead to an increase of T cell infiltration into the tumor. It has been shown that the expression of CCR2 in CAR T cells increases tumor infiltration and anti-tumor efficacy in preclinical models [20]. Therefore, multiple mechanisms can be generated to allow the infiltration of an armored CAR T cell into a solid tumor and abrogate the suppressive tumor microenvironment. An example would be to have small molecules that induce endogenous CCR2 expression as an alternative for

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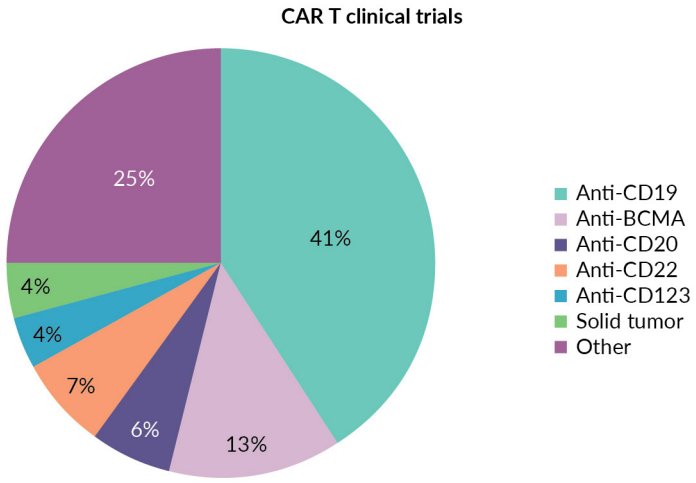
FIGURE 3

Chimeric antigen receptor-based therapies worldwide.



Data were obtained from 'clinicaltrial.gov' on June 07, 2020. Filters applied: search: condition or disease: no entry; other terms: chimeric antigen receptor; country: no entry; status: recruiting, enrolling by invitation and active, not recruiting; other filters CAR T cells CD19; CD20; CD22; BCMA; CD123; solid tumor.

the transduction of *CCR2* gene in mesothelin CAR T cells [20]. This could increase the safety of CAR T-cell therapy by eliminating any risks related to the insertion of the *CCR2* gene. While this technology would possibly be increasing the efficacy of the products, as a result of the boosted T cells' activity, it will likely increase the likelihood of remission for patients on their last line of treatment (CAR T cell therapy only being prescribed following several rounds of chemotherapy and monoclonal antibody immunotherapies) by using it in combination with checkpoint inhibitors (reviewed in Titov *et al.* [21]).

On the other hand, the infiltration of a solid tumor may require the use of CAR cell therapies based on alternative cell types to conventional αβT cells. Interest is growing in exploring the potential of other immune cells, including natural killer (NK) cells [22], gamma delta (γδ) T cells [23] and macrophages [24]. All of these cell types have been armed with CARs to test their efficacy in treating solid tumors [22–24]. NK cells are particularly suitable for immune ACT as they can acquire antigen specificity via the CAR technology, while still retaining their natural cytotoxicity through their ability to recognize target cells via the detection of lack of HLA expression – referred to as 'missing self-recognition'. Recently, HLA-mismatched anti-CD19 CAR NK cells derived from cord blood were administered to 11 patients with relapsed or refractory CD19-positive lymphoid tumors. It was observed that 7 of the patients had complete remission and without any side effects (cytokine release syndrome, neurotoxicity, or GvHD) [22]. For all of the reasons listed above, NK cells are currently viewed as a good candidate cell type for allogeneic therapies. However, certain challenges remain when working with NK cells for immune ACT. For example, it is difficult to scale up NK manufacturing to support 'off-the-shelf' allogeneic treatments [25]. *In vitro* expansion is necessary for any NK cell-based cell therapy as the cells constitute only 5–15% of peripheral blood monocyte cells (PBMCs). Alternatively, NK cells can be differentiated from cord blood or stem cells. This could reduce variability between batches and increase the quality of the cell product, but in this case NK cells must be differentiated as well as expanded. Protocols for expansion of NK cells rely on cytokines and/or feeder cells, but overall *in vitro* expansion of NK cells tends

to be modest, and often NK cells require an additional *in vivo* maturation step to acquire full functionality. These complexities have pushed researchers to search for alternative sources, such as the NK-92 cell line, which has been used in clinical trials, and was most recently engineered to express CARs directed towards liquid and solid tumors [26,27]. Although safety and pre-clinical data for efficacy [28] have been demonstrated, clinical efficacy is still to be confirmed, and the cells have to be irradiated before use, greatly shortening their lifespan after transplant.

An alternative approach to CARs is the generation of T cells in which the α and β chains of a T cell receptor (TCR) specific for a cancer antigen or neoantigen are expressed as an addition to, or in replacement of, the endogenous TCR [29]. Such α and β chains are usually identified from T cell clones enriched in patient biopsies. T cells genetically engineered to express the chosen TCR recognize the cancer antigens through the classical antigen presentation pathway, which processes cytoplasmic as well as surface proteins. To be effective, TCR engineered T cells rely on antigen presentation in the tumor microenvironment, which is often downregulated in cancer cells as an escape mechanism. HLA matching between the donor and the recipient is also required, making this an autologous ACT, although some degree of HLA-mismatching may be tolerated and could potentially be used in allogeneic ACT. The number of clinical trials that employ TCR-engineered T cells in cancer treatment is much lower compared to those for CAR T cells [30], perhaps due to the complexity of identifying good 'universal' target antigens, and the challenges to produce a TCR with the optimal affinity and avidity for the cognate antigen.

The field of cell therapy for immuno-oncology is rich in possibilities for both autologous and allogeneic treatments, using different modalities such as CARs or TCR engineered receptors, and different cell types, like T cells, NK cells, and others [8]. Moreover, the synergy of combinatory approaches such as cell therapy and immune checkpoint inhibitors may enable the immune system to disrupt and destroy the tumor microenvironment. Much knowledge will be obtained from the data generated by ongoing and new clinical trials, providing a solid basis for ever safer and more effective cell-based therapies. Moreover, the lessons learned in immuno-oncology are now rapidly applied to GvHD and to the other side of the coin in immunology, autoimmunity. The exciting possibility to use technologies like CARs to induce immunological tolerance to treat and prevent organ rejection, or to restore immunological balance in autoimmune diseases, has already started to be explored, and promises to have a big impact on the lives of many people living with chronic conditions.

MANUFACTURING ASPECTS OF CELL THERAPY

Traditionally, pharmaceutical development follows a defined pathway that covers all stages of a therapeutic product. This is summarized by the first phase of discovery and development, followed by preclinical and clinical research phases that lead to drug approval, then by post-market safety monitoring. Starting from the clinical research phase, all processes must adhere to Good Manufacturing Practice (GMP), which ensures that fully characterized, controlled and consistent manufacturing processes are in place to guarantee the safety and efficacy of products in accordance with pre-determined quality standards.

ACT has questioned and even bypassed traditional pharmaceutical development, challenging the status quo. In cell therapy, early development has mainly taken place in the clinic, frequently under hospital exemption. Moreover, the preclinical phase is challenging due to the lack of relevant animal models that truly recapitulate human disease. Finally, the first therapies to reach the immuno-oncology market are autologous, made from cells collected from one patient for the treatment of the same patient, epitomizing the concept of 'personalized medicine' [4,5]. This model does

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not fit with production scale-up processes in the same way as, for example, small molecules or even biologics. Among the many variables that characterize this model, the quantity and quality of the starting material are notable, as these heavily depend on each patient's medical history. ACT in immuno-oncology is currently approved for patients who have undergone two or more lines of systemic therapy, meaning that the starting material from which the therapeutic product is manufactured can be extremely variable between patients, and even within the same patient if the cell therapy product has to be made on more than one occasion for additional dosing. Depending on the quality and quantity of the starting material, the manufacturing process must be adjusted for each batch.

In addition, the manufacturing process is often still manual, although appropriate automated solutions have been developed and have started to be implemented. In the current ACT setting, the clinical and manufacturing teams have to work closely to be able to synchronize product manufacturing and patient treatment (reviewed in [31]). While the manufacturing team generates the cell product, the clinical team has to assist the patient to undertake a conditioning treatment to 'make space' for the ACT after infusion. To accommodate this model, ACT manufacturing has been kept in close proximity to the clinic, often with manufacturing suites located at the hospital site or nearby. Analytical, quality control and quality assurance teams are also likely to be located close to the hospital for the same reasons. This modus operandi is typically more complicated and more expensive to manage administratively, and it is referred to as 'scaling out'. Scaling out requires a different manufacturing set up compared to classical drug manufacturing, and this model has been adopted by most cell therapy companies. Bigger companies have started to move away from scaling out and adopted the classical 'scaling up' model by setting up manufacturing centers in strategic locations served by appropriated transport facilities. In this case, logistics and operations become even more crucial to the completion of the tight vein-to-vein turnaround allocated for manufacturing and product release. As this sometimes involves shipping items over considerable distances, suitable transportation systems have been developed to guarantee that cell therapy products are delivered safely and on time [31]. This also demands that the chain of custody and identity of the product are maintained throughout the entire process, as a failure to document the identity and integrity of the product could have fatal consequences for the patient.

Currently, one of the main drawbacks of ACT is its high cost, which is reported to be $475,000/dose for YESCARTA®. Due to the 'ad hoc' manual or semi-automated manufacturing process, one of the major contributors to the cost of goods is labor [32]. The rate of optimization, and implementation of automation and process simplification will therefore determine the speed at which ACT becomes affordable. Significant advances have already been made with the development of modular automated systems that reduce the 'hands-on' time required for product manufacturing. Nevertheless, due to the limitation imposed by the fact that one batch is made for one patient, it is unlikely that the cost of cell therapy will suddenly drop. The cost of GMP-grade raw materials is also high, but it is likely that as the industry continues to grow new solutions will become available. An example is illustrated by the shortage of animal-derived serum for the growing cell therapy sector that was forecasted in 2012 [33] – eight years later, although serum is still in high demand, serum-free alternatives are available and have already been implemented by some.

Most of the issues described above relate to autologous cell therapy, mainly due to the personalized nature of the treatment. In contrast, the manufacture of allogeneic products is less challenging [8]. The paradigm of 'one batch for one patient' that characterizes autologous therapy is replaced with 'one batch for several patients' in allogeneic therapies. In allogeneic therapy, the pathway for manufacturing is

more aligned with the traditional model of scaling up, mainly thanks to the uncoupling of the vein-to vein turnaround. To meet demands, innovative and more efficient systems are being developed, such as the use of suspension cells instead of adherent cells for viral production, or large volume bioincubators and automated closed systems. One of the major challenges for scaling up off-the-shelf manufacturing is the availability of the large volume and consistently high quality of cells required. Innovative solutions and beginning to be identified and some have now been developed and started to be tested in clinical trials.

The discovery of *in vitro* methods for differentiating as well as modifying the differentiation status of cells has come to the rescue of cell therapy manufacturing. A good source of cells for further differentiation and genetic manipulation are embryonic stem cells (ESCs), for which substantial knowledge has already been accumulated in other fields, mesenchymal stem/stromal cells (MSCs) and induced pluripotent stem cells (iPSCs). Figure 4 shows the usage of ESCs, iPSCs and MSCs in clinical trials worldwide.

Although these cell types are currently used mainly in transplantation and regenerative medicine, their usage as starting materials to manufacture clinical-grade cell therapy products is also foreseeable in the near future, which will require the development of cell differentiation methods compliant with GMP. The availability of iPSCs has also opened new possibilities in ACT for immuno-oncology. The employment of iPSCs as starting material to be differentiated into the desired cell type will allow the generation of large batches of identical cells obtained from suitable donors and enable the manufacturing of 'off-the-shelf' cell therapies. Moreover, research around the world is concentrating on developing even more advanced tools, such as a hypoimmunogenic universal donor cell line [34]. The latter is as challenging as it is desirable, and could provide a 'blank canvas' on which to add further properties to create a new 'artificial cell' that does not cause GvHD, and is poised to recognize and kill tumors with high specificity as well as safety.

THE EMERGING LANDSCAPE OF NEXT-GENERATION MODALITIES IN SINGLE-CELL ANALYSIS & THEIR APPLICATION IN IMMUNE CELLULAR THERAPIES

Many methods have been used to characterize and understand immune cells over the past century, from morphology and tissue distribution through to modern flow cytometry capable of measuring the expression of >20 proteins. This has given us a deep understanding of the markers and functions of different cell types in a range of tissues and in response to different stimuli. This synergy between immunology and emerging technologies ensures that our paradigms are continually updated to consider new methods and information.

Biology has often been limited by the fact that approaches that give information about many genes or proteins are limited to a few samples of large numbers of cells. However, recent years have seen an explosion of technologies available to study cells at the single-cell level, and the application of these technologies to immune cells [35–39]. These have largely been based around sequencing technologies, including transcriptome profiling using RNA sequencing and single-cell RNAseq (scRNAseq) [40], and epigenomic studies using assay for transposase-accessible chromatin (ATAC)-seq [41–44]. Additionally, cytometry by time-of-flight (CyTOF) [45] has expanded the number of proteins we can analyze compared with flow cytometry by tagging antibodies with heavy metals and passing stained cells through a mass spectrometer to detect protein expression by molecular weight, rather than fluorescence. These methods are now frequently used in combination on the same cells [46,47], for example, to look at changes in the transcriptome [48,49] or chromatin accessibility [50] in response to gene knockdown in CRISPR

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screens, or coupling protein expression with transcriptomics by using DNA-tagged antibodies for immunophenotyping (CITEseq) [51]. There are also many resources comparing these different technologies and methods to analyze data [52,53].

These technologies have allowed researchers to assimilate a lot of new knowledge without a priori assumptions, such as screening for immune cell subsets present in a variety of different tissues and species [54] and how that changes in autoimmune diseases [55], harmonizing the markers used to identify cell types across different species [56], and to better understand the ontogeny of immune subsets [36,54]. There are efforts underway to map the entire human body at single-cell resolution using these technologies, in the Human Cell Atlas project [57].

Due to the somatic recombination that the T and B cell receptor genes undergo during differentiation, we can also track cell clones through TCR and BCR sequencing at the single-cell level (so-called V(D)J sequencing) [58,59]. This allows the monitoring of clonal evolution in infection and disease settings, and through aging processes. MHC-dextramers tagged with DNA barcodes have also been used to probe the antigen-specificity of T cells [60]. Coupling transcriptomics with V(D)J sequencing, CITEseq, CRISPR screening and antigen-specificity has allowed up to five modalities to be analyzed from the same cells using next-generation sequencing [61]. These technologies are allowing us to interrogate the complexities of the immune system at unprecedented scale and resolution [38].

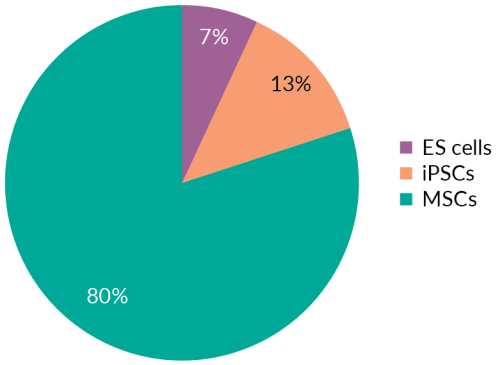
Selectively programing cell fate

A key aim of these new technologies is to better understand cell ontogeny and mechanisms of cell fate decisions [62–64], accompanied by computational techniques for identifying differentiation trajectories [65–67]. This could translate into improved cell therapies by directed differentiation from pluripotent cells by identifying key transcription factors (TFs) or signaling pathways required during differentiation. This is of particular interest in immuno-oncology where autologous CAR T and NK cell products are expensive and time-consuming to make, and fraught with issues, so the search for allogeneic 'off-the-shelf' alternatives is intensive with many companies developing iPSC-derived products [8,68]. Many of these protocols are also very long and costly as they replicate normal human development, so there is interest in bypassing normal differentiation by overexpressing TFs, which are often viewed as the master regulators of cell fate. Additionally, cells derived from ESCs or iPSCs often have an immature, fetal phenotype and lack full adult function, so improvements are required to fully realize their potential. Papers describing the reprograming of cell fate using the overexpression of transcription factors have spanned decades [69–71], but it has been challenging to identify optimal combinations of TFs for conversions, usually involving trial and error. Rational, data-driven selection of TFs and scalable screening methods are required to accelerate discovery in this area, and new technologies are aiding this process.

One strategy, Reprogram-Seq [72], predicts candidate TFs by identifying genes that are differentially expressed between source

FIGURE 4

Usage of ESCs, iPSCs and MSCs in clinical trials worldwide.



Data were obtained from 'clinicaltrial.gov' on May 29, 2020. Filters applied: search: condition or disease: no entry; other terms: ESCs, iPSCs or MSCs; country: no entry; status: recruiting, enrolling by invitation and active, not recruiting.

and target cells in scRNAseq data. Pools of TFs are then overexpressed in the source cells, such that each cell will receive a different, random combination of factors. The converted cells are then surveyed using scRNA-seq for transcriptional signatures that match the target cell of interest, and the transgenes that were overexpressed can be identified as they lack a 3' UTR compared with the endogenous transcripts for the same factor. MOGRIFY® can efficiently navigate a combinatorial space of >500 billion possible TF combinations to find the optimal set of TFs controlling the genetic programs required to be switched for a given cell conversion [9]. This results in few transcription factors with the highest and non-overlapping regulatory influence. Initially, the algorithm was run to generate predictions for cell conversions and has successfully demonstrated multiple cell conversions, including keratinocytes from fibroblasts, and endothelial cells from keratinocytes [9].

An alternative method, SEUSS (scalable functional screening by sequencing), addressed the effect of overexpressing a library of 61 developmentally important TFs in iPSCs, including modified versions of genes that could not be predicted by Reprogram-seq [48]. The effect of TF overexpression was screened by scRNAseq, coupled with a fitness readout of cell growth in multiple culture conditions. In contrast to Reprogram-seq, a barcode associated with each transgene was used to identify useful TFs from the sequencing data. These data were used to construct a genetic co-regulatory network based on transcriptomic changes, identifying key factors for early fate specification. This included identifying ETV2 as a reprograming factor for an endothelial-like state, a useful validation of the method as this TF was already known as an important regulator of early blood and endothelial specification during embryogenesis.

While these are important proof of concept studies, it is difficult to screen the whole transcriptome this way as the number of possible TF combinations scales rapidly, and it would be prohibitively expensive to sequence and analyze the required number of cells. It is also important to consider that each converted cell in a pooled screen is not an independent experiment, and the effects of paracrine and juxtracrine signaling may be significant. Better computational methods to narrow down the set of TFs used will have great value. CellNet assesses the quality of cell differentiation or conversion experiments by comparing transcriptome information to reference data and identifying genes that can be modulated to enhance conversion [73]. By contrast, MOGRIFY® can predict *de novo* the combination of TFs required to induce direct cell conversion from any cell type to any other cell type using a combination of transcriptome data, protein-protein, and protein-DNA interaction databases to identify the network of genes to activate, and the TFs that regulate them, removing the guesswork from direct cell conversions [9]. Most screens also focus on overexpression of TFs as this gives strong and stable expression of the gene of interest and control over the isoforms used. However, the regulatory impact of non-coding RNAs and the need to downregulate particular genes are important factors that should be included in future studies. An overview of the different algorithms and their approach to identifying TFs for cell conversion is summarized in Table 1 and in [9,73–76].

New methodologies also offer the potential to better understand the role of the tissue microenvironment in development, which could aid in improving culture conditions for directed differentiation or direct cell conversions. A paper from the Human Cell Atlas project surveyed the dynamics of human thymic development from the fetus through to >30 years postnatal life [77]. T cell development requires a complex interaction of T cells with the thymic stroma and dendritic cells to direct fate towards the multiple functional lineages, in combination with the rearrangement of the TCR genes that define antigen specificity. The study included scRNAseq analysis of T cells and other immune and stromal cells, to show

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TABLE 1

Summary of methods.

Platform name	Computational steps for TF prediction					Reference
	Input data type required	Data used for identifying cell identity profiles	Strategy used to identify TF influence	Criteria to prioritize TFs	Prediction	
MOGRIFY®	RNA-Seq, CAGE	Fantom CAGE dataset (274 cell types)	Build cell type-specific regulatory network of TF and target genes	Cell type specificity in the target cell of the regulatory network and upstream TF regulators	Non-redundant set of core TFs	Rackham *et al.* [9]
JSD	Microarray	GEO Microarray database (233 cell types)	Target cell versus background cell types	By JSD specific score	Core of 10 TFs	D'Alessio *et al.* [75]
CellNet	Microarray	GEO Microarray database (16 cell types)	Differential expression	By number of regulated gene and TF expression fold changes	Target Cell specific network	Morris *et al.* [73]
TranSyn	Single-cell RNA-Seq	Single cell RNA-Seq data clustered by subpopulation	Multivariate mutual information (MMI) starting from the most expressed TFs	Maximize MMI value	List of TFs	Okawa *et al.* [76]

Each row represents the method for predicting TFs in transdifferentiation. Each column represents the computational stages involved in the TFs set prediction which are input requirement, generation of differential expression profile, identifying the influence of each TF in cell conversion, criteria to prioritize the TFs and finally the predictions.

how such interactions shape T cell development and repertoire, as determined using TCR sequencing. The study observed early emergence of innate-like T lymphocytes (including γδT cells and CD8αα$^+$ T cells), with conventional αβT cells developing later [77], in line with reports that T cells derived *in vitro* from iPSCs show a tendency towards an innate-like phenotype or do not fully recapitulate the typical properties of their phenotype [78]. Computational tools were used to predict the trajectory of cell differentiation, identifying waves of TCR recombination and sets of stage-specific TFs regulating differentiation. This analysis is important as recent differentiation protocols have highlighted the importance of TCR expression in differentiation, with iPSCs genetically edited to carry a particular transgenic TCR or CAR undergoing superior differentiation, compared with unedited cells, in the absence of a thymic microenvironment [79]. To understand the role of stromal and dendritic cells, the study made use of CellPhoneDB, the authors' previous work that uses a statistical framework and known receptor-ligand pairs to predict enriched cellular interactions from scRNAseq data [80]. This identified chemokine signatures promoting migration of T cells from one area of the thymus to another during differentiation. Single molecule fluorescent *in situ* hybridization (smFISH), a technique that identifies mRNA expression at single-cell resolution in tissue slices, was used to validate the sub-thymic localization of cell subsets and the predicted intercellular interactions [77]. Lastly, spatial transcriptomics can allow for cellular transcriptional sequencing *in situ*, for informing on ACT for solid tumors. While not being strictly at the single-cell level in the case of 10x Visium, when used in conjunction with scRNAseq, the transcriptional signatures can be deconvoluted. These approaches have already been applied to understand the cellular microenvironment of solid tumors [81,82].

Next-generation sequencing and high throughput data approaches will play a key role in the identification of gene regulators or soluble factors that can increase the quality of

ACT products. As an example, MOGRIFY® leverages datasets such as the FANTOM5 consortia data, which uses Cap Analysis of Gene Expression (CAGE) to map the sets of transcripts, transcription factors, promoters and enhancers active in the majority of mammalian primary cell types, making it amenable to in-depth transcriptomic analysis. The technology uses a big-data algorithm to compare gene expression and identify the optimal combination of transcription factors required to directly convert any cell type into any other [9]. In addition, the same dataset has made it possible to identify enhancers and promoters that are important in T cell and macrophage differentiation by profiling human T cells and monocytes [10,11]. This type of large-scale data could be used to identify the regulatory molecules needed to overcome resistance in ACT, to bypass lengthy differentiation protocols from pluripotent stem cells, and to reduce variability between batches of cellular products. These types of approaches can also be applied to improve infiltration in solid tumors or to identify and engineer switch receptors that transform suppression signals and increase CAR T-cell resistance to the tumor microenvironment.

Multi-omic & screening approaches to tackle T cell exhaustion

As well as interest in programing cell fate, the era of genome engineering also provides opportunities to enhance cell function or overcome roadblocks to cell therapies. A large focus has been placed on circumventing the issue of T cell exhaustion, where repeated stimulation leads to cellular dysfunction and impaired immune response.

In one recent study, T cells from patients with basal cell carcinoma were analyzed by scRNAseq coupled with TCR sequencing before and after treatment with anti-PD-1-antibody. Interestingly, this study identified a spectrum of T cell phenotypes in the tumors, but showed that checkpoint blockade does not reinvigorate tumor-infiltrating leukocytes, as previously thought, but allows novel cell clones to enter the tumor from the circulation [83]. Combining TCR sequencing and ATACseq of single cells [42] in a similar set of patients identified an enhancer within the *PDCD1* locus, encoding PD-1, which becomes activated during exhaustion [84]. Investigating the TF binding motifs in such regions could help identify targets to regulate exhaustion, in the context of our new understanding of clonal dynamics.

Another study combined a variety of 'omics techniques to search for factors that could overcome exhaustion [85]. T cells expressing different CARs were treated with several different stimuli to induce exhaustion profiles and compared to identify differentially expressed genes driving exhaustion. Exhaustion is associated with epigenetic changes, and scATACseq identified differentially accessible regions of chromatin near exhaustion-associated genes such as *CTLA-4* in exhausted T cells, and a decrease in accessibility at genes associated with memory, such as *IL7A*. DNA motifs for the AP1 complex were enriched among the newly open regions in exhausted T cells. The canonical AP1 complex of c-JUN and c-FOS drives expression of IL2, but can be antagonized by other family members, and such factors were found to be upregulated in the transcriptomes of exhausted cells. Overexpression of c-JUN was shown to restore T cell function, and experiments using modified c-JUN proteins lacking functional domains showed that its interaction with other proteins was important for this function rather than its DNA binding capacity. Additionally, overexpression of c-JUN also rendered the CAR T cells more sensitive to lower levels of antigen, which could help in tumors with low antigen expression and where the selective pressure from CAR T cells leads to antigen down-regulation [85]. However, c-JUN is potentially oncogenic, so the potential of such modifications to produce to unwanted side effects in the modified T cells needs to be thoroughly assessed.

These studies illustrate how modern technologies can be used to understand the

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mechanisms behind phenomena such as T cell exhaustion and inform the rational design of strategies to circumvent these mechanisms. However, while they provide a deeper understanding of exhaustion, they have limited throughput for discovering and validating targets to enhance the therapeutic effect of adoptively transferred cells. The development of CRISPR technology has greatly facilitated genome-wide knockout screens for target discovery across biology. Combining this with scRNAseq has provided a balance between the high-dimensionality of arrayed screens, where knockouts are considered one-by-one, with the throughput of pooled screens [49,86]. This technology depends on sequencing either a barcode associated with the CRISPR guide RNA [49,86] or by sequencing the guide RNA itself after capture onto microbeads [87]. Several CRISPR knockout screens coupled with scRNAseq and tumor infiltration models have identified regulators of $CD8^+$ T cell fitness in mice, including REGNASE-1 [87–89].

Roth et al. used a targeted approach to examine both wild type and novel constructs that could be introduced to cells to enhance $CD8^+$ T cell function [90]. The system used CRISPR-mediated homologous recombination to introduce a transgene carrying a constant transgenic TCR targeting the NY-ESO-1 antigen into the endogenous *TRAC* locus, along with one of the genes used in the screen, and a transgene-specific barcode. Modified cells were challenged in a number of assays to identify transgenes that could enhance tumor infiltration and cytotoxicity towards NY-ESO-1-expressing cancer cells in humanized mouse models, under stimulation with anti-CD3/CD28, the immunosuppressive cytokine TGFβ, or other factors. After functional readouts, the cells were sampled for scRNAseq and targeted sequencing of the knock-in barcode to couple transgene expression to the transcriptome. Interestingly, while the authors found that knocking in either TCF7 or a synthetic TGFbR2-41BB receptor increased T cell abundance in solid tumors, the latter promoted accumulation of cells expressing key effector cytokines while the TCF7-expressing cells failed to function, highlighting the importance of using multiple functional assays to assess phenotypes. While very interesting, this approach is of course limited to transgenes selected a priori rather than representing an unbiased screening approach.

CRISPR holds great promise, but there are concerns around off-target editing events and long-term safety of edited cells. An important component of clinical studies is, therefore, tracking the fate of adoptively transplanted cells. Results of the first clinical study with CRISPR-edited T cells were published in early 2020 [91]. T cells were modified to remove the endogenous genes encoding the T cell receptor (*TRAC* and *TRBC*) and introduce a cancer-specific TCR, a strategy thought to enhance transgene function by preventing the mispairing of endogenous and exogenous TCRα and β chains. The *PDCD1* gene was also removed to limit exhaustion and enhance anti-tumor immunity, resulting in a total of three genomic edits. scRNAseq of cells from patient samples was used to assess changes in the transcriptome and the frequency of edited cells over time, showing that after an initial decline in the frequency of edited cells after transplant, they remained stable for several months. Up to 40% of peripheral blood T cells carried at least one edit, but there was a low frequency of cells carrying all three edited loci and the transgenic TCR, due to a lack of selection for these cells before transplant. The frequency of cells with the *PDCD1* knockout, in particular, decreased over time, consistent with mouse studies indicating that these cells are less able to establish immunological memory, while cells with the *TRAC/TRBC* knockouts had transcriptional signatures of central memory in contrast to previous studies using only the knock-in of the transgenic TCR, which resulted in T cell exhaustion. This is an important first step in the development of next-generation immune cell products for personalized cell therapies.

REGULATORY ASPECTS OF CELL THERAPY

The European Medicines Agency (EMA) directive 2001/83/EC and Advanced Therapeutic Medicinal Products Regulation EC No 1394/2007 reads: 'Somatic cell therapy medicinal product means a biological medicinal product which contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor'. Similarly, the Food and Drug Administration (FDA), in Guidance for Human Somatic Cell Therapy and Gene Therapy – Guidance for Industry, March 1998, states that 'Somatic cell therapy is the administration to humans of autologous, allogeneic, or xenogeneic living cells which have been manipulated or processed *ex vivo*'. Interestingly, the FDA document is guidance, which represents 'the current thinking of the FDA on [a particular] topic'. This perhaps highlights the fact that, due to its relative novelty, each cell therapy product is evaluated on a case-by-case basis rather than regulations set in law.

Among the definition of 'manipulations' is genetic modification of the cells, and this implies that the regulations for gene therapy also have to be followed for cell therapy, as viral and non-viral vectors may be integral parts of the final product. Different territories are governed by different agencies, e.g. the Pharmaceutical and Food Safety Bureau (PFSB) in Japan, and the National Medical Products Administration (NMPA) in China, in addition to the FDA in the USA and the EMA in the EU. This results in different regulations regarding quality, safety, and efficacy for the same product. Thus, it is important to consider this when developing a pharmaceutical product - not a trivial task for small companies. Even the terminology can vary between agencies, and so a standardized medical terminology, MedDRA, has been developed by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) – an organization that aims to bring together agencies and the pharmaceutical industry, providing guidelines to reduce differences between territories and facilitate sharing of regulatory information internationally. From a consumer point of view, i.e. the patient, the existence of different regulatory agencies and jurisdictions can make the difference between having access to a life-saving treatment or not. For this reason, any effort that aims to further align the regulatory agencies from different countries must be encouraged, promoted and supported. For example, the European Union (EU) has mutual recognition agreements (MRAs) of GMP inspections and batch certification of human medicines with countries including Australia, Canada, Japan and the US. This facilitates market access and international harmonization of standards, while reducing duplication of GMP inspections and costs for manufacturers. Unfortunately, cell therapy is excluded from the MRAs, therefore a product commercialized in one of the countries mentioned above will not be available in the EU unless the necessary inspections and batch testing are carried out by the EU, adding time and costs.

In cell therapy, the starting material consists of cells, most often obtained from human donors. This process requires strict adherence to the directives of the regulatory authorities, such as the obtaining of informed consent that must be provided by eligible donors and data protection that must be guaranteed by the manufacturer. An alternative scenario is represented by the case in which the starting material consists of a cell line. The regulations for the manufacturing of the cell line are expected to follow GMP regulations for clinical samples, but perhaps the most interesting aspect of this approach is related to the *in vivo* behavior of the cell line. Thorough pre-clinical studies must be carried out to demonstrate safety and efficacy. The first cell line to be used to provide the starting material for cell therapy is the cell line NK-92. This

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cell line, isolated from a patient with malignant non-Hodgkin's lymphoma, has shown a high level of safety and efficacy in preclinical studies [92,93], although the cells have to be irradiated before use because of their origin in lymphoma. NK-92 has been used as a starting material to manufacture the anti-HER2-CAR-CD28zeta-expressing allogeneic NK-92 cells, and anti-CD33 CAR NK cells, used in clinical trials NCT03383978 and NCT02944162 respectively (from clinicaltrials.gov). The data currently available indicate no issues with safety and no or mild reactions post-infusion, but unfortunately these products have not so far shown significant improvement in efficacy compared to the standard of care. Nevertheless, these clinical trials have paved the road to the use of cell lines as starting material, an approach that is central for the off-the-shelf model for cell therapy.

There are numerous challenges ahead for the development and manufacture of cell therapies, the most prominent being reduction of costs. This will be achieved through a combination of process optimization, automation, and the production of off-the-shelf alternatives. Innovation driven by the computation of large-scale data sets is expected to play a central part in the delivery of new solutions, ensuring quality and accessible products for a larger number of patients. Mogrify®, using its suite of platform technologies for direct cellular conversion and maintenance of cells, offers a transformative approach to the development of 'off-the-shelf' cell therapies, reducing the processing time of cell differentiation, and reducing variability between batches, whilst at the same time increasing the scalability of the cell products and facilitating an entirely new class of *in vivo* reprograming therapies.

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EXPERT INSIGHT

Immunological tolerance: scanning a barren landscape for signs of sustained growth

Harry Petropoulos, Timothy J Davies, Paul J Fairchild

The immune system is finely poised to respond to the challenge of infection but occasionally lacks discernment, mounting inappropriate responses to agents that pose no threat, thereby driving pathologies as diverse as autoimmune disease, allergy and allograft rejection. Although our understanding of how immunological tolerance is established and maintained and the circumstances that lead to its failure has improved substantially over the past 20 years, translation of these principles into effective treatments has proven unexpectedly difficult to achieve. Here we explore how the underlying principles of antigen recognition have inspired novel approaches to the induction of tolerance and review progress made in their use to pacify an aggravated immune system. While many challenges undoubtedly remain, for those dedicated to commercialising the opportunities that biologicals and cell therapies have begun to provide, the rewards are likely to be substantial.

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INTRODUCTION

The past few decades have witnessed unexpected advances in our understanding of the pathophysiology of human disease and the development of novel approaches to intervention, from the treatment of infections, to the management of cardiovascular disease and the cure of certain forms of cancer. One subset of diseases that has, however, proven intractable is those induced by an inappropriate immune response, the available treatments being described as blunt or even palliative by some [1].

A growing appreciation of the principles of self/non-self discrimination that lies at the very heart of the immune system, inspired early approaches to intervention in immune pathology. These typically sought to impose sanctions on the entire immune system in response to the iniquities of a small number of lymphocytes. While proving enabling for solid organ transplantation and life-changing for those with chronic autoimmune conditions, the advent of immune suppression and the judicious use of steroids to reduce inflammation, failed to halt the pathological immune response, merely serving to restrain its impact. Furthermore, their side effect profiles often presented more of an immediate health hazard than the pathologies they purported to treat. The subsequent development of monoclonal antibodies (mAbs) such as Alemtuzumab and Rituximab, permitted the indiscriminate killing of populations of T and B lymphocytes respectively in the hope of eliminating the aggressors but, like immune suppression, lacked discernment, treating all lymphocytes as equally culpable. Recent advances in our understanding of antigen recognition at the cellular and molecular level (Figure 1) have, however, suggested new targets for immune intervention that in some cases introduce a welcome element of antigen specificity into treatment options [2,3]. These emerging technologies present a new paradigm that represents a disruptive technology to the widespread use of immune suppression, creating a unique therapeutic market, ripe for commercialization [4]. Although interventions such as the use of altered peptide ligands (APL) and the adoptive transfer of regulatory T cells (Treg) have had significant impact on the field, here we focus on the rationale underlying four emerging technologies at varying stages of development, that seek to induce or re-establish a state of operational tolerance. Furthermore, we discuss evidence from animal models and human trials supporting their safety and potential efficacy.

TARGETING CD3 WITH MONOCLONAL ANTIBODIES

Early studies of antigen recognition by T cells revealed the accumulation of CD3 at the very centre of the immunological synapse they form with dendritic cells (DCs) presenting cognate antigen (Figure 1). The CD3 complex is responsible for initiating T cell activation upon ligation of the T cell receptor (TCR), suggesting mAbs targeting this co-receptor may either disrupt antigen recognition altogether, with potentially immunosuppressive consequences, or may modify its outcome, promoting tolerance rather than T cell activation. Furthermore, the expression of CD3 by all T cell subsets, whether $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells, makes it an attractive universal target for intervention in pathologies such as type 1 diabetes (T1D) [1].

As proof of concept, anti-CD3 mAbs were first used in non-obese diabetic (NOD) mice, a murine model of T1D. While early studies suggested a poor side-effect profile, including induction of a cytokine storm, administration of an aglycosylated version of anti-CD3 prevented Th1-mediated insulitis while avoiding pro-inflammatory cytokine release. Furthermore, at high enough therapeutic concentrations, it showed the ability to reverse established T1D by selectively depleting pathogenic T cells while sparing regulatory T cells (Treg), known to be protective [5,6].

Given the high selectivity of this therapy, it was hypothesised that CD3 mAbs might prove to be efficacious, not only in the

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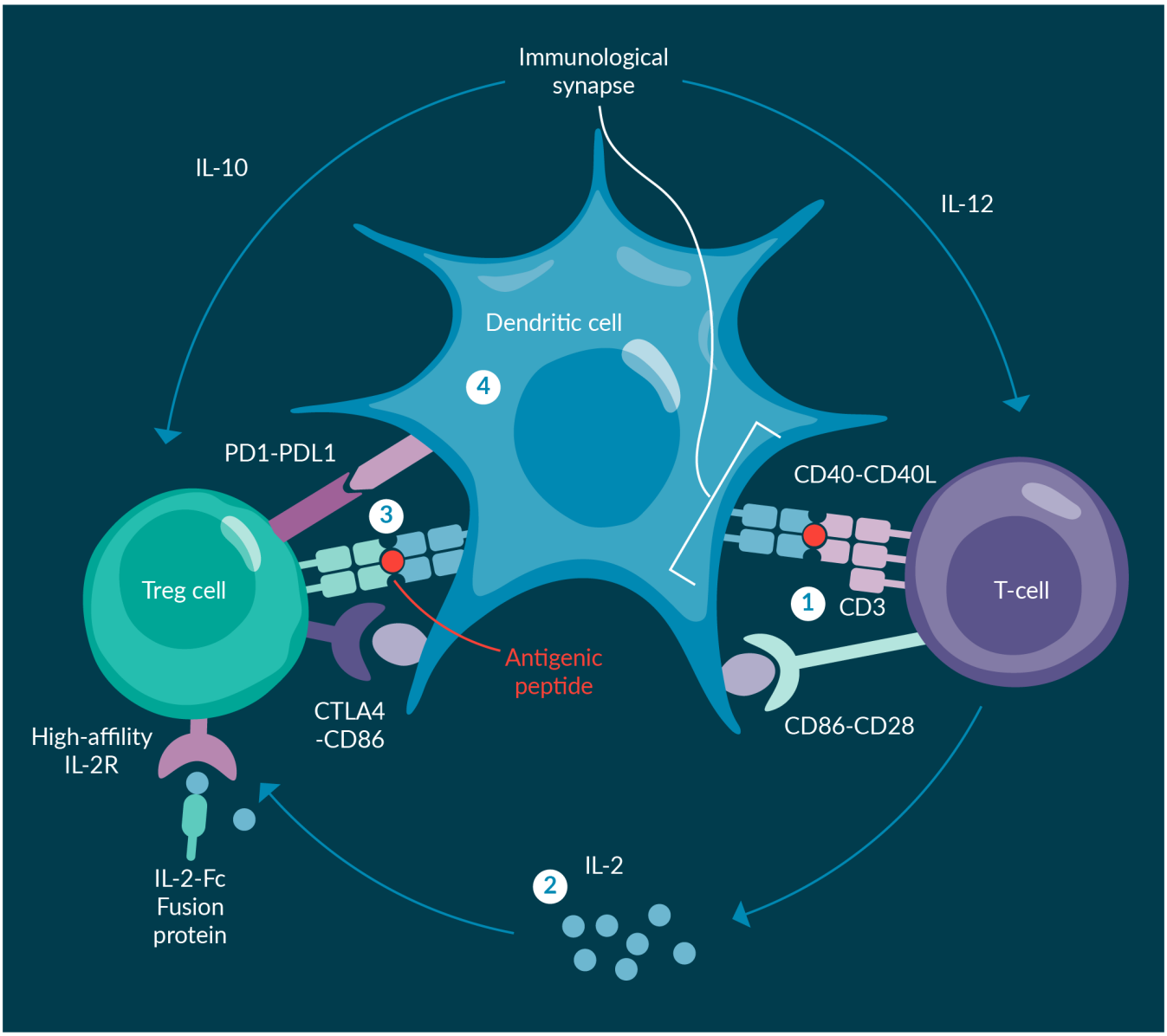
treatment of T1D but also in its prevention. Accordingly, NOD mice treated prior to the spontaneous development of disease, were found not to progress to overt T1D [6]. Furthermore, histopathological investigations revealed that anti-CD3 treatment prevented T cell infiltration into the pancreatic islets while distinctive molecular signatures suggestive of tolerance could be identified among treated T cells, including suppression of interferon (IFN)-γ, increased secretion of interleukin (IL)-10 and the up-regulation of PD-1 [7]. However, in mice in which the onset of T1D had been induced through administration of anti-PD-L1 mAbs, progression of the disease was halted following administration of anti-CD3 only during the course of treatment, the pathological process resuming following discontinuation of the therapeutic for a period of up to 7 days [6].

FIGURE 1

The dynamic interaction between dendritic cells and either effector or regulatory T cells provides numerous opportunities for intervention in the pursuit of immunological tolerance.



Potential targets that have been identified are (1) the CD3 co-receptor, (2) IL-2, (3) the antigenic peptide itself and (4) the dendritic cell responsible for antigen presentation.

Subsequent clinical trials were performed to assess the safety and efficacy of anti-CD3 monoclonal antibody therapy in patients with newly diagnosed T1D [1]. C-peptide serves as an important surrogate marker for monitoring beta cell function in the pancreas and, by inference, patients' progression to T1D. Early trials showed that a single course of anti-CD3 therapy given after recent diagnosis of T1D correlated with an improved C-peptide response one year later. Follow-up trials, designed to compare multiple dosing regimens, revealed a 75% higher mean C-peptide level and lower insulin use in the treatment arm [8]. Furthermore, overall treatment was found to be safe with only 8% of patients experiencing adverse events related to cytokine release. Post-hoc analysis revealed that trial participants which responded best to treatment were those with lower exogenous use of insulin and lower numbers of autoreactive Th1 cells at baseline, indicating that such therapy would be best licenced for use early in diagnosis of T1D [9]. Long-term follow up for up to 9 years revealed that anti-CD3 treatment had lasting effects on C-peptide levels and persistent changes in the T cell population, most notably increased expression of PD-1, as seen in animal models, as well as the induction of anergy among autoreactive $CD8^+$ T cells [10]. These effects were not, however, consistent in all participants, suggesting interpatient variability requiring optimisation of patient selection criteria and dosing regimens. Nevertheless, despite the inevitable variability inherent in human trials compared to animal models, anti-CD3 has been shown to have potential clinical utility in a select group of patients by reducing inflammation and preserving beta cell function, especially among younger patients, resulting in a delay in onset of T1D. Furthermore, a recent study of the relatives of patients with T1D, known to be at high risk of likewise developing the disease, showed a significant delay in onset of clinical symptoms from 24.4 to 48.4 months following a short course of anti-CD3 delivered prophylactically, thereby greatly extending the potential reach of this treatment regimen [11].

INTERLEUKIN-2: A DOUBLE-EDGED SWORD

IL-2 has attracted much interest since its discovery in 1976 when it was shown to play an integral part in the clonal expansion of activated T cells, sustaining the response to infection and contributing to immune surveillance against neoplastic cells [12]. Subsequent findings have, however, revealed a double life, the same cytokine also contributing to immune homeostasis and the maintenance of tolerance through its activity on Treg cells *in vivo* [13,14]. This dual allegiance may be attributed, in part, to the unique structure of the IL-2 receptor.

Research into the structural properties of the IL-2 receptor have revealed three different forms displaying low, intermediate and high affinity for its ligand. Low affinity receptors are comprised of monomeric α-chain subunits, also referred to as CD25, while the combination of β (CD122) and γ chains (CD132), which are constitutively expressed, yields a functional receptor with intermediate affinity: only when this structure associates with CD25 upon its up-regulation due to T cell activation, is the IL-2Rαβγ trimer formed which serves as a high affinity receptor [15]. Accordingly, Daclizumab, a mAb specific for the high affinity receptor through its interaction with CD25, has been shown to be effective in the treatment of multiple sclerosis by selectively targeting autoreactive T cells, indeed, phase I trials have yielded promising results in terms of safety, pharmacodynamic and pharmacokinetic properties as well as treatment efficacy [16]. However, the high affinity receptor is also constitutively expressed by Treg cells suggesting that, in the steady state, Treg cells may depend on low concentrations of IL-2 for their differentiation, expansion and viability to which resting effector T cells fail to respond, making it an attractive candidate for targeted immunotherapies.

Early studies of the role of IL-2 in the immune system revealed just such a dose dependency, high doses appearing responsible for the activation of effector T cells while lower doses were effective at recruiting Treg

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cells for the induction of tolerance [12]. It was hypothesised that animals deficient in IL-2 might have impeded Treg cell development and function, rendering them susceptible to autoimmune disease. This was initially confirmed by Malek and colleagues who demonstrated not only that autoimmunity was a consequence of IL-2 deficiency, but that it could be prevented by the adoptive transfer of $CD4^+CD25^+$ Treg cells from wild type mice [17]. Armed with this knowledge, animal models were used to test varying dose regimens of IL-2 on diseases such as diabetes in which low doses showed a long-lasting impact on progression, secondary to an influx of Treg cells into the pancreatic islets [17]. Low dose IL-2 also showed potential utility in modulating the response to food allergens, suggesting that IL-2 therapy may be effective in the treatment of a broad range of indications [18].

In human trials, IL-2 was first utilised for its anti-cancer properties, high doses of IL-2 being used to treat metastatic melanoma and metastatic renal cell carcinoma in patients with intact immune systems. These trials showed tumour regression not only by expanding activated T cells but by augmenting Natural Killer (NK) cell activity [12]. Unfortunately, this strategy exhibited a very narrow therapeutic window, resulting in adverse events such as cytokine release syndrome. Despite the relatively high incidence of such events, FDA approval was granted for the use of high dose IL-2 as an anti-cancer drug. That low doses of IL-2 were better tolerated by patients, suggested, nevertheless, that the cytokine might be better deployed in regimens for the induction of tolerance [18].

Initial clinical trials of low dose IL-2 focussed on its use in haematopoietic stem cell transplantation in which its administration led to a 1.9-fold increase in $CD4^+CD25^+$ Treg cells which improved the effect of transplantation while avoiding the toxicity observed at higher doses [19]. Since then, IL-2 therapy has been trialled in vasculitis, secondary to Hepatitis C infection, and autoimmune conditions such as diabetes, alopecia areata and systemic lupus erythematosus, all of which have demonstrated a similar increase in Treg cells and varying degrees of efficacy [18]. Recent developments have paved the way for next generation therapeutics that are less dependent on narrow dosing regimens and are highly specific for Treg cells, inducing their polyclonal expansion *in vivo*. An engineered form of IL-2 capable of selectively binding the high affinity IL-2R constitutively expressed by Treg cells, was fused to the Fc portion of human IgG1 to prolong its half-life *in vivo*. The resulting fusion protein induced a 10-14-fold expansion of Treg cells in cynomolgus monkeys and humanized mice, auguring well for its use in the treatment of a broad spectrum of inflammatory and autoimmune conditions [20]. IL-2 therapy therefore continues to present an emerging paradigm in the quest for immunological tolerance, showing promise for the treatment of a broad range of disease states.

EXPLOITING PEPTIDE MIMETICS

An emerging technology which has sparked interest in the field of neuroimmunology is that of ATX-MS-1467, a cocktail of four peptides based on epitopes derived from myelin basic protein (MBP), known to drive the pathogenesis of multiple sclerosis (MS). Such an approach, borrowed from the field of allergy medicine, was formulated to try and reinstate tolerance to MBP, a rather more nuanced approach than the ablation of entire T and B cell subsets that is currently favoured. The cocktail of peptides, of which ATX-MS-1467 is composed, mimics the naturally-processed epitopes of MBP capable of interacting with MHC class II molecules on the surface of immature DCs [21]: presentation of these so-called 'antigen-processing-independent epitopes' (apitopes) to naïve T cells in the absence of DC maturation has been shown to polarise responses toward a regulatory phenotype, characterised by abundant IL-10 secretion [22]. Given that IL-10 has a profound impact on DCs, further inhibiting

their maturation, apitopes are thought to achieve tolerance through a mechanism that is inherently self-reinforcing [23] while also expanding the tolerant state to encompass additional epitopes and autoantigens through a form of infectious tolerance [24].

Pre-clinical studies in transgenic mice expressing the human MHC class II molecule, HLA-DR2, demonstrated how administration of therapeutic doses of ATX-MS-1467, either early or late in the disease process, was able to halt disease progression, resulting in a reduction in inflammation within the central nervous system, reduced T and B cell infiltration and fewer signs of demyelination [21]. Although murine models of MS fail to faithfully recapitulate human pathophysiology [22], first-in-human trials of ATX-MS-1467 have been conducted in patients with relapsing-remitting (RRMS) and secondary progressive MS (SPMS). In Phase I trials, ATX-MS-1467 proved to be well tolerated amongst participants with only one serious adverse event which promptly resolved following brief hospitalisation. No antibody response to the treatment was observed and radiographic investigations showed a significant decrease in the appearance of new foci of demyelination. Phase II studies continued to demonstrate safety as well as a statistically-significant decrease in enhancing lesions compared to baseline, although this did not translate to improvements in disability scales [25]. Further study is, therefore, required to assess whether this novel approach to intervention in the pathogenesis of MS is able to re-establish a durable state of tolerance that persists beyond the cessation of treatment and has an objective impact on quality of life.

Antigen-specific immunotherapy using appropriate vehicles for the delivery of disease-associated apitopes is attractive as a concept due to its application to a broad range of autoimmune conditions, providing a more targeted therapy and reducing the potential adverse effects of non-specific immunosuppression. Peptide based therapies have shown significant growth within the global therapeutic market, with an average annual growth of 9.8% in the past decade which looks certain to continue, paving the way for the introduction of personalised peptide therapy [26].

CELLULAR THERAPIES: AN EMERGING PARADIGM

The ease with which peptide-based therapeutics can be manufactured makes them attractive candidates for commercialisation. Nevertheless, the relevant epitopes from key autoantigens have been identified for only a small fraction of the MHC molecules expressed within the human population, greatly limiting the cohort of patients that might benefit from such an approach to those expressing the most common MHC determinants, such as HLA-DR2. An alternative approach which has begun to gain traction over recent years, has, therefore, exploited the properties of DCs to select appropriate epitopes from autoantigens they have acquired or with which they have been pulsed *ex vivo*: by using DCs directly as a cell therapy, the identity of epitopes presented by them need never be fully defined.

DCs may be derived in an autologous manner from a patient's own peripheral blood monocytes by culturing them *in vitro* for 7 days with granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4. Although highly immunogenic under normal circumstances, the addition of anti-inflammatory cytokines, such as IL-10 or TGF-β, or pharmacological agents including vitamin D_3, rapamycin or dexamethasone [27], is known to render monocyte-derived DCs (moDCs) more tolerogenic by causing their developmental arrest at an immature stage associated with low expression of the costimulatory molecules needed for full T cell activation and clonal expansion (Figure 1). Indeed, early work demonstrated that immature tolerogenic DCs (tolDCs) had the ability to inhibit effector T cell function in humans through deletion and the induction of T cells with regulatory properties [28,29]. Such tolDCs have, therefore, been pursued,

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not only as a potential treatment for autoimmune diseases, but in the context of solid organ transplantation [30,31].

Development of closed cell-culture techniques now make it feasible to employ the principles of good manufacturing practice (GMP) in a step towards clinical application and commercialisation. Multiple Phase I trials have been conducted to investigate the safety profile of tolDC treatment in a number of autoimmune conditions including MS, rheumatoid arthritis and T1D [32]. In a Phase I dose escalation trial, patients with inflammatory arthritis were administered autologous tolDCs within the intra-articular capsule of affected joints to assess feasibility of the treatment and its safety. TolDCs were exposed to culprit autoantigens from autologous synovial fluid and injected at varying doses into the joints of affected individuals [33]. Data from the study showed the treatment to be safe, those patients receiving higher doses also demonstrating stabilisation of their symptoms with no systemic sequalae. Stabilisation of symptoms was seen throughout the trial period of 91 days, warranting further investigation into use of tolDCs as a treatment modality in inflammatory arthritis [33]. Insight into the identity of autoantigens responsible for such complex pathologies is currently incomplete, making it difficult both to source appropriate autoantigens with which to pulse tolDCs and monitor the antigen-specificity of subsequent immune responses *in vivo*. A recent study has, however, circumvented these limitations by pulsing tolDCs with peptides derived from heat shock proteins (HSP), known to be abundant in inflamed synovia. When presented by tolDCs, these surrogate autoantigens induced antigen-specific Tr1 cells capable of secreting copious IL-10 and perpetuating a tolerogenic microenvironment [34], results that augur well for forthcoming clinical trials.

The field of neuroimmunology has also taken particular interest in the use of tolDCs for the prospective treatment of MS and neuromyelitis optica, both autoimmune pathologies of the central nervous system. Recently, Phase I trials were conducted to investigate the use of tolDCs to establish antigen-specific tolerance in both these conditions with favourable results being reported with respect to both safety and efficacy [35]. The cellular product was loaded with peptides specific for either disease and administered in a dose escalation pattern to recipients with no severe adverse events noted. No relapses were observed in any of the patient groups and no worsening of the condition in imaging studies in the neuromyelitis optica group. Two MS patients were seen to have one new lesion each twelve weeks into the trial, however this may be attributed to the refractory period between prior therapies. Interestingly, an increase in IL-10 secretion was reported together with a decrease in prevalence of $CD8^+$ memory T cells [36]. This approach therefore holds promise in the developing field of personalised medicine as rare and even orphan diseases may now benefit from a customized therapy, tailored to the patient's individual needs.

Despite these positive signs, the use of DCs as a cell therapy has encountered a number of practical challenges. For instance, the only source of DCs currently available from patients has been the differentiation *ex vivo* of peripheral blood monocytes, other populations of DCs proving inaccessible in sufficient numbers. Given that moDCs are inherently pro-inflammatory, most closely resembling DCs recruited to tissues in response to local infection, their use for the induction of tolerance may run counter to their normal role. An alternative source has recently emerged, however, inspired by the landmark achievement of Yamanaka and colleagues in reprogramming adult cells to a pluripotent state through the introduction of key transcription factors [37]. Indeed, induced pluripotency has spawned a new paradigm in the field of cell therapy, raising the prospect of second-generation DC vaccines [38,39]. Accordingly, it has proven feasible to direct the differentiation of mouse induced pluripotent stem cells (iPSCs) into DCs with regulatory properties capable of intervening

in the rejection of organ allografts through the induction of alloantigen-specific Treg cells [40]. Furthermore, protocols have been developed for the differentiation of iPSCs of human origin into subsets of DCs, such as the CD141$^+$ population, implicated in the maintenance of tolerance *in vivo* [41]. Importantly, the tractability of iPSCs for genome editing may also permit the rational design of next generation therapeutics whose gene expression profile is fully conducive to the establishment of tolerance [38], securing DCs as a credible new player in the field of tolerance induction.

The market for cell therapies is currently varied due to the spectrum of different methodologies in development, from mesenchymal stem cells and embryonic stem cells to the emergence of induced pluripotency [42]. Although there is no unifying approach for this highly variable product, cell therapies clearly have the potential to create a market of their own rather than capitalise on pre-existing demand. With global revenues surpassing $1 billion annually, it seems that cell therapies represent an attractive option for future investment [43].

TRANSLATIONAL INSIGHT

The induction of immunological tolerance in a clinical setting has historically proven difficult to achieve, despite the evident benefits that reliable protocols would offer for the treatment of a broad spectrum of disease states. This impasse may be attributed to a number of issues, not least of which is the need to re-establish tolerance in an already primed immune system in order to intervene in autoimmune disease, a task significantly more challenging than the induction of tolerance *de novo* to antigens that have not previously been encountered. Other issues are more practical in nature: the identity of many autoantigens remains obscure and is greatly confounded by the process of epitope spreading during the course of the disease, significantly amplifying the specificities of T cells whose activity must be brought back under control. Clinical trials of tolerance induction are also complicated by the chronic nature of most autoimmune conditions, it being difficult to reach a primary endpoint within an acceptable timeframe. Furthermore, the need to improve on immune suppression represents a daunting obstacle, given that its use is frequently life-changing for patients, despite the unfavourable long-term consequences. Notwithstanding these hurdles, some of the emerging approaches to the induction of tolerance offer the alluring prospect of operating in an antigen-specific manner, allowing them to preserve the activity of all law-abiding lymphocytes. Far removed from the blunt instruments of immune suppression, such approaches show early signs of proving to be disruptive technologies with the potential to capture a lucrative market.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY: NEXT-GEN PLATFORMS & MODALITIES

SPOTLIGHT

COMMENTARY

Is a second wave of (CAR T) cell based cancer therapies a mirage or reality?

DR DAVID GILHAM first joined Celyad Oncology in April 2016 as a member of the Scientific Advisory Board. As of September 1, 2016, David became Vice-President R&D, heading the implementation of our research and development strategy for our programs in immuno-oncology. Dr. Gilham received his Ph.D. in Molecular Pharmacology at the University of Dundee prior to moving to Bristol University in 1996 to work on CAR T cells with Professor Robert Hawkins. The group moved to Manchester in 1998 where his research activity has focused on engineering T-cells for cancer therapy and developing the necessary pre-clinical studies to support translation of this therapy into Phase 1/2 clinical trials in Manchester. Prior to joining Celyad Oncology, David was a Reader in the Institute of Cancer Sciences, University of Manchester, UK and led the Clinical and Experimental Immunotherapy Group based within the Manchester Cancer Research Centre.

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By the end of 2017, CAR T cell therapy was major headline news for two main reasons. The first was the regulatory approval of two CD19-targeted CAR T products (Kymriah® produced by Novartis and Yescarta® produced by Kite Pharma) for the treatment of certain B cell malignancies on the back of frankly stellar clinical responses in relapsed / refractory patients. The second corporate-focused reason was the acquisition of Kite Pharma by Gilead Sciences for $11.9 billion, which certainly turned a few heads.

The enthusiasm for CAR T cell therapy exploded during this time with many companies entering the space to deliver this new form of immune-based therapy. Many focused (and continue to focus) on developing the nuts and bolts of CAR T cell therapy with CD19 / B cell malignancies since the use of a validated target removes one variable from the equation, facilitating the testing of the CAR or cell technology itself. Indeed, a rapid search on the clinicaltrials.gov website on 22 July 2020 using the search term 'CAR T cell' identified 807 ongoing trials while the term 'CD19 CAR T cell' identified 314 trials. Adding the term 'BCMA' or 'CD20' to 'CAR T cell' identified a further 118 trials. Hence, on this crude search at one specific point in time in 2020, approximately 54% of ongoing clinical trials identified by the term 'CAR T cell' focus on targeting B cells.

However, going into 2020, the CAR T field is in a somewhat different position to that of the end of 2017. Kymriah and Yescarta failed to deliver the levels of sales that were anticipated by many analysts. The challenges of delivering a just-in-time, individualized cell therapy are evident (as reviewed in CGTI). The next CAR T product to be licensed will likely emerge from the red-hot battlefront of multiple myeloma where several companies are developing BCMA targeted CAR Ts. However, the expectations of a flood of follow up CAR T products for indications outside of B cell malignancies that achieve licensing based on small scale trials driven by extreme clinical responses have yet to materialize.

Does this translate to CAR T being a failed experiment? Absolutely not.

Novartis and Gilead / Kite are the groundbreakers here by bringing a complicated therapy to commercialization and dealing with the hurdles beyond as they arise. The clinical responses in B cell acute lymphoblastic leukemia (B-ALL) and non-Hodgkin lymphoma (NHL) confirm the paradigm-changing nature of the therapy, yet the hurdles appear to be primarily related to manufacturing and business models that include how to reimburse for an expensive, one-off personalized therapy. While these specific challenges (among others) were predicted, real-world experience is crucial to fully understand all nuances to support the achieving solutions. Moreover, considering the apparent lack of non-B cell targeted CAR T products in advanced development, does this reflect the focus on using CD19 as the model target antigen of choice thereby skewing the CAR T knowledge base to B cells and failing to fully explore other indications, especially solid tumor indications?

The challenge of targeting malignant cells not of B cell origin and developing commercially relevant platform systems to facilitate that targeting is what drew me to work in the life sciences industry. During my academic time, primarily at the University of Manchester, the challenges of targeting solid tumors with early iterations of CAR T were clear. Several forerunner clinical trials using CAR T in ovarian cancer [1], renal cell carcinoma [2] and metastatic colorectal cancer [3] showed safety but no evidence of clinical response. At the time of these trials, the potential of pre-conditioning chemotherapy had not been explored in the CAR T context. However, seminal studies arising from the Surgery Branch of the National Cancer Institute (NCI) showed that combining pre-conditioning with tumor infiltrating lymphocyte (TIL) therapy dramatically improved therapeutic responses in patients with malignant melanoma [4].

There are many mechanisms by which pre-conditioning may impact T cell therapy including eliminating cytokine sinks, enhancing the availability of homeostatic cytokines, transiently eliminating immune suppressor elements and creating space for the adoptively transferred T cells [5]. However, this combination is also likely to impact the tumor burden itself, dependent upon the indication with hematological malignancies likely to be susceptible to the lymphodepletive effects of pre-conditioning. Finally, and not often mentioned is that this transient lymphodepletion may help CAR T cells avoid immune recognition of the CAR construct itself as has been

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observed in patients receiving CAR T cells in the absence of pre-conditioning chemotherapy [6].

Combining cyclophosphamide / fludarabine with first generation CAR T cells in Manchester did induce clinical activity in patients with B cell malignancies along with persistence of the engineered T cells [7]. However, there was little evidence of clinical efficacy nor persistence in an initial trial testing pre-conditioning with carcinoembryonic antigen (CEA) CAR T cells in patients with CEA^+ solid tumors [8].

Against this background, the move to Celyad Oncology with a focus on a differentiated natural killer receptor, Natural Killer Group 2D (NKG2D), used in the context of CAR T along with intellectual property on the broad allogeneic CAR T concept and specific IP relating to non-gene edited allogeneic CAR T cell technology was attractive. These critical aspects of broad tumor targeting coupled with platform technologies to deliver the therapy were extremely interesting to an academic researcher who had been working on the challenges of targeting solid tumors with CAR T for decades.

NKG2D is a receptor that binds eight known in human ligands (MICA, MICB and ULBP1-6) whose expression is up-regulated in response to cellular stress. Natural Killer (NK) cells use NKG2D as one of an array of activating receptors that enables NK cells to target and eradicate cells that are subject to pathogen infection and transformation. The attraction of using a natural receptor that targets eight independent ligands whose up-regulated cell surface expression is found across most hematological malignancies and solid tumor indications is clear and obvious. The challenge is to use such a receptor in a therapeutically relevant context which requires a strong safety profile coupled with therapeutic activity.

Fusing the NKG2D receptor to the CD3ζ intracellular domain generated a CAR construct that, when expressed in T cells, re-directed the activity of the T cells against cells expressing NKG2D ligands. Extensive pre-clinical studies documented the potential of the approach but the concern moving into clinical testing was the potential for on-target, off-tissue toxicity given that the target ligands were not tumor specific. The company has taken a stepwise approach to clinical testing initially testing three infusions of the T cells alone which resulted in some very encouraging signs of anti-tumor activity in patients with relapsed or refractory acute myeloid leukemia / myeloid dysplastic syndrome (AML/MDS) [9]. In this trial, patients received no concomitant therapy including no preconditioning chemotherapy for at least five weeks prior to receiving the T cells, given in three doses over a month in order to avoid the potential of chemotherapy inducing target ligand expression on healthy tissues. The safety and tolerability profile proved to be very good with no evidence of on-target, off-tissue toxicity. Moreover, the absence of concomitant therapy clearly indicated that it was the T cells alone and no other factor driving the reductions in tumor burden observed in this challenging to treat patient population, an observation close to being unique in the CAR T cell space since the vast majority of therapies include some form of therapy immediately prior to infusion of the T cell product.

The THINK trial continues to evaluate the clinical activity of the NKG2D CAR T cells (called CYAD-01) in the AML/MDS population. However, given the safety profile seen in this trial, a subsequent trial combined CYAD-01 with cyclophosphamide / fludarabine pre-conditioning in the AML/MDS population again showed no evidence of on-target, off-tissue toxicity [10]. The encouraging safety profile of the NKG2D approach has led to further iterations that aim to increase the potency of the CAR T cells in the next generation approach that is CYAD-02 [11] which incorporates a short-hairpin RNA (shRNA) to control the gene expression of NKG2D ligands on T cells and is now in clinical testing.

The NKG2D CAR T cell story is now 'taking off' based on our clinical trials performed to date demonstrating the very encouraging

safety and tolerability of the therapeutic approach. This supports the view that increasing the potency of the cell approach is warranted since the over-riding concern around on-target, off-tissue toxicity has receded. Indeed, it would appear that this has resonated with the field for NKG2D as a means to target cancer is now being actively explored by various companies and academics in the T cell and NK cell area that is at least in part supported by the safety profile we have observed in our clinical trials to date.

However, the field is also moving on. Autologous CAR T remains the mainstay of the current engineered cell therapy field but the appreciation of the difficulty in commercializing the approach is now top of mind, which has promoted the concept of the 'off the shelf' approach. This concept is now becoming a reality with entrants showing that at least in the early stages of clinical testing, allogeneic cell therapy is feasible [12].

As mentioned above, one of the attractions of moving to Celyad Oncology was its allogeneic CAR T concept where the first product has now entered clinical testing. This concept is a little different to many other allogeneic CAR T players in the field since the company focuses on a 'non-gene edited' approach. The predominant potential toxicity associated with allogeneic T cell therapy is graft versus host disease that is mediated by the T cell receptor (TCR) present on the donor T cells recognizing the recipient patient as 'foreign' and thereby mounting an assault on the foreign tissue leading to potentially life threatening toxicity. Therefore, abrogating the activity of the TCR is the common approach to generate allogeneic CAR T cells. Most are using gene editing approaches that eliminate expression of the TCR alpha chain. Celyad Oncology is taking a different approach using non-gene edited approaches based on either a peptide based competitive inhibition approach to interfere with TCR signalling [13] or expressing shRNA to knockdown expression of key TCR components [14] that involve expressing all of the elements needed to generate the CAR T from a single vector, avoiding the multiple manipulations required to generate gene edited allogeneic CAR T. The first candidate employing the peptide-based method in the CYAD-100 series, CYAD-101, has shown very encouraging clinical activity in patients with advanced metastatic colorectal cancer with no signs of graft versus host disease [13].

Where does this background from an individual researcher bring us? Personally, I believe we are at a pivotal time for the field. Autologous CAR T cell therapy has set the commercial stage and there is now a breadth of activity to deliver this paradigm further, either through technological developments, clinical trial design (combining in some form with a checkpoint inhibitor being one prime example) or by adding more 'bells and whistles' to the basic concept, including cytokines and other immune modifiers. There is also a significant degree of revisiting past concepts to test these within the current environment. For instance, the first generation CAR concept is being revisited [15] using lessons learnt in the past about the optimal signalling of a CAR involving incorporation into the TCR complex [16].

Nevertheless, the delivery of autologous therapies beyond the relatively small disease indications, such as hematological malignances, becomes a much greater challenge. These challenges stem from requiring manufacturing capacity to deliver a just-in-time product for thousands of patients coupled with the logistical challenges of track and tracing individualized therapies from manufacturing center to clinical center, a scale that is daunting in size. With manufacturing options, such as decentralized manufacturing, these issues do not disappear. Rather they are likely expanded in number given the need to have multiple regulated Good Manufacturing Practice (GMP) compliant production centers handling a slightly smaller logistical load, which would test a finance model. The point of care paradigm has slipped away from mainstream thinking despite the potential advantage of a shorter travel distance for patient samples outweighed by the regulatory challenge of providing all that is needed for

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compliant cell production, including critical reagent transport and storage and personalized data collection management at multiple remote sites without compromising data security. Data security depends on systems that vary from clinical site to clinical site, enabling qualified personnel sign off and waste management which is a major issue for clinical centers not used to working with gene modified organisms (GMO) and requires bespoke waste management. There is also the question of familiarity; hematology units are very comfortable with the concept of cell therapies. However, the paradigm is somewhat alien to oncologists at the front line dealing with multiple solid cancer indications.

Against this understanding, allogeneic CAR T cell therapy is emerging as one solution to the challenge of providing a cell therapy for larger indications. As discussed above, Celyad Oncology along with colleagues in the field are rising to this challenge and testing the first iterations of allogeneic CAR T in the clinic. The benefits of an off-the-shelf approach to treat more patients will be an important early readout. Additionally, in manufacturing, generating cell banks and managing stocks will be ever-important features of cell therapy - both are now logistical challenges readily recognized by colleagues in the pharmaceutical industry. This would represent a shift in the understanding of cell therapies from being a bespoke, niche therapeutic area to entering pharmaceutical mainstream consciousness.

Referring to the title of this commentary, it seems clear that a second wave of CAR T therapies is a reality but the arrival of this wave with respect to commercial products still feels a little way off given the efforts working in both targets and the allogeneic platform. The field of cell therapy is now rapidly expanding among renewed vigor with different cell platforms being explored including γδ T cells, NKT cells, natural killer cells, cytokine induced killer (CIK) cells and monocyte / macrophages. With these 'new' cell therapy platforms, CARs are likely to play an important role. NK cells tend to poorly infiltrate solid tumors and have a relatively short life span. Moreover, NK activation is based on the balance of activatory receptor signals, a key one coming from NKG2D, overcoming the activity of inhibitory receptor activity which are specifically exploited by tumors through the high expression of HLA-E that binds the NK cell inhibitory receptor NKG2A [17]. Exploiting a CAR may overcome some of these deficiencies that could improve the poor activity NK cell therapy demonstrated in the solid tumor context [18]. As such, the second wave of CAR T cell therapy may ride on an αβ T cell backbone (likely an allogeneic version) and could arrive on the back of other cell types. My personal perspective is that the newer cell types still have major challenges to overcome to enter the early stages of clinical trials while the αβ T cell is the current workhorse of most ongoing CAR T cell therapies. Allowing for my obvious bias, I'll keep my money on those specific T cells as the platform aligned with a solid target and likely additional immune modulating elements as the head of CAR T cell therapy's second wave. I'll not say how much money in print in case of being held to it.

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY: NEXT-GEN PLATFORMS & MODALITIES

SPOTLIGHT

EXPERT INSIGHT

From a clinician's perspective: road to safer CAR-T cell immunotherapies

Chenggong Li & Heng Mei

Chimeric antigen receptor (CAR)-T cell therapy represents a paradigm shift in cancer treatment, especially in hematological malignancies. To date, three CAR-T cell products have received FDA-approval for clinical application, including axicabtagene ciloleucel for B-cell lymphoma, tisagenlecleucel for B-cell leukemia and lymphoma, and brexucabtagene autoleucel for mantle cell lymphoma. This groundbreaking success has stimulated exponentially increasing preclinical researches and clinical investigations of CAR-T cell therapy. Nevertheless, toxicities associated with CAR-T cell therapy are common and can be fatal, hampering its widespread use. With more optimized CAR-T cells being developed and tested, rational evaluation and scientific management of the relevant toxicities is urgently needed to ensure their safe use and clinical benefit for the patients. From a clinician's perspective, this review summarizes prominent CAR-T cell-related toxicities and the road to safer CAR-T cell immunotherapies in the following three aspects: patient selection, CAR-T intrinsic factors, and post-infusion monitoring.

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INTRODUCTION

Chimeric Antigen Receptor (CAR)-T cell therapy has emerged as a novel genetically engineered cellular immunotherapy that has transformed the cancer treatment landscape. CD19-directed CAR-T cell therapy

has yielded impressive remission rates and dramatically improved outcomes in patients with relapsed or refractory B-cell malignancies [1–5]. However, CAR-T cell therapies are associated with significant toxicities like cytokine release syndrome (CRS), neurotoxicity, and on-target off-tumor toxicity, which can be fatal if not identified early and treated appropriately. Infections are common under the conditions of neutropenia and hypogammaglobulinemia. Rare complications include tumor lysis syndrome (TLS), macrophage activation syndrome(MAS)/hemophagocytic lymphohistiocytosis (HLS) and genotoxicity. Hematotoxicity and multi-organ toxicity can occur independently or as part of syndromes. These adverse events impede the road to widespread application of CAR-T cell therapy in more patients with advanced tumors.

In order to remove these roadblocks, researchers have developed a wide range of engineering strategies to generate safer CAR-T cells, such as adding suicide genes, utilizing synthetic Notch receptor, or designing on-switch CAR, which have been comprehensively reviewed previously [6,7]. As more CAR-T cell products have been or are going to be approved for commercial application and more optimized CAR constructs are being tested, safe administration appears more critical to alleviate toxicities and ensure patients' therapeutic benefits.

Patients must meet the strict inclusion criteria before enrollment; CAR-T cell products must meet the rigorous releasing criteria before infusion; post-infusion monitoring has been recommended before discharge [8,9]. Identifying the appropriate patients, correctly assessing the safety features of CAR-T products, and early recognition and management of relevant complications can create the opportunity to substantially improve patients' outcomes. In this review, we as clinical investigators sum up the prominent CAR-T cell-associated adverse events and discuss how to improve safety from the following three aspects: patient selection, CAR-T intrinsic factors and post-infusion monitoring.

CAR-T RELATED ADVERSE EVENTS

CRS

The most common and prominent CAR-T cell-associated nonhematologic toxicity is CRS, a systemic inflammatory response characterized with a spectrum of clinical manifestations and transient elevations of various cytokines [10,11]. Currently, there are several grading systems available for clinical trials to evaluate the severity of CRS, including CTCAE scale [12], Lee scale [13], Penn grading scale [14], CARTOX criteria [15] and ASTCT consensus [9]. The safety and grading criteria of three FDA-approved CAR-T cell products are summarized in Table 1. A better understanding of the underlying mechanisms is beneficial to guide the administration. A new mode divides the progression of CRS in B-cell lymphoma into four stages: CAR-T cell local expansion, CAR-T overflow and inflammatory cytokine surge, CAR-T redistribution and organ damage, and recovery stage. The mode also indicates that local CRS is the "trigger" of subsequent systemic CRS, and timely management of local CRS can effectively prevent and control serious systemic CRS. In this mode, tumor burden and bone marrow suppression are considered determinants of CRS and it will help us to more effectively predict, identify, and manage it [16].

Neurotoxicity

Neurotoxicity is the second major side effect, which was previously referred to as CAR-T cell-related encephalopathy syndrome [8], and is now named immune effector cell-associated neurotoxicity syndrome (ICANS) [9]. It manifests as a spectrum of neurological signs, ranging from low grade with headache, aphasia, and tremor to high levels with life-threatening seizure and fatal cerebral edema. For example, in the pivotal ROCKET study of JCAR015 for adult patients with B-cell acute lymphoblastic leukemia

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TABLE 1

Safety and efficacy of three FDA-approved CAR-T cell products.

CAR-T products		Axicabtagene ciloleucel (KTE-C19)	Tisagenlecleucel (CLT019)		Brexucabtagene autoleucel (KET-X19)
CAR structure	Target	CD19	CD19		CD19
	Hinge+TM	CD8	CD28		CD8
	Costimulatory	4-1BB	CD28		4-1BB
Cell production	Vectors	Retrovirus	Lentivirus		Retrovirus
	Source	PBMCs	PBMCs		PBMCs deleting $CD19^{+}$ cells
Phase 2 clinical trial		ZUMA-1	NCT02435849	JULIET	ZUMA-2
Conditioning chemotherapy		Cyc 500 mg/m^2/d + Flu 30 mg/m^2/d for 3 days	95%; Cyc 250 mg/m^2/d + Flu 25 mg/m^2/d for 3 days or Ben 90mg/d for 2 days	Cyc 250 mg/m^2/d + Flu 25 mg/m^2/d for 3 days or Ben 90mg/d for 2 days	Cyc 300 mg/m^2/d + Flu 30 mg/m^2/d for 3 days
Dose		2x10^6 CAR-T cells/kg	Median 3.1x10^6 CAR-T cells/kg; range (0.2–5.4) x10^6 CAR-T cells/kg	Median 3.0x10^6 CAR-T cells/kg; range (0.1–6.0) x10^8 CAR-T cells/kg	2x10^6 CAR-T cells/kg
Indication		RR LBCL	Children/young adults RR B-cell ALL	Adult RR DLBCL	RR MCL
Bridging therapy		Not allowed	Allowed	Allowed	Allowed
Efficacy		101pts	75pts	93pts	60pts
ORR/CR		82%/54%	81%/60%	52%/40%	93%/67%
Follow-up		12-month PFS 44%; 12-month OS 59%	12-month EFS 50%; 12-month OS 76%	12-month PFS 83%; 12-month OS 49%	12-month PFS 61%; 12-month OS 83%
Safety		101pts	75pts	111pts	68pts
CRS		Lee scale	Penn grading scale	Penn grading scale	Lee scale
Overall		93%	77%	58%	91%
Severe		13%	46%	22%	15%
ICANS		CTCAE v4.03	CTCAE v4.03	CTCAE v4.03	CTCAE v4.03
Overall		64%	40%	22%	63%
Severe		28%	13%	12%	31%
Hematological toxicity	Neutropenia	84%; 78%	/: 9%	34%; /	87%; 86%
	Thrombocyopenia	58%; 38%	/: 9%	33%; /	74%; 51%
	Anemia	66%; 43%	/: 4%	48%; /	68%; 50%
Infection		/	43%; 24%	Severe 20%	Severe 32%
Reference		[1]	[3]	[4]	[5]

Ben: Bendamustine; Cyc: Cyclophosphamide; Flu: Fludarabine; LBCL: Large B cell lymphoma; MCL: Mantle cell lymphoma.

(B-ALL), cerebral edema caused five deaths, which reinforced the challenge to manage the ICANS [17]. The exact pathophysiology of CAR-T cell-mediated neurotoxicity is increasingly recognized but poorly understood. Single-cell analysis shows that CD19

is expressed in human brain mural cells that are critical for blood-brain-barrier integrity, suggesting CD19$^+$ mural cells may contribute to neurotoxicity of CAR-T therapy [18]. Severe neurotoxicity was linked with endothelial activation, including disseminated intravascular coagulation, capillary leak, and increased blood-brain barrier permeability [19]. Substantial CAR-T cells and cytokines trafficked into the central nervous system (CNS), as potential mechanisms to account for its development [20]. Clinical trials demonstrated that patients with neurologic toxicity have significantly increasing CAR-T cells and elevated cytokines in the cerebrospinal fluid (CSF) [21,22]. Steroids are generally used to control ICANS due to its excellent CNS penetration. However, their use may hamper CAR-T activity, suggesting the need to develop novel agents to prevent ICANS. In a mouse model, the interleukin (IL)-1 receptor antagonist anakinra exhibited the potency to abolish both CRS and neurotoxicity [23]. Patients with large B-cell lymphoma who received anakinra for the management of high-grade ICANS after infusion of axicabtagene ciloleucel attained clinical benefit [24]. Anakinra also could limit the extent and duration of CRS as a valuable adjunct to IL-6 antibody, tocilizumab, following anti-BCMA CAR-T cell therapy in myeloma [25].

On-target off-tumor toxicity

Because the target antigens recognized by CAR-T cells aren't strictly restricted to malignant cells, on-target off-tumor toxicity is an unavoidable adverse effect. Although CAR-T cell therapy has achieved remarkable efficacy in B-cell malignancies, the target antigens (CD19, CD20, CD22) are also expressed on normal B cells, and hence B-cell aplasia is a major on-target off-tumor toxicity in the relevant trials [26]. The B-cell aplasia persists from 1 month to 4 years among these studies. BCMA is also expressed on mature B cells and normal plasma cells, and hypogammaglobinemia is an accompanying on-target off-tumor toxicity in reported BCMA-directed CAR-T trials [27–30]. These adverse events make patients susceptible to infections, and empiric antimicrobials and immunoglobulin supplementation are recommended for these patients. Fatal on-target off-tumor toxicity was reported in a patient with colon cancer metastatic to the lungs and liver, who rapidly experienced respiratory distress after anti-ERBB2 CAR-T cells infusion and died on day 5, for the reason that CAR-T cells recognized little ERBB2 on lung epithelial cells [31]. Therefore, reasonable target selection and appropriate patient selection are imperative issues for further CAR-T cell therapy development.

Infections

Patients treated with CAR-T cell therapy are at high risk of infections, including basic malignancies, prior antitumor treatment, lymphodepletion-induced cytopenias, B-cell aplasia and hypogammaglobinemia, and tocilizumab or steroids administration.

Infections have features that overlap with CRS and even ICANS, and a causative microorganism is only identified in 20–30% of cases [32]. Therefore, it is crucial to monitor patients' symptoms and infection-related indicators, and to utilize appropriate imaging for differential diagnosis. Infections have been reported in 23–42% of CAR-T cell recipients and occurred most commonly in the first month after infusion, declining sharply thereafter [33–35]. Bacterial infections predominate within the first month, and fungal and herpesviridae infections are uncommon. The updated Clinical Practice Guideline provides infection prophylaxis, diagnosis, and treatment for management of febrile patients during the neutropenic period [32]. Antibiotic prophylaxis with a fluoroquinolone and antifungal prophylaxis with an oral triazole or parenteral echinocandin are recommended for patients at high risk for febrile neutropenia [36].

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TLS

TLS is a relatively rare toxicity, caused by tumor cell death and the rapid release of intracellular substances, resulting a series of metabolic disorders such as hyperuricemia, hyperkalemia, hyperphosphatemia, hypocalcemia and metabolic acidosis. The metabolic disturbance further leads to severe arrhythmia or acute renal failure and even death [37]. TLS was observed in three patients in the exploratory trial of a biepitopic CAR-T targeting BCMA in multiple myeloma [29]. All the patients presented with laboratory metabolic abnormalities. TLS could overlap with the symptoms of CRS and one patient had significantly elevated serum IL-6 and TNF-α, and anti-IL-6 antibody tocilizumab and anti-TNF-α drug etanercept were successively and successfully applied. TLS is related to high tumor burden, and close monitoring of metabolic profiles and management with cytokine blockers are recommended.

MAS/HLS

HLH/MAS is a rare but potentially life-threating complication that can occur concurrently with CRS [38]. MAS, characterized by severe immune activation, lymphohistiocytic tissue infiltration, and multi-organ injuries, tends to occur more commonly in children [39]. Patients with MAS have been reported in CD19-directed CAR-T therapy for ALL, and they had elevated cytokine profiles similar to those observed in severe CRS [40,41], indicating the necessity to closely monitor serum cytokines and block immune cascade reactions.

Hematotoxicity

Hematotoxicity is the most frequently reported CAR-T cell-associated adverse effect, and is to a great extent attributed to lymphodepleting chemotherapy [1,3,4,42]. Meta-analysis demonstrated that the most frequently reported grade ≥3 adverse effects were anemia (34%), thrombocytopenia (30%), and febrile neutropenia (19%) in B-cell lymphoma [43]. In multiple myeloma, the most common grade ≥3 toxic effects were neutropenia (85%), thrombocytopenia (70%), and leukopenia (60%) [44]. Most severe hematologic toxicities recover within one month after infusion [3,4,42]. Consequently, patients with cytopenia are needed for supportive treatment, including platelet and erythrocyte transfusion, to avoid uncontrolled bleeding and hypoxia. Leucocyte-stimulating drugs aren't recommended due to the risk to induce cell over-activation and cytokine storms.

Major organ-toxicity

Major organ-toxicity, like liver dysfunction, renal injury and cardiac toxicity, can occur independently or as part of systemic syndrome after CAR-T cell infusion. Elevation of aspartate aminotransferase and alanine aminotransferase, and renal insufficiency are relatively common in patients with BCMA-directed CAR-T cell therapy [28,29]. Based on our experience [45,46], organ-toxicity is associated with baseline organ functions, thus baseline patient evaluation is critical for toxicity management.

Genotoxicity

The majority of CAR-T cells used in clinical trials to date are transduced using lentivirus or retrovirus. Viral vectors possess an ideal transduction efficiency and stable transgene expression, but to some extent, they also correlate with a potential risk of insertional mutagenesis and malignant transformation [47,48]. Although genotoxicity hasn't been reported in patients with CAR-T cell therapy, insertional leukemogenesis, caused by lentivirus integration into a single leukemic B cell, was reported in a patient relapsing 9 months after tisagenlecleucel infusion, raising the importance to explore safer alternative methods of gene delivery [49].

HOW TO ALLEVIATE TOXICITY AS A CLINICIAN?

Patient selection

Identifying patients at high risks of developing severe CRS or neurotoxicity is helpful for patients to weigh the pros and cons before receiving this therapy, and is also beneficial for clinicians to recognize early and prevent or treat subsequent toxicity. Meta-analysis indicates that CRS was significantly prevalent in patients (n=896) with hematologic malignancies compared to solid malignancies (67% vs 35%), and the prevalence of neurotoxicity was slightly higher (9% vs 6%) [50]. Tisagenlecleucel has been approved for B-ALL and lymphoma (Table 1). In the global Phase 2 study in patients with ALL (n=75), CRS occurred in 77% of patients (severe 46%), and ICANS occurred in 40% of patients (severe 13%) [3]. While in the pivotal JULIET study for B-cell lymphoma (n=111), 64% of patients experienced CRS with 22% of grade 3–4; 23% suffered neurotoxicity with 12% of grade 3–4 [4]. CAR-T cell-associated CRS and neurotoxicity seem more frequent in patients with ALL compared with lymphoma. Therefore, more attention is needed for patients with hematologic cancer, especially ALL.

High tumor burden has been a widely-recognized predictor of high-grade CRS [21,27,51–54] and severe neurotoxicity [19,53,54]. Bulky tumor is also related to TLS [55]. Disease burden correlates with the peak and duration of CAR-T cell expansion as well as elevation of serum cytokines, thus possibly explaining the underlying relationship [19,21,52]. Baseline thrombocytopenia were associated with severe CRS [51] and neurotoxicity [56]. ALL, high tumor burden, CRS, and preexisting neurologic comorbidities were high-risk factors of ICANS in the analyses of 133 adults treated with CD19-directed CAR-T cells [19]. Patients with evidence of endothelial activation (serum angiopoietin-2 and von Willebrand factor) before lymphodepletion are also at higher risk of neurotoxicity [19]. Patients' baseline organ function, including cardiac, pulmonary, hepatic and renal function, has an important impact on whether patients will experience organ toxicity [57,58]. From our experience [45,46,59,60], age is also an influencing factor. Children are susceptible to severe neurotoxicity due to immature blood-brain barrier, whereas the old are susceptible to severe CRS and organ toxicities. Evaluation of patients' baseline characteristics is of importance for toxicity management.

CAR-T intrinsic factors

The classical CAR construct contains an antigen-specific single-chain variable fragment(scFv), a hinge and a transmembrane domain, one or two costimulatory domains, and an intracellular CD3ζ domain. The extracellular scFv determines the antigen-binding specificity and affinity, showing influence on CAR-T cell-related toxicities. Different antigen-specific scFv is accompanied by different on-target off-tumor toxicities, such as B-cell aplasia in CD19-targeted CAR-T cell therapy [3,14,21,53], hypogammaglobulinemia in BCMA-specific CAR-T cell therapy [29,30], and hematopoietic suppression in CD38-directed CAR-T cell therapy [61,62]. scFv avidity is a critical determinant in the balance between safety and efficacy of CAR-T cell therapy. High-avidity CAR possesses faster and better response to antigen recognition but also trigger T cell exhaustion and excessive cytokine production; whereas low-avidity CAR has better selectivity but a weak anti-tumor activity [63]. T cells expressing a low-affinity CD19 CAR show enhanced CAR-T cell expansion and prolonged persistence without severe CRS in pediatric patients with ALL [64]. Hinge and transmembrane domain sequences play an important role in CAR-T cell activation and function [65,66]. CD19-BBz(86) CAR-T cells, with longer CD8α extracellular hinge and intracellular domains in the prototype of CTL019, are effective in treatment of refractory B-cell lymphoma and do not cause neurological toxicity or

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severe CRS, representing a safe and potent anti-CD19 CAR T cell therapy [67]. Hu19-CD828z T cells, containing anti-CD19 scFv derived from a fully human antibody and CD8α hinge and transmembrane domains plus a CD28 costimulatory domain, show lower levels of cytokines and neurologic toxicity compared with KTE-C19 [68]. CD28 and 4-1BB are the most widely used costimulatory molecules in CAR design. CD28 co-stimulates CARs activate faster with larger-magnitude changes in protein phosphorylation, correlated with an effector T cell-like phenotype and function. In contrast, 4-1BB based CARs preferentially express T cell memory-associated genes and exhibit sustained anti-tumor activity with a more favorable safety profile [69]. In a Phase 1/2 trial for lymphoma, 4-1BB-CAR19-T cells were well tolerated with only 1/2 grade adverse events, whereas severe CRS and neurotoxicity occurred in the CD28-CAR19-T group [70]. Based on the second- and third-generation CAR constructs, a variety of safer strategies to generate next-generation CAR have been investigated to reduce these adverse effects [6].

CAR-T cells, as self-replicating drugs, own individual and personalized compositions, phenotype, and *in vivo* proliferation and persistence. High infused dosages of CAR-T cells have been proven to hold a strong association with severe CRS [42,51,53] and ICANS [19]. High infused dosages contribute to high peak expansion of CAR-T cells, resulting in significant activation of T cells and excessive production of various cytokines. High peak expansion is a reliable indicator of great risk and severity of CRS [22,42,54,71] and ICANS [22,54], and *in vivo* expansion only correlates with the frequency of a $CD8^+CD45RA^+CCR7^+$ subset within the infused product [72]. Patients with grade ≥3 CRS or ICANS have significantly more poly-functional T cells capable of deploying multiple cytokines and chemokines in the pre-infusion products [73]. CAR construct, infused cell dose, and compositions are critical considerations to evaluate the safety of individual CAR-T cell products. Ibrutinib could improve CAR-T cell antitumor efficacy and reduced CRS in preclinical studies, and CD19-targeted CAR-T cells with concurrent ibrutinib lead to high rates of MRD-negativity and low CRS severity in patients with CLL. Combination therapy could be a beneficial area of exploration for improved safety of CAR-T cell therapy [74].

Post-infusion monitoring

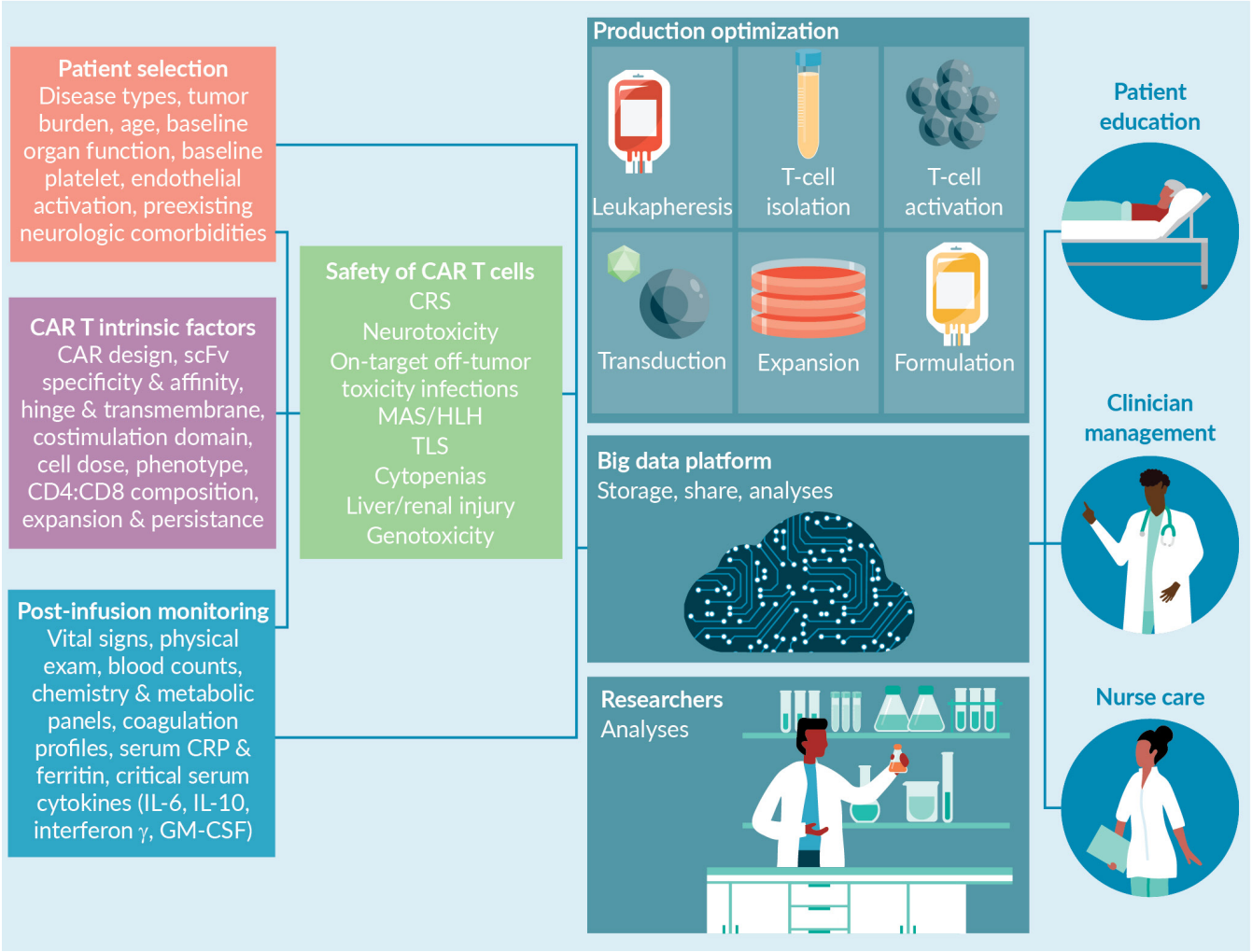
The most recent guidelines, CARTOX recommendations [8] and ASTCT consensus [9], have integrated multidisciplinary suggestions and provide a comprehensive and practical guide for monitoring and grading of CAR-T-cell-therapy-associated toxicities. CRS usually occurs within 14 days following CAR T-cell infusion, and peaks and starts to resolve within 7 days [57]. ICANS typically occurs simultaneously or after CRS [75]. Patient hospitalization with close monitoring is recommended for 14–21 days after CAR-T cell infusion. For patients with bulky diseases, monitoring time should be appropriately extended in case of TLS. Monitoring indicators include vital signs, physical exam, blood counts, chemistry and metabolic panels, coagulation profiles, serum CRP and ferritin levels, and critical serum cytokines.

Fever ≥38°C is the first objective sign of CRS and thus, body temperature is a critical reference indicator for current CRS grading systems [8,9,12–14]. Patients with severe CRS exhibit hemodynamic instability and capillary leak including hypotension, tachycardia, and tachypnea. If the situation deteriorates further, patients will suffer pulmonary edema and respiratory distress. Vital signs, including body temperature, blood pressure, blood oxygen saturation, and heart rate are recommended for continuous monitoring for high-risk patients and close monitoring every 4 hours for standard-risk patients. Because ICANS manifests with a series of neurological symptoms, Immune Effector Cell Encephalopathy screening tool [8] and CARTOX-10 neurological assessment tool [9], containing orientation,

writing, attention and/or following commands, have been developed for assessment of potential neurotoxicity. Cytopenias at different degrees occur in almost all the patients after CAR-T cell therapy and complete blood counts are required daily in case of life-threating hemorrhage, infection, or asphyxia. The chemistry and metabolic panels are necessary for monitoring organ toxicity and differential diagnosis of TLS. The severity of coagulopathy is positively correlated with CRS grades with elevated D-Dimer, fibrin degradation products and activated partial thromboplastin time and low fibrinogen and platelet [51,76]. C-reactive protein (CRP) and ferritin have a positive association with the occurrence and progression of CRS [1,3,4,11,14,17,21,27–30,40,42,51–53,71], thus daily monitoring of serum CRP and ferritin is helpful to assess CRS in the absence of cytokine assistance. A spectrum of serum cytokines associated with CRS and neurotoxicity was summarized previously [77,78]. Preclinical studies demonstrate that serum IL-6 and IL-1 are critical determining factors causing CRS and ICANS [23,79]. Clinical investigation indicates that changes of IL-6, IL-10, interferon γ, and GM-CSF coincide with onset and resolution of CRS [40]. For patients with neurological symptoms, a head CT scan is recommended as the first option, followed by an MRI if it is available. If patients have symptoms of seizures, electroencephalogram is needed. Cerebrospinal fluid

FIGURE 1

Influential factors on safety of CAR-T cell therapy and application of cloud platform for further improvement.



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examination for CAR-T cells and relevant cytokines is recommended if the patient can coordinate with it. *In vivo* expansion of CAR-T cells has been regarded as an indicator of severe CRS and neurotoxicity, and daily monitoring of CAR copies in peripheral blood is recommended before discharge.

BIG DATA PLATFORM

As we enter the Era of Internet Plus, a rational, scientific, and efficient organization of medical information will be crucial in determining whether it can be applied to the health system. Digitalization, machine learning, and artificial intelligence have been successfully used in many fields of science, technology, and medicine. With more and more novel CAR-T cell products entering single-arm, single-center, Phase 1 trials, establishing a big data platform integrating patients' information is a promising and powerful tool for patient education, nurse care, physician management, decision support, pharmacist innovation, and constructing predictive models of safety, efficacy, and cost (Figure 1).

CONCLUSION & OUTLOOK

CAR-T cell therapy has undoubtedly revolutionized the treatment of malignant diseases, but safety issues substantially limit its wide application. In this review, we summarized the prominent adverse events and discussed how to effectively prevent, predict, and monitor these toxicities from a clinician's perspective. Establishing a big data sharing platform is critical for further advancement.

TRANSLATION INSIGHT

From a clinician's perspective, this review summarized prominent CAR-T cell-related toxicities and the road to safer CAR-T cell immunotherapies in the following three aspects: patient selection, CAR-T intrinsic factors, and post-infusion monitoring.

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY: NEXT-GEN PLATFORMS & MODALITIES

SPOTLIGHT

INTERVIEW

Driving lupus out with CARs

MARKO RADIC is an Associate Professor in the Department of Microbiology, Immunology and Biochemistry at the University of Tennessee Health Science Center in Memphis, TN. He studied Genetics at the University of California in Davis and Biological Sciences at the University of California in Irvine. He completed postdoctoral training with the research team of Professor Martin Weigert at the Fox Chase Cancer Center in Philadelphia. Dr. Radic established his own laboratory at the Medical College of Pennsylvania before moving to Memphis. His research interests are in molecular and cellular immunology of autoimmune diseases and the development and functioning of the innate and adaptive immune systems. The research from his lab has been funded by private and federal sources for nearly three decades and published in over 100 publications. His current interests are in the cellular immunotherapy of lupus in pre-clinical models and the regulation of innate immunity in COVID-19.

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Q What are your working on right now?

MR: As your readers may know, CAR T cells are generally used in oncogenesis and cancer immunotherapy. However, we have chosen to use this technology to address problems of autoimmune disease. We have adopted an approach that uses

CAR T cells that are directed by means of their chimeric antigen receptor to bind to a surface molecule on B cells, called CD19.

As shown my many others, anti-CD19 CAR T cells are quite effective in treating B cell leukemias. We treat an autoimmune disease called lupus in pre-clinical models. Lupus is a disease, in which B cells are the main culprits by initiating the disease, and then perpetuating it. In our studies to date, we have shown that anti-CD19 CAR T cells are quite effective in treating lupus in mouse animal models. [Figure 1]

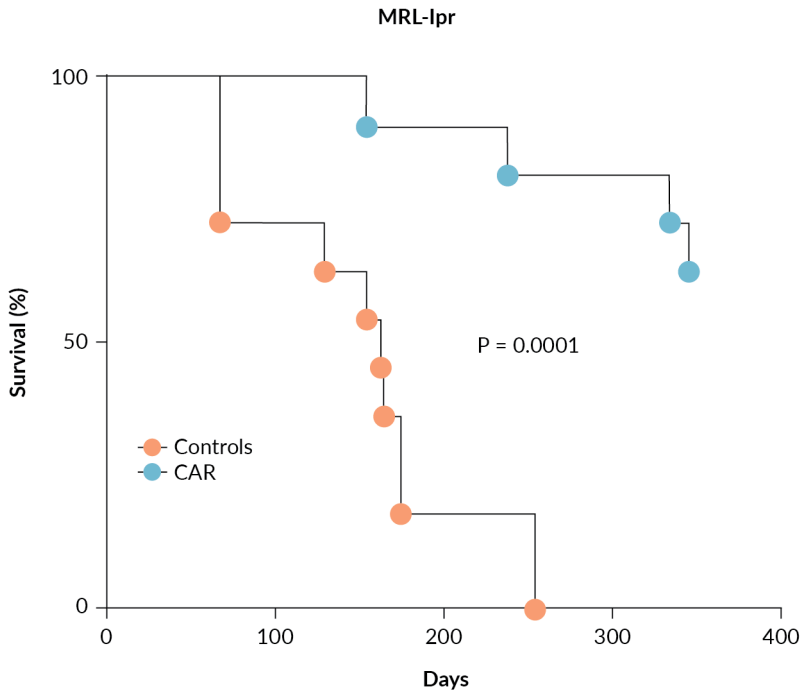
Our current efforts focus on two specific objectives: firstly, to demonstrate how different and effective this therapy is compared to standard treatments with non-specific anti-inflammatory or immunosuppressive medications, and secondly, to explore how this therapy can be adapted to the specific conditions that exist in patients with an autoimmune disease.

To broaden the perspective here, it is useful to remember that quite a large segment of autoimmune diseases (which by some estimates affect anywhere between 5-7% of the world's total population) are driven by B cells. More specifically, B cells may play different roles in an autoimmune disease: in addition to producing auto-antibodies, or antibodies against cell antigens, B cells are very important as antigen presenting cells - in other words, they help activate T cells and break immune tolerance. B cells are also an important source of cytokines, which can further amplify inflammation.

We consider our approach an important contribution towards the treatment of lupus, and as a model for a variety of other B cell dependent autoimmune diseases.

FIGURE 1

Extension of MRL/lpr mouse lifespans by anti-CD19 CAR T cell treatment.



Autoimmune mice were treated at 4 months of age with the CAR T cells described in this article and survival was plotted according to days elapsed since treatment. Further details can be found in the original article [1].

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Q What are the key challenges facing you and others seeking to develop and apply this technology in the autoimmune disease space?

MR: The patent landscape that currently exists around CAR T cells is one of the practical obstacles to the broader application of this technology, as many of your readers from the oncology space can appreciate. The initial patents covering CAR T cells were granted in a very broad way - even though essentially all of them were dealing with applications in cancer, the patents were sufficiently broad to also cover applications in other therapeutic areas, such as autoimmune diseases. Due to this, it becomes very difficult for people interested in actually developing and investing in this technology area to face the additional burden of not having the important intellectual property foundations on which to base and defend this immune application. However, we are confident we have avenues that will allow us to carve out unique variations on CAR T cell therapy, which will help us develop unique IP valuations.

A second obstacle we are currently dealing with is the need to find more unique ways to apply this technology - to tailor the CAR T cell therapy approach to the needs of an autoimmune patient, and to explain its features to his or her clinician. This problem may take a bit longer to solve but we are actively engaging clinicians and patient groups to define problems and shape solutions.

Regarding lupus specifically, it is a very tricky disease both to diagnose and to treat because it can manifest in many different ways. In fact, some people think that lupus is really not a single disease but may be a group of related disorders. Because of this, defining even the initial group of patients for which this therapy might be particularly applicable is a difficult proposition - certainly, a good number of lupus patients will be perfectly well served by available therapies. But you need to remember that lupus can be a devastating and potentially lethal disease, which can affect organ systems such as the central nervous system and severely impact quality of life. I think that at that point in the disease's progression, it becomes quite important to have a specific, effective treatment at hand, such as a CAR T cell therapy.

Q What does the data you've generated to date tell you in terms of promising future development pathways and also potential new areas of application for cellular immunotherapy?

MR: What's most exciting is that we continue to be surprised and impressed by the efficacy of CAR T cells - at least in the animal models of lupus that we are using. And the important characteristic here is that the approach is both highly specific as well as long-lasting, such that really all of the symptomology we can assess in animal models is greatly improved by this approach.

> "...we have shown that anti-CD19 CAR T cells are quite effective in treating lupus in mouse animal models."

"...the approach is both highly specific as well as long-lasting, such that really all of the symptomology we can assess in animal models is greatly improved by this approach. We are confident that future applications of this technology will prove useful in other diseases beyond lupus - for example, anti-phospholipid syndrome, or various types of vasculitis."

We are confident that future applications of this technology will prove useful in other diseases beyond lupus - for example, anti-phospholipid syndrome, or various types of vasculitis. Also, B cell depletion should prove valuable in multiple sclerosis and rheumatoid arthritis, amongst other applications.

Q Are there any considerations relating to dosing that you're exploring as this approach/application continues to advance towards the clinic?

MR: I think that's an important question. And in some ways, we are trying to get to it.

I would break this down into two aspects. One is that currently, administration of CAR T cells requires a patient preconditioning procedure, which is usually a high-dose chemotherapy treatment. This is of relevance because one of the most effective, or at least transiently effective, treatments for lupus is immunosuppression. So in fact, drugs like cycloheximide are used in severe cases of lupus already, which means they would not only be a reasonable agent for preconditioning before administering CAR T cells, they might also allow for lower doses of CAR T cells than might otherwise be needed.

Secondly, an active area of research in my lab is to try to define endogenous genes in CAR T cells that make them particularly resilient and long-lasting. Once the candidate genes are confirmed, we will be able to genetically engineer CAR T cells to endow them with better survival capability in the patient, and potentially achieve better efficacy at lower dose levels.

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

MR: I've touched on some of the goals we are currently pursuing. We are constructing and testing variations in the anti-CD19 CAR T cells such that we can develop more unique and novel intellectual property with patent protection of this approach in mind.

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We also have certain ideas we're working on, in which we can make the CAR T cells more specific towards autoantibody producing B cells - meaning to target with CAR T cells those B cells that are really at the root of the autoimmune process. Rather than targeting all B cells, which may lead partial immunodeficiency, we are learning about the unique features of autoreactive B cells that may give us a more specific handle to eliminate those B cells that directly mediate autoimmunity.

In addition, there's a PR role I'm trying to play, which is to broaden the understanding and appreciation of the unique benefits that CAR T cells bring to treatment in the field of autoimmune research. I'm actively trying to spread the message - to get more converts on board and generate more enthusiasm for this field of application because for me, there are quite unprecedented benefits that come from using CAR T cells in autoimmunity, and in lupus in particular.

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CELL & GENE THERAPY INSIGHTS

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EDITORIAL

Leading the way in bringing gene therapy to the CNS with conditioning: a renaissance for busulfan?

EDITORIAL

Chris Mason

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Effective treatment of the central nervous system (CNS) through gene therapy is a promising approach for the many genetic diseases with neurological manifestations. It's a particular hurdle for diseases with both CNS and systemic involvement, where an effective therapy would need to work on both sides of the blood–brain barrier. Prominent among this group are lysosomal disorders (LDs), a family of monogenetic degenerative diseases which we focus on at AVROBIO, some of which have severe neurodegenerative aspects [1].

Effectively arresting or perhaps even reversing this degeneration could spare parents the pain of watching their child fail to progress

or even regress through milestones – as seen in young boys with Hunter syndrome [2], for example. It could prevent many adults from developing genetically linked dementias such as GBA-Parkinson's disease – seen disproportionately in people with Gaucher disease [3]. The medical need is inarguable and is driving intense research. My colleague Geoff MacKay recently reviewed the breadth of efforts to treat CNS symptoms he saw at 2020 WORLD*Symposium*™, the leading LD conference [4].

At AVROBIO, we are pursuing lentiviral gene therapy, an approach whereby hematopoietic stem cells (HSCs) are genetically modified. Lentiviral gene therapy is unique in its potential to impact symptoms from head to toe via all the nucleated components of blood, as well as the microglia in the CNS. Microglia are multi-functional and widespread throughout neuronal tissue and offer a compelling avenue to investigate the potential for their own functional correction and/or therapeutic enzyme delivery after lentiviral transduction of HSCs. Others in the industry also use this modality; across the field, transformative efficacy from a one-time treatment with lentiviral gene therapy has been demonstrated across a growing list of indications and clinical trials [5].

Our investigational gene therapies are designed to combine optimized lentiviral vectors and personalized busulfan conditioning deploying state-of-the-art precision dosing. Busulfan is an established conditioning agent in the lentiviral gene therapy field [6] – and it is key to the "head-to-toe" benefits we hope to demonstrate in our therapies.

Indeed, after more than 70 years of clinical use, principally in blood cancers, human data now show that busulfan is finding a new career – as a shepherd to the CNS for the descendants of transplanted HSCs, in particular the monocytes that become microglia cells (see Box 1) [6]. We are leveraging this 'shepherding' facility of busulfan in our own clinical programs and hope to expand in the future through continued innovation around this remarkable drug, as I recap below (Box 1).

FROM TEAM PLAYER TO SOLO OPERATOR: A NEW ROLE FOR BUSULFAN SHOWCASES SPECIAL TALENTS

Busulfan is a clinically validated conditioning agent that has been administered as a single agent in a single cycle to hundreds of patients treated with investigational lentiviral gene therapies [7]. A therapeutic alkylating agent, busulfan has conventionally been used as part of a cocktail of drugs for treating blood cancers.

But emerging data support busulfan as a potentially powerful tool for brain conditioning. This novel feature is due to the low level of busulfan binding to proteins in the blood; as a small molecule unencumbered by larger proteins, it can readily cross the blood–brain barrier [8].

We believe that long-term engraftment of HSC-derived "daughter" cells following gene therapy may lead to the reconstitution of a microglial network, where the new gene-modified microglia have the potential to be functionally corrected and/or drive cross-correction of neuronal tissue via protein secretion and uptake. The key questions leading up to such a potentially outcome: Does busulfan enable genetically modified microglia to engraft in the CNS? And once there, do the microglia produce a functional enzyme? There are now early human data providing

BOX 1

Defining microglia: A primary immune cell within the CNS, and a type of macrophage. Neurologists tend to only use the term 'microglia' when the cell in question is derived from primitive macrophages in the yolk sac, rather than from an HSC in the bone marrow. For most intents and purposes, however, referring to them as resident macrophages, microglia-like cells or microglia is interchangeable in relation to their overall phenotype and function.

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insight into these questions, drawing from clinical trials in the devastating disease metachromatic leukodystrophy (MLD), which has led the way in this area in many respects [6,5].

AN MLD CLINICAL STORY CONFIRMS A ROLE FOR BUSULFAN IN ADDRESSING CNS DISEASE

A recent study of two patients who had received allogeneic hematopoietic stem cell (HSC) transplants for MLD with the use of busulfan showed donor-derived macrophages distributed throughout the entire white matter [7]. Although there was no evidence of enzyme cross-correction (which would likely require the amplification of expression to supraphysiological levels), the transplanted patients had local tissue changes that suggested the donor-derived cells were making a therapeutic impact. This is encouraging histopathological evidence underpinning HSC transplantation for addressing CNS pathophysiology, and raises the exciting question of what further benefits will be achieved with the presence of gene-modified macrophages.

Happily, over the last ten years, there has been supporting evidence on that front, too. For example, in a clinical study of lentiviral gene therapy-treated patients with MLD employing busulfan conditioning, researchers observed durable, raised levels of active enzyme in the cerebrospinal fluid of treated patients, as reported by Alessandra Biffi et al [5]. The levels and activity of the enzyme reported in the study were comparable to healthy individuals and were completely absent prior to treatment. A reduction in the toxic substrate in peripheral nerve samples was also seen. Overall, pre-symptomatic treatment was associated with protection against disease and resultant comparability on motor skills to normally developing children: the best possible result. This program is now being advanced by Orchard Therapeutics.

In summary, these case studies tell a compelling story of busulfan-supported lentiviral gene therapy making a difference in the CNS of patients. And these human studies are of course additive to many more years of supportive preclinical work. In our own Pompe disease preclinical program, we recently demonstrated in a mouse model the ability of transduced hematopoietic stem cell transplants, supported by busulfan conditioning, to reduce levels of glycogen (the pathological substrate that accumulates) to wild-type mouse levels in the brain [9].

How does this migration across the blood–brain barrier occur? We don't yet know. Perhaps busulfan temporarily disrupts the barrier [10]. Perhaps monocytes cross on their own to some extent. What we do know is that in other than in very young children, adeno-associated viral (AAV) vector gene therapies, administered intravenously, do not efficiently cross the blood–brain barrier. And that large macromolecules, such as enzyme replacement therapy (ERT), also do not. ERT is the standard of care for many lysosomal disorders, but the blood–brain barrier prevents its migration through the blood–brain barrier, so the CNS symptoms develop unchallenged.

NEW OPPORTUNITIES ABOUND

It is clear conditioning is a powerful tool for supporting lentiviral gene therapy, potentially opening the door to addressing a wide range of diseases with CNS involvement. Over the years, busulfan has benefited from an ever-evolving pool of clinician experience, and numerous improvements, including: patient education; an intravenous option; new capsule dosing to reduce pill burden; protocols for at-home and out-patient dosing (which has been reported to be associated with equivalent or superior outcomes compared to inpatient care); and more effective management, including advanced antiemetics.

It's important to note that even with this personalized medicine approach, in our

clinical trials to date we have observed anticipated side effects from busulfan, including nausea, mucositis, fever, rash and hair thinning/loss, which typically came on quickly with a peak of three to five days and resolved quickly. Busulfan, indicated to be dosed in combination with cyclophosphamide to treat chronic myeloid leukemia, may cause temporary or permanent infertility when injected prior to allogeneic (or donor) HSC transplantation. In our lentiviral gene therapy trials, we use personalized busulfan as a single agent for a single cycle followed by infusion of the patient's own genetically modified HSCs cells, and the potential risk of infertility due to busulfan in this setting is still being studied.

And there's even more we can do to advance the potential of this conditioning agent. Specifically, precision around cumulative tissue exposure is crucial, with a target range of 78–101 mg.hr/L to maximize the potential for long-term engraftment while avoiding toxicities associated with out-of-range exposure [11]. Our target is a cumulative AUC of 90 mg.hr/L, a regimen we refer to in shorthand as "Bu90." Achieving this target range is a challenge because busulfan is metabolized differently from patient to patient, and even from day to day within the same patient. AVROBIO is leading the way by not just aiming to be in range but targeting the actual midpoint in order to further enhance safety and efficacy. We deploy a personalized medicine approach involving daily blood monitoring to assess the rate of busulfan metabolism and precisely adjust the next day's dose accordingly in order to hit our cumulative target of 90 mg.hr/L. The challenge with busulfan is the high variable relationship between the dose and resulting blood concentration. The degree of intra- and inter-patient pharmacokinetic variability can be as much as 10x.

This type of precision targeting of the cumulative exposure resulting from a single agent, which takes place over four days, differs from the 'traditional' use of alkylating agents as part of potent drug cocktails aimed at eliminating cancer cells, with the risks associated with polypharmacy. A personalized medicine approach aims to achieve a more consistent targeted exposure through a combination of readily available point-of-care busulfan plasma concentration measurement and targeting the mid-point of the range. This method significantly refines traditional therapeutic drug monitoring (TDM), which typically targets a range, hence there is the potential if at the outer edges of the range to increase the risk of out-of-range toxicity (upper end) or reduced engraftment (lower end).

We recently undertook a collaboration with the aim of enabling the widespread deployment of precision targeting for cumulative busulfan exposure through a collaboration with Saladax Biomedical [12]. This collaboration is intended to deliver a novel nanoparticle-immunoassay kit compatible with automated analytical devices commonly used at hospitals and clinics. It is designed to enable much faster, on-site analysis of busulfan metabolism – minutes as opposed to hours. We hope this will greatly expand access to personalized busulfan conditioning deploying state-of-the-art precision dosing, for use not just in the field of lentiviral gene therapy, but across a wide range of HSC transplants for conditions ranging from hematological cancers to autoimmune disease.

It has been incredibly exciting to see the emergence of a technology that can work both sides of the blood–brain barrier and potentially halt, or potentially in some instances reverse, CNS and systemic changes across a range of devastating diseases. Busulfan's role in that process, after decades of unrelated use in hematological oncology, is a reminder that established drugs can often have new uses, and that biology is a strange and wonderful thing. Ultimately, there's increased potential for busulfan to serve as a shepherd of gene-modified cells to impact CNS diseases, and AVROBIO is excited to have the opportunity to be a leader in this next frontier for lentiviral gene therapy.

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CELL & GENE THERAPY INSIGHTS

EXPERT INSIGHT

Process development and scale-up of pluripotent stem cell manufacturing

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Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), together human pluripotent stem cells (PSCs), have tremendous potential for production of cellular therapies for regenerative medicine. The final therapeutic dose of differentiated PSCs varies according to application; however, most indications have production requirements that cannot be met by traditional static tissue culture methods. Stirred-tank reactor expansion of PSCs represents a scalable solution to meet this developing demand. Here we present the process development of a 10 L single-use stirred tank bioreactor platform with the ability to generate > 10^{10} PSCs per production run and its applicability across both hESC and hiPSC cell lines. Manufacturing advancements are also presented. High-density seed bank inoculation of the process is demonstrated to decouple the adherent tissue culture requirement and normalize the cellular process input, as well as shorten the time requirement of the suspension seed train. Process correlations of pH and dissolved oxygen to viable cell densities are presented to remove or minimize the sampling requirements during expansion. Automated downstream processing, with volume reduction and washing, is demonstrated with 80–90% cell recovery and >94% viability. Together, these works represent seminal steps in the development of a controlled, automated and defined manufacturing process for PSCs.

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INTRODUCTION

Human pluripotent stem cells (PSCs) have the capacity to become the source of many cell therapies in the regenerative medicine space due to their unique characteristics of unlimited self-renewal and differentiation potential to repair and regenerate diseased or damaged cells, organs and tissues [22,25,26]. The possible applications for these cells are extensive, and their therapeutic potential is being investigated for use in a wide array of clinical areas, including those associated with high morbidity and mortality rates on a global scale, such as heart failure [8,13], Parkinson's disease [7] and type I diabetes [6,10].

Currently, PSC-based clinical trials have been initiated for therapies targeting age-related macular degeneration of the retina, spinal cord injury, type I diabetes and myocardial infarction, among others [27,28]. Yet, despite a large and ever-growing body of preclinical research supporting the regenerative potential of PSCs, most clinical trials remain small-scale and run through academic groups [14]. While clinical trials using human embryonic stem cells (hESC) to treat ocular degenerative diseases have proceeded with cell dosage numbers in the 1 x 10^5 cell range, it is estimated that most proposed PSC-based cell therapies, including those targeting some of the leading causes of morbidity and mortality—heart disease, type I diabetes and Parkinson's disease for example—could require as many as 2 x 10^9 PSCs per patient dose [9,29]. Given the large cell numbers required, traditional strategies of PSC propagation, which rely on 2D adherent culture, are neither practical nor cost efficient enough to meet the cell numbers required for PSC-based therapies.

To enable translation of these therapies into the clinic and to attract the interest of business partners willing to fund the emerging cell therapy industry, the field must meet the challenge of large-scale production, manufacturing and commercialization of these cells at clinically relevant titers [14]. As such, one of the limitations facing the implementation of these powerful cells as a tool to fight disease is the development and standardization of a robust, scalable and cost-effective production solution able to meet good manufacturing practices (GMP) and regulatory approval guidelines [27,28].

Historically, cell scale-up and manufacturing efforts have employed stirred tank reactors (STRs) to maximize cell expansion by ensuring optimal mixing of gasses, nutrients and growth factors, while allowing for tight control and monitoring of key culture parameters and minimizing costs associated with production [3,29]. Based on previous work done with murine embryonic stem cells (ESCs), studies have shown that human PSCs are amenable to suspension culture under defined and growth factor-supported conditions, maintaining key phenotypic markers, pluripotency and proliferative abilities required for stem cell identity. These studies saw cell concentrations in the 0.5–2 x 10^6 cells/mL range in spinner or shake flasks at the 5–25 mL scale, through cell aggregate formation [1,2,17,19]. Since this important early work, the process has been optimized by moving PSC cultures into stirred tank reactor vessels with pH and dissolved oxygen (DO) control abilities, allowing for scale-up and the production of up to 2 x 10^8 cells in a 100 mL volume (2 x 10^6 cell/mL) [18]. The addition of an effective perfusion system with cell retention capabilities compatible with these cells allowed for even higher cell concentrations, in the 3 x 10^6 cell/mL range at the same volume scale [11].

While these cell titers show great promise in the scalability of pluripotent cultures, final scale-up of this process will entail culture of these cells at much larger volumes, requiring new optimization and control of parameters to overcome large scale-specific challenges such as oxygen and nutrient transport, homogeneity of cell aggregate sizing and shear sensitivity [11].

Recently, Kwok and colleagues have demonstrated a suspension culture for PSC propagation using single-use bioreactors, able to produce 2 x 10^9 PSCs with full

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pluripotency retention, for the first time establishing the scalability of PSC culture to clinically relevant cell numbers [12]. Yet, while this study demonstrated the production of cell numbers sufficient to meet the needs of a single patient dose, new research is driving the field towards the possibility of allogeneic PSC therapies, introducing the need for a PSC culture process able to produce enough cells to treat hundreds or even thousands of patients at a manufacturing scale [4,23]. As such, the field still requires process intensification and optimization to (1) continue the scale-up of these PSC cultures towards clinical manufacturing volumes and (2) achieve large-scale PSC manufacture that meets regulatory specifications and is compatible with GMP manufacture and clinical use.

In the present study, we demonstrate a reproducible protocol for the scaled-up expansion of pluripotent stem cells in 10 L single-use stirred tank bioreactors. This could lend itself to further scale-up, as the platform has a demonstrated, incremental path to 2000 L in traditional bioprocess applications (i.e. monoclonal antibody production). Here, we detail the process development approach taken to develop this protocol and scale these cultures to produce $>10^{10}$ cells per batch, providing clinically and industrially relevant quantities towards their use in therapeutic regenerative medicine applications. With attention to future GMP compliance and regulatory considerations for clinical manufacture, our use of detailed monitoring of in-process parameters and closed processing protocols allowed for minimal in-process sampling, which will lend itself to future automation and reduced contamination risk. Finally, we have demonstrated a downstream processing approach for volume reduction and washing of the final PSC product and present cases where this product was successfully used either directly, or as a cryopreserved high-density seed bank (HDSB), as inoculum for further stirred tank bioreactor expansion and a suspension-based downstream differentiation.

The high-density cryopreservation of PSCs provides a consistent inoculum to the manufacturing process. These HDSBs remove the need for upstream adherent tissue culture, normalize the manufacturing process input and reduce the overall time required to produce a 10 L batch. Direct inoculation of suspension-based differentiation serves as a game-changer in process development and eventual clinical manufacturing by decoupling the expansion and differentiation processes.

MATERIALS AND METHODS

Maintenance of human PSCs in 2D culture

ESI-017 (hESCs, ESI BIO, Alameda, CA, USA) and NCRM1 (iPSCs, National Institutes of Health, Bethesda, Maryland, USA) were cultured at 37°C, 5% (v/v) CO_2 and maintained in mTeSR™1 medium (STEMCELL Technologies, Vancouver, BC, Canada) on Matrigel® - coated (Corning, New York, NY, USA) flasks. Cells were cultured in T-75 flasks (BD Falcon, Fisher Scientific, Carlsbad, CA, USA), in a 12 mL volume. The medium was refreshed daily. When reaching approximately 75% confluence, cells were washed with PBS (Gibco, Grand Island, NY, USA) and dissociated in Gentle Cell Dissociation Reagent (GCDR, STEMCELL Technologies) for 6–8 min. Cell colonies were then detached by scraping with a cell scraper. A uniform suspension of cell aggregates was obtained by carefully pipetting the mixture up and down. The cell aggregate suspension was plated at a density of 20,000 cells/cm^2 (or 1.5 x 10^6 cells/flask) on the pre-coated flasks with Matrigel and maintained in mTeSR1.

Expansion of PSCs in stirred tank bioreactors

PSCs were cultured adherently on Matrigel-coated flasks as described above. When

they reached ~75% confluence in flasks, cells were harvested by treatment with TrypLE™ Select 1X (Thermo Fisher Scientific) for 5 min at 37°C. Dissociated cells were quenched with DMEM/F12 (Thermo Fisher Scientific) and centrifuged at 300 *x g* for 5 min at room temperature. The cell pellet was gently resuspended in 40 mL mTeSR1. The cell suspension was strained through a 70 µm cell strainer (Thermo Fisher Scientific) to remove undissociated clumps. Cells were counted and viability was determined using a NC-200™ NucleoCounter™ (Chemometec, Allerod, Denmark) and inoculated at a cell density of 2.5 x 10^5 cells/mL into a 250 mL DASbox™ vessel (Eppendorf, Hamburg, Germany). Cells were grown in mTeSR1, supplemented with 10µM rho-associated protein kinase inhibitor Y27632 (Tocris Bioscience, Bristol, United Kingdom), for the first 24 hours of batch suspension culture. The bioreactor was maintained at 37°C, pH 7.2, 50% DO, stirred at the optimized dynamic agitation profile (specified in results), and supplied with sterile-filtered air and CO_2 via an overlay. Perfusion was initiated 24 hours post-inoculation with either a fixed perfusion rate or enhanced perfusion rate (see Results section for detailed perfusion protocols), via a stock micro-sparger used in reverse orientation. Process control and continuous data acquisition were executed by DASGIP™ software (Eppendorf). Daily sampling included cell counts, aggregate sizing by Multisizer 4e (Beckman Coulter, Brea, CA, USA) and metabolite (glucose, lactate) analysis by Vi-Cell™ Metaflex Bioanalyzer (Beckman Coulter). After counting, cells from each sample were fixed for flow cytometric analysis as described.

Stirred suspension culture of PSCs

When viable cell density reached between 1.0 – 2.0 x 10^6 cells/mL in either the DASbox or BioFlo™ 320 (Eppendorf) platforms, cells were harvested, dissociated to a single-cell suspension and passaged to the next larger vessel (BioFlo 320 or Xcellerex™ XDR-10 single-use bioreactor (Cytiva, Marlborough, MA, USA)). This cell density range was chosen to align with our aggregate size limit of approximately 300 µm and was typically achieved by day 5 of suspension culture. A single cell passaging method, described below, was used. After dissociation, cells were counted and inoculated at a density of 2.5 x 10^5 cells/mL in either the BioFlo 320 or XDR-10. Cells were grown in mTeSR1, supplemented with 10 µM rho-associated protein kinase inhibitor Y27632, for the first 24 hours of suspension culture without perfusion. The reactors were maintained at 37°C, pH 7.2, dissolved oxygen (DO) 50%, stirred at the optimized dynamic agitation profile, and supplied with sterile-filtered air, O_2 and CO_2 through an overlay. 100% DO calibration was performed relative to atmospheric levels. Perfusion started at 24 hours post-inoculation with either fixed perfusion rate (50% medium refreshment per day) or enhanced perfusion, depending on the growth of cells. Perfusion in the BioFlo 320 and XDR-10 platforms was performed by a separate acoustic mini-BioSep system (Applikon Biotechnology, Delft, Netherlands). Daily sampling was performed as previously described.

Cell passaging

To passage cells between reactor vessels, aggregates were dissociated into a single cell suspension. The passaging process required five cell bags. An identical procedure was used to passage between the various bioreactor platforms (DASbox into BioFlo 320, and BioFlo 320 into XDR-10). For passage from DASBox to the BioFlo 320 we used five 600 mL bags (Baxter, Fisher Scientific) and for passage from the BioFlo 320 to the XDR-10 we used five 2 L bags (Sartorius Stedim North America, Toronto, Canada). Dissociation occurred inside of each respective bioreactor vessel. Cells destined for downstream

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processing were grown and then dissociated inside of the XDR-10 vessel.

Bioreactor controls such as agitation, heat and gas flow were turned off, and cell aggregates were collected into a cell collection bag, Bag #1 using air delivered through the sterile filtered exhaust line to slightly pressurize the reactor vessels. After the cells were collected, Bag #2 containing PBS (80 mL for the DASbox, 800 mL for the BioFlo 320) was sterile-welded to an input line and gravity-fed into the vessel to rinse and collect any remaining aggregates. This rinse was then added to the cell collection bag (Bag #1). The cell collection bag was then removed and brought into a biosafety cabinet. The cell suspension was then transferred into a 250 mL conical tube and the aggregates were centrifuged at 100 *x g* for 5 minutes. After centrifugation the supernatant was carefully decanted and the cells were washed with 250 mL of pre-warmed PBS and centrifuged again. During this time Bag #3 containing TrypLE (50 mL for DASbox, 500 mL for BioFlo 320) + 1% DNase I (v/v) (Millipore Merck KGaA Darmstadt, Germany), was infused into the reactor via sterile weld. Temperature control (37°C) was restarted and agitation was set to 80 RPM. Next, the supernatant from the centrifuged cells was removed, cells were resuspended in 50mL of PBS + 1% DNase, and loaded back into Bag #2, and infused back into the reactor. Bag #2 was left attached to the reactor vessel during this time. The reactor was operated for 10 minutes at 80 RPM to begin dissociating the aggregates. After the initial dissolution the reactor agitation was set to 100 RPM for 1 minute to completely dissociate any remaining clumps. The single-cell suspension was then collected back into the attached Bag #2 using positive pressure from air through the sterile-filtered exhaust line. Next, Bag #4 containing DMEM (150 mL for DASbox, 1L for BioFlo 320) + 1% DNase was infused into the reactor and allowed to rinse the vessel for 1 minute. The cell suspension in Bag #2 was then infused back into the reactor vessel and the contents allowed to mix to quench the dissociation reaction. The quenched cell suspension was then collected back into Bag #2, brought into the biosafety cabinet, and transferred into a fresh 250 mL conical tube. The single cell suspension was then centrifuged at 300 *x g* for 5 minutes. During centrifugation Bag #5 of mTeSR1 (400 mL for BioFlo 320, 2 L for XDR-10) with 10 µM rho-associated protein kinase inhibitor Y27632 was welded on to the recipient reactor vessel, and the media was infused to begin warming with agitation at 75 RPM. The centrifuged cells were resuspended mTeSR1 media (40 mL for BioFlo 320, 300 mL for XDR-10). Next, the cell suspension was passed through a 70-micron strainer to remove any residual clumps. A cell count was performed as described. Using the procedures outlined here, cells were sequentially passaged from the DASbox into a BioFlo 320 vessel and then from the BioFlo 320 into an XDR-10 bag (thus forming a complete seed train). An inoculation density of 2.5 x 10^5 cells/mL was targeted across all vessels. For the first 24 hours, mTeSR1 supplemented with 10 µM rho-associated protein kinase inhibitor Y27632 comprised the media system. After 24 hours, cells were expanded in suspension using mTeSR1 only.

Downstream processing

To concentrate and wash dissociated single cells, we harvested an XDR-10 using a closed, single-use, continuous centrifugation instrument, (Sefia™ Cell Processing System, Cytiva). First, aggregates were digested into a single-cell suspension within the XDR-10 as described for cell passaging. PBS was used throughout the downstream process to wash the cells. As a proof of concept, two separate runs with single cell suspension aliquots between 2–3 L (targeting 7 x 10^9 cells total) were processed using the FlexCell protocol and CT-800.1 consumable kit, washed and volume reduced to approximately 250 mL. Initially, approximately 120 mL of cell suspension was loaded into the chamber at 100

mL/min. This was reduced to 60 mL at 300 *x g* for 2–3 minutes. The chamber was then filled to 200 mL, spinning at 300 *x g* at a feed rate of 100 mL/min and the culture was concentrated for a period of 45 minutes. Once the entire input volume was concentrated, the chamber volume was reduced to 50 mL. A wash of 150 mL of PBS was added and the cells were spun at 300 *x g* for 10 minutes. This PBS wash and spin was repeated one additional time. Once cell washing was complete, the concentrated cells were harvested, and an additional 50 mL of PBS was used to wash the chamber for a total final volume of 250 mL.

Metabolite analysis

Cell aggregate samples were collected from culture vessels at the same time interval (every 24 hours) without interrupting the cultures and perfusion. Samples were centrifuged at 100 *x g* for 3 min at room temperature to remove single cells and debris. The glucose and lactate concentrations of the supernatant were analyzed with the Vi-CELL MetaFLEX™ Bioanalyzer.

Mean aggregate diameter sizing

Aggregate sizing was performed on a Multisizer 4e particle analyzer. Samples of aggregates were collected daily. Sizing was performed using a 560 µm aperture on days 1–3 of bioreactor culture, and then on a 1000 µm aperture on days 4–7 of bioreactor culture, utilizing 3 mm Hg of vacuum pressure for the 560 µm aperture, and 6 mm Hg of vacuum pressure for the 1000 µm aperture. Briefly, 2 mL of sample was added to 200 mL of Isoton II solution (Beckman Coulter) in the sample chalice. Sizing data were collected over a 40 second run time. Sizing data were exported from the supplied software as the mean of all measured events +/- standard deviation. A total of 200–1000 events were captured. A time course of mean aggregate size versus time was generated and a linear fit was applied to determine the rate of mean aggregate size increase in µm/day.

High-density seed bank preparation and application

To shorten the length of time required to carry out the 10 L PSC seed train, high-density seed banks (HDSBs) were prepared that could be used to directly seed a stirred tank reactor at the 700 mL – 1 L scale. To accomplish this, we carried out our seed train process beginning in static culture and moving through the 100 mL to 1 L and ending up at the 10 L scale. The vessel agitation was turned off, the aggregates were allowed to settle, and media was aspirated out of the vessel via a dip tube. The aggregates were then dissociated to single cells as previously described. Dissociated single cells were removed from the bioreactor into a cell bag using positive pressure from air through the sterile-filtered exhaust line, then transferred to 500 mL conical bottles for centrifugation at 300 *x g* for 5 minutes. The supernatant was removed, and the cells were resuspended in 7 mL of CryoStor™ CS-10 (STEMCELL Technologies) cryoprotectant at 25 x10^6 cells/mL, which was sufficient for direct seeding of a 700 mL volume at 2.5 x 10^5 cells/mL. Individual 10 mL cryovials (Fisher Scientific) were manually filled and the vials were frozen at -80 °C overnight in a CoolCell SV10 (VWR, Toronto, Canada) to ensure controlled-rate freezing. Frozen vials were subsequently placed in vapor-phase liquid nitrogen for long-term storage. Cell pluripotency was verified by flow cytometry as described. A validation expansion run was performed after the banking was completed, whereby a vial was used to directly seed a BioFlo 320 at a 700 mL volume. The target initial cell density of 2.5 x 10^5 cells/mL was achieved, with growth and aggregate size kinetics as expected. Cell pluripotency was verified at the end of the run by flow

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cytometry and a sample was prepared for karyotype analysis as described.

For the application runs in Figure 6C, a frozen vial of the HDSB was thawed and inoculated at a cell density of approximately 2.5 x 10^5 cells/mL into a 1 L Minibio bioreactor (Applikon Biotechnology, Delft, Netherlands) with a 750 mL working volume. Cells were expanded as described previously, although maintained at 90% DO with 100 RPM. On the third day of the expansion, the stirrer speed was increased to 120 RPM until the end of the culture. Perfusion was performed with a mini-Biosep acoustic cell retention system (Applikon Biotechnology) and was initiated 24 hours post-inoculation with a continuous fixed perfusion rate of 50% media exchange per day, as this work was performed in parallel to determination of the dynamic perfusion strategy.

Flow cytometry

PSC pluripotency was evaluated by flow cytometry. All wash steps were performed using wash buffer (2% FBS, (Thermo Fisher Scientific) and 2 mM EDTA (Life Technologies, Carlsbad, CA) in Hank's Balanced Salt Solution (HBSS, Life Technologies)). For PSC pluripotency, aggregates were collected, digested into a single-cell suspension with TrypLE, and fixed in 2% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) for 10 min at room temperature. Samples were then washed in wash buffer (filter sterilized 2% FBS-HBSS with 2 mM ETDA) and stored at 4°C until stained. For the evaluation of cell-surface and intracellular pluripotency markers, 2 x 10^5 cells were prepared in V-bottom plates and permeabilized with a 0.1% (v/v) solution of wash buffer with Triton™-X 100 (Sigma Aldrich, St. Louis, MO, USA) by incubating for 5 min at room temperature. For evaluating PSC pluripotency, cells were next stained with antibodies against Nanog, Oct3/4, Sox2, SSEA-4 (BD Biosciences, San Jose, CA, USA), and Tra-160 (Biolegend, San Diego, CA, USA) diluted in permeabilization buffer (filter sterilized 2% FBS-HBSS with 2 mM ETDA and 0.1% v/v Triton X-100).

Single-stained compensation beads (BD Biosciences) were prepared in parallel. Compensation controls and stained samples were incubated for 30 min at room temperature, in the dark. Unstained cell samples were incubated with permeabilization buffer alone. After staining, sample plates were centrifuged at 500 *x g* to pellet the cells, and the supernatant was removed. Samples were washed twice in wash buffer and then analyzed on a CytoFlex™ flow cytometer (Beckman Coulter). Flow cytometry data were analyzed by FlowJo™ software (BD Biosciences).

Karyotyping analysis

Cell lines were routinely subjected to karyotype analysis at the end of the suspension expansion protocols. Cells were prepared according to the requirements of the Cambridge University Hospital Cytogenetics Laboratory (Cambridge, UK). Briefly, at the end of the seed train, cell aggregates were dissociated as previously described and plated onto Matrigel-coated 6-well tissue culture plates. Cells were cultured as previously described with daily media exchange (see maintenance of PSC in 2D culture) until 50 – 70% confluent. At this point, KaryoMAX™ Colcemid in PBS (Thermo Fisher Scientific) was added to the growth media to make a final concentration of 100 ng/mL, and the plate was incubated for 1 hour under normal culture conditions. After incubation, the culture supernatant was collected, the cell monolayer was washed with PBS, and the wash was collected with the supernatant. Cells were then detached from the wells using 1 mL TrypLE Select per well and incubation for 4 minutes at 37°C. Cells were triturated into a single cell suspension, and the dissociation reaction was quenched with mTeSR1 media. After centrifuging at 200 *x g* for 5 minutes and aspirating off the supernatant, the cell pellet was resuspended in 300 µL of fresh media. Next, 10 mL of 0.075 M KCl

solution (KaryoMAX KCl solution, Thermo Fisher Scientific) was added dropwise to the cell suspension while vortexing. The cells were then incubated at room temperature for 20 min to allow swelling. After this incubation, cells were centrifuged at 200 *x g* for 5 minutes, and the KCl solution was aspirated. A 3:1 solution of methanol (Thermo Fisher Scientific) and glacial acetic acid (Thermo Fisher Scientific) was made in-house and used to fix the cells. The fixative was added dropwise to the test tube with simultaneous vortexing. The cells were centrifuged again, and the fixative removed. The cells were then resuspended in 2 mL of fresh fixative solution and stored at -20°C until analyzed. Samples were sent to Addenbrooke's Hospital, Cambridge University Hospital Cytogenetics Laboratory, for analysis.

RESULTS & DISCUSSION

Parameter optimization for a 10 L seed train

We set out to develop a cell expansion protocol, or seed train, to generate > 10^{10} human pluripotent stem cells (hPSC) in the Xcellerex 10 L single-use stirred bioreactor (XDR-10), as this platform has a scalable, incremental path to 2000 L and a history of GMP manufacturing. We performed a survey of small-scale (<10 L), single-use platforms suitable for GMP manufacturing [20,21] and selected the Eppendorf DASbox (60 - 250 mL) and BioFlo 320 (400 – 1000 mL) as the intermediate steps to the 10 L scale, with the DASbox acting as the small volume, de-risking platform.

The seed train was developed starting with the DASbox bioreactor at 160 mL, passaging to the BioFlo 320 platform at the 1 L scale, which generated enough cells to seed the XDR-10 at an 8 L operational volume. To ensure successful expansion at each scale, set points for fundamental process parameters for PSC suspension culture were determined, using studies performed in small volume DASbox bioreactors and with a commercially available hESC line (ESI-017) as a model. In these initial experiments, agitation, DO, and perfusion feeding strategy were investigated, and enabling profiles for each parameter were specified.

With single-use, GMP use as the driver for platform selection, we encountered differences in vessel geometry/aspect ratio, volume turndown ratios and impeller design. Traditional bioprocess scale-up parameters such as mixing time, power input per volume (P/V), oxygen mass transfer (kLa), tip speed (V_{tip}), impeller to reactor diameter ratio (D/Tv) were calculated and reviewed to guide the process development. However, these were not always directly applicable due to the platform differences and the unique constraints and characteristics of culturing PSCs in stirred tanks (for example, aggregate size limitations, shear sensitivity, quality concerns, downstream differentiation ability). Therefore, rational experimental designs to explore the operational space were utilized as required in the small volume de-risking platform.

Agitation rate was first investigated in the DASbox bioreactors. Initial experiments were performed to determine the minimum enabling agitation rate, sufficient to maintain a suspension culture. This was achieved by testing increasing agitation rates of 30, 45, 65 and 80 RPM. It was demonstrated that a rate greater than 65 RPM was required to maintain the PSC aggregates in suspension culture. Agitation rates of less than 65 RPM resulted in aggregate settling (data not shown). A second study with agitation setpoints of 75, 80, and 85 RPM as well as with a dynamic strategy of increasing agitation at 75, 80, and 85 RPM on days 0, 1, and 2, respectively, was performed to determine if a fixed or dynamic stirring strategy would be more suitable. In this experiment, cells were cultured in the DASbox bioreactor system at 160 mL, with one vessel for each of the four agitation conditions, for five days per passage. Data from the first passage were not included, as the cells were adapted to suspension culture.

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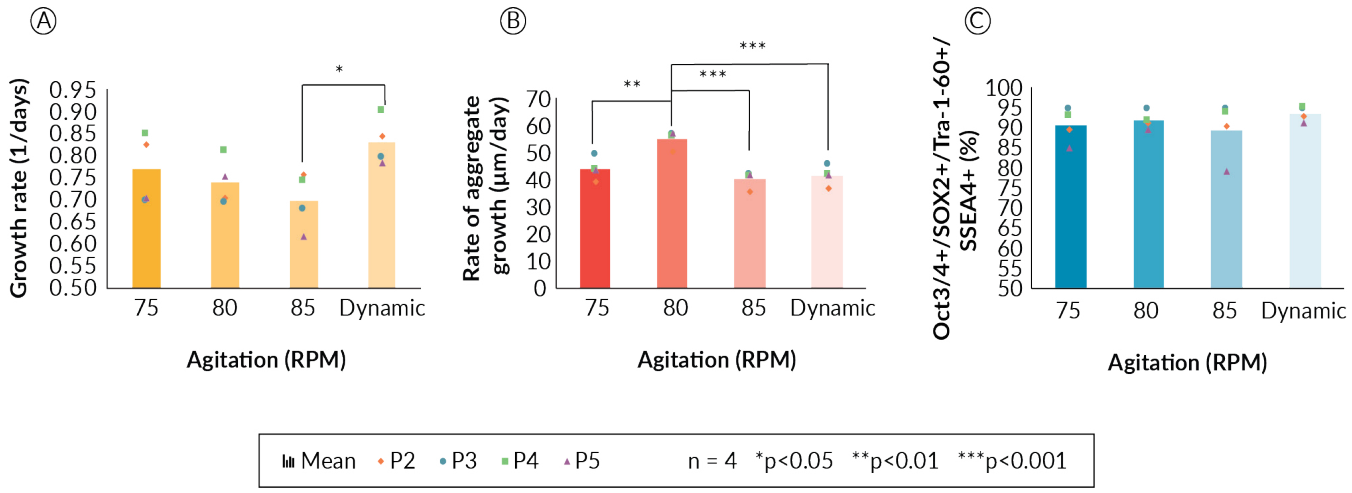
Data from passages 2–5 were collected and are depicted in Figure 1. When determining growth rate, an exponential fit was applied to determine the maximum specific growth rate.

It was hypothesized that a dynamic mixing strategy would be most beneficial for PSC suspension culture because a lower agitation rate at the beginning of the culture would promote aggregation and enhance cell survival during the initial stages of the culture, and an increase would be beneficial to maintain suspension as cell aggregates grew in size. We also sought to use agitation to control aggregate size as the culture expanded. In order to maintain adequate and biologically relevant oxygen availability to PSCs in aggregates, we referred to suggested oxygen diffusion limits from blood vessels of approximately 100–200 µm [5]. With this in mind, we aimed to develop an agitation scheme able to control the mean aggregate diameters to below 300 µm on the day of cell harvest. These experiments demonstrated that PSC cellular growth rate (Figure 1A) may be affected along with the rate of aggregate size increase at 85 RPM and in the dynamic agitation profile (Figure 1B).

Expansion kinetics (growth rate, Figure 1A) decreased with increasing agitation rate, but did not show statistical significance across the fixed agitation conditions in our experiments. However, the growth rate was significantly lower for the 85 RPM condition as compared to the dynamic condition ($p < 0.05$). Presumably, 85 RPM imparted a higher shear during the initial aggregation, which may have prevented formation of aggregates and resulted in slower growth. In general, a trend of decreasing growth rate was observed as the agitation rate was increased. The rate of aggregate growth was significantly higher for the 80 RPM condition (Figure 1B) as compared to all other conditions, but interestingly, the rate of aggregate size increase does not correlate with specific growth rate. The 80 RPM condition stands alone and in contrast to the three other conditions in this respect. More experiments would be required to elucidate the nature of the rate of aggregate size change at the 80 RPM condition. Despite differences in growth kinetics and aggregate size, cells displayed high levels of viability and pluripotency maker expression for all four mixing conditions (Figure 1C). Greater than 91%

FIGURE 1

Impact of agitation on growth and quality attributes of ESI-017.



Experiments were performed to assess the impact of different mixing strategies on various metrics of PSC growth and quality, using 160 mL cultures in the Eppendorf DASbox mini bioreactor system. (A) Growth rate, (B) rate of aggregate size increase and (C) pluripotency via flow cytometry (depicted as percent of total cell population 4-marker positive for Oct3/4, Sox2, SSEA-4 and Tra-1-60) were measured and are reported here for passages 2-5. Bars represent values averaged over the four passages. Values for each of the individual passages are depicted with individual dots. Data were analyzed for significant difference by one-way ANOVA, followed by post-hoc Tukey test. Significant differences are highlighted, and levels are denoted with stars.

of the cell population was four-marker positive (Oct3/4, Sox2, Tra- 1–60 and SSEA-4—as assessed by flow cytometry) in all cases, and the dynamic agitation strategy produced the highest pluripotency data at 95% four-marker positive. From the experiments completed, the dynamic mixing strategy was carried forward as it gave the highest growth rate, did not negatively impact aggregate size increase, and cells retained a high level of pluripotency marker expression.

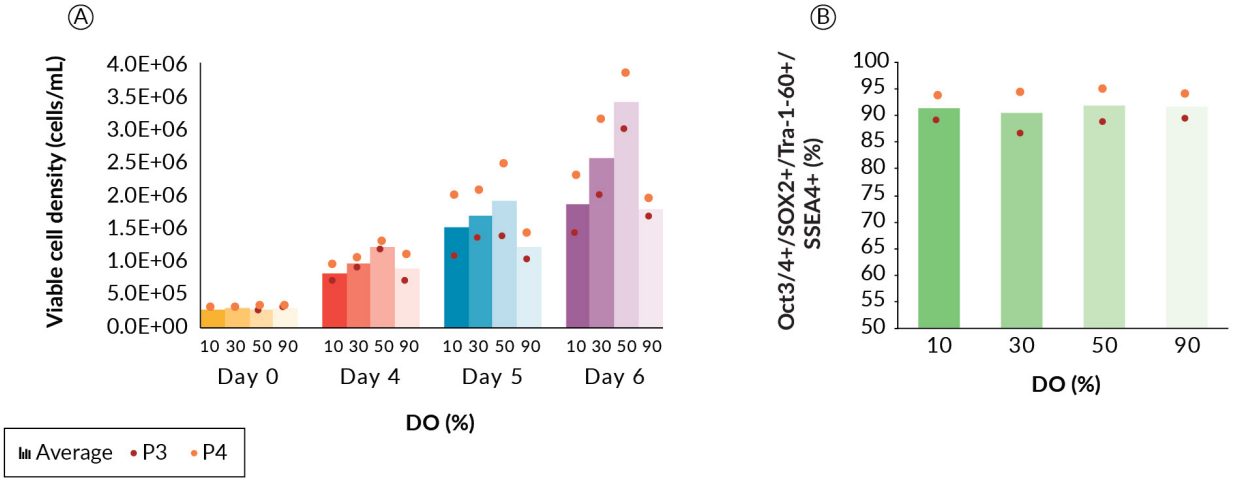
The second parameter investigated was DO, which are related to the percent of oxygen saturation at 37 °C and normal pressure. DO has physiological [5], diffusional and cost implications in large-scale suspension culture. Thus, understanding the impact of DO and identifying the minimum DO necessary to maintain growth rate and pluripotency will impact scaling strategy and may impact manufacturing costs at scale. This is especially important for cell types that are particularly shear sensitive and consequently require obligate headspace gassing as opposed to sparging, such as PSCs [16].

In this experiment, a single DASbox was cultured at 90% DO (passage 1). This DASbox generated enough cells to split into four DASboxes at DO settings of 10, 30, 50, and 90%, respectively. Each DASbox was run for three further passages. The first passage at each DO condition (passage 2) was taken as an adaptive one, and the final two passages were used for comparative data analysis (passages 3 and 4). The cells from the DO study were then pooled and used to seed a 1 L BioFlo 320 vessel, which was then passaged to the first XDR-10 run (Seed Train 1).

Viable cell density (VCD) data from passages 3 and 4 (Figure 2A) both indicate that the lower and higher DO set points of 10 and 90%, may negatively impact growth rate. Further studies would be required to understand if the apparent reduction in VCD is statistically significant. However, some reports [15, 24] support the possibility that lower and higher DO conditions may negatively impact stem cell growth. A DO setting of 50% appears to be most favorable of the conditions tested. DO had little impact on the aggregate diameter (data not shown) or day 6 pluripotency (Figure 2B). A DO set point of 50% was carried forward given that it appeared to be beneficial from a growth perspective, did not

FIGURE 2

Impact of dissolved oxygen (DO) on growth and quality attributes of ESI-017.



Cells from one DASbox run at 90% DO were split into four DASbox vessels, one at each of four DO conditions—10%, 30%, 50, and 90% and passaged three further times. Data from passage 2 was not used as cells were equilibrating to the new DO conditions. (A) Viable cell density and (B) pluripotency via flow cytometry are depicted. Passage 3 and 4 are respectively represented as dots and their average as bars.

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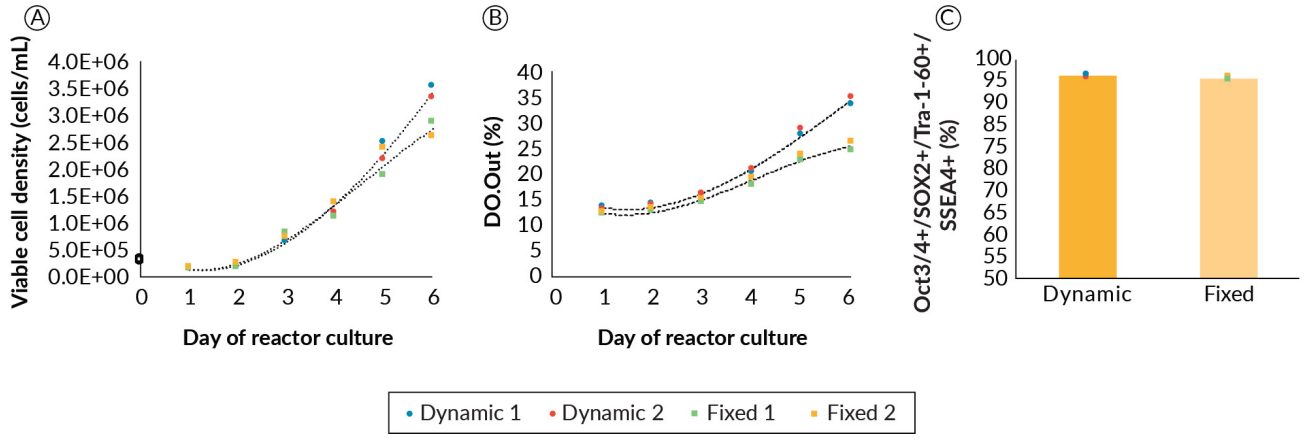
negatively impact pluripotency, would reduce the oxygen requirement from the 90% starting point and would be a reduced burden to maintain in a headspace-aerated bioreactor.

The final parameter optimized was perfusion feeding strategy. Perfusion was chosen as the culture mode to continuously supply temperature-labile media components such as growth factors and cytokines, and to wash out inhibitory byproducts such as lactic acid. We examined the differences and effects of a fixed versus dynamic/responsive perfusion strategy on PSC growth and quality. Four DASbox reactors, two with fixed perfusion rate and two with dynamic perfusion, were run. Perfusion was initiated at 50% of the culture volume per day on day 1, on the condition that aggregation was confirmed microscopically for all bioreactors. From this point, whenever referring to the perfusion rate, it is a percentage of the culture volume per day unless otherwise stated. The two reactors with fixed perfusion conditions remained at 50% for the remainder of the culture. For the two dynamically perfused bioreactors, we chose to increase perfusion rate when pH decreased to 6.8 (typically day 3 or 4). When this pH drop was reached, perfusion rate was manually increased by 30% over the previous set point. From that point forward, 30% increases from the previous set point were made daily (50%, 80%, 110%...etc). We did not optimize the point or rate of perfusion increase further but suggest there would be additional operational space to examine. Figure 3A indicates that growth appears to increase in the dynamically perfused DASboxes from day 4 onward. This difference is made even clearer when observing the DO output (DO.Out) signal from the probe (Figure 3B). The probe output is the signal the DO probe sends to indicate the culture's demand for oxygen. From these data, we can see that the higher growth in the dynamically perfused reactors corresponded with an increased demand for oxygen. Perfusion strategy did not impact pluripotency (Figure 3C).

In the fixed perfusion conditions, lactate concentration reached 15 mM on day 4 and continued to climb to 16 mM by day 6. Dynamic perfusion kept lactate concentration below 15 mM (data not shown). In parallel, glucose level dropped to 4.6 mM and then 2.7 mM, respectively in these reactors (data

FIGURE 3

Impact of perfusion strategy on growth and quality attributes of ESI-017.



Two replicate DASboxes were run in each of two perfusion conditions; fixed perfusion (50% reactor volume/day starting on day 1) and dynamic perfusion (50% reactor volume/day on day 1, increased by 30% reactor volume in response to culture first reaching pH 6.8 and increased by 30% each day thereafter) strategies were tested. Data collected included (A) viable cell density of the cultures as well as (B) the culture's demand for oxygen as described by the output signal (DO.Out %) on the in-line DO probes and (C) pluripotency via flow cytometry.

not shown). As high levels of inhibitors and low levels of nutrients generally do not favor PSC growth [11], it was hypothesized that growth rate would improve if the perfusion rate was increased throughout the expansion. While levels of glucose and lactate may affect expansion, there is insufficient data to determine whether these alone are responsible, due to provision of other growth factors, cytokines and nutrients and dilution of other potentially inhibitory by products as the perfusion rate is increased. Nonetheless, the higher rate of media exchange in the dynamic perfusion strategy promoted better cell expansion comparatively, which became noticeable from day 4. On day 6, dynamic perfusion led to an average 25% increase in cell density compared to the fixed perfusion rate bioreactors. Despite ample potential for further optimization, we progressed with the current perfusion strategy.

The influence of agitation, DO, and perfusion rate on the expansion of ESI-017 as aggregates in process-controlled bioreactors was studied. Based on these data, a protocol for the expansion was developed. A set point of 50% for DO, a dynamic agitation profile starting at 75 RPM upon inoculation and increasing by 5 RPM/day to 85 RPM on day 2, and a perfusion strategy starting at 50%/day on day 1 with rate increases of 30% over the set point every day once culture pH first drops to 6.8 was specified. These parameters were carried forward and formed the basis for further scale-up work.

Application of the optimized parameters to the expansion of a hiPSC line

Once the agitation, DO and perfusion strategies and setpoints were specified, the robustness of the process was evaluated using a second PSC line. The human induced pluripotent stem cell (hiPSC) line NCRM1 was compared to the human embryonic stem cell (hESC) line ESI-017 used previously. Both ESI-017 and NCRM1 were suspension-cultured in one DASbox each for a single passage. Each cell line was then split into two bioreactors. The cells were cultured in DASbox bioreactors for three additional passages. From this, a total of seven passage datasets were collected. Datasets contained growth, viability, metabolite, and aggregate size kinetic data as well as pluripotency.

While we agree that passage-based data is not a sufficient replacement for biological replicates, this approach demonstrates the stability of the process across the multiple passages required to scale the expansion process. From these data, the two cell lines showed no significant differences in growth rate, and no metabolic differences were noted in terms of lactate yield from glucose (Figure 4A and Figure 4B, respectively). Both cell lines averaged >90% four marker-positive for pluripotency at the end of each passage with no significant difference between cell lines (Figure 4C), and both cell lines showed no genotypic abnormalities upon karyotypic analysis following the final passage (Figure 4F).

Aggregate formation appeared to be different between the two cell lines (Figure 4D and Figure 4E). Aggregate size was significantly different on day 1 with ESI-017 cells forming aggregates with an average diameter of 74.9 µm and NCRM1 cells forming aggregates with an average diameter of 92.4 µm ($p < 0.05$). This size difference persisted throughout the culture duration. When we quantified the rate of aggregate size growth, this difference narrowly missed achieving significance at the $\alpha = 0.05$ level ($p = 0.062$). ESI-017 aggregates grew at an average rate of 41 µm/day compared to NCRM1 at 48 µm/day. It is hypothesized that the difference in the initial aggregate formation could be due to cell line differences in response to the shear experienced on transition to suspension culture.

Despite the small differences in the rate of aggregate size increase, we proceeded with the ESI-017- determined process parameters for scale-up of NCRM1. For cell lines that are not successful following this scale-up protocol, we propose that day 1 aggregate

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sizing may prove to be a key datapoint highlighting differences that could influence process parameters such as agitation and culture length due to the potential impact of aggregate diameter on aggregate settling and oxygen availability. With respect to process robustness, aggregate formation and day 1 aggregate size may prove important sources of line-to-line variability and may be key parameters when transferring additional cell lines to this process.

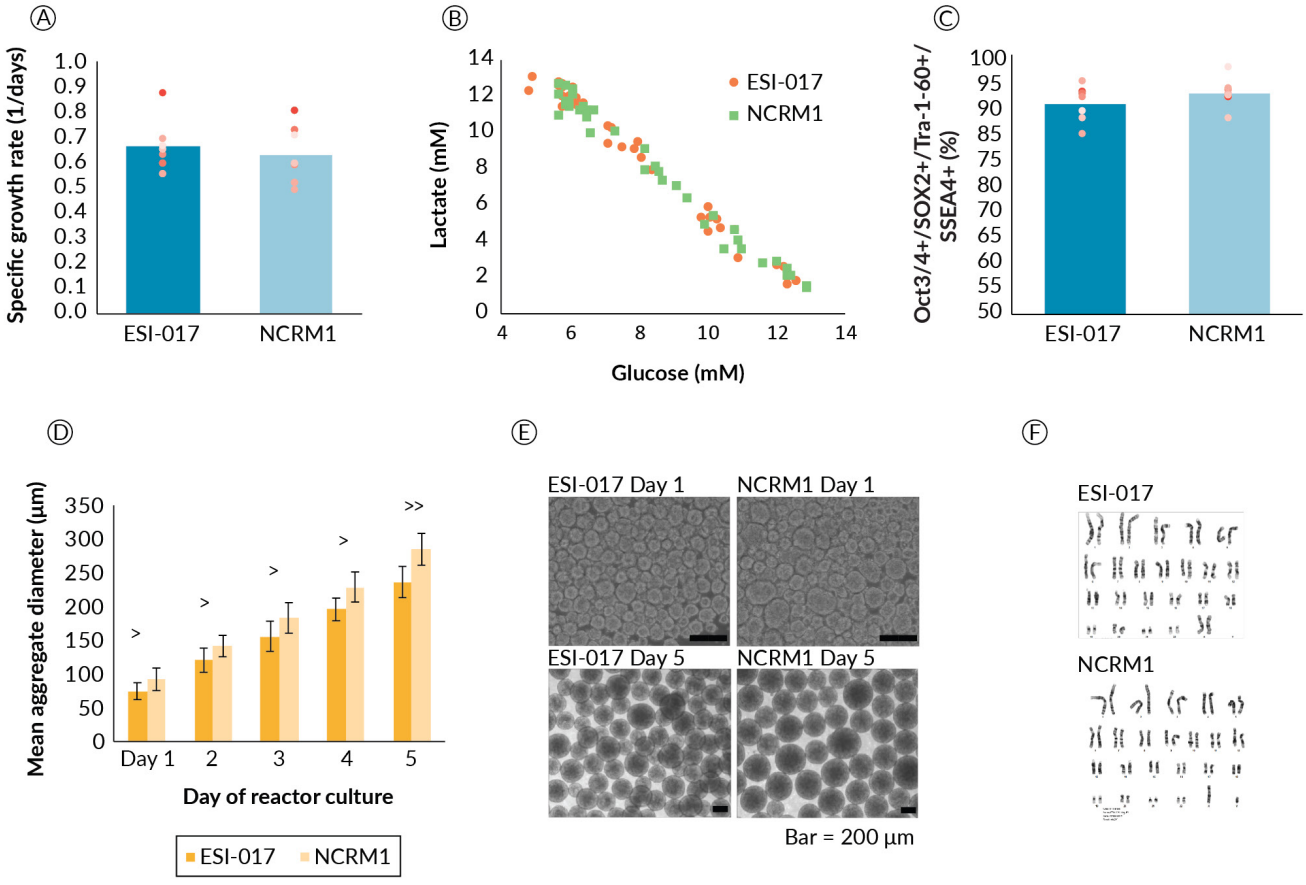
Correlation of hPSC growth kinetics with stirred tank bioreactor process parameters

As PSCs expand, they produce lactate and consume oxygen and glucose. Correlations between process values and outputs from installed pH and DO probes were investigated with discrete sample data such as viable cell density, lactate production and glucose consumption. Any lactate and CO_2 produced will acidify the medium, thereby lowering the pH. As DO was being controlled, the rate of change of the probe output signal (DO.Out), which reflects the culture's demand for oxygen, was also investigated for correlation to growth.

Growth data from the ESI-017 and NCRM1 comparison study were plotted against both pH and DO probe output

FIGURE 4

Comparison between embryonic (ESI-017) and induced (NCRM1) cells lines expanded using process developed in DASbox platform.



Human embryonic stem cell line ESI-017 and human induced pluripotent stem cell line NCRM1 were each grown in one DASbox for a passage, then passaged into two DASboxes each for three subsequent passages, generating 7 total passage datasets for each cell line. Passage data were used to determine (A) specific growth rate for the exponential phase of each passage, (B) lactate yield from glucose, (C) pluripotency by flow cytometry analysis, (D) aggregate size throughout the culture from their formation at day 1 through day 5. (E) Example aggregate images are depicted for each cell line at the beginning and end of culture and (F) chromosome spreads from karyotype analysis.

(Figure 5A and Figure 5B, respectively). Since the perfusion rate was being increased in response to the increase in cell density, the pH did not decrease below 6.75 while cells continued to expand in the bioreactor. Therefore, to correlate pH with viable cell density, we did not include the growth data after pH plateaued. A relatively good linear correlation was found between pH and the viable culture density for both hPSC lines (Figure 5A). The similar slopes derived from the linear trendlines also indicate that the viable culture density must be higher than 1 x 10^6 cells/mL to drive down the pH value close to or below 7.0 with 50% daily medium exchange. Similar to the pH:VCD correlation, a good linear relationship was also found for the dissolved oxygen probe output (Figure 5B).

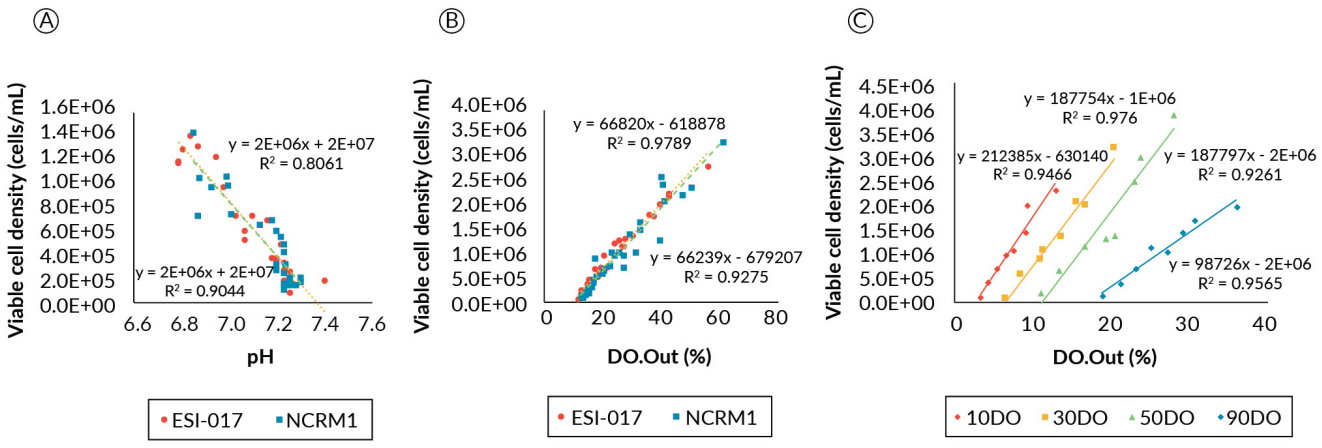
We additionally investigated if a good linear correlation could be found for the cells cultured at different DO setpoints from those used in the preliminary DO determination study (Figure 5C). Among the different DO conditions, DO90 had a shallower slope compared to the low DO conditions, whose slopes appeared essentially parallel. We do not believe this to be a physiological response, but rather it may be related to a reduction in driving force to dissolve oxygen as the saturation point is approached at the higher setpoint; whereas the parallel linear correlations at the lower DO set points indicate that the oxygen demand to cell density correlation in this system is unaffected at ≤ 50% DO.

Taken together, the correlation analyses suggest that both pH and the oxygen demand value from the online probes can be used to estimate the hPSC growth condition in the bioreactors. Though the pH correlation can be linked to the viable culture density irrespective of the bioreactor geometry, it will be impacted by how fast medium is exchanged and the buffering capacity of the medium. On the other hand, DO correlation is less influenced by the perfusion rate and the medium type, but will be influenced by the parameters that will affect the oxygen transfer coefficient. Interestingly, different DO conditions are likely to impact the oxygen consumption rate of the cells. Therefore, to have a more accurate estimation of viable cell density in the bioreactor, both pH and DO probe signals should be considered and analyzed.

Since we have established a correlation between the bioreactor process parameters and viable cell counts, we propose the dynamic feeding regime could be cascade-controlled via integration with the pH and DO continuous

FIGURE 5

Correlative analysis of online process parameters to discrete data to demonstrate automation of the expansion process.



Linear correlations between viable culture density and (A) pH and (B) DO probe values (DO.Out) are shown for ESI-017 and NCRM1. (C) For ESI-017, under varying DO conditions ≤ 50%, the relationship between DO and viable culture density is consistent, where under high (90%) DO conditions the relationship appears to be skewed potentially due to DO saturation.

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process data [20,21]. This would enable metabolic feedback of the system to control feed rates, providing a level of physiological control and automation of the manufacturing process. Additionally, with proxy measures of viable cell density via pH and DO correlations, the need to extract samples from the bioreactor during manufacturing would be removed, reducing labor and contamination risk.

Scale-up of suspension aggregate culture to 1 L and 10 L reactors

Parallel to the DASbox process development efforts, scale-up was also being performed at the 1 L BioFlo 320 scale and the 10 L XDR-10 scale. As process improvements were being realized in the DASbox bioreactors, they were being applied to the scale-up efforts occurring in parallel. Numerous parameters were evaluated in the scale-up activities including agitation, DO and pH control strategies, perfusion strategy, closing of the passaging and harvest process, and demonstration of scalability. We found that agitation, DO and perfusion rates scaled nearly directly between the DASbox and BioFlo 320 platforms, with only a small decrease in agitation required to maintain mixing time in the 1 L BioFlo 320. Agitation rates to maintain mixing time in the XDR-10 were reduced even more, due to more significant differences in platform and impeller design. A total of 9 expansion runs at the 10 L scale were made. Two critical quality attributes (CQA) for passaging have been identified, with aggregate size being primary and cell number being secondary.

The first attempt at the 10 L scale was seeded from the cells generated as part of the DASbox DO study. Cells from all four DASboxes were pooled and used to inoculate a single BioFlo 320. The BioFlo 320 was cultured for 7 days generating enough cells to seed an XDR-10 at 5.5 L at the target seeding density of 2.5 x 10^5 cells/mL. A perfusion technology solution had not been developed at this point, so a combined fed-batch/perfusion-based approach was used to enable the culture. Despite not having an ideal culture mode, a total of 7.75 billion cells were generated. Perfusion was required to maintain low levels of inhibitory by-products, but the Applikon BioSep used could only achieve 1500 mL/day, which is 28% volume/day at the starting volume and 19% volume/day at the final volume. It was demonstrated earlier that 50% volume/day increases in perfusion rates were required to maintain exponential growth. To overcome this, mTeSR1 supplement was bolus-fed into the XDR-10 on days 2, 3 and 5 using 500 mL for the first two feeds and 1750 mL for the last feed, resulting in a total volume of 8250 mL.

Seed trains 2, 3 and 4 were executed to identify a perfusion solution for the XDR-10. Seed train 2 used the dip tube in the XDR-10 Pro Plus bag as a gravity settling device. The intent was to allow the heavier aggregates to be retained while spent media was removed. This was not successful as significant aggregate loss was observed in the waste media collection. Seed train 3 used the Applikon 10 L BioSep, which has a larger capacity, which is not an in-bag solution but rather a retrofit. It is also more complicated and requires two pumps to operate. The first is a higher flow-rate pump to supply the BioSep with culture and the second removes the waste. Despite multiple attempts to configure the setup correctly, aggregates settled at various low points in the setup. Additionally, continuously exposing PSC aggregates to a peristaltic pump head is not advisable, as significant viability loss and growth impacts were observed (data not shown). Seed train 4 utilized Repligen's ATF (alternating tangential flow filtration) system. Based on in-house, single-pass shear assessments of aggregates through hollow fibers in a tangential flow filtration (TFF) system, and recommendations made by the vendor, a shear rate of 2700 1/s was used to recirculate the culture and remove waste via perfusion. This perfusion set-up was abandoned due to adverse impact on cell viability and, like the high flow rates in seed train 2, was hypothesized to be too harsh for PSC aggregates.

To date, a commercially available, large-scale, single-use solution for perfusion of PSC aggregates has yet to be developed. As such, a prototype perfusion device for PSC expansion was developed and tested. PSCs grown as aggregates in bioreactors have a reasonably high settling velocity, but not quite enough to resist removal through a dip tube. Based on this, a perfusion device composed of spiral tubing coupled with a top-mounted 20 µm filter was devised. Through inclusion of a filter element and a pump having a flush-back cycle, the aggregates are preferentially returned to the bioreactor environment while waste media is removed. To demonstrate the device's utility, NCRM1 cells, grown as part of an iPSC experiment to demonstrate robustness of the expansion protocol, were passaged to a 1 L BioFlo 320 retrofitted with this settling device, twice (P2 and P3). Cells were then passaged a fourth time (P4) in the BioFlo 320 and in parallel, to an XDR-10 also retrofitted with the same settling device.

The BioFlo 320 was seeded at 2.5 x 10^5 cells/mL at 700 mL with an agitation setting of 70 RPM on day 0; culture volume was increased to 1 L on day 1 and agitation rate increased to 75 RPM once aggregation was confirmed. Perfusion was started on day 1. On day 2 the agitation rate was increased to 80 RPM.

To determine if NCRM1 aggregate morphology was impacted by use of the settling device, it was run only intermittently during the first passage in the BioFlo 320. Samples of the culture were taken before and after use of the device and were analyzed microscopically, with no observable impact on aggregate morphology. The culture was then passaged two additional times (P2-P3) into the same BioFlo 320, now with the settling device running in continuous mode for the entire expansion. Pluripotency was examined via flow cytometry after each of P1, P2 and P3 and was found to exceed our minimal requirements (97%, 94% and 93%, respectively). On the final passage (P4), the 1 L culture was used to seed the XDR-10 bioreactor (seed train 1). Pluripotency for the P4 expansion in the XDR-10 was 82% positive for the four-marker panel. We recognize the downward trend of pluripotency seen for NCRM1 through these passages, however we have not seen this for ESI-017. Additionally, on previous, repeated passaging of NCRM1 (Figure 4C) we did not observe a linear decrease in potency through P2-P4 in bioreactor culture (replicate 1: 93%, 93%, 92% and replicate 2: 88%, 93%, 97%).

The XDR-10 working volume was 8 L. Agitation speed was 55 RPM on day 0, increased to 60 RPM on day 1 and 65 RPM on day 2 with perfusion starting on day 1. To enable the back flush of the aggregates accumulating in the settling filter, the waste pump was placed under the control of the BioSep controller. Enabling settings for the back-flush time and overall cycle frequency were identified to be 10 seconds and 7 minutes, respectively. Masterflex L/S size 25 tubing was used for the spiral settling tubing. For the BioFlo 320 a single spiral tubing setup was used while for the XDR-10 a double spiral setup was applied. Masterflex L/S size 16 tubing was used in the pump head for the BioFlo 320 expansions and Masterflex L/S size 25 tubing for the XDR-10 expansion. Figure 6A illustrates how the culture grew with continuous settling device perfusion in the BioFlo 320 (2 passages, P2 and P3) and in the XDR-10 (1 passage, P4, seed train 5)

Scale-Up to the XDR-10

For the successful ESI-017 XDR-10 runs (seed trains 1, 7 and 8), differences in growth at increasing scale were observed; however, this did not prevent growing over 10 billion cells at the XDR-10 scale. Seed trains 7 and 8 exceeded our target (25 and 37 billion PSC's, respectively); whereas the first, which did not have a suitable perfusion solution, was slightly short of our goal (7.7 billion PCSs). The NCRM1 scale-up run, from development of the large-scale perfusion device (seed train 5), also exceeded our target by producing 15 billion PSCs. The four successful scale-up runs in the XDR-10 (Figure 6B) maintained acceptable pluripotency (86%, 82%, 81% and 97%

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for seed trains 1, 5, 7 and 8, respectively) with no karyotypic abnormalities.

The cause of variability in final pluripotency from the 10 L runs is unknown. Certainly, these scaled up expansions describe developmental work, and the standard operating protocols for clinical manufacturing have not been locked down. However, the state of analytics (flow cytometry and karyotype analysis) and their suitability for application to cell populations in the billions deserve some consideration. Nonetheless, we considered these runs successful as they were above our internal CQA for pluripotency (≥ 80% positive for Oct3/4, Sox2, SSEA-4 and Tra-160) and returned normal chromosome G-banding results compared to master cell banks.

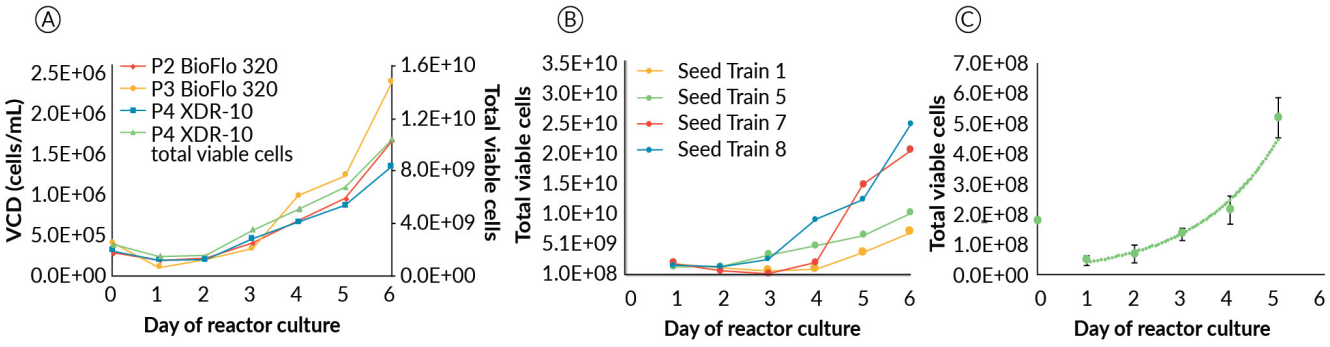
A drop in viable cell density is observed across all expansions regardless of scale or culture platform during the first 24–48 hours. We hypothesize this is a result of multiple stress factors as opposed to a dilution effect associated with a volume change due to initiation of perfusion or bolus feed of media, as both viable cell density and total cell number are shown to drop (Figure 6a). Harsh passaging conditions (treatment with TrypLE, trituration, multiple centrifugation steps, etc.), acclimatization to a new cell culture environment, and efficiency of aggregate formation (i.e. not all of the inoculated single cells will form aggregates), may all contribute to loss of viable cell density in the initial phase.

Perfusion was started in all cases on day 1, and rates were increased at 30%/day of the previous set point, once the culture pH dropped to our internal target of 6.9. The pH did not drop below 6.8 throughout any of the expansions. Lactate did not exceed 14 mM at any scale. The average growth rate across the two ESI-017 seed train runs with the prototype perfusion device (seed trains 7 and 8) was 0.761 ± 0.131/day. Average lactate yield was 1.533 ± 0.162 mol lactate produced/mol glucose consumed. The average rate of aggregate size increase was 32 ± 12 µm/day.

As a final note on the last scale-up run performed, this process was accomplished completely closed, with in-reactor aggregate dissociation and tube-welded transfers. The intent was to demonstrate that large-scale PSC expansion is possible in a completely closed system and to provide a potential starting point for large-scale, clinically relevant production of pluripotent stem cells. We recognize there are many potential

FIGURE 6

Scaled up PSC growth.



(A) Expansion kinetics from two BioFlo 320 1 L runs and the first 8 L XDR-10 run enabled by the large-scale perfusion device using cell line NCRM1. Secondary axis depicts total viable cells for the XDR-10 run (B) Growth curves for the four successful XDR-10 runs, represented as total viable cells. Seed trains 1, 7 and 8 were the embryonic cell line ESI-107 and seed train 5 the induced pluripotent cell line, NCRM1. (C) Growth curves (n=7) of ESI-017 directly inoculated into 1 L suspension culture from the HDSB. Growth rates were calculated from day 1 and were comparable across seven attempts with an average of 0.59 +/- 0.1 day^{-1}.

opportunities to improve, and to continue scaling of this manufacturing workflow, and by no means present a final commercial-ready solution. However, we feel with the scale achieved and approach taken, this work represents the most advanced biomanufacturing process for PSCs reported to date.

High-density seed bank for direct inoculation of 1 L stirred tank bioreactor

With success reaching > 10^{10} cells produced in the 10 L Xcellerex platform, we hypothesized these cells could be banked at high density for use as an inoculum, as in traditional bioprocesses. Cryogenically preserved, high-density seed banks (HDSB) were made to target the cell numbers required to directly inoculate a 1 L bioreactor at 2.5 x 10^5 cells/mL. The HDSBs had a post thaw viability of 89.1 +/- 1.9 % and were successfully used to inoculate seven 1 L bioreactors, resulting in very consistent growth rates with an average of 0.59 +/- 0.1 day^{-1} (Figure 6C). These growth rates were not significantly different (p = 0.569) than those in the cell line comparison study for ESI-017 of 0.66 +/- 0.1 day^{-1}. However, both growth rates were slightly lower than the average of the two 10 L runs (0.633 day^{-1}), likely due to differences in perfusion technologies and overall reactor set-up and operation. Day 5 pluripotency in the HDSB expansions averaged 87.6 +/- 12.6% positive for the four-marker pluripotency flow cytometry panel. While the average was above our target CQA of 80% positive, we noted one replicate experiment was unusually low at only 64% positive. The cause of low pluripotency from this run is not known and unexpected, as the bioreactor run conditions were held constant for all HDSB – 1 L expansions. On removal of this outlier, the day 5 pluripotency averaged 91.8 +/- 6.4%. Whether or not this data point was excluded, the HDSB pluripotency was not significantly (p = 0.624 or 0.578) different than that found in the cell line comparison work at 90.4 +/- 3.5%.

We did note differences in the amount of cell loss following inoculation. With the tissue culture inoculated expansion, cell counts reduced by 50% on day 1 compared to a 70% loss on day 1 in the HDSB inoculated expansions. Operationally, we note that the HDSB expansions were perfused at 50% per day and were not fed with the dynamic perfusion regime developed herein. Together, as a result, the day 5 cell counts for the cell line comparison study were on the order of 10^6, while the HDSB expansions only reached 10^5 in the same amount of time. Nevertheless, we feel that the maintenance of specific growth rate and end-point pluripotency positions the use of HDSBs as an appropriate replacement for tissue culture-based inoculum for bioreactor PSC expansion.

The decoupling of adherent tissue culture and small-scale (200 mL) stirred tank bioreactor expansion from large-scale (1 L) stirred tank bioreactor expansion is a significant advancement in the PSC manufacturing paradigm. The manual, adherent tissue culture process requires a high level of skill and consistent technique. With the use of HDSB as inocula, this source of process variability can be eliminated through normalization of cellular input. Additionally, for the 10 L scale manufacturing process, this process improvement reduces the overall process time by 50%.

Downstream volume reduction and concentration

With demonstration of the XDR-10 scale-up, we turned our attention to downstream processing of the cells. In order to demonstrate a closed and automated solution, we subjected aliquots of 7 x 10^9 cells (2–3 L) of a 10 L expansion to processing through the Sefia™ Cell Processing System. We made an initial attempt to process PCS aggregates with the stock Sefia protocols. This was unsuccessful and resulted in clogging or

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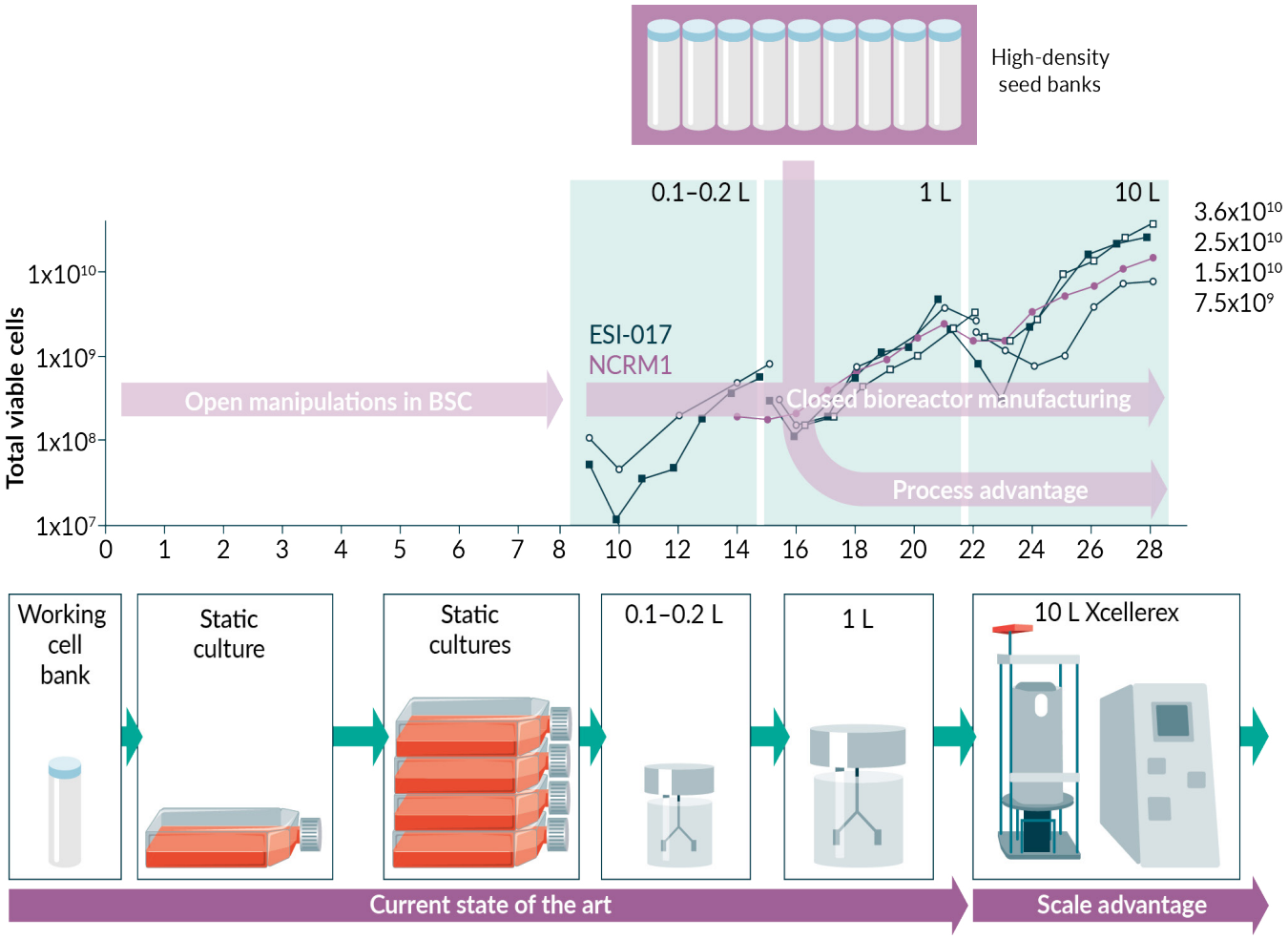
fouling of the consumable kit, presumably due to the density differences between aggregates and single cells. Rather than exploring the operational space that may enable processing of cellular aggregates, we decided to process a 10 µM rho-associated protein kinase inhibitor Y27632-mediated, single-cell solution and disaggregated with our passaging protocol as described. Originally designed and marketed for processing single-cell blood cell products, we demonstrated the utility of Sefia in processing PSCs. Run #1 reduced 2.0 L (7.39 x10^9 cells) to 240 mL in 1h 15min with 89% cell recovery (6.48 x10^9 cells) and 94% viability. Run #2 reduced 2.8 L (7.0 x10^9 cells) to 250 mL in 1h 27min with 79% recovery (5.55 x 10^9 cells) and 98% viability. These runs were not optimized in any way, and we feel recoveries could be further improved with additional replicates and fine-tuning of the processing protocol software by adjusting the operational parameters.

Conclusions

FIGURE 7

Process overview.



Images across the bottom depict the current state of the art in scaling up PSC expansion, from adherent flask growth to small and medium bioreactors. The work described here offers a scale advantage of a 10 L platform. The time axis depicts the typical duration of each stage and indicates which portions of the process require open manipulations in a biosafety cabinet. The use of high-density seed banks offers a direct route to bioreactor expansion with normalized cellular input without highly skilled labour for adherent culture and reduces the overall process time by 50%. Data depicted are the total viable cells from the seed trains and from direct HDSB inoculation for NCRM1 (purple) and ESI-017 (dark blue).

As regenerative medicine advances to clinical manufacturing, the need for closed, automated and scaled pluripotent stem cell manufacturing will increase. This work has presented process development and process improvements for a XDR-10 based, 10 L PSC manufacturing process, capable of producing >10^{10} cells per batch, with downstream concentration and volume reduction using the Sefia Cell Processing System.

We chose the hardware platforms with single-use, GMP manufacturing modality in mind. Since scalable stirred tank bioreactors from 0.1 L to 1 L are not commercially available, this meant choosing mixed hardware with differing configurations and properties. This presented some challenges to the direct application of traditional bioprocess engineering scale-up approaches. Choosing an appropriate small-volume, de-risking system was invaluable for process definition and examination of the unique sensitivities of culturing PSCs in stirred tank reactors, prior to attempting 10 L manufacturing runs.

Innovative solutions have been presented with implications for manufacturing, automation and process closure. The development of high-density seed banks offers many opportunities for process improvement, removing manual manipulation steps, shortening the expansion by 50% and decoupling subsequent suspension differentiation (Figure 7). The use of HDSBs may also contribute to the reduction of manufacturing variation by normalizing cellular input to the process. Demonstration of process correlations to viable cell densities have the potential to remove sampling and reduce both labor costs and contamination risk. The presented process correlations may also be exploited to control perfusion feed rates in response to cellular growth and metabolism. This way, changes to the extracellular media through growth and cellular metabolism would drive changes in feed rates, rather than a time-based schedule or a sample-based datapoint. This aspect could be important for cell lines with different growth rates entering the manufacturing workflow. Perfusion technology was identified as a gap in the available scale-up hardware, so an innovative yet simple solution was devised with a spiral settling chamber.

Taken together, this work demonstrates the relevant unit operations, process requirements and potential manufacturing improvements to set the stage for commercial production of PSCs. We expect this technology application will be further optimized and used to provide PSCs to differentiation workflows. Our hope is to enable the allogeneic cell therapy industry through facilitating the eventual clinical application of PSC-derived therapeutic cell types in advanced therapeutic products.

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

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Oct 2020

Clinical Trends

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Clinical Trends

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Oct 2020
Volume 6, Issue 9

COMMENTARY

Major clinical stories and read-outs from key conferences for the cell and gene therapy space

Sven Kili

1549–1554

CELL & GENE THERAPY INSIGHTS

Clinical Trends

-

COMMENTARY

Major clinical stories and read-outs from key conferences for the cell and gene therapy space

Sven Kili

2020 has certainly been a testing year for all of us! One particular headache for the life sciences world has been the loss of live conferences. For so many of us, they represent a great opportunity to take the pulse of the field we work in: to make new contacts, catch up with friends, identify and track trends that might impact our work, and get updated on the latest scientific/technical breakthroughs.

Whilst many of the key conferences for the cell and gene therapy space have persevered online, it can be challenging to immerse oneself in these virtual events as much as one might wish, due to time zone differences and the pressures of the day-to-day workload being that much closer to hand. This final *Clinical Insights* edition of 2020 highlights some of the notable clinical development-related trends, stories, and data readouts from two important recent conferences for cell and gene therapy, which you might have missed.

Cell & Gene Therapy Insights 2020; 6(9), 1549–1554

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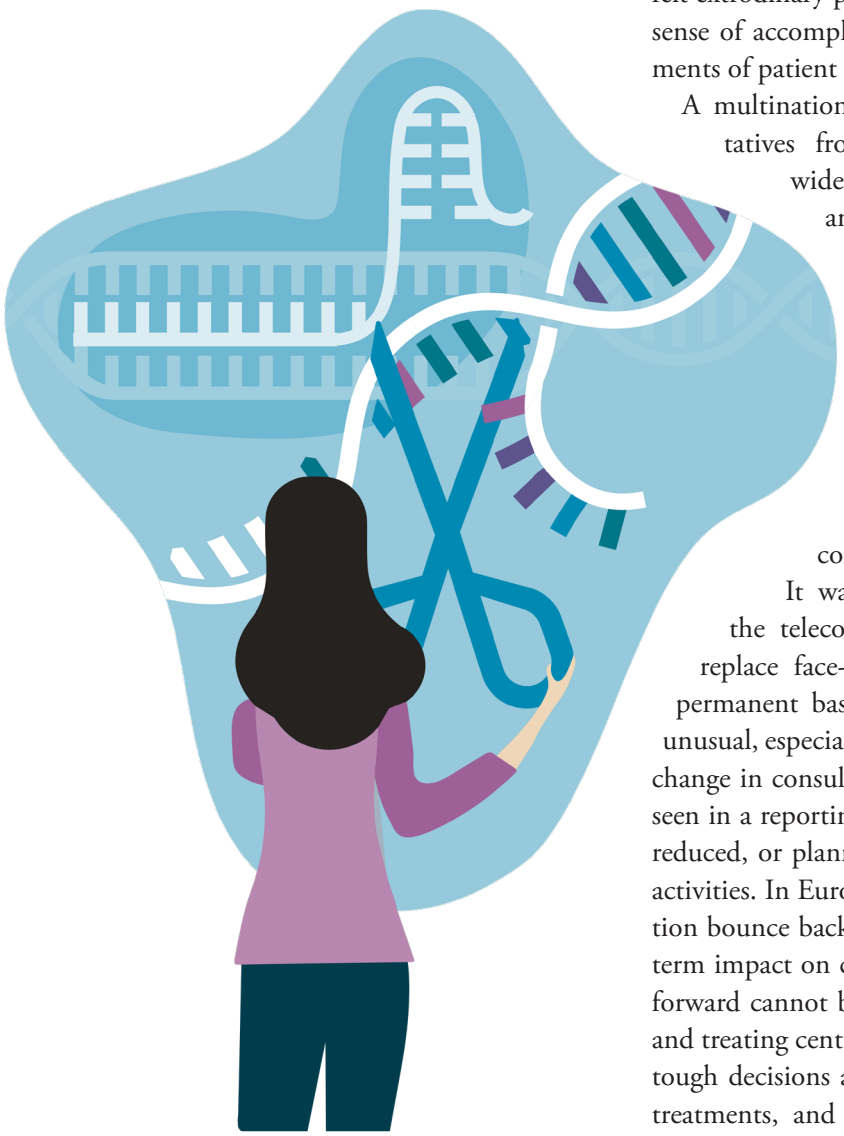
ESMO 2020

The European Society for Medical Oncology's annual congress took place in September. Unsurprisingly, there was much discussion and analysis of the impact of the COVID-19 pandemic on cancer drug trials [1].

The impact of COVID-19 continues to be felt globally and by all patients, not just those suffering from the disease. There were a number of studies presented at ESMO describing the various challenges faced by patients and clinicians during the initial months of the pandemic. A prospective study from France revealed that more patients experiencing modifications to their treatment regime for hematological and solid cancers reported suffering symptoms of post-traumatic stress than those not undergoing changes [2]. Perhaps not surprising given the stress of being treated for cancer is enourmous alone, not to mention in the middle of a pandemic. Perhaps of more interest was that the perceived stress scores were higher amongst caregivers compared to patients. This was again further confounded by the overall high level of professional accomplishment reported by caregivers. This may be partially explained by the fact that only 45% of oncology professionals redeployed during the first wave received appropriate training [3]. We have seen many medical professionals with expert knowledge and skills being redeployed to specialities and areas for which they received little or no additional training since qualifying many years previously. It is hardly unexpected that they felt extrodinary pressure – and likely a strong sense of accomplishment in those brief moments of patient improvements.



A multinational survey of 109 representatives from 18 countries reported widespread delays in surgery and chemotherapy as a result of various lockdown procedures [4]. This resulted in many making the change to teleconsultations. What is interesting here is that 82% of respondants reported plans to continue telemedicine consultations going forward. It was not made clear whether the teleconsultations are planned to replace face-to-face consultations on a permanent basis, which would be highly unusual, especially in the oncology field. The change in consultation mechanisms was also seen in a reporting of 37% of centers having reduced, or planning to reduce, clinical trial activities. In Europe we have seen this reduction bounce back in the main, but the long-term impact on clinical development movng forward cannot be overstated. Trial sponsors and treating centres are again having to make tough decisions about patient consultations, treatments, and clinical studies, as Europe

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> "The impact of COVID-19 continues to be felt globally and by all patients, not just those suffering from the disease. There were a number of studies presented at ESMO describing the various challenges faced by patients and clinicians during the initial months of the pandemic."

and the US appear to be in the midst of a new wave of infections.

Just as we as cell and gene therapy professionals have had to adapt to remote working, conferences, and even happy hours, so too will the clinical trial activities. We must now leverage our learning from the first wave to better serve our patients during the coming challenging months. Perhaps we should be thinking about adapted treatment schedules, remote monitoring, utilizing various communication platforms, and even remote biomarker acquisition, where possible. Last but certainly not least, we will need to look after the clinical teams treating the patients by supporting them with resilience training programmes and other targeted mechanisms to ensure they are able to continue serving their patients.

ESMO 2020 also provided a platform for discussion of challenges to the IP around CAR T cell therapy [5].

Following the explosion in research and development in the CAR-T space, we have now seen one of the first high profile challenges to IP in this field with the successful challenge and revocation of patent EP3214091 covering Tisagenlecleucel, held by Novartis and University of Pennsylvania. The challenge was brought forward by Médecins du Monde and Public Eye, both EU advocacy organisations. The basis of this challenge was the perceived monopoly by therapeutics developers leading to abusive monopolies in cancer care.

This was but one successful challenge and it will not open the floodgates to biosimilar versions of Tisagenlecleucel, but it does set a precedent in this space. With so much new IP being created, will the review process become more stringent, or will we simply see more challenges in future? Perhaps all it will take is more approved products on the market to create sufficient IP diversity?

Clearly, monopolies are not in the best interests of patients or clinicians, but companies must also be rewarded for their innovation and risk taking in developing these amazing life-saving therapies. We need to be vigilant to ensure equitable access to these amazing therapies at all times.

ASGCT POLICY SUMMIT

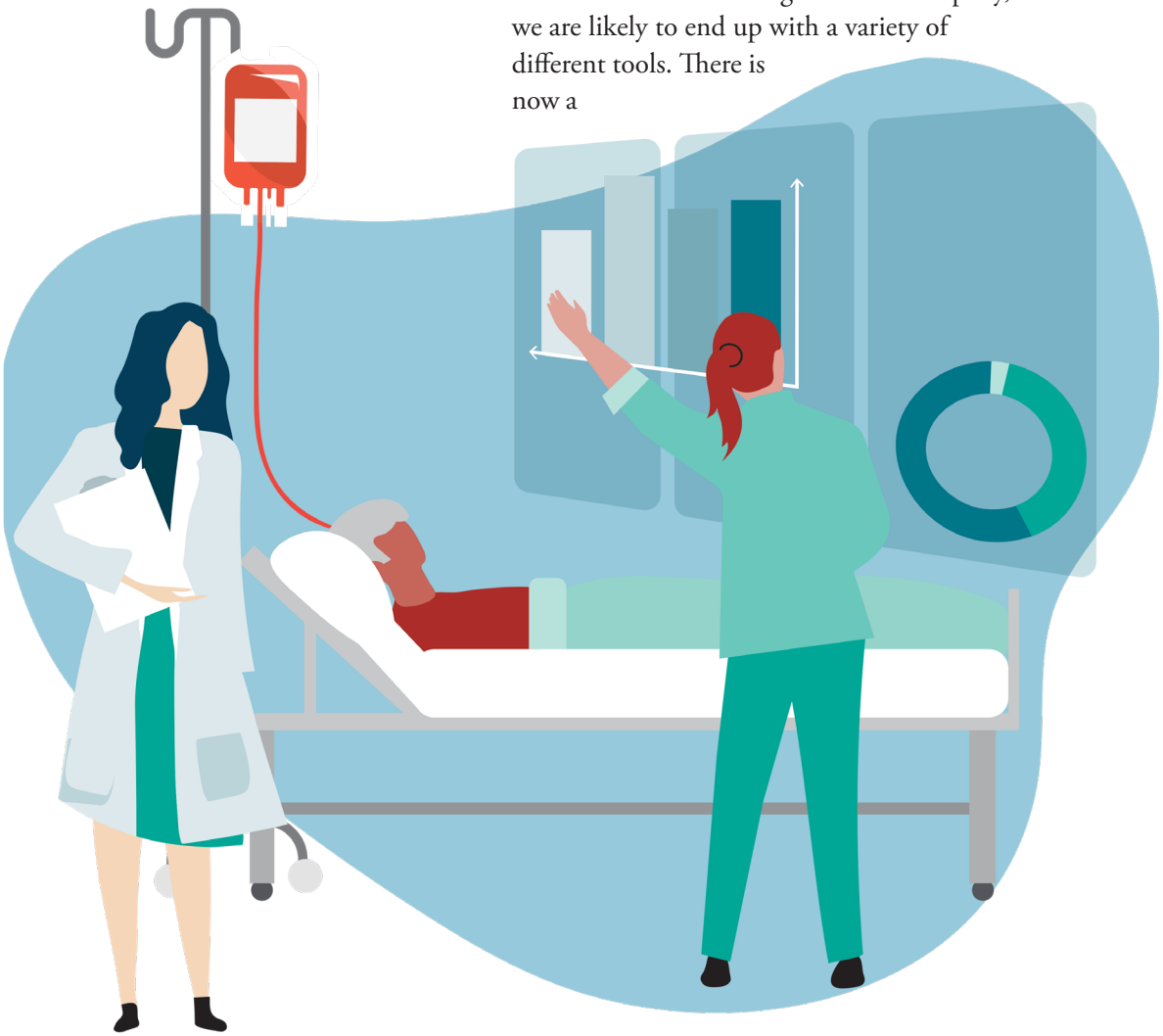
The American Society of Gene & Cell Therapy's annual Policy Summit featured an interesting discussion on ethics by George Daley, MD, PhD of Harvard Medical School [7].

Dr Daley explored the challenges associated with the rapidly moving science and clinical programmes in advanced therapies, especially around gene editing, and even the more recent COVID-19 pandemic. He raised the issue of needing to educate the public on the technologies being used to develop these new therapies, and that this should ideally precede therapy development so that the ethical implications

are not trying to catch up to the science. As might be expected, the topic of germline editing came up. Following a meeting convened by the US National Academy of Medicine, the US National Academy of Sciences, and the UK Royal Society, a report was issued on 3 September 2020 where experts representing 10 countries conclude that germline editing of human embryos still poses too high a risk [8]. They described a number of unresolved ethical and scientific issues, which need to be better understood before being able to move forward, and proposed 11 recommendations. The group's realization that a simple condemnation would not stop certain actors from pursuing germline editing led to the introduction of guidance stating that if it is allowed in any country, it should only be used to fix mutations that are known to cause serious disease. Notable positives include the proposal of an international mechanism whereby investigators are able to report suspected editing that contravenes the recommendations, as well as a new call for education on a wider level. To this end, a further report from the WHO is expected soon, which will address ethical and societal issues of germline editing.

The aforementioned group's report has been met with cautious optimism by researchers globally. What the community needs now is for all countries globally to recognize these recommendations and put in place laws preventing further unscientific misadventures that put patients at risk.

More broadly, as we are now utilizing gene editing clinically and making often permanent genetic changes to patients, it is critical that we monitor our patients over the long-term. This is already a requirement (for up to 15 years) for integrative gene therapies, but with the onus being on each company, we are likely to end up with a variety of different tools. There is now a



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sufficient groundswell to consider an international standard that developers could sign up to. A standard system would make follow-up and monitoring patients who tend to be globally mobile much simpler and more cost-effective in the longer-term. It would also facilitate data related to safety to be collected from a much larger global population as opposed to the current approach taken by some gene and cell therapies, where there are separate systems for the US and Europe. Whilst this approach may be simpler and faster, it is not in the best interests of patients in the longer term.

On the regulatory side, the US FDA's Andrew Byrnes, PhD acknowledged the need for improved communications between the agency and cell and gene therapy developers, notably highlighting the rapidly increasing workload as the chief underlying reason rather than the current pandemic. Dr Byrnes also confirmed that new guidance on CAR T cell therapy would come out by the turn of the year, and that this guidance would include a small section on comparability - encouraging news for the field in light of recent high-profile CMC-related issues encountered by bluebird bio/Celgene and others.

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SVEN KILI provides specialist strategic consulting services to innovative Regenerative Medicine companies. His clients include small and medium sized companies from company formation through to clinical development and commercialisation. He was previouslythe Head of Development for the Cell and Gene Therapy division of GSK Rare Diseaseswhere he led teams developing ex-vivo Gene Therapies for a variety of rare genetic disorders including Strimvelis®, the first ex-vivo gene therapy to be approved for children with ADA-SCID; Wiskott – Aldrich syndrome (WAS); Metachromatic Leukodystrophy (MLD) and Beta-Thalassemia. Prior to this, he was Senior Director, Cell Therapy and Regenerative Medicine for Sanofi (Genzyme) Biosurgery where he led the clinical development, approval and commercialisation activities of the first combined ATMP approval in the EU for MACI®. His team also prepared and submitted Advanced Therapy regulatory filings for Australia and the US, including health technology assessments and he was responsible for late stage developments for Carticel® and Epicel® in the US. Before joining Genzyme, Sven led the cell therapy activities and oversaw all UK & Irish regulatory functions and was the QPPV for pharmacovigilance for the Geistlich Pharma. Sven trained as an Orthopaedic surgeon inthe UK and South Africa and since leaving full-time clinical practise has developed expertise Cell and Gene Therapy in clinical development, regulatory compliance, value creation, risk management and product safety, product launches and post-marketing activities. He sits on the board of CCRM in Canada; Xintela – a Swedish Stem Cell company and is the chair of the CGTAC as part of the UK BIA and the VP of the Standards Co-ordinating Body for Regenerative Medicine. Additionally, he still maintains his clinical skills in the UK NHS and serves as an ATLS Instructor in his spare time.

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INTERVIEW

Driving disruptive innovation in the ATMP field

KERSTIN PAPENFUSS started her PhD at German Cancer Research Centre and then moved to a lab at Imperial College London to work on novel treatment options within the field of tumor immunology. After a Post Doc in academic drug discovery, she since has spent almost ten years in leadership roles at impact-driven organizations advancing medicine and therapeutics, while also securing an executive MBA and a Women in Business award. Before joining DSV to develop science companies designing more effective therapies, Kerstin was transforming ideas for cutting edge cell and gene therapies into investable propositions at UK's Cell and Gene Therapy Catapult.

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Q Can you give us some background to Deep Science Ventures and its involvement in the advanced therapies space in particular?

KP: Deep Science Ventures (DSV) has been going for three and a half years. We are a small fund and a venture builder with a big vision for the future – to generate companies that have the potential to disrupt whole industries.

We believe that there is an alternative way to creating life science ventures, by approaching life science as an engineering problem. This approach creates cross-disciplinary innovation which lends itself to innovation in the cell and gene therapy space, leveraging bioinformatics and AI.

The advanced therapy space is particularly suited to our approach as it allows you to combine multiple components into novel solutions. Initial prototypes and proof of concept (POC) necessary to attract follow-on investment can be generated rather quickly and cheaply, if you compare this to small molecule drug discovery programs, which are investment heavy up-front including screening campaigns and medicinal chemistry programs.

Good examples of our approach in action are the DSV portfolio companies Reflection Therapeutics and Immtune Therapies. Reflection Therapeutics is a company built around a CAR Treg platform – regulatory T-cells that are expressing chimeric antigen receptors, with the addition of a logic gate that ensures that these cells are only ever active at the site of the target tissue. Initially, the company is going after neuron inflammation, which is the underlying mechanism across multiple neurodegenerative diseases, including Alzheimer's, Parkinson's and more. For strategic reasons we are prioritizing Amyotrophic lateral sclerosis (ALS) first as the lead indication but there is also a lot of potential to branch this out into other indications.

Immtune Therapies is also adopting our engineering-style thinking. They have been asking the question: why do CAR T cells have to be generated outside of the body? This is essentially the problem that leads to the long, complicated manufacturing process that is driving up the cost of CAR T therapies. It makes them prohibitively expensive and is a barrier to CAR T becoming a mainstream modality that is used across other markets.

There is actually a lot of evidence in the literature that CAR Ts can be generated *in vivo*, at least in mice. But the existing solutions that have been used in the literature are restrained by lots of bottlenecks such as immunogenicity, oncogenicity, and insufficient loading capacity. More creative approaches are also needed in order to tackle issues such as solid tumors. A solution that overcomes all of these issues has the potential to turn a CAR T approach into an off-the-shelf gene therapy approach that is robust, cheap to manufacture, and requires no personalization.

Immtune are currently working on their POCs, so hopefully we will be able to report some exciting results at the end of the year. Importantly, their solution has been built by combining existing components that have already been individually validated in different contexts, but have never been combined in this novel way. Therefore, their approach is somewhat technically de-risked in comparison to normal early-stage drug discovery projects.

Q Could you go into a little more depth on what differentiates DSV's approach to biotech funding and founding?

KP: As I mentioned, we here at DSV build ventures from scratch. Unlike most venture capital investors that build companies, we do not follow the traditional approach of trying to spot novel technologies coming out of universities and then building experienced management teams around that. While this is an approach that is of course valid, we believe

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"...why do CAR T cells have to be generated outside of the body? This is essentially the problem that leads to the long, complicated manufacturing process that is driving up the cost of CAR T therapies. It makes them prohibitively expensive and is a barrier to CAR T becoming a mainstream modality that is used across other markets."

that there is a whole different space of companies that can be built by approaching science innovation as an engineering project.

What we do is look at a potential market, and then look at what the problems are in this market which are holding the sector back and apply a first principles thinking approach. By way of example, if you apply this to cancer, you find out that the problem in cancer is mostly linked to heterogeneity of the cancer itself. This is actually the thing that is creating resistance, that is creating redundancy, and that allows cancer cells to metastasize.

If you came up with an approach that tackles heterogeneity, you would have something that has the potential to disrupt the whole industry. That is the process we go through. It is important that you always question assumptions – just because something has always been a certain way, doesn't mean that the assumption is correct.

This holds especially true when you are considering potential innovation in other fields. We take a really wide look across technology in other areas to see if there are technological innovations that we can apply in this novel context. Once we have identified an opportunity area where we believe our thinking can be applied, we hire what we call founding analysts. These are entrepreneurial scientists who are very good technically, but who also have all the other qualities that you need in order to set up a start-up company: they have to be resilient; they have to be the type of people that can get other people to follow them just based on their ideas.

For us, it is not necessarily important that they have done this before. We are not just working with serial entrepreneurs, we are also tapping into this pool of talent; of people who can actually do this if they have an ecosystem to support them. That is the sort of ecosystem that we are looking to provide within DSV to help these new entrepreneurs through the early stages of company creation, until they are at the stage where they can raise a proper seed or series A round of funding. This is where other later players come in that can then supplement with all the other functions.

Q Your background is in oncology – what particularly excites you in the way of emerging ATMP innovation in that particular field at the moment?

KP: It is amazing to see the speed of innovation that is currently occurring in the cancer field. People are now willing to try out novel modalities and technologies that have the potential to generate actual cures rather than extending lifespan for just a couple of months.

Coming from a small molecule and antibody background, I have personally reviewed around 100 different drug discovery projects involving kinase inhibitors for cancer. In doing that, you can already foresee all the usual problems of specificity, redundancy, and resistance development. I was really starting to question what I was doing when these ground-breaking results of the first CAR T therapies began coming in. That definitely influenced my decision to become involved in the ATMP space at the time.

What currently excites me is the realization that we are working within a system – the cancer itself, the microenvironment, and then the immune system. We should be considering all the different components that make up the immune system in order to beat cancer.

I am delighted to see that other cell types are now coming into play such as natural killer cells, macrophages, and neutrophils. Adding these to the arsenal will definitely help us address the heterogeneity issue that I mentioned earlier.

And of course, using synthetic biology approaches in a smarter way to make them even more powerful is something that I'm really excited about.

Q What lessons did you learn during your time at the Cell and Gene Therapy Catapult that you have taken forward into your present role?

KP: Quite a few, but I will focus on my top three here.

The main lesson is that I now have a better understanding of the additional commercialization challenges for ATMPs. They differ quite a lot from traditional small molecule or antibody projects, where you just assume that if you have a small molecule and there is a compound, that you can somehow make it. That is not necessarily the same if you are talking about a cell therapy.

> "Just because you can make something on a small scale doesn't mean that you can make it on a large scale. Scale-up and manufacturing considerations need to be examined very early on."

Just because you can make something on a small scale doesn't mean that you can make it on a large scale. Scale-up and manufacturing considerations need to be examined very early on. Also, even if you can make it at a scale and at a sufficient quality that you end up with a representative product, can you make it cheaply enough in order to still be competitive in the market? These issues need to be considered extremely early on, and I have seen a lot of university start-ups that had not considered these questions. That was definitely a learning for me that we are using going forward here at DSV.

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In addition, there is a need for a large network that brings all of these different areas of expertise to the really early stage of the innovation process. You can't leave these things for later, so that is something that I have been trying to do. I am still working here at DSV with a lot of contacts I have established at the Catapult. For example, Keith Thompson, the former CEO of the Cell and Gene Therapy Catapult, has just joined me to be the chair of my advisory board. I am currently building a board of advisors that will allow our new ATMP companies to move from ideation to POC as quickly as we possibly can.

> "...there is a lot of untapped potential in UK academia, which we are trying to leverage."

The third main lesson is that there is a lot of untapped potential in UK academia, which we are trying to leverage. In my roles, a lot of the time I was talking to hugely gifted post-docs and senior scientists who are very talented and have really good ideas. However, they are currently not enabled by the UK ecosystem. Unless you work for a Principal Investigator who has commercial ambition and wants to spin out a company, there are very few ways to do that yourself. Here at DSV, we are looking to change that environment in the UK.

Q How has the pandemic affected DSV and its portfolio companies to date, and how are you helping those companies prepare for further challenges ahead?

KP: It has definitely resulted in a lot of rollercoaster moments, and a lot of grey hair for me! Different things have affected our portfolio companies in the times of Covid. The most obvious one is fundraising. There was a time where fundraising was essentially frozen. We had a few DSV companies that had closed their funding rounds, but they were then finding themselves in a position where just 24 hours before the document was signed, the investors walked away.

In this context we had to come up with creative solutions to keep them going for the couple of months longer that it took to find alternative investors. Innovate UK funding has helped – they introduced a scheme where if you already had an Innovate UK grant, you were given additional funding to get you through this period. The companies that had that were definitely better off. Lab work has also been delayed quite a lot – for example, Immtune Therapies have been hit particularly hard by this. Their POC experiments that are necessary for fundraising were delayed by at least four or five months. Now they are starting up again we are hoping for some good news soon.

It definitely has not been easy. Most of the schemes that were put in place by the government are not necessarily aimed at therapeutic start-ups; in order to qualify for these schemes, you have to be revenue-generating. Obviously, if you are a therapeutic biotechnology company, you won't generate revenue until you sell. We found ourselves in a tough spot that we had to navigate but overall, I think that we have weathered the situation pretty well. All of our companies are still going, and some of them are doing better than before.

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Q What ATMP investment trends do you expect to emerge or evolve over the course of the next year?

KP: I anticipate that we will see the trend of 2020 continuing, which has seen unprecedented amounts of investment going into the ATMP space. Many of the more established players are advancing their products and are moving through clinical trials, hopefully successfully.

I think we will see more and more investment into perceived standards, such as for therapies involving adeno-associated viral (AAV) vectors – I get asked about these a lot.

What I am personally hoping for – although this might be wishful thinking – is that the positive developments in the sector will serve as a de-risking function for the sector as a whole, and we will see more investment into the start-up space, too.

It is still difficult to get cell and gene therapy start-ups off the ground in the UK unless you have extremely deep pockets. There isn't necessarily the realization yet that cell and gene therapy start-ups need a lot more money upfront than traditional small molecule and antibody projects in order to get all the way through preclinical development to the clinic, as you need to address the additional challenges that I was talking about earlier.

Q What "do's and don'ts" would you give a cell therapy or gene therapy biotech seeking investment in the current environment?

KP: Do's: go for it. There is such an impetus in the whole sector and investment community for cell and gene therapies at the moment. I would take advantage of that – don't be shy, go and talk to as many people as you can.

In terms of don'ts, this is probably slighted biased by the very early stage start-up environment that I operate in. I see a lot of companies or start-ups that approach us saying that they are able to make these cells, so let's create a company around it. However, they haven't put real thought into what the potential market is, and what the best path to market would be. Figuring out where you actually have a product market fit for these types of cells is crucial. That is what investors want to see.

Also, there is usually a very narrow look at the competition. "Nobody else is doing this exactly the way that I am doing it" – yes, that is fair, but there are a lot of different ways to solve the same problem. Taking a wider look at the competition is necessary for some of the approaches that we see, especially because cell therapies are very expensive. If there are other solutions in the pipeline that use a much cheaper modality, that is something you definitely need to be aware of.

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Investor Insight

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COMMENTARY

Boldly de-risking development of impactful cell and gene therapies: The California Stem Cell Agency's $3B funding model

Shyam Patel, Sohel Talib & Maria T. Millan

The California Institute for Regenerative Medicine (CIRM) is California's Stem Cell fund-ing agency [1]. Since its inception in 2004, CIRM has deployed $2.7B to advance develop-ment of stem-cell based regenerative medicine therapies for patients with unmet medical needs. This perspective highlights 3 elements of CIRM's funding model that have enabled California academic researchers and companies to de-risk development of novel regenera-tive medicine therapies and attract biopharma industry support. To date, CIRM has funded over 1000 projects including 64 regenerative medicine clinical trials; over half the clinical trial projects have secured biopharma industry support. Overall, CIRM funding has enabled the launch of 44 companies and CIRM-funded projects have drawn in a cumulative $9B in industry investments.

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The California Institute for Regenerative Medicine is a unique state agency tasked with deploying $3B in California state funds to support research of regenerative medicine treatments and cures based on pluripotent stem cell, progenitor cell and other vital medical technologies. Since its inception in 2004 after voter approval of Proposition 71, CIRM has allocated $2.7B in grant funding to over 1000 projects that have supported basic biology research, development of novel stem cell-based technologies and therapies, stem cell education, workforce training and strategic infrastructure development. CIRM currently supports over 132 active projects and expects to fund several more before fully allocating its research funding.

The active portfolio consists of 118 active therapeutic development projects that span all disease areas including oncology, cardiovascular disease, diabetes, ophthalmology, neurodegeneration, rare immune disorders, sickle cell disease and, most recently, COVID-19 [1]. While a majority of the projects are cell therapies and gene-modified cell therapies the portfolio also includes small molecules, biologics and gene therapies (Figure 1). To date, CIRM has directly funded 64 clinical trials and has enabled an additional 31 non-CIRM funded clinical trials through its support of earlier stage research in those programs. This perspective will describe how CIRM's unique public funding model has de-risked the discovery and development of stem cell-based treatments until they are ready to be partnered by the biopharma industry.

Over the years, the CIRM de-risking model has attracted robust biopharma industry investment into CIRM-funded projects including: partnering of 50% of CIRM-funded clinical trials, spinout of 44 companies, and overall commitment of $9B.

Over 50% of CIRM-funded clinical trials are backed by venture capital, public capital markets and/or strategic biopharma partners. CIRM funding of California academic R&D projects have enabled the launch of at least 44 spinout companies. Recent company launches include Jasper Therapeutics with $50M in Series A funding to enable antibody-based bone marrow conditioning for cell and gene therapies and Aspen Neurosciences with $70M in Series A funding to develop induced pluripotent stem cell-based therapies for Parkinson's Disease. CIRM has tracked a total of $9B in industry funding committed to CIRM-funded projects since 2014. The industry dollars cover the entire spectrum from angel investments to public offerings and biopharma acquisitions. Over the past 2 years, three CIRM-funded companies have issued IPOs including Forty Seven, Inc., Orchard Therapeutics & Poseida Therapeutics, and two CIRM-funded companies have been acquired including Forty Seven's acquisition by Gilead Sciences and Asterias Therapeutics' acquisition by Biotime, Inc. (now Lineage Therapeutics). In addition, a recent economic impact report [2] prepared by University of Southern California researchers estimated that CIRM funding has generated 56,000 jobs and added $10B to the California economy.

Based on CIRM's experience, the three critical elements of CIRM's de-risking approach are:

1. Ensuring that funding mechanisms bridge the entire translational "Valley of Death"
2. Constantly optimizing funding models to meet the needs of a rapidly evolving industry
3. Championing the portfolio and proactively engaging potential industry partners

BRIDGE THE ENTIRE VALLEY OF DEATH

At the time of Proposition 71 approval in 2004, the field of human embryonic stem cells was in its infancy with just over 132 scientific publications comprising the collective knowledge. The discovery of human induced pluripotent stem cells, which overcame several of the ethical and practical challenges of embryonic stem cell research, wouldn't be

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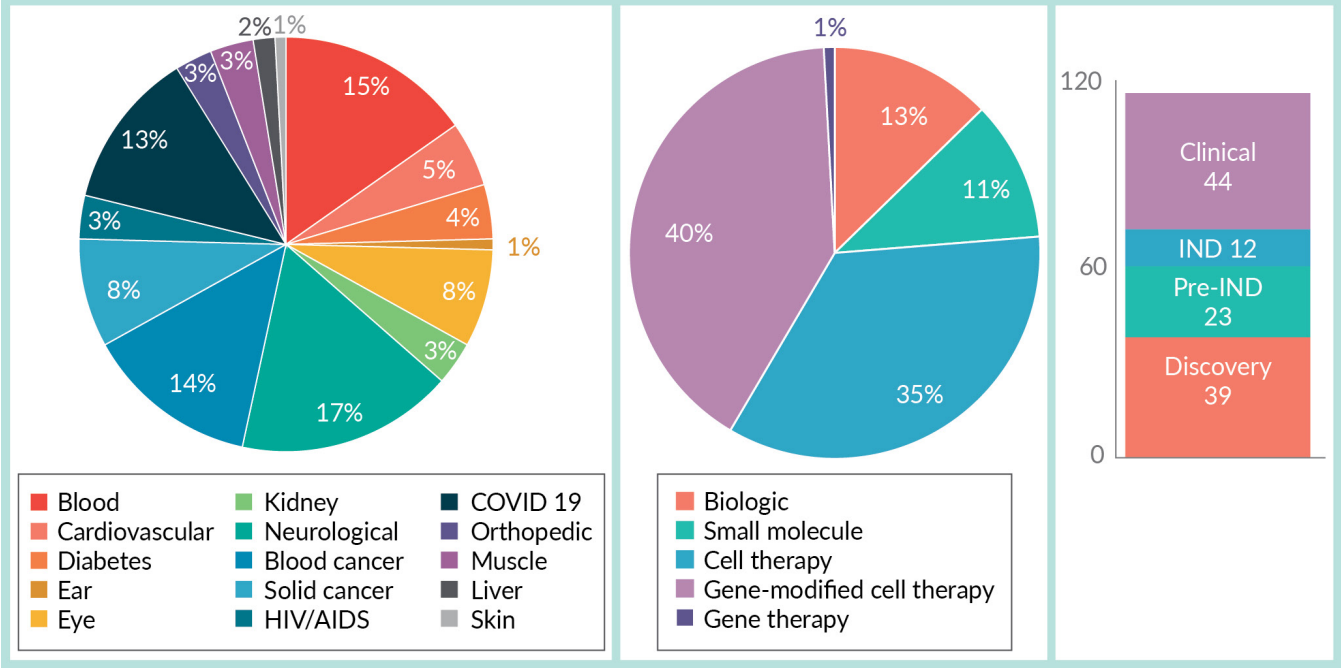
FIGURE 1

CIRM currently supports 118 active therapeutic development projects across discovery, pre-IND, IND and clinical trial stages.



A majority of the projects are developing cell therapies or gene-modified cell therapies but small molecules, biologics and gene therapies are also represented. The 118 active projects cover a broad range of disease areas including COVID-19.

published for another 3 years [3]. The seminal preclinical proof of concept of genetically engineered CD19 CAR-T cells had been published just a year earlier [4]. The field of gene therapy, a core pillar of regenerative medicine today, was still struggling to learn the ethical, scientific and clinical lessons from the tragic death of Jesse Gelsinger in a gene therapy clinical trial just a few years prior [5]. Suffice it to say, when CIRM first became operational and started allocating grant funding in 2006, the immediate focus was on seeding the field to build a critical mass of expertise, resources, and infrastructure for fundamental pluripotent stem cell and regenerative medicine research in the state [6].

Having said that, CIRM's foray into translational research and development occurred relatively early in the agency's lifecycle when it launched a funding initiative to support candidate discovery of stem cell-based therapies in 2008. From 2008-2013, CIRM issued two sets of funding initiatives encompassing the entire pathway of therapeutic development from candidate discovery, IND-enabling preclinical studies as well as clinical trials. Early stage research on candidate discovery and translational bottlenecks was supported by the Early Translational Research funding initiative. The bulk of the translational research pathway from late-stage candidate discovery research through clinical trials was covered by Disease Team funding initiatives. The Disease Team awards were envisioned as collaborations of multi-disciplinary teams including scientists, clinicians, manufacturing experts and regulatory advisors to tackle development of disease-modifying therapies for a particular disease.

A crucial hallmark of both initiatives was commitment of substantial long-term funding to support the research and development activities. For example, the Early Translational Research awards provided up to $6.7M over 3 years and the Disease Team awards provided up to $20M over 4 years. Contrast these funding levels to the National Institutes of Health's (NIH) Small Business Innovation Research (SBIR) funding programs, which collectively represent the largest source of

seed funding in the country. The two phases of SBIR awards, which are roughly analogous to CIRM's aforementioned funding initiatives, currently provide up to $250K for 6 months of initial R&D and up to $1.7M for two years of later stage R&D.

CIRM's Early Translational and Disease Team initiatives were offered a combined 7 times between 2008-2013 and committed $643M in research funding. The Early Translational awards resulted in the discovery of at least 7 therapeutic candidates that would go on to clinical studies and at least 18 candidates that are currently in preclinical development. The Disease Team initiative helped progress at least 14 preclinical candidates to clinical trials. Combined, both initiatives helped launch 17 spinout companies.

Both these initiatives uniquely enabled California academic researchers to de-risk their therapeutic candidate deep into the development pathway at their own academic institutions thereby setting them up to attract significant industry investments. In several instances, CIRM funding supported preclinical discovery and development in a California academic institution followed by successful launch of a spinout company. The company then leveraged CIRM, industry and other funding sources to progress the therapeutic candidates through clinical studies. For example, a combined $19M in Early Translational and Disease Team award funding enabled Dr. Henry Klassen's team at UC Irvine to discover, develop and complete a phase 1 study of a retinal progenitor cell therapy for the rare blinding disease retinitis pigmentosa. The UC Irvine team launched the spinout company jCyte, which went on to secure an additional $8M in clinical trial award funding from CIRM to complete a phase 2b study. On May 8th, 2020, jCyte entered an ex-US licensing and commercialization agreement with Santen Pharmaceutical and will receive up to $252M, which includes $62M in up-front cash. CIRM Disease Team award funding also enabled Dr. Irving Weissman and the Stanford University team to discover, develop and obtain first-in-human clinical data for the innovative anti-CD47 antibody immunotherapy approach to cancer. The spinout, Forty Seven, Inc., then leveraged CIRM funding as well as venture and public market financing to progress clinical development of the lead candidate until its acquisition by Gilead Sciences in April 2020 for $4.9B.

These funding initiatives also enabled small companies to de-risk truly novel stem cell technologies such as Viacyte, Inc.'s, embryonic stem cell-derived cell replacement therapy for diabetes. In fact, Viacyte has utilized $72M in CIRM funding to drive two generations of its cell-device combination therapies to the clinic and to initiate early stage research of a universal cell therapy. In 2018, the company reported strategic collaborations with Gore & CRISPR Therapeutics as well as an $80M Series D venture financing round to support progression of all three pipeline programs.

CONTINUAL OPTIMIZATION OF THE FUNDING MODEL

Over the last decade the cell and gene therapy industry has undergone rapid growth. Both biopharma investment and venture capital investment have grown steadily year over year between 2010 and now. Analyses by Smith, et al. of large biopharma investments between the period of 2010 and 2016 showed that almost every major biopharma company had committed capital to cell and gene therapy development [7]. Similarly, venture capital investment in regenerative medicine saw 34% year over year growth between 2011-2016 [8]. The 2019 annual report from the Alliance for Regenerative Medicine (ARM), an industry advocacy group, showed that global venture capital investment in regenerative medicine had reached $4.9B in 2019 [9]. This same period saw the groundbreaking US Food and Drug Administration (FDA) approvals of chimeric antigen receptor T (CAR-T) cell therapies such as Kymriah,

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Yescarta and Tecartus and gene therapies such as Luxturna and Zolgensma. Along with the multibillion-dollar acquisitions of late-stage companies such as Kite Pharma and Avexis, there were also billion-dollar acquisitions of preclinical stem cell companies such as Semma Therapeutics and Bluerock Therapeutics suggesting robust biopharma appetite for these advanced technologies.

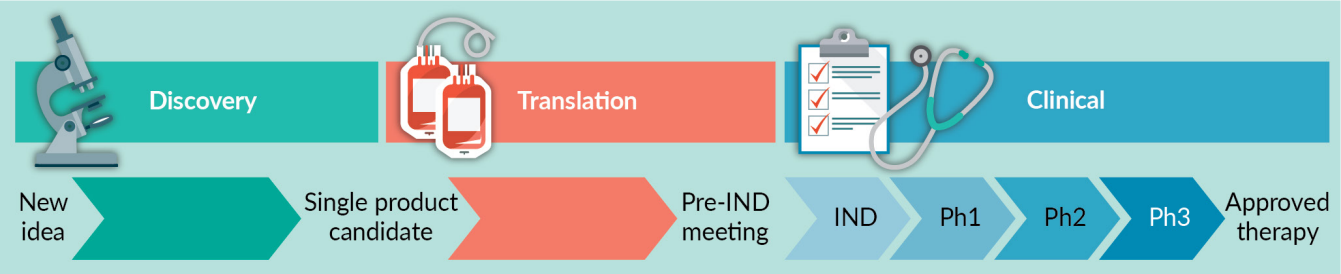
After assessing the state of the industry in 2015, CIRM overhauled its entire operational model to better serve cell and gene therapy development and to anticipate the industry's future needs. CIRM innovated a systems-based funding approach to move projects efficiently and rapidly through the development pathway from candidate discovery to phase 3 clinical trials (Figure 2). The systems-based approach was designed to overcome several limitations of the previous initiatives-based model such as slow, discontinuous funding opportunities and the financial and project risks associated with large denomination, multi-year awards. The former was addressed by three always-on funding programs covering candidate discovery (DISC), translational development (TRAN), and clinical trials (CLIN) that rapidly reviewed and approved scientifically meritorious projects for funding. By gating the funding to well-established regulatory milestones such as pre-IND meeting, IND clearance, etc., both the expected outcomes and eligibility criteria were clearly defined. The financial and project risks were mitigated in two ways:

1. By adopting a milestone-gated disbursement model similar to that utilized by the venture capital community and
2. By requiring all for-profit grantees and late-stage non-profit grantees to have "skin in the game" through commitment of co-funding to the project.

Combined, these were designed to promote timely execution of project tasks in a capital efficient manner.

CIRM envisioned these awards as true partnerships between CIRM and the grantee. As such, it also established strategic infrastructure and advisory services to further accelerate achievement of project milestones and outcomes. The strategic infrastructure funding programs established the Alpha Clinic Network and the IQVIA Cell and Gene Therapy Center. The Alpha Clinic Network comprises six leading California medical centers that have utilized CIRM funding to establish specific expertise in cell and gene therapy clinical trials. The network works collectively to implement solutions such as rapid trial startup, efficient patient recruitment, broader patient access and coordinated manufacturing support [10]. The Alpha Clinic Network has supported over 130 trials, both CIRM-sponsored and industry-sponsored, in more than 40 disease indications. The cell and gene therapy trials supported by the network range from phase 1 trials for small companies such as Angiocrine Biosciences to Kite Pharma's ZUMA-9 study. The CIRM-funded

FIGURE 2



CIRM's systems-based funding model divides the funding opportunities into three stages that span candidate discovery through phase 3 clinical trials. Therapeutic development projects may enter at any stage and are expected to progress from one stage of funding to the next.

IQVIA Cell and Gene Therapy Center comprehensively supports both preclinical and clinical development of cell and gene therapies by leveraging the expertise and resources of IQVIA, Charles River Laboratories, Wuxi Apptec and City of Hope. It serves the needs of CIRM-funded and non-CIRM funded clients in areas such as project management, pre-clinical research, assay development and manufacturing and process development support.

To foster a collaborative approach toward achievement of CIRM-funded project milestones, CIRM utilizes a unique advisory panel support structure for both its TRAN and CLIN programs. The advisory panels leverage both internal CIRM expertise as well as external domain-specific expertise to help CIRM portfolio projects overcome bottlenecks and execute project tasks. The advisory panels have provided specific guidance and solutions to help project teams overcome manufacturing delays, design clinical trials with patient input, enhance trial enrollment, and apply for expedited regulatory designations.

This systems-based funding approach, which deployed $863M through 2019, has dramatically shifted CIRM's portfolio toward therapeutic assets. It added 98 discovery stage projects, 36 pre-IND stage projects, 21 IND-stage projects and, most notably, 46 clinical trial projects. One of the first programs to utilize the CLIN funding mechanism was a lentiviral gene therapy trial for adenosine deaminase severe combined immunodeficiency (ADA-SCID) at University of California, Los Angeles. This enabled the launch of Orchard Therapeutics, which went on to raise several rounds of venture financing before issuing a $225.5M IPO in 2018. Similarly, Poseida Therapeutics received an $18M CLIN award to fund the first-in-human phase 1 clinical of its next generation P-BCMA-101 CAR-Tscm cell therapy for multiple myeloma. P-BCMA-101 incorporated several innovations including a non-viral genetic engineering method, a fully human CAR construct and a high percentage of stem central memory T cells. The company leveraged its progress in the CIRM-funded clinical trial to secure three rounds of venture financing totaling $282.5M, including a $75M Novartis investment, and then issued a $204.8M IPO in July 2020.

CIRM's funding mechanisms have also enabled academic researchers and their institutions to continue de-risking therapeutic candidates after securing a licensing partner early in development. For example, Mustang Bio launched after licensing City of Hope's CAR-T cell technology in 2015 based on candidates discovered as part of a CIRM Early Translational Award. City of Hope continues to drive development of the CAR-T candidates by leveraging CIRM CLIN awards for clinical trials in glioma and brain-metastatic breast cancer. Similarly, AVROBIO licensed UC San Diego researcher Dr. Stephanie Cherqui's genetically engineered HSC therapy for the rare disease cystinosis at the preclinical stage. Dr. Cherqui and her team at UCSD progressed the candidate through IND-enabling studies and are currently studying it in a phase 1/2 clinical trial with CIRM CLIN awards.

The systems-based funding model has enabled CIRM to continually adapt its programs to the agency's funding situation as well as to emerging biomedical research needs and opportunities. In early 2019, as CIRM started deploying the last of its research funds, it took the opportunity to extend the impact of its research funds and address the unmet medical need in sickle cell disease. CIRM partnered with the National Heart, Lung and Blood Institute (NHLBI) on its initiative to cure sickle cell disease in 5-10 years. CIRM committed $30M to cost-share gene and cell therapy IND-enabling and clinical trials projects with NHLBI. The partnership also enabled NHLBI to adopt CIRM's accelerated, milestone-driven funding model. As of this writing, the program is actively soliciting and funding gene and cell therapy projects.

In 2020, CIRM had suspended soliciting new applications for all its funding opportunities save the sickle cell program. As the COVID-19 pandemic took hold in Spring

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2020, CIRM again was presented with a challenge to address the urgent need. It launched a rapid funding opportunity for COVID-19 therapy development that promised to evaluate and fund applications within 30 days. Between April-July 2020, the program funded 3 clinical trials and 17 drug discovery projects. Finally, as it recovered funds from closed awards through the first 6 months of 2020 CIRM rapidly re-opened its DISC and CLIN programs to fund additional therapeutic development projects, which are being reviewed for funding at the time of this writing.

On a broader level, CIRM has played a proactive role in engaging both the public and federal agencies to advocate for safe and effective stem cell-based therapies. It launched a "Stem Cell Champions" campaign to engage the patient community and to advocate for efficient yet safe regulatory approaches for stem cell-based treatments at the FDA. CIRM was also an early and strong advocate for the 21st Century Cures Act, which introduced the Regenerative Medicine Advanced Therapies (RMAT) designation for cell, gene and regenerative medicine therapies. RMAT designation has enabled early and often engagement from the FDA to efficiently guide clinical development of these innovative therapies. To date, 7 CIRM-funded clinical stage therapeutic candidates have received RMAT designation.

While the current operational model prioritizes support of therapeutic development programs, CIRM recognizes the importance of education and workforce training in supporting the future growth of the California regenerative medicine economy. For the past 11 years, it has funded stem cell research training for over 1400 undergraduate and masters students, many of them first-generation, at 16 California universities and colleges. The training programs incorporate coursework, laboratory internships as well as patient engagement to prepare students for careers in stem cell research and development. In addition, over the past 8 years, CIRM has funded stem cell research internships for almost 500 high school students at 10 leading California research institutions. The internships provide high school students with meaningful exposure to stem cell coursework, hands-on research, and engagement with patients.

CHAMPION THE PORTFOLIO

To further enhance industry partnership opportunities for its portfolio projects, CIRM launched the Industry Alliance Program (IAP) in 2018 [11]. The IAP leverages CIRM's resources to proactively engage the biopharma industry about its portfolio, and to facilitate one-on-one interactions between industry partners and its portfolio projects. The IAP leverages CIRM resources to facilitate and assist both CIRM grantees and industry partners at all stages of a prospective partnership interaction. For industry partners, IAP will initially provide curated access to CIRM's project portfolio based on specific criteria. Then, it will facilitate introductory meetings with CIRM grantees. As the interaction progresses, the IAP accelerates the partnering process by coordinating with academic technology licensing offices, helping CIRM grantees prepare data rooms, advising all parties on CIRM regulations and advising CIRM grantees on deal terms.

The CIRM Business Development team routinely engages a broad range of biopharma industry stakeholders including family offices, angel networks, venture capital firms, small and large companies and incubators/accelerators. To date, the IAP has also formally enrolled 8 partners with demonstrated commitment to cell and gene therapy development: Bluerock Therapeutics, Vivo Capital, Panacea Venture, Novo Nordisk, Vera Therapeutics, Frequency Therapeutics, ElevateBio and Bayer. The enrolled IAP partners represent companies both small and large, multi-national venture firms and innovative accelerators.

Over the past 18 months, the IAP program has enabled over 50 one-on-one partnership interactions across CIRM's portfolio from discovery stage pluripotent stem cell therapies to clinical stage engineered HSC therapies. CIRM's broader vision for the Industry

Alliance Program includes applying the IAP partners' resources into CIRM's funding model and to facilitate partnering opportunities between IAP members that will broadly benefit the California regenerative medicine economy.

Forward Looking Conclusion:

The cell and gene therapy industry in the state of California is showing little signs of significant slowdown even in the current economic environment. Looking forward, as the industry grows and matures, manufacturing challenges will continue to pose significant risks both for individual projects and the industry as a whole. Truly collaborative approaches are needed to solve the dual bottlenecks of manufacturing expertise and capacity. In particular, cross-functional partnerships that deeply integrate the operational experience of contract manufacturers with the technical expertise and innovative capacity of academic institutions and cell and gene therapy companies may be required at all stages of manufacturing process development. These public-private partnerships should also be utilized to educate and train a robust manufacturing workforce capable of immediate contribution at both the technical and leadership levels.

Similar coordination of funders, investors and strategic partners may also be beneficial. There are several sources of capital for cell and gene therapy researchers and developers. These include the prominent sources such as federal agencies, venture capital, public capital markets and biopharma but also emerging sources such as state funding agencies, disease foundations, angel investor networks and university systems. While any individual cell and gene therapy developer will thread several of these capital sources along its long march to the market, there exists relatively little coordination between the funding sources themselves. This presents an opportunity for a coordinated approach that leverages both the capital and domain expertise of all stakeholders to drive a portfolio of cell and gene therapy technologies from discovery to commercialization. Along the way, the coordinated effort could probably even realize impactful solutions to systemic challenges such as manufacturing, regulatory, pricing, and real-world patient access.

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