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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:
New horizons for cell therapy: emerging platforms

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SPOTLIGHT

EXPERT INSIGHT

CAR T cell immunotherapy of epithelial ovarian cancer: past, present and a view of the horizon

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Women diagnosed with ovarian cancer often face a poor prognosis, particularly owing to late diagnosis. The treatment of this highly lethal malignancy has remained largely unaltered over the last few decades and novel strategies to combat the disease are urgently needed. As it is considered an "immunologically cold" tumor, harnessing strategies that overcome immunosuppressive barriers that operate in this cancer may lead to improved therapeutic impact. One such approach entails the use of chimeric antigen receptor (CAR) T cell therapy. In brief, T cells are re-targeted to selected tumor-specific antigens in a human leukocyte antigen (HLA)-unrestricted manner, endowing the cells with potent signaling capacity. Impressive success has been achieved using CAR T cells to treat selected hematological malignancies. However, these cells encounter several additional hurdles when targeting solid tumors. Nevertheless, numerous studies have designed strategies to improve responses to CAR T therapy of solid tumors. Outlined in this review are CAR T cell-based approaches that have been evaluated in the treatment of ovarian cancer. We discuss both pre-clinical and available clinical data on the effects of these therapies against a range of ovarian cancer targets that include folate receptor a, mesothelin, MUC16, the ErbB family of receptors and ligands of the NKG2D receptor.

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INTRODUCTION

As the most lethal gynecological malignancy, treatment of ovarian cancer remains a major challenge. Ninety percent of tumors are of epithelial origin (EOC), of which the most common and prognostically unfavorable subtype is high-grade serous ovarian cancer (HGSOC). Current standard-of-care comprises initial debulking surgery followed by adjuvant taxane- and/or platinum-based chemotherapy [1,2]. Though complete clinical responses are observed frequently, recurrence rates reach 75% for those diagnosed at stage III or IV [3]. Although immunotherapy has revolutionized the treatment of other cancer types, ovarian tumors are generally immunologically 'cold' and responsiveness to immune checkpoint inhibition remains disappointing [4,5].

An alternative approach to harness the immune response entails adoptive cell therapy, particularly the use of chimeric antigen receptor (CAR) T cells [6]. Immunotherapy using CAR T cells has proven remarkably successful in the treatment of selected hematological malignancies, and cell therapies in general have surpassed the achievements thus far seen with immune checkpoint inhibitors [7]. Broadly, patient-derived T cells are engineered to express a synthetic fusion receptor that engages one or more cancer-specific cell surface antigens in an HLA-independent manner. This is especially relevant in ovarian cancer where HLA downregulation correlates with disease severity [8]. The CAR targeting domain typically consists of a single chain variable fragment (scFv) derived from immunoglobulin variable domains, although a number of alternatives such as peptides and ligand derivatives have also been used. The targeting moiety is tethered via a spacer and transmembrane domain to a T cell activating module, such as CD3ζ. These 1st generation CARs were further modified to contain either one (second generation) or two (third generation) co-stimulatory units, such as CD28 or 4-1BB, leading to improved activation and persistence respectively [9–12]. Nonetheless, CAR T cell treatment of solid tumors has proven disappointing. The highly hostile and immunosuppressive tumor microenvironment (TME) and surrounding stroma hinder tumor penetration and T cell persistence. Moreover, suitable targets are difficult to select, due to heterogeneous expression and the ability of cancer cells to downmodulate expression. Target selection is further hindered by the lack of 'true' tumor-specific antigens [13,14]. Nevertheless, there are over a hundred studies actively recruiting patients, and many completed studies, evaluating the efficacy of CAR T cells in solid tumors (ClinicalTrials.gov, April 2022).

Given the poorly immunogenic nature of ovarian cancer, CAR T cell immunotherapy presents as an exciting therapeutic opportunity in this context. Ovarian tumors express a large number of tumor-specific antigens, with 62 markers identified through high-throughput screening, thereby offering a variety of potential targets [15]. Thus far, five of these targets have been extensively studied in the context of CAR T cell therapy, namely the folate receptor/folate binding protein (FR/FBP), MUC16, αvβ6, mesothelin, and the ligands of the natural killer cell NKG2D receptor [16]. Moreover, since ovarian cancer primarily undergoes locoregional dissemination, there is a possibility to administer CAR T cells intraperitoneally (i.p.), as opposed to the standard intravenous (i.v.) route.

CANDIDATE TARGETS FOR OVARIAN CANCER CAR T CELL IMMUNOTHERAPY

Folate binding protein (FBP)/folate receptor (FR)

The first attempt at CAR T cell treatment of ovarian cancer was directed against folate-binding protein (FBP), also known as folate receptor alpha (FR/FRα). This receptor mediates cellular uptake of folic acid and is upregulated on ~90% of EOCs, whilst undetectable on healthy somatic tissue [17–19]. The CAR consisted of the MOv18 scFv

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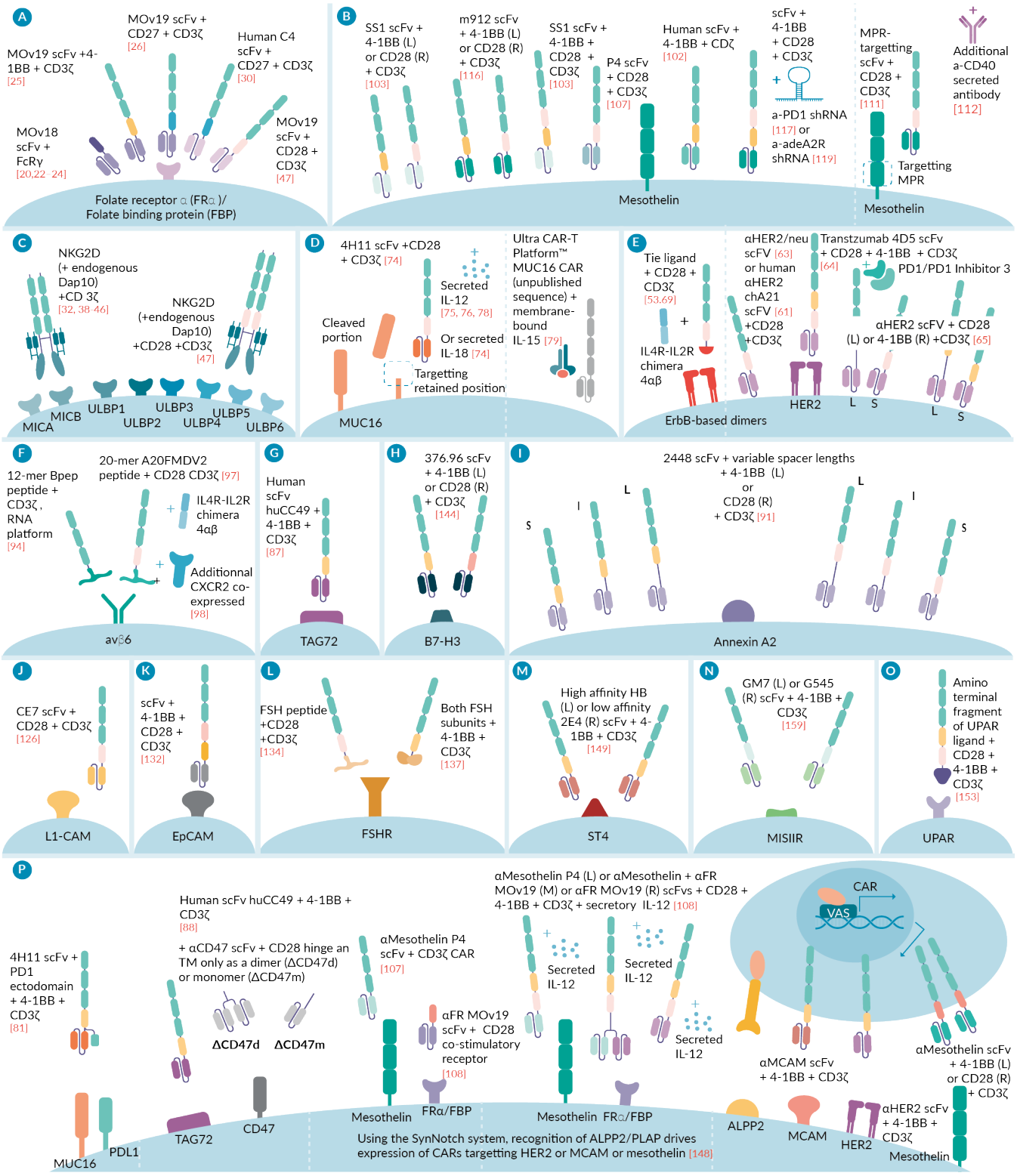
coupled to the Fc receptor-associated γ-subunit, termed MOv-γ (Figure 1A). Melanoma-derived CD8+ tumor-infiltrating lymphocytes (TILs) were retrovirally transduced with MOv-γ, enabling lysis of the IGROV-1 human ovarian carcinoma cell line and cytokine release [20]. This approach was further assessed *in vivo* in a fully murine model in which control of pulmonary metastasis was achieved. In an intraperitoneal (i.p) IGROV-1 model in nude mice, MOv-γ TILs significantly extended survival compared to untransduced TILs or those with irrelevant specificity, illustrated by the 100% survival rate at ~day 50 post MOv-γ TIL infusion, compared to ~20% for the latter groups [21]. Impressively, patient T cells transduced with MOv-γ maintained functional activity against ovarian cancer for up to several months [22]. In a separate study, the same group expressed the MOv18 CAR in alloreactive T cells. Mice that were subsequently immunized with allogeneic splenocytes prior to tumor challenge were effectively protected, in contrast to mice that were only immunized or treated with MOV-γ cells alone. The group also showed that human dual-specific CAR T cells could be generated similarly, demonstrating responsiveness to allogeneic peripheral blood mononuclear cells (PBMCs) and the IGROV-1 cell line [23].

These encouraging outcomes prompted the evaluation of MOv-γ T cells in a two-cohort Phase I clinical trial (ClinicalTrials.gov: NCT00019136) in relapsed/ refractory FBP+ EOC. The first cohort (8 patients) received escalating doses of intravenous (i.v.) MOv18 CAR T cells in combination with high dose of IL-2. The second cohort (6 patients) received autologous dual-specific T cells using the same dose escalation regimen and which were stimulated *in vitro* with allogeneic PBMCs prior to infusion. This was followed by s.c immunization with allogeneic PBMCs from the same donor, without IL-2. The modified T cells were well-tolerated, although some grade 3 or 4 toxicities were attributed to high dose IL-2. However, administration of 111In-labelled CAR T cells demonstrated poor T cell trafficking to the tumor site and no therapeutic efficacy was observed. Moreover, poor T cell persistence was indicated by loss of detectable cells after day 5. Importantly, unlike in their murine model, T cells did not expand *in vivo* following s.c. immunization with allogeneic PBMCs. This was compounded by a human anti-mouse inhibitory response against the murine scFv [24].

These disappointing results prompted efforts to optimize the CAR using a Mov19 scFv, fused onto either CD3ζ alone or with additional co-stimulation through 4-1BB (Figure 1A) [25]. The provision of co-stimulation enhanced IFNγ secretion without alteration of cytotoxicity. In a s.c SKOV3 xenograft model established in NOD/scid/ IL2rγ-/- (NSG) mice, intratumoral (i.t) treatment with MOv19ζ only marginally delayed tumor outgrowth, whereas administration of MOv19BBζ T cells mediated complete tumor regression. A comparison of i.t., i.v. or i.p. administration of these T cells showed little difference, where the latter two methods had slightly delayed results. Importantly, T cells equipped with additional co-stimulation persisted for longer and numbers inversely correlated with tumor burden. This phenomenon could be partly attributed to enhanced expression of the anti-apoptotic marker Bcl-XL. While anti-tumor activity required scFv engagement, persistence was solely attributed to the presence of 4-1BB. Administration of MOv19BBζ T cells also significantly extended survival in a xenogeneic i.p model of metastatic ovarian cancer and a model of pulmonary metastatic spread [25] Notably, the group noted very similar results when CD27 was used as a source of co-stimulation [26]. The T cells displayed a central memory phenotype (CD45RO+CD62L+CD28+CD27+) and achieved a survival advantage due to Bcl-XL upregulation. They secreted higher quantities of IFNγ, TNFα and IL-2 and lower amounts of anti-inflammatory IL-4 and IL-10 compared to first-generation counterparts. When compared *in vivo* using a s.c SKOV3 model all three second-generation CAR T treatments (e.g. CD28, CD27 or 4-1BB

FIGURE 1

The design of ovarian cancer-targeted CARs, either as a stand-alone therapy or in combination with cytokine or antibody secretion, antibody-based antagonists, or chemokine receptors.



Panels A-E illustrate CARs against well-studied targets in the context of ovarian cancer, namely folate receptor α/folate-binding protein (A), mesothelin (B), NKG2D ligands (C), MUC16 (D) and the ErbB receptors (E). Panels F-O represent CARs generated against novel, less well characterized ovarian tumor antigens, namely αvβ6 (F), TAG72 (G), B7-H3 (H), Annexin A2 (I), L1-CAM (J), EpCAM (K), FSHR (L), 5T4 (M), MISIIR (N) and UPAR (O). Panel P depicts dual-targeting strategies employed in CAR T cell-targeting of ovarian cancer using either a dual-scFV CAR, separate CARs, or using the SynNotch system to induce CAR expression upon recognition of the initial target antigen.
I: Intermediate; L: Long; L: Long hinge; MPR: membrane proximal region; S: Short; S: Short hinge.

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co-stimulation) induced rapid tumor regression, in contrast to 1st generation T cells. These collective findings emphasized on the importance of co-stimulation in CAR T cell therapy against ovarian cancer [26,27].

Production of CAR T cells typically involves the use of either retroviral- or lentiviral-based vectors. However, these methods incur high cost as well as risks of immunogenicity and insertional mutagenesis [28,29]. Therefore, one group has generated anti-FR CAR T cells using a non-integrating RNA-based platform (Figure 1A) [30]. A further benefit of this method is the reduced risk of on-target off-tumor toxicity, since RNA degradation eliminates the need for a CAR T cell suicide switch. This study was also the first to utilize a fully humanized anti-FR CAR, reducing the risk of immunogenic toxicity. The CAR was equipped with the C4 scFv and a CD27+CD3ζ endodomain. Higher CAR expression and function was observed when cells were electroporated with a codon optimized RNA and expression was maintained up to 10 days post transfer, although a rapid decrease in transduction was observed post exposure to antigen-expressing SKOV3 cells. Tumor cell lysis was demonstrated using a panel of FR+ ovarian cancer cell lines. This was accompanied by high Th1 cytokine secretion owing to a synergistic interaction between CAR+ CD4 and CD8 T cells. Impressive responses were observed when these CAR T cells were subject to *in vivo* testing, particularly with repeated T cell dosing. In an i.p model of SKOV3 ovarian cancer, 100% durable complete responses were obtained under a dosing regimen of one higher dose followed by two lower doses. In the s.c/i.v model, significant attenuation of tumor progression was observed although complete responses were not achieved.

The LeY carbohydrate antigen

The carbohydrate antigen Lewis-Y (LeY) is overexpressed on a high proportion of epithelial tumors, including ovarian cancers. To target this, a CAR was engineered in which a humanized anti-LeY scFv was fused to a CD28 + CD3ζ endodomain. T cells that expressed this CAR lysed LeY+ cell lines, proliferated and secreted IFNγ in proportion to the antigen expression. Importantly, on target off tumor toxicity was not observed against neutrophils, which bear low levels of LeY expression. In a s.c *in vivo* model of LeY+ OVCAR3 ovarian cancer, i.v. delivered CAR T cells achieved 89% complete remission, although tumor outgrowth was eventually observed due to a lack of T cell persistence [31].

NKG2D ligands

Sentman et. al pioneered the design of CARs using the natural killer cell receptor, NKG2D [32]. This activating receptor recognizes eight stress-associated ligands, namely the MHC class I chain-related genes MICA and MICB, and the UL-16 binding proteins 1–6 in humans and Rae1, Mult1 and H60 in mice [33,34]. These ligands are commonly expressed on malignant or virally infected cells, with low to minimal expression on healthy tissue. This approach theoretically circumvents two key barriers to the application of CAR T cells to solid tumors: heterogeneous antigen expression and the downmodulation of antigen expression. NKG2D ligands (NKG2DL) were found to be expressed on ovarian cancer cell lines and as well as human tumor ascites samples [35]. An increased expression of these "stress" ligands has also been shown upon the induction of DNA damage, raising the prospect of synergistic effects of chemotherapeutic agents with NKG2DL-targeted therapies [36, 37]. Primary human T cells transduced with the chNKG2D CAR, consisting of full-length NKG2D fused to the cytoplasmic domain of CD3ζ, lysed ovarian cancer cell lines in a ligand-dependent manner (Figure 1C). Moreover, chNKG2D-engineered CD8 T cells isolated from patient ascites produced large amounts of pro-inflammatory cytokines upon co-culture with autologous primary cancer cells, in contrast to controls. In an ID8

murine i.p model of ovarian cancer, chNKG2D mouse T cells significantly decreased tumor burden compared to T cells that expressed NKG2D alone [38,39]. Importantly, the mice developed a host immune response as evidenced by a complete rejection of a re-challenge with the same tumor cells [39]. The *in vivo* efficacy of chNKG2D T cells was attributed to perforin-mediated cytotoxicity and the secretion of IFNγ, indicated by deficient responses in mice that lacked these mediators [39].

Subsequent studies illustrated the impressive ability of chNKG2D T cells to transform the ovarian cancer milieu from an immunosuppressive to an immunostimulatory one. Despite the poor long-term persistence of chNKG2D T cells *in vivo*, this treatment was shown to induce a long-lived systemic immune response [40]. Splenocytes isolated from chNKG2D-treated mice bearing i.p ID8 tumors could secrete IFNγ in culture for up to 10 weeks after T cell injection, with a peak at day 7. The source of the IFNγ was not attributed to the chNKG2D T cells, but rather host CD4 and CD8 T cells as well as NK cells. Notably, this IFNγ response was reliant on chNKG2D-secreted IFNγ, GM-CSF and perforin, as well as host cell expression of the IFNγ receptor. Intriguingly, the systemic response following treatment with chNKG2D T cells was shown to be at least partially due to an increase in tumor antigen presentation, an effect which was abrogated in T cells deficient in IFNγ, GM-CSF and perforin. *In vivo* efficacy was similarly shown to be wholly dependent on host cell-derived IFNγ and perforin, whilst tumor eradication was only attenuated in mice lacking GM-CSF. The presence of host T cells, B cells and NK cells was likewise required for complete tumor eradication [40].

chNKG2D administration appeared to increase tumor antigen-specific host T cell trafficking and survival, both around the tumor site and in the draining lymph nodes [40,41]. This recruitment of host CD4 and CD8 T cells was dependent on the interaction between CXCR3 and cognate ligands CXCL9 and CXCL10. chNKG2D treatment induced a high secretion of these ligands by macrophages which in turn increased the accumulation of endogenous T cells, as this effect was diminished in CXCR3-deficient mice. The presence of either host or transferred CD4 T cells was necessary for tumor elimination by chNKG2D cells. The treatment of wild-type mice with purified CD4+ chNKG2D cells mediated an identical outcome to control T cells, whereas purified CD8+ chNKG2D T cells reduced tumor burden to the same extent as total chNKG2D T cells. However, the treatment of MHC Class II-deficient mice with purified CD8+ chNKG2D T cells yielded an inferior outcome to total chNKG2D T cells, albeit still an improvement to control T cells. These results indicate that whilst sufficient, host CD4 T cells are not necessary for tumor elimination, and the provision of transferred CD4 T cells is required for optimal efficacy in the absence of host cells. Nevertheless, despite long-term tumor free survival conferred by chNKG2D in the absence of host CD4 T cells (100% survival up to day 130 post tumor inoculation, compared to 0% for the untreated group), these mice demonstrated a suboptimal CD8 T cell memory response, highlighting the necessity of these host cells in memory formation. Unsurprisingly, host CD8 T cells were shown to be crucial for optimal tumor burden reduction. Although substantial efficacy was still observed with chNKG2D T cells in CD8-deficient mice, the systemic anti-tumor response was diminished, and inhibition of tumor growth was impaired. Collectively these findings highlight the vital roles host T cells play in complementing the anti-tumor effects of chNKG2D T cell therapy against ovarian cancer [41].

These results prompted the group to further evaluate the efficacy of this therapy in tumors with heterogeneous ligand expression [42]. ID8 tumor cells, which lack expression of Mult1 and H60, were engineered to knockdown 90% of Rae-1 expression (ID8 shRae1 cells). When inoculated i.p into mice, treatment with i.p chNKG2D T cells failed to

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inhibit tumor growth and less IFNγ secretion was detected compared to a challenge against wt ID8 cells. Nevertheless, tumor cell lysis was still higher compared to treatment with wtNKG2D cells. A subsequent challenge with a mixture of Rae1+ and Rae1- ID8 cells yielded more promising results, with complete tumor elimination at a 1:1 ratio respectively, and significant cytotoxicity observed at a 4:1 and even 13:1 ratio. The knock-on effect of the elimination of ligand-negative cells can be attributed to the recruitment of host leukocytes and myeloid cells, and the decreased secretion of immunosuppressive cytokines. Impressively, chNKG2D T cells were able to reject a re-challenge with ID8 shRae1 cells, 250 days after the initial tumor inoculation. The number of tumors and tumor cell count in the ascites of the tumor-surviving mice were significantly decreased compared to with naïve mice. Splenocyte function from the tumor-free mice was confirmed through the secretion of high amounts of IFNγ upon co-culture with both ID8 and ID8 shRae1 cells. This response was not observed with Rae1-expressing lymphoma cells, indicating the generation of an ID8-specific immune response [42].

A human version of chNKG2D has been clinically evaluated in a several Phase I/II assessments by the biotechnology company, Celyad Oncology. The therapy named CYAD01 has been evaluated in both hematological malignancies and solid tumors, summarized in [43]. Preliminary results have primarily illustrated a favorable safety profile as well as some evidence of anti-tumor efficacy, mainly against leukemias [44,45]. In the first trial to include a solid tumor arm, the THINK (Therapeutic Immunotherapy with NKR-2) trial, CYAD01 was to be assessed in seven clinical indications: AML, multiple myeloma, pancreatic cancer, urothelial cancer, colorectal cancer, triple-negative breast cancer and ovarian cancer (ClinicalTrials.gov: NCT03018405). CYAD01-transduced autologous cells are administered i.v with three infusions at 2-week intervals, without lymphodepletion. From the last published update in 2018, two ovarian cancer patients had been enrolled and one had achieved stable disease. Peak peripheral expansion of CYAD01 cells was shown to correlate with the dose-level and response [46].

Song et al. sought out to compare targeting NKG2D ligands to FR as a means of tackling ovarian cancer using CAR T cells. The NKG2D receptor (NKG2D-CAR) and the scFv targeting FR (FR-CAR) were fused to 4-1BB and CD3ζ as second-generation constructs (Figure 1C). Despite NKG2D-CAR T cells expanding significantly slower than FR-CAR cells, strong CAR enrichment was observed over time, whilst CAR expression of FR-CAR cells remained the same. Both these phenomena were attributed to T cell fratricide which occurs due to the expression of NKG2D ligands on the T cell surface itself. Both CAR constructs were only stimulated when recognizing their cognate ligand across a panel of ovarian cancer cell lines, secreting high levels of IFNγ. Moreover, NKG2D-CAR T cells were also able to recognize primary ovarian cancer cells. Interestingly, the treatment of tumor cells expressing low to moderate levels of NKG2DLs with the histone deacetylase (HDAC) inhibitor sodium valproate (VPA) increased their level of expression, an increase was consistently seen with the ligands MICA/B and ULBP-2. This subsequently further sensitized NKG2D-CAR T cells to activation by the treated cell lines [47].

The ErbB family of receptor tyrosine kinases

The epidermal growth factor (EGF) family of receptors are commonly overexpressed on a wide range of epithelial cancers [48–51]. Specifically on ovarian cancer, the expression of all four ErbB receptors (ErbB1, ErbB2, ErbB3, ErbB4) has been detected to a variable extent and to be associated with prognosis [52–56]. The HER2 (ErbB2/neu) receptor is upregulated in breast and ovarian cancer and is one of the most extensively studied tumor antigens for immunotherapy

[57–59]. Despite this, the rationale for targeting HER2 in ovarian cancer remains debatable, primarily due to the range of expression reported by different studies [58,60,61]. Despite some promising *in vitro* and *in vivo* studies, treatment of ovarian cancer with the HER2-specific mAb, trastuzumab, yielding disappointing clinical responses, with a 7% response rate as a single agent, attributed to resistance [60,62]. Nevertheless, HER2 was still investigated as a target for CAR T cell therapy owing to its amplification in some ovarian cancers. RNA encoding a CAR composed of an anti-HER2/neu scFv fused to CD28 and CD3ζ was used to electroporate peripheral blood lymphocytes, achieving efficient transduction (Figure 1E). These cells were able to effectively lyse HER2+ SKOV3 ovarian cancer cells, as well as secrete high levels of pro-inflammatory IL-8, GM-CSF and IFNγ, primarily mediated by CD8 T cells. In a s.c SKOV3 xenograft model, treatment with CAR+ cells significantly reduced tumor burden compared to mock-transduced cells. Tumor growth was delayed at a higher rate than in mice treated with trastuzumab, although this difference could be attributed to the low dosage used [63]. A subsequent study evaluated the efficacy of a humanized CAR, comprising the anti-HER2 scFv chA21 fused to CD28 and CD3ζ. Transduced T cells lysed HER2+ ovarian cancer cell lines in an antigen-dependent manner, secreting high levels of IFNγ and IL-2. These T cells were also able to reduce tumor burden significantly better than untransduced cells when administered i.v. in a s.c. xenograft of a HER2+ breast cancer cell line [61]. Trastuzumab-based anti-HER2 CAR T cells were also shown to be efficacious in a more clinically relevant model in which the SKOV-3 cell line was embedded in a hypoxic three-dimensional (3D) hydrogel, thereby recapitulating tumor cell-extracellular matrix interactions. When the CAR T cells were delivered via microfluidic channels, they effectively lysed the tumor cells, albeit mainly at the periphery of the 3D structure. Since infiltration into the tumor bulk was negligible, cytotoxicity was postulated to occur via a bystander effect such as the secretion of granzymes or through metabolic competition [64]. After uncovering superior efficacy of CD28-bearing versus 4-1BB-bearing CAR T cells targeting L1CAM in pre-clinical models of neuroblastoma, Textor et al. confirmed these findings through HER2-targeting CARs against ovarian cancer (Figure 1E). The efficacy of CARs designed with a short spacer domain and either of these co-stimulatory molecules was equivalent in a s.c. model of SKOV3 when T cells were administered i.v. However, when incorporating a long spacer domain, CARs signaling through CD28 achieved superior tumor eradication [65]. There is one Phase I clinical assessment which has been initiated evaluating anti-HER2 CAR T cell therapy in several HER2+ indications, one of which is ovarian cancer (ClinicalTrials.gov: NCT04511871). Despite all this encouraging efficacy, the route of administration and dosage of T cells targeting ErbB2 are crucial factors in the safety of these therapies, as the recognition of low levels of this antigen on lung tissue has previously resulted in the premature death of a patient [66].

The dynamic and complex nature of the ErbB receptor-ligand network as well as the possibilities for both homo- and heterodimerization has been exploited using an alternative CAR T cell approach. A CD28 + CD3z-containing CAR named TIE28z was designed which incorporates a chimeric polypeptide named T1E as targeting moiety (Figure 1E) [67]. This CAR was shown to recognize ErbB1- and ErbB4-based dimers, as well as the ErbB2/3 heterodimer. T1E28z T cells were also equipped with a chimeric cytokine receptor termed 4αβ, a fusion of the IL-4Rα ectodomain to the IL-2R and IL-14R β chain [68], collectively named T4. As a result, IL-4 delivers a potent and selective growth signal to the CAR-engineered T cells only. Analysis of epithelial ovarian cancer patient tumor samples revealed heterogeneous ErbB expression patterns, although at least one of the ErbB receptors was overexpressed on most samples [53]. T4 T cells demonstrated impressive killing of autologous tumor samples

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and tumourspheres generated from these cells, as well as the IGROV1 and SKOV3 cell lines, accompanied by high secretion of IFNγ in a CAR-dependent manner. *In vivo* assessment of T4 immunotherapy was undertaken using an i.p SKOV3 xenograft model in SCID-Beige mice. The administration of 1x107 CAR+ T4 cells resulted in rapid tumor regression, however with subsequent progression. This was attributed to poor T cell persistence, rescued to a certain extent by the repeat administration of the therapy (two doses spaced one week apart). However, the pre-treatment of mice with carboplatin combined with a lower CAR+ T cell dose of 2.5x106 significantly improved tumor growth control by T4, similarly enhanced through repeat administration of both the drug and CAR T cells, whereby 3/5 mice had undetectable tumor [53]. Pre-treatment with either carboplatin or paclitaxel sensitizes ovarian cancer cells to T4 through various mechanisms including inducing enhanced apoptotic pathways, induction of G2/M arrest and shuttling the mannose-6-phosphate receptor to the cell surface, improving T cell cytolytic killing. The additional introduction of PD-1 blockade further enhances the efficacy of this chemo-immunotherapy combination [69]. Nevertheless, the use of anti-ErbB CAR T therapy operates within a therapeutic window, since administration of larger doses induces profound toxicity due to cytokine release syndrome [70].

Targeting mucins in ovarian cancer

Mucins are a family of highly glycosylated proteins playing a critical role in forming a protective and hydrating mucus barrier on the surface of epithelial cells [71]. Their expression has been found to be upregulated on epithelial cancers, including breast, prostate, lung, pancreatic, and ovarian cancer, promoting tumor growth [72]. It is thereby unsurprising that they have been selected as potential targets for cancer therapy. Two of these transmembrane proteins, MUC1 and MUC16, have primarily been implicated in cancer prognosis and progression, and have therefore been evaluated in the context of immunotherapy. Several antibody-based therapeutics, bi-specific T cell engagers (BiTEs) and CAR T cell therapies against either MUC1 or MUC16 are being assessed in early-phase clinical studies, well summarized in [71].

MUC16 is found overexpressed on most ovarian tumors, and contains a cleavable soluble domain known as CA-125, considered a gold-standard serum marker for ovarian cancer detection [73]. This antigen can be found expressed at low levels on healthy uterine, endometrial, fallopian and ovarian cells, making it an attractive target for CAR T cell therapy. The initial mAbs designed against MUC16 all targeted CA-125 which would render these therapies inefficient against the retained extracellular portion. The first study evaluating CAR T cells against MUC16 generated a CAR bearing an scFv against the retained antigen (termed 4H11), fused to CD28 and CD3ζ (4H1128z), and compare this to a 1st generation counterpart bearing only CD3ζ (4H11z) (Figure 1D) [74]. 4H1128z retrovirally-transduced T cells showed marked expansion and secretion of IL-2 and IFNγ upon a co-culture with MUC16+ artificial antigen-presenting cells, compared to 4H11z cells and those bearing a CD19-targeting CAR. The T cells also demonstrated CAR+ fraction enrichment upon re-stimulation with these cells. 4H1128z T cells were only able to target the ovarian cancer cell lines OVCAR3 and SKOV3 when genetically modified to express the retained MUC16 antigen. Intriguingly, T cells derived from patient ascites transduced to express 4H1128z were able to lyse autologous primary ovarian cancer cells. The *in vivo* activity of these cells was assessed in several experiments using an orthotopic MUC16 genetically modified OVCAR3 xenograft in SCID-Beige mice, a model known to induce ascites formation and multiple peritoneal tumors. The *in vitro* expanded cells were all shown to retain a central memory phenotype prior to infusion. Dose-escalating quantities of T cells were administered i.p, yielding a

dose-dependent response against the tumor, whereby a minimal dose of 1x107 CAR+ cells was required for long-term survival. When 3x107 CAR+ T cells were administered, both the 4H11z- and 4H1128z-treated groups showed a significant survival advantage compared to controls, however there was no significant difference between the two groups (a survival rate of 15% and 40% respectfully at the end of the study, 120 days post tumor-injection). In addition to early tumor stages, these T cells were shown to be effective against more well-established tumors, however most treated mice eventually relapsed, despite the retention of antigen expression. Although these cells could be detected in peritoneal washes for up to 28 days after administration, their numbers were on the decline, and therefore the relapses could be attributed to poor T cell persistence. Interestingly, the infusion of the T cells i.v had little impact on the anti-tumor efficacy observed with the i.p route, and these cells were shown to traffic to the peritoneum. Crucially, this study showed the lack or minimal expression of the target antigen on a range of healthy tissues, using immunohistochemical staining with the 4H11 antibody [74].

To further enhance the efficacy of this therapy, 4H1128z T cells were additionally engineered to secrete IL-12, in an effort to overcome the immunosuppressive microenvironment within ovarian cancer (Figure 1D) [75]. IL-12 is known to stimulate T cells to secrete IFNγ, and inhibit suppressive mechanisms. The CAR was also equipped with a truncated EGFR gene (EGFRt) to enable cetuximab-mediated depletion of the cells. Neither of these alterations appeared to affect the cytolytic capacity of these cells, termed 4H1128z-IL12. Low amounts of IL-12 were detected in the supernatants of these T cells stimulated with antigen-negative cells which increased to 4-fold compared to the parental 4H1128z cells when stimulated with antigen-positive SKOV3. Similarly, IFNγ secretion upon antigen stimulation was 27-fold higher compared to T cells expressing 4H1128z alone, and the presence of IL12 enhanced the *in vitro* expansion of the CAR T cells. In an *in vivo* assessment of an i.p. SKOV3 orthotopic xenotransplant model, 4H1128z T cells equipped with IL-12 induced complete tumor eradication and improved survival compared to the parental CAR T cells, with a survival rate of 100% at the end of the study 90 days post tumor injection, versus ~20% for the parental cells. A higher percentage of these T cells and higher IFNγ secretion was detected in the peripheral blood 6 days post T cell injection compared to the 4H1128z cells; however, these findings were not present at day 35 post-treatment. High serum IL-12 was detectable in mice treated with the IL-12 expressing CAR T cells until day 20, which was similarly diminished by day 35. Notably, T cells expressing an irrelevant CAR targeting CD19 (19–28z) appeared to induce an anti-tumor response, with subsequent outgrowth of the tumor at a higher rate than 4H11-targeting T cells. 19–28z cells equipped with IL-12 mediated improved tumor control and survival compared to the parental cells. In this model, enhanced efficacy was observed when the T cells were administered i.p compared to i.v owing to the improved trafficking of the cells. Importantly, successful elimination of CAR+ T cells was observed upon treatment with cetuximab [75]. A follow-up study aimed at uncovering the mechanism behind the improved efficacy of 4H1128z-IL12 cells [76]. When cultured in the presence of cell-free pooled ascites, IL-12 secreting 4H1128z cells maintained superior proliferation and cytotoxicity against ID8-Muc16ecto cells, which was diminished in parental CAR T cells. The secretion of IL-12 also appeared to confer resistance to apoptosis in this inhibitory microenvironment. These 4H1128z-IL12 cells were also assessed in an advanced syngeneic ID8-Muc16ecto model of peritoneal carcinomatosis in C57BL/6 mice. 2x106 CAR+ T cells were administered i.p and exerted significantly enhanced anti-tumor effects and survival compared to the original 4H1128z cells (100% survival until ~day 90 for mice treated with IL-12 secreting cells vs 0% at

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day 56 for the group treated with parental cells). This effect was at least partly attributed to the autocrine activity of IL-12, as efficacy was diminished when mice were treated with CAR T cells in which IL-12 receptor had been knocked out. Between 24h and 48h after T cell administration, marked *in vivo* expansion of the CAR T cells was observed in peritoneal washes with both 4H1128z-IL12 and 4H1128z cells and this was significantly higher for the IL-12-secreting T cells. Production of IL-12, IFNγ and TNF-α was significantly increased; however, this was not observed with IL-2 suggesting a steady-state level of its production and usage. In contrast, IL-2 levels decreased in control and 4H1128z parental cells. RNA sequencing of a panel of 770 immune-related genes revealed decreased expression of genes encoding exhaustion markers (Eomes, FoxP3, Ctla4, Lag3, HAVCR2(TIM3), CD274(PD-L1)), chemokines involved in monocyte recruitment (Ccl2, Ccl7, Ccl12), as well as genes implicated in T cell metabolism (Ido1, Nos2). These T cells expressed significantly higher levels of Tbx21 which regulates Th1 cytokine production, as well as Fas and FasL which mediate T cell cytotoxicity. Intriguingly, treatment with 4H1128z-IL12 T cells did not appear to recruit endogenous T cells, and the efficacy was unaffected in CD8 (CD8 -/-) or IFNγ (IFNγ -/-) knock-out mice, suggesting that the host T cell response was dispensable. This study also revealed that 4H1128z-IL12 T cells deplete F4/80+ CD11b+ tumor-associated macrophages (TAMs) in a Fas/FasL-dependent manner, illustrating another mechanism for the impressive efficacy. Marginally enhanced efficacy and survival was observed when PD-L1 (PD-L1 -/-) knock-out mice were treated with 4H1128z-IL12 T cells, indicating that their efficacy is attenuated in the presence of surface PD-L1. The pre-treatment of mice with an anti-PD-L1 antibody prior to CAR T cell administration only had a significant effect on survival when a lower dosage of T cells was used, although impressive efficacy was still achieved without the depletion of PD-L1. Crucially, no toxicity was observed owing to either the CAR itself or IL-12 release [76]. Overall, these results confirmed the superior anti-tumor efficacy and persistence of MUC16-targeted CAR T cells when further equipped with IL-12 and uncovered several potential mechanisms underlying its superiority. Interestingly, similar improvements in efficacy were observed when 4H1128z T cells were engineered to secrete IL-18 (Figure 1D) [77]. Together with the safety data and the co-expression of the tEGFR suicide switch, the efficacy of these IL-12 secreting MUC16ecto CAFR T cells were set to be assessed further in a Phase I clinical trial for patients diagnosed with platinum-resistant advanced ovarian cancer [75,76,78]. In this trial, patients would be administered half of the dose i.p and half i.v, with or without a prior lymphodepletion regimen using cyclophosphamide. The trial was designed as a dose-escalation study with the primary objective to assess safety and subsequently the efficacy and persistence of these CAR T cells. The study has not been completed to date (NCT02498912) [78] although interim data has been presented [161]. Another Phase I/Ib trial evaluating anti-MUC16 CAR T cells is underway. This study is being conducted by PGEN Therapeutics, harnessing their Ultra-CAR-T™ platform, a non-viral gene delivery system which utilizes a decentralized and rapid manufacturing process (Figure 1D). Impressively, (as seen on a poster presentation at the 2020 Annual Meeting of the American Association for Cancer Research) this process allows for the infusion of genetically-modified T cells only one day post gene delivery. These T cells bear a CAR targeting MUC16, membrane-bound IL-15 to improve *in vivo* expansion, and a kill switch for safety purposes, and were termed PRGN-3005. PRGN-3005 cells presented with a stem cell-like memory phenotype and demonstrated impressive *in vitro* and *in vivo* cytotoxicity against the SKOV3 cells and derived tumors. The presence of membrane-bound IL-15 was shown to be critical for expansion, particularly the T stem cell memory population, as well as for anti-tumor efficacy [79,80]. These findings

promoted the initiation of the Phase I/Ib trial, a dose-escalation study assessing the efficacy of PRGN-3005 cells at treating advanced, recurrent, platinum-resistant ovarian, fallopian tube, or primary peritoneal cancer (ClinicalTrials.gov: NCT 03907527).

In an effort to minimize tumor immune escape and off-target toxicity, a more recent study evaluated the dual targeting of MUC16 and PD-L1 (Figure 1P). Impressively, despite comparable *in vitro* efficacy, dual-targeting CARs outcompeted CARs targeting either antigen alone in an i.p OVCAR3 xenograft model. This disparity between *in vitro* and *in vivo* efficacy could be attributed to the targeting of PD-L1 on non-neoplastic components of the tumor microenvironment [81].

MUC1 also presents as an attractive target owing to not only its elevated expression, but also its altered glycosylation and its widespread expression in a cancer setting, whereby it is normally limited to the luminal epithelium [82–84]. The initial study evaluating CAR T cells against MUC1 demonstrated the ability to target this antigen in the context of cancer treatment, and showed that steric hindrance and heterogeneous glycosylation are barriers to the efficacy of this therapy. They overcame these obstacles through the further incorporation of both the IgD and IgG1 Fc+ hinge into the CAR, as well as the use of a MUC1-targeting scFv which has a broad capacity to bind to differentially glycosylated MUC1 [82]. Since this study, MUC1-targeted CAR T therapies have been evaluated in the context of cancers of the breast, prostate, head and neck, esophagus, pancreas, liver, and the lung. However, to date no studies have assessed targeting ovarian cancer with CAR T cells through MUC1.

TAG72

Akin to MUC1 and MUC16, TAG72 is another cell surface protein alternatively glycosylated in ovarian cancer, and often these biomarkers are co-expressed [85]. High TAG72 expression is detected on 90% of epithelial ovarian cancers, and it appears to correlate with tumor stage and prognosis [85,86]. A CAR incorporating a humanized anti-TAG72 scFv fused to 4-1BB and CD3ζ demonstrated antigen-dependent *in vitro* cytotoxicity against ovarian cancer cell lines, as well as patient-derived ascites tumor cells (Figure 1G). Regional i.p administration of these T cells in OVCAR3 tumor-bearing mice induced an impressive anti-tumor response which was not attained through i.v. delivery of the T cells. In the early days post-treatment, CAR T cell counts in the peripheral blood and ascites of the mice was significantly elevated in mice receiving i.p. compared to i.v. T cells. However, these values equalized at later time points, highlighting the delayed kinetics of expansion of i.v administered T cells. Repeat i.p inoculation of T cells further improved the anti-tumor response. Unfortunately, these responses were short-lived, as the tumors recurred after 3–4 weeks, bearing decreased expression of the antigen. Interestingly, tumor recurrences at later time points retained high TAG72 expression [87]. To overcome antigen downregulation, a separate study evaluated the dual targeting of TAG72 and CD47, a surface protein ubiquitously expressed in ovarian cancer which functions to suppress macrophage-mediated phagocytosis. The anti-TAG72 CAR was endowed with either CD28 (TAG-72.CD28) or 4-1BB (TAG-72.4-1BB) to provide co-stimulation, and CD3ζ as an activating signal, whilst the CD47 CAR was truncated to remove the signaling component, as targeting CD47 through CAR T cells has previously been shown to impair T cell expansion and efficacy as a cause of fratricide from low expression levels on the T cells themselves (Figure 1P). The anti-TAG72 CARs on their own mediated effective cytotoxicity against ovarian cancer cell lines *in vitro*, although CD28-bearing T cells induced quicker killing compared to those incorporating 4-1BB. Nevertheless, comparable levels of pro-inflammatory cytokines and chemotactic factors were secreted by these cells. Surprisingly, cytotoxicity was equivalent between isolated CD4 and CD8

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CAR T cells. The introduction of the truncated CD47 CAR (ΔCD47) reduced both the expansion and transduction efficiency of these CAR T cells, which was marginally rescued by the disruption of the CD47 dimerization domain through point mutations to prevent dimerization with the anti-TAG72 CARs (ΔCD47m). Nevertheless, this dual CAR expression enabled the T cells to eliminate TAG72low/CD47+ ovarian cancer cell lines. Unfortunately, cytotoxicity was observed with TAG-72.CD28 + ΔCD47 and TAG-72.4-1BB + ΔCD47 T cells against healthy human fibroblast cells. Only the TAG-72.4-1BB + ΔCD47m T cells demonstrated favorable safety in this context and were advanced for *in vivo* evaluation. Efficacy was assessed in s.c models of both TAG72high OVCAR3 and TAG72low MESOV xenografts. In the OVCAR3 model, both single- and dual-targeting CAR T cells were able to suppress tumor outgrowth, although superior efficacy was seen in the former group. However, in the TAG72low model, only dual-targeting CARs demonstrated delay in tumor growth, although this efficacy was not significant [88]. TAG72 is set to be clinically evaluated as a target for CAR T cell therapy in patients with recurrent platinum resistant EOC but the trial is yet to commence recruitment (ClinicalTrials.gov: NCT05225363).

Annexin A2

Following on from the idea of targeting aberrantly glycosylated proteins, one recent study evaluated the targeting of Annexin A2 (ANXA2) via its N-linked glycoepitope. ANXA2 has been shown to play a role in epithelial-mesenchymal transition (EMT) and metastasis in a number of cancers, including HGSOC [89–91]. It has also been advanced as a potential novel biomarker for ovarian cancer [92]. Leong et al. constructed CARs using the scFv of a previously characterized anti-ANXA2 2448 mAb targeting this glycoepitope [93], bearing spacers of a short, intermediate or long length, fused to CD28 and CD3ζ (Figure 1I). As T cells were transduced by nucleofection of CAR mRNA, peak transduction occurred after 12h and all assays were carried out at this early time point. In an initial *in vitro* cytotoxicity screen, the CAR bearing the longest spacer demonstrated highest efficacy and was chosen as the lead candidate for further assays. T cells bearing this 2448-CAR mediated antigen-specific tumor cell lysis and pro-inflammatory cytokine secretion, sparing healthy cell lines. At low effector-to-target ratios, a 2448-CAR bearing CD28 in place of 4-1BB mediated improved killing and higher secretion of IFNγ, IL-2, TNFα and GM-CSF. However, only the 4-1BB-containing CAR was assessed for efficacy *in vivo*, chosen because of lower cytokine secretion (perceived as a safety advantage) and improved T cell persistence. Although treatment with the 2448-BBz CARs reduced tumor burden in a s.c SKOV3 model, these tumors eventually relapsed. Concerningly, weight loss was observed with this treatment and relapsed tumors had greatly reduced ANXA2 expression. Whilst the targeting of aberrant glycans shows promise for the targeting of ovarian cancer with CAR T cells, further evaluation of antigen choice is still required to improve the safety and durability of this therapy [91].

The αvβ6 integrin

Another attractive target is the integrin αvβ6, which is highly expressed on epithelial carcinomas including pancreatic, colonic, hepatic, oral squamous, as well as ovarian tumors [94,95]. As a regulator of collagenase production, the targeting of this integrin would further prevent tumor invasion and metastasis [95,96]. Pameijer et al. initially explored targeting αvβ6 in ovarian cancer using a combinatorial approach of phage display and CAR design, expanding the repertoire of CAR specificities beyond scFvs and receptor-ligand interactions (Figure 1F) [94]. Ligand expression was confirmed on both the ovarian cancer cell line OVCAR-3 as well as primary ovarian tumor cells. A 12-mer

peptide entitled 'Bpep' was used as the targeting moiety, fused onto the IgG4 hinge, CD4 transmembrane (TM) domain and intracellular CD3ζ. Cytotoxic T lymphocytes (CTLs) electroporated with the CAR mRNA were shown to effectively lyse αvβ6+ tumor cell lines and secrete high amounts of IFNγ in a ligand-dependent manner, albeit at a high effector-to-target (E:T) ratio of 50:1. Nevertheless, this approach paved the way for expanding CAR targeting specificities, particularly with the potential for multi-specificity targeting using multiple peptides. In a separate study, Whilding et al. assessed the CAR T cell targeting of αvβ6 in multiple solid tumor indications including ovarian cancer [97]. Using a 20-mer peptide named A20FMDV2 as the targeting moiety, CARs were generated incorporating CD28 and CD3ζ (A20–28z) (Figure 1F). T cells were further equipped with the same chimeric cytokine receptor 4αβ as in [53] for selective expansion of CAR T cells. A20–28z T cells selectively lysed a panel of solid tumor cell lines including those of ovarian cancer origin, secreting high amounts of IFNγ and IL-2 which correlated with the degree of αvβ6 expression. Efficacy was further confirmed in a SKOV3 i.p xenograft model, where A20–28z T cells significantly reduced tumor burden and extended survival. These results were mirrored when A20–28z T cells were evaluated in pancreatic and breast cancer xenografts. Most importantly the treatment was well tolerated despite the cross-reactivity of the 20-mer peptide with murine αvβ6. However, when a high dose of T cells was administered i.v, significant toxicity was observed, although this was shown to be transient and reversible [97]. When these T cells were further equipped with the CXCR2 chemokine receptor, improved intra-tumoral migration and disease control was observed in both pancreatic and ovarian tumor xenografts [98]. These findings were recapitulated in a separate study evaluating the efficacy of CD70-targeting CAR T cells in pre-clinical models of different solid tumors, including ovarian cancer [99].

Mesothelin

As a protein directly implicated in ovarian cancer progression and metastasis, mesothelin was naturally selected as another potential target for CARs. This 40 kDa glycoprotein is endogenously expressed on mesothelial cells at very low levels but is overexpressed on several tumors. These include 70% of ovarian cancers, primarily the epithelial serous subtype [100,101]. Crucially, expression can be detected on both primary tumors and metastases [101]. It is thought to play a role in cancer cell migration and metastatic spread through its interaction with MUC16/CA125 [102]. Murine mesothelin peptide-specific CD8 T cells generated from C57BL/6 mice were able to significantly reduce tumor burden in a model of murine ovarian surface epithelial cancer (MOSEC), upon an intraperitoneal adoptive transfer, with 100% survival 16 weeks post transfer compared to 0% for untreated mice [103]. This prompted the further development of CAR T cells targeted against mesothelin, circumventing the requirement to generate tumor-specific CTLs. The initial study used the SS1 scFv as the targeting moiety for the CAR, owing to its high affinity for human mesothelin, and favorable safety profile when administered to patients as an immunotoxin (Figure 1B) [104]. A panel of SS1-based CARs were assessed for their ability to target mesothelin-expressing tumor cells – including 2nd generation constructs combining CD3ζ with either CD28 or 4-1BB as co-stimulatory components (BBz, 28z), as well as a 3rd generation receptor incorporating both aforementioned sources of co-stimulation (28BBz) [102]. T cells transduced with the CARs efficiently lysed mesothelin-expressing cells, including those derived from primary ovarian and mesothelioma tumors, and secreted large amounts of Th1 cytokines. The lytic ability of these T cells was shown to be both ligand- and CD3ζ dependent, albeit not enhanced through the provision of co-stimulation. However, the importance of co-stimulation in these constructs was evidenced by the higher expansion

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of those T cells in culture and their polyfunctionality. Immunocompromised NSG mice were challenged s.c with both mesothelin-expressing and mesothelin-negative A431 cells on opposite flanks prior to i.t. administration of the CAR T cells. T cells incorporating the co-stimulatory components were able to efficiently eliminate ligand-positive tumors, particularly when CD28 was present, whilst CD3ζ-only CAR T cells only delayed tumor growth. Surprisingly, the greatest persistence of peripheral T cells was noted in mice treated with T cells expressing 28BBz, whilst the level of 28z T cells was comparable to that of CD3ζ-only T cells. This study also compared the anti-tumor effects of these CAR T cells through different routes of administration. Although i.t, delivery mediated greatest tumor reduction, greatest T cell expansion was noted when T cells were injected i.v. These initial findings collectively demonstrated the ability to target mesothelin using CAR T cells, and highlighted the relevance of co-stimulation, where CD28 appears to enhance the lytic ability of CAR T cells, and the presence of 4-1BB improves persistence [102]. Using a different high-affinity scFv, P4, Lanitis et al. similarly constructed mesothelin-targeting CAR T cells bearing CD3ζ as the source of activating signaling (P4-z) (Figure 1B) [105]. Out of a panel of six ovarian cancer cell lines, three expressed detectable human mesothelin. When targeted against these mesothelin+ cell lines, P4-z T cells secreted high levels of IFNγ, IL-2, MIP-1α and TNF-α. IFNγ secretion correlated with the level of mesothelin expression and was not inhibited by high concentrations of soluble mesothelin, which is frequently detected in serum and ascites from ovarian cancer patients. The P4-z cells also expressed high levels of CD69 and CD107 indicative of activated, degranulating cells, and effectively lysed only ligand-positive tumor cells. Furthermore, an immunohistochemical analysis of patient HGSOC samples revealed that 93% of samples expressed mesothelin to a certain extent. However, some samples contained tumor regions with no detectable mesothelin, which may promote the emergence of therapeutic resistance. Nevertheless, P4-z T cells were able to elicit bystander killing of mesothelin-negative cells in a mixed tumor cell culture of ligand-positive and -negative cells. When P4-z T cells were provided with additional CD28 co-stimulation (P4–28z), they secreted higher levels of pro-inflammatory cytokines compared to their first-generation counterparts. In a s.c. model of the ovarian cancer cell line A1847, tumor outgrowth was only modestly delayed by the i.v. administration of P4-z cells, whereas rapid and significant tumor regression was achieved by the treatment with P4–28z cells. In a more physiologically relevant xenogeneic model of i.p metastatic ovarian cancer, the i.v infusion of P4–28z cells similarly swiftly eliminated tumor burden and significantly higher levels of peripheral blood CAR T cells were detected after 3 weeks, compared to P4-z cells [105].

As mesothelin can be detected on healthy pleura and peritoneum, as well as on fallopian, tracheal and tonsil epithelial cells, as detected by immunofluorescence [106], there remains a great risk of on-tumor off-target toxicity from mesothelin-targeted CAR T cell therapy. In an effort to minimize this, CAR T cells were generated in a tandem fashion targeting both mesothelin and FR, separating the two components of a second-generation car across these receptors – the mesothelin scFv P4 fused to CD3ζ (Mz) and the FR MOv-19 scFv fused to CD28 (F28) (Figure 1P) [107]. This would allow the T cells to undergo maximal activation in the presence of both ligands, which are frequently co-expressed in ovarian cancer. The efficacy of the first-generation Mz and the co-stimulatory Mz/F28 was compared to that of a P4-targeting second-generation construct bearing CD28 and CD3ζ (M28z). Upon exposure to cells engineered to express mesothelin alone (C30-M), M28z produced significantly higher amounts of IFNγ compared to Mz and Mz/F28, accompanied by a significantly greater expression of the degranulation marker CD107a and higher cytolytic capacity. These outcomes were further enhanced within Mz/F28 T cells when stimulated with tumor

cells engineered to co-express both ligands, although they remained highest for M28z cells. When cultured with ovarian tumor cells endogenously co-expressing mesothelin and FR (A1847), Mz/F28 T cells secreted cytokine comparable levels to M28z, but significantly higher levels of both IFNγ and IL-2 compared to Mz. These effects were shown to be antigen-specific, and dependent on the presence of CD3ζ within the construct. Cytotoxicity against both C30-M and A1847 cells remained highest for M28z, followed by Mz and subsequently Mz/F28. The presence of CD28 within the construct appeared protective against activation-induced cell death (AICD), regardless of the signaling structure, in cis (M28z) or trans (Mz/F28). The initial *in vivo* experiment assessing the efficacy of these CARs revealed superior tumor burden suppression and T cell persistence in mice treated with either M28z or Mz/F28 compared to Mz. To assess the effect of these CAR T cells against tumor cells expressing only mesothelin, an shRNA knockdown of FR was performed on the A1847 cell line. IFNγ secretion was significantly reduced when Mz/F28 cells were cultured with this A1847M+/F- cell line compared to the parental line expressing both ligands. For a subsequent *in vivo* comparison of the cis- (M28z) vs trans-signaling (Mz/F28) CARs, mice were challenged with a s.c dose of either the A1847M+/F- or parental A1847M+/F+ cell line on opposing flanks. Upon i.v administration of the CAR T cells, a significant attenuation of anti-tumor efficacy was observed with Mz/F28 T cells compared to M28z T cells against the A1847M+/F- tumor, whilst the control of A1847M+/F+ tumors was comparable between the constructs. Moreover, immunohistochemical analyses revealed a higher infiltration of trans-signaling CAR T cells in dual antigen-expressing tumors, whilst the abundance of cis-signaling CAR T cells was comparable between the different tumors. These results collectively confirm the necessity to stimulate both receptors in trans-signaling Mz/F28z T cells for potent activation and provide evidence for the enhanced safety of this approach [107]. The dual targeting of these two antigens was similarly assessed in a more recent study, whereby mesothelin and FR were chosen through gene expression mining, based on their highest overexpression by malignant ovarian cells compared to healthy counterparts [108]. Out of 160 patient samples, only 18 were negative for both antigens, highlighting the broad scope of this approach. Single- and dual-targeting third-generation CARs, bearing CD28, 4-1BB and CD3ζ were compared (Figure 1P). These CARs were further armored with IL-12 to enhance *in vivo* anti-tumor efficacy (Figure 1P). Both single- and dual-targeting T cells proliferated and lysed ovarian tumor cells in an antigen-dependent manner, although these effects were marginally higher with the tandem CAR T cells. The dual-targeting cells also had higher expression of perforin and granzyme B, and secreted larger amounts of IFNγ, TNFα, IL-2 and IL12, although these differences were not large. Concerningly, some lysis of mesothelin- and FR-negative cells was also observed at higher effector-to-target ratios. In an *in vivo* s.c model of the dual antigen-expressing SNU119 xenograft, tandem CAR T cells mediated significantly improved tumor regression and extended survival compared to mesothelin-targeting CARs. Tandem CARs mediated 100% survival until day 64 post-tumor inoculation, versus a 0% survival rate of mice treated with mesothelin-targeting CAR T cells by day 60. These differences were less prominent when compared to FR-only targeted CARs, with 100% survival until day 62. T cell infiltration numbers mirrored these results, with equivalent infiltration between tandem and FR-only targeting CARs, which was significantly higher than in the control or mesothelin-only groups. These findings indicate that some improvements in anti-tumor efficacy can be achieved through dual antigen targeting of ovarian cancer; however, the greatest benefit of this strategy is the safety of limiting the destruction of healthy tissue [108].

In an alternative approach to limit off-tumor toxicity, mesothelin-specific CAR T cells were generated using mRNA electroporation,

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a method endowing T cells with transient CAR expression. This study was also the first to utilize a human anti-mesothelin scFv, fused to 4-1BB and CD3ζ and importantly demonstrated similar efficacy to the murine CAR (Figure 1B). Using an i.p. model of mesothelin-expressing ovarian cancer cells, the group highlighted the improved potency of this therapy through repeat weekly doses of T cells. This study also crucially confirmed comparable transduction and efficacy of fresh versus cryopreserved mRNA CAR T cells, providing rationale for the use of this product in clinic [109]. Through *in silico* approaches, Banville et al. uncovered that the dual targeting of mesothelin and CA125/MUC16 should target the majority of HGSOC cells, although this combination has not been evaluated in practice so far [110].

The ectodomain of mesothelin is composed of three regions, the N-terminal membrane-distal region (MDR) I, an intermediate region II and the C-terminal membrane-proximal region (MPR) III [111]. The studies described above had assessed CAR T cells bearing scFvs targeting the MDR. However, due to the nature of mesothelin engagement with other endogenous proteins, such as CA125/MUC16, the efficacy of CAR T cells targeting this region could be hindered. As such, one study compared the effects of targeting the MDR versus the MPR by CAR T cells (meso1 and meso3 CAR T cells respectively) (Figure 1B). A higher proportion of Meso3 T cells expressed the degranulation marker CD107a compared to Meso1 T cells upon a co-culture with mesothelin+ ovarian and gastric cancer cell lines. These T cells also secreted larger amounts of pro-inflammatory cytokines IFNγ, IL-2 and TNFα and lysed a larger proportion of these cell lines *in vitro* compared to Meso3 cells. In a s.c model of SKOV3, meso3 T cells administered at Day 7 mediated complete tumor eradication compared to mock-transduced cells. When T cells were administered at a later time point against more established tumors (Day 14), Meso3 T cells significantly attenuated tumor growth, although treatment with both Day 7 and Day 14 T cells mediated 100% survival at the end of the experiment, which was 60% and 40% for the mock T cells and saline controls respectively. However, the efficacy of Meso1 cells was not assessed in this model, so the translation of the *in vitro* findings could not be confirmed [111]. To further improve efficacy, Meso3 CAR T cells were engineered to secrete anti-CD40 agonist antibodies (Figure 1B) [112], previously demonstrated to elicit anti-tumor efficacy in various cancers particularly in combination with alternative therapies [113–115]. To prevent immune-mediated cell death of the CAR T cells themselves which express CD40, the anti-CD40 scFv was fused to a mutated human IgG4-Fc. Plasmids encoding the CAR and secretory anti-CD40 were co-electroporated into T cells using the PiggyBac transposon system, yielding efficient CAR expression and stable and continuous anti-CD40 antibody secretion. This modification bestowed T cells with a higher central memory population and significantly higher IL-2 and IFNγ secretion compared to the parental Meso3 cells. CD40-secreting Meso3 cells demonstrated a marginal but significant improvement in *in vitro* cytotoxicity. However, differences were more prominent in an s.c *in vivo* SKOV3 tumor model, where this treatment decreased tumor flux and improved survival greater than seen with parental CAR T cells. Most importantly, no toxicity was observed with either CAR [112].

More recently, Shoutrop et al. compared CD28- (M28z) versus 4-1BB-containing (MBBz) anti-mesothelin CAR T cells and uncovered mechanisms underlying their differential efficacy and persistence (Figure 1B) [116]. Comparable transduction, antigen-specific *in vitro* cytotoxicity and IFNγ secretion was observed between both CAR T cell types when co-cultured with mesothelin+ OVCAR3 and SKOV3 cell lines. Subsequently in an orthotopic SKOV3 model, M28z mediated a quicker initial response than MBBz T cells; however, this effect was transient, and the tumors quickly outgrew. The proportion of responders in the MBBz group

was lower although these responses were long-lived and resulted in improved survival compared to the M28z group (20% vs 0% survival at the end of the study). Using the same approach in the more aggressive HG-SOC OVCAR4 model, survival was comparable between the two groups. Importantly, treatment with either CAR T cell approach was followed by loss of antigen expression on tumors due to cell surface downregulation/internalization and recycling. This decline was more rapid in M28z-treated mice while antigen re-expression was observed upon ex vivo culture. This phenomenon could also be explained by the induction of mesothelin expression on the CAR T cells themselves as a cause of trogocytosis of the antigen-positive tumor cells. Moreover, the remaining mesothelin-positive cells were shown to gradually acquire the expression of PD-L1 and HLA-DR, with the effect being quicker with the M28z treatment. Crucially, although there was a higher proportion of tumor-infiltrating M28z T cells compared to MBBz T cells, these cells presented with a more exhausted phenotype, with a higher upregulation of TIM3 and LAG3, whilst PD-1 expression was comparable. Trafficking T cells expressed lower levels of these markers compared to those which penetrated the tumor. Notably, tumor-isolated MBBz cells were shown to be functional when co-cultured with SKOV3 cells ex vivo with an efficiency higher than that of M28z cells; however, these differences were not found to be significant. Overall, the differential kinetics between CD28- and 4-1BB-bearing CARs illustrated in this study provide a rational for combination approaches, particularly with checkpoint blockade inhibitors [116]. Indeed, the silencing of PD1 expression on third generation mesothelin-targeted CAR T cells bearing both CD28 and 4-1BB significantly improved the anti-tumor efficacy in pre-clinical models of both ovarian and colon cancer (Figure 1B) [117]. Another inhibitory factor secreted by dying tumor cells as a means of inducing immune suppression is adenosine [118]. Using the same shRNA strategy to silence PD1 on these anti-mesothelin CAR T cells [117], this group also demonstrated improved *in vitro* and *in vivo* cytotoxicity in SKOV3 models when the adenosine A2 receptor was downregulated (Figure 1B) [119].

Clinical assessment of mesothelin-targeted CAR T cells

The first in-human study evaluating anti-mesothelin CAR T cells in ovarian cancer (ClinicalTrials.gov: NCT02159716) entailed an SS1 scFv-based CAR incorporating 4-1BB and CD3ζ (CART-meso). Fifteen patients were recruited, comprising 5 each with malignant pleural mesothelioma, pancreatic ductal adenocarcinoma, or ovarian adenocarcinoma. Patients were treated i.v with lentivirally-transduced cells and split into four cohorts; cohorts 1 and 2 received 1–3x107 CART-meso cells/m2 whilst cohorts 3 and 4 received a higher dose of 1–3x108 CART-meso cells/m2. Cohorts 2 and 4 also underwent cyclophosphamide lymphodepletion two days prior to T cell infusion. Ovarian cancer patients had the most consistently upregulated levels of mesothelin across the cohorts. The best overall response was stable disease, seen in 11 of the patients. One patient within the ovarian cancer arm in cohort 2 achieved a considerable reduction in tumor burden although it was insufficient to be classified as a partial response using RECIST criteria. Upon infusion, the CART-meso cells expanded within the peripheral blood, with peak expansion 6–14 days post administration. However, persistence was limited, and CAR T cells were only detectable in two patients 6 months post treatment. Lymphodepletion improved initial expansion but had no effect on T cell persistence. Poor persistence could be attributed to immune-mediated elimination owing to the murine nature of the scFv. Although no human anti-mouse antibodies were detected, 10 out of 14 evaluated patients had detectable human anti-CAR antibodies. Moreover, CART-meso DNA could be detected at low levels within five post-treatment

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biopsies obtained, suggesting some infiltration into the tumor site. Generally, the treatment was well tolerated, with the main toxicities being nausea and low-grade fatigue. However, one patient in the pancreatic cancer arm experienced a dose-limiting toxicity (abdominal pain, jaundice, and fatigue 34 days after T cell infusion). Importantly, no off-tumor toxicities were observed. Although this study highlighted the safety of CART-meso cells up to a dose of 3x108 cells/m2, little anti-tumor efficacy was observed which could be attributed both to poor persistence and inadequate tumor infiltration [120,121]. In order to minimize CAR immunogenicity, the same group is recruiting patients on trials assessing a fully humanized anti-mesothelin CAR in different indications, including ovarian cancer (ClinicalTrials.gov: NCT03054298 and NCT03323944). Several mesothelin-targeting CAR T cell trials from other groups are also currently recruiting patients diagnosed with refractory epithelial ovarian cancer either alone (ClinicalTrials.gov: NCT04562298, NCT03799913, NCT03916679, NCT02580747, NCT03814447) or in combination with anti-PD1 secreting nanobodies (NCT04503980) or with the oncolytic adenovirus VCN-02 (NCT05057715).

L1-CAM

The L1 cell adhesion molecule (L1-CAM) was similarly identified as a diagnostic marker for serous ovarian carcinoma, where expression was shown to correlate with disease severity. Importantly it was undetectable on healthy tissue [122,123]. Its upregulated expression has been detected on several solid tumor subtypes and has previously been targeted by CAR T cells directed against neuroblastoma [124]. Moreover, mAbs targeting L1-CAM were shown to inhibit the growth and dissemination of an i.p SKOV3 tumor in nude mice, revealing prospects for targeting ovarian cancer via this antigen [125]. In the initial study evaluating anti-L1-CAM CAR T cell efficacy against ovarian cancer, primary human T cells were transduced with a CAR bearing the same scFv targeting the CE7 epitope of L1-CAM as used in the neuroblastoma study [124], but further modified to include CD28 alongside CD3ζ (Figure 1J) [126]. The resultant CE7R+ T cells were enriched for CD45RA-CD62L+ central memory T cells (TCM) prior to transduction, a subpopulation shown to confer enhanced therapeutic function. CE7R+ T cells were shown to effectively target a panel of L1-CAM+ cell lines in an antigen-dependent manner, including SKOV3, CAOV3, OVCAR3 and MADH2744, accompanied by production of high levels of IFNγ and TNFα. Intriguingly, level of surface LI-CAM1 expression correlated with cytokine secretion but not with cytolytic capacity. These T cells were then assessed *in vivo* in a SKOV3 i.p xenograft model in NSG mice. Mice received two i.p doses of 5x106 CAR+ T cells on days 5 and 12 post tumor inoculation. Complete tumor outgrowth and dissemination into multiple nodular tumors was observed in mice treated with PBS or mock-transduced cells, with mice developing ascites and requiring euthanasia within two months. On the other hand, 3/6 mice of mice treated with CE7R+ T cells remained alive, one of which had a complete response. Impressively, tumor outgrowth in the remaining 3 mice treated with CE7R+ was not accompanied by ascites. However, decreased L1-CAM expression was detected, highlighting at least one mechanism of immune escape in this model. Impressively, these T cells lysed primary ovarian cancer cells from patient malignant ascites samples, accompanied by production of large amounts of IFNγ and TNFα [126].

EpCAM

The epithelial cell adhesion molecule (EpCAM) is a well-recognized marker of epithelial cancer cells, driving their proliferation and differentiation, as well as regulating adhesion, and has been extensively evaluated as an anti-tumor target mainly using antibody-based methods [127,128]. Moreover, the targeting

of this antigen using CAR T cells showed some promise in preclinical testing against colorectal cancer [129–131]. One study thus far has evaluated the targeting of this antigen in ovarian cancer, revealing encouraging *in vitro* and *in vivo* cytotoxicity against SKOV3 cells (Figure 1K) [132]. Nevertheless, similar to the ErbB family, the broad expression of EpCAM on healthy epithelial cells and stem cells may limit its use as a target for CAR T cell therapy [133].

Follicle-stimulating hormone receptor (FSHR)

An underexplored target for CAR T therapy against ovarian cancer is the follicle-stimulating hormone receptor (FSHR). This G-protein-coupled receptor is mainly expressed on the Sertoli cells of the ovary, and it is thought to play a role in neoplastic transformation [134,135]. FSHR is expressed in most serous subtypes of ovarian cancer [136,137] and ectopic expression has also been detected on tumor-associated blood vessels, including those found within ovarian tumor microenvironments [138]. This provides a rationale for targeting FSHR with the aim of eliminating both cancerous cells and surrounding vasculature. Moreover, given that most patients undergo an oophorectomy, and given the abundant expression of FSHR on ovarian cancer compared to somatic tissues, the on-target off-tumor toxicity risk of this therapy remains limited. Similarly to the ErbB-binding T4 CAR [53], a panel of immunoreceptors were generated utilizing peptides derived from FSH subunits (Figure 1L) [134]. The anti-FSHR peptides were fused to either CD3ζ alone or in conjunction with CD28, and the two CARs with the greatest functional activity were selected based on the secretion of multiple Th1 cytokines (IFNγ, IL-2, MIP-1α and TNFα) upon co-culture with FSHR+ ovarian cancer cell lines. Importantly, these peptides were also shown to recognize murine FSHR, although accompanied by lower-level cytokine production. Nevertheless, this provides an opportunity to assess the toxicity of this therapy. Two doses of i.v. T cells that expressed either anti-FSHR CARs significantly suppressed the growth of a s.c CaOV3 tumor, established in NSG mice. T cell persistence was observed for up to 5 weeks post treatment [134]. In a separate study, both full-length subunits of the FSH were used to re-target T cell specificity against FSHR, further incorporating CD3ζ and 4-1BB (Figure 1L) [137]. Impressive *in vivo* efficacy of these T cells was demonstrated in both a CaOV3 xenograft model in immunodeficient mice, as well as against ovarian patient-derived xenograft (PDX) tumors. Complete rejection of the FSHRhigh PDX tumors was observed, whereas tumor growth was only delayed in models with low expression of the receptor. Efficacy was further demonstrated in an orthotopic PDX model using autologous patient T cells, inducing prominent tumor necrosis. The safety of this construct was demonstrated in a fully murine model of the ID8 tumor cell line using murine CAR T cells. Two i.p. doses of 1–1.5x106 cells were sufficient to significantly enhance survival, an effect which was not observed when T cells were administered i.v. Survival rate dropped to 0% at day 70 post tumor challenge for i.p administered CAR T cells versus day 62 for mock-transduced cells. However, for i.v administered CAR T cells this occurred at day 58 versus day 57 for mock-transduced cells. Importantly, there was no evidence of toxicity. Isolated CD4+ CAR T cells maintained similar efficacy to a mixed CD4/CD8 population, whereas treatment with CD8+ CAR T cells was suboptimal. This group also showed evidence of the induction of a host anti-tumor response induced by the transfer of anti-FSHR CAR T cells. Intriguingly, these T cells were shown to be persistent in the peritoneal cavity, with a peak at 5 days after administration. T cells were similarly detectable in the spleen and tumor draining lymph nodes even at the later stages of tumor progression, with a peak at day 10. However, T cells were not found present in tumor ascites, and the group postulated that in the presence

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of advanced ascites, a membrane-bound form of FSHR in exosomes secreted from tumor cells activates distal CAR T cells, impairing the efficacy of this therapy within the tumor beds. It would therefore be preferential to administer these T cells in patients with low volume ascites or following drainage [137].

B7-H3

Originally thought to be a T cell co-stimulatory molecule [139], B7-H3 has subsequently been shown to act as a suppressor of CD8 T cell-mediated anti-tumor responses [140–142]. It is highly expressed on various human cancers including breast, lung, ovarian and brain cancers whilst expression remains low on healthy cells [143]. In addition to mediating immune suppression, this protein has been shown to play a role in promoting tumor migration and invasion, EMT and chemoresistance [143]. In the context of CAR T cell therapy, this target has mainly been evaluated in glioblastoma [143]. Du et. al evaluated the efficacy of anti B7-H3 CAR T cells primarily in pre-clinical models of pancreatic ductal adenocarcinoma but also in ovarian carcinoma. CAR targeting was mediated by the B7-H3 376.96 mAb scFv, fused to either CD28 or 4-1BB and CD3ζ (Figure 1H). 90% of ovarian tumor specimens stained positive with the B7-H3 376.96 mAb. Both B7-H3.CAR-28ζ and B7-H3.CAR-BBζ T cells lysed ovarian cancer cell lines and secreted comparable levels of pro-inflammatory cytokines *in vitro*, against three different cell lines; SW626, SKOV3 and CaoV3. Both constructs similarly controlled tumor growth when evaluated in a SKOV3 i.p. xenograft model, with the CD28-bearing CAR resulting in a marginally improved survival rate (100% vs 80% at the end of the study). The safety of this therapy was confirmed by the lack of impact on any healthy immune or hematopoietic cells, except peripheral blood monocytes, and the lack of any healthy tissue damage. This minimal off-tumor toxicity could however be attributed to the lower density of B7-H3 expression on murine tissues and the lower affinity of the B7-H3 376.96 mAb for murine B7-H3. Nevertheless, this promising preclinical data was supportive of clinical assessment of B7-H3 CAR T cell therapy [144]. There are two trials recruiting patients for the evaluation of autologous B7-H3 CAR T cells in recurrent epithelial cancer (ClinicalTrials.gov: NCT04670068) as well as the use of a fully humanized anti-B7-H3 CAR for recurrent malignant ovarian cancer (ClinicalTrials.gov: NCT05211557).

Placental alkaline phosphatase (PLAP)

The membrane-bound placental alkaline phosphatase (PLAP) is another potential solid tumor cell surface target, overexpressed on a number of indications including ovarian cancer [145,146] where it has been shown to induce tumor aggressiveness [147]. Using a SynNotch circuit system, anti-PLAP CAR T cells co-targeting either melanoma cell adhesion molecule (MCAM), mesothelin, or HER2 demonstrated superior pre-clinical *in vivo* efficacy against both mesothelioma and ovarian cancer xenografts (Figure 1P) [148]. A Phase I/II study evaluating anti-PLAP CAR T cells in patients with ovarian and endometrial cancer is underway (ClinicalTrials.gov: NCT04627740).

5T4

The oncofetal antigen 5T4 was identified as a potential ovarian cancer target due to its abnormal expression compared to healthy tissue and its link to disease stage and progression [149]. Only one study thus far has assessed the targeting of this antigen in the context of CAR T cell therapy. Two CARs bearing scFvs of different affinities were compared, both bearing 4-1BB and CD3ζ, the higher affinity H8-CAR and lower affinity 2E4-CAR (Figure 1M). Both healthy and patient-derived PBMCs were efficiently transduced and

secreted high levels of IFNγ upon co-culture with antigen-positive target cell lines. Most patient-derived CAR T cells were similarly activated when cultured with autologous tumor cells. The higher affinity H8-CAR T cells secreted significantly larger amounts of cytokine compared to the 2E4-CAR. However, IL-2 secretion was only detected at moderate levels in cell line co-cultures and was not detectable when CAR T cells were stimulated with autologous tumor. This was attributed to the differential and overall lower antigen expression on tumor disaggregates compared to the high uniform expression on cell lines. In an i.p. SKOV3 model, mice were treated i.v. with H8-CAR T cells using a dose-escalation regimen evaluating between 0.03–1x107 total T cells. Although transduction efficiencies were low, both doses of 0.3x107 and 1x107 total T cells mediated rapid tumor regression, accompanied by survival for >100 days in the latter group. This study also found that i.p. administration of T cells shows improved efficacy compared with the i.v route [149].

Urokinase plasminogen activator receptor (uPAR)

The urokinase plasminogen activator receptor (uPAR) is another potential candidate which has been evaluated as a CAR T cell targeting antigen. This GPI-linked protein is upregulated in ovarian cancer tissue and has been shown to play a role in peritoneal metastasis and ascites development [150,151]. Interestingly, expression is also detected on tumor-associated stromal cells, providing an attractive means of targeting both the primary tumor and the microenvironment [152]. A third generation CAR was generated using amino-terminal fragment (ATF), the natural ligand for uPAR, as the targeting moiety, fused to CD28, 4-1BB and CD3ζ (Figure 1O). These ATF-CAR T cells specifically lysed ovarian cancer cells in an-antigen dependent manner, as demonstrated through the inability of these cells to target uPAR shRNA-silenced cell lines. Nevertheless, background tumor cell killing was also observed with control T cells [153].

Müllerian inhibiting substance type II receptor

The TGF-β receptor family member Müllerian inhibiting substance type II receptor (MISIIR) is another emerging target for CAR T cell therapy against gynecological malignancies including ovarian and endometrial cancer due to its overexpression on these tumors [154,155]. The engagement of this receptor with its endogenous ligand MIS has been shown to induce neoplastic cell death. However, efforts to stimulate MIS signaling have not reached the level of clinical assessment yet [156–158]. Therefore, this receptor was chosen as a target for CAR T therapy in one study [159]. A panel of four CARs bearing different anti-human MISIIR scFvs fused to CD27 and CD3ζ were screened for efficacy against MISIIR-bearing ovarian tumors (Figure 1N). Only two of the CARs, bearing the GM7 and GS45 scFvs, were able upregulate CD69 expression on T cells and mediate the secretion of large amounts of IFNγ upon co-culture with ovarian C30 cells engineered to ectopically express the ligand. However, only GM7 demonstrated antigen-specificity and was chosen as the lead CAR. GM7 CAR T cells upregulated expression of the degranulation marker CD107a and secreted high levels of IFNγ, IL-2 and TNF-α when co-cultured with C30.MISIIR cells. Impressive complete tumor eradication was observed *in vivo*, when GM7 CAR T cells were administered i.v into 3/5 mice bearing s.c C30.MISIIR tumors, with delayed tumor progression in the remaining mice. However, when assessed in xenografts endogenously expressing MISIIR, OVCAR3 and OVCAR5, progressive and rapid tumor growth was observed, although tumors were smaller in the GM7 CAR T-treated group compared to control groups. T cells bearing a signaling-deficient GM7 CAR were unable to elicit any cytotoxicity against ovarian cancer cell lines *in vitro*,

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despite engagement with MISIIR. Therefore, the anti-tumor effects were solely attributed to CAR-mediated T cell activation. Impressively, GM7 CAR T cells were activated by and lysed patient-derived HGSOC samples. Importantly, no on-target off-tumor toxicities were observed in any of the pre-clinical xenograft models despite the cross-reactivity of the GM7 scFv for murine MISIIR. Moreover, these T cells did not elicit any lysis against a range of different healthy primary human cells bearing low expression of MISIIR, apart from some low-level cytotoxicity of aortic smooth myocytes at a high effector-to-target ratios [159].

CONCLUSIONS

The treatment of ovarian cancer remains a challenging obstacle due to the nature of the disease commonly resulting in late diagnosis and poor survival outcome. Clinical responses to immunotherapeutic agents have been discouraging, owing to the "immunologically cold" nature of these tumors. However, the emergence and initial successes of CAR T cell therapy for the treatment of cancer have opened up exciting opportunities to combat this disease. Encouragingly, great strides have been made in identifying targetable antigens and optimizing CAR design to enhance anti-tumor responses (summarized in Figure 1). Here, we have reviewed the pre-clinical studies which have evaluated the targeting of a range of targets in ovarian cancer by CAR T cells. Some of these impressive results have prompted the initiation of Phase I clinical trials; however limited results have been published to date. Target selection remains a key priority in the design of CARs, and a truly ovarian cancer-specific target has yet to emerge. Moreover, we believe that simultaneous targeting of multiple antigens will drive improved successes with these therapies, circumventing antigen escape mechanisms and affording opportunities to deliver complementary signals to the CAR T cells [160]. Furthermore, it is likely that armoring and/ or combinatorial therapeutic strategies, perhaps with immune checkpoint inhibition, will be required in order to enable CAR T cells to home to, infiltrate and operate within the highly immunosuppressive TME generated by these tumors. From a safety perspective, approaches that involve the fine-tuning of T cell activation and optimal antigen engagement will be crucial in limiting the toxicity arising from CAR T cell treatment. It should be noted however that safety has not proven as large an issue in the treatment of ovarian cancer with CAR T cells as has been seen in hematological malignancies. Therefore, our view is that the primary focus should remain on efforts to boost efficacy and functional CAR T cell persistence as these therapeutic approaches are advanced. Nevertheless, the collective findings summarized in this review reveal great prospects for the treatment of ovarian cancer using re-targeted T cell therapies.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY

SPOTLIGHT

In vivo engineering of CAR T cell therapies

Haig Aghajanian
Capstan Therapeutics & University of Pennsylvania

"How far away is *in vivo* CAR T cell therapy? We at Capstan Therapeutics believe it is closer than many people think."

VIEWPOINT

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On July 19, 2022, David McCall, Commissioning Editor, *Cell & Gene Therapy Insights*, spoke to Haig Aghajanian about the rise of *in vivo* cellular immunotherapy. This article has been written based on that interview.

THE SILVER LINING OF COVID-19: LNP & MRNA IN ADVANCED THERAPY APPLICATIONS

The COVID-19 pandemic has been a tragedy for everyone. However, one of the biggest 'silver linings' has been the acceleration of both mRNA technology and lipid nanoparticle (LNP) technology.

Drew Weissman and Katalin Karikó discovered the modifications to mRNA that paved the way for its use as a therapeutic, though it took many years for that discovery to reach the clinic. With billions of patients now dosed with mRNA-LNPs, the technology has seemingly been de-risked and accelerated. This mRNA and LNP combination technology has been used and built upon by adding ligands to specifically target certain subsets of immune cells in the body in order to reprogram them.

IN VIVO ENGINEERING APPROACHES TO CAR T CELL THERAPY

Chimeric antigen receptor (CAR) T cell therapy has been a revolution in oncology, with high cure rates in refractory blood cancers. There are currently six different products on the market, and these therapies are changing patients' lives. The remaining issues lie in scalability, manufacturability, and access to these lifesaving therapies. In addition, not all patients achieve a durable response and some experience life threatening toxicities. Thus, there are various aspects to the patient access challenge, including cost, geographic location, access to major medical facilities, as well as the current requirement for therapy administration in the inpatient setting.

In vivo engineering aims to solve many of these limitations to access. Allogeneic cellular immunotherapy is a novel approach, but it still has many limitations that are similar to those found with autologous therapies, including manufacturing requiring cell handling, and lymphodepletion prior to administering the cells. *In vivo* takes the field one step further by potentially providing a drug-like approach for a cell therapy-type treatment. This reprograming approach can increase access in many ways, including potentially lowering cost and making a stable, off-the-shelf product with the prospect for use in earlier lines of therapy and in outpatient settings.

Much of our early proof-of-concept work for *in vivo* CAR T cell therapy is in heart disease (cardiac fibrosis). In this setting, we were able to show that ablating activated cardiac fibroblasts in a model of cardiac injury can reduce fibrosis and restore cardiac function. This is a novel area for CAR T cell therapy, with the field having initially gained traction in hematologic malignancies, where it has up to 40–50% cure rates in some indications and products.

This serves to illustrate a key limitation of the incumbent *ex vivo* CAR T cell therapy methodology. At this point, it is not feasible for the CAR T cell therapy field to scale from tens of thousands of patients a year to the millions of potential patients a year with cardiac fibrosis. UPenn's Bruce Levine has commented that there is not enough AB serum in the world to make enough CAR T cells to treat heart disease. We need another approach.

In addition to allowing us to reach diseases with larger patient populations, including solid tumors, the *in vivo* approach can help us reach other therapeutic areas. For example, almost every organ system can suffer from fibrosis, from the lungs to the liver to the kidneys. It is a broad-reaching pathology that affects many disease processes and is an important part of progression to heart failure and other endpoints in these diseases. If we can scale this up and have an off-the-shelf approach, we can reach the types of indications that have previously been off limits to CAR T cell therapies from a commercial perspective. This includes indications in which *ex vivo* CAR T cells have been already shown some promise, such as autoimmune disorders like systemic lupus, and metabolic diseases such as type 1 diabetes. Additionally, many researchers are targeting cellular senescence and other

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age-related diseases. Furthermore, the very first indications considered for CAR T cell therapies were infectious diseases. There were many early clinical trials for human immunodeficiency virus (HIV), for instance. Today, CAR T is once again gaining traction in large infectious disease indications including HIV, hepatitis C, tuberculosis, and invasive aspergillosis. The *in vivo* approach can make these therapeutics a possibility for the many rather than the few in the not-too-distant future.

THE VERSATILITY OF *IN VIVO* REPROGRAMING OF IMMUNE CELLS

There are several different viral and non-viral delivery vehicles for *in vivo* reprograming. Non-viral platforms include LNPs, nanocarriers, and polymer-based vehicles. There is also a whole host of different viral platforms that can be used for *in vivo* reprograming, each with its own benefits and downsides. With the non-viral approach comes the ability to re-dose, to not illicit an immune reaction, and to have a transient effect. The viral approaches carry the potential to have a more permanent effect with the integration of a gene into the cells of interest.

However, whichever type of delivery approach is taken, *in vivo* cellular immunotherapy can offer versatility. In our case, using LNPs, we are employing an antibody-targeting platform to reach specific cells of interest. We have shown effectively that it is possible to use CD4/CD5 antibodies to target either pan-T cells or a subset of T cells.

This is not limited to T cells, though – we could potentially target any immune cell subset, including NK cells, monocytes, macrophages, and T regulatory cells. We could also target other cells in the body, including hematopoietic stem cells that give rise to immune cell lineages.

The payloads are also highly versatile. In an LNP, various types of nucleic acid can be added, including mRNA, modified mRNA, DNA, or a combination of these. For instance, an mRNA encoding for CRISPR nuclease and a guide RNA could be placed within the same LNP. There is also the possibility for protein and viral payloads, though with viral-based approaches, there is more of a limit on size.

NEXT STEPS FOR *IN VIVO* GENE IMMUNOTHERAPY

Many of these delivery components have already proven to be effective in the clinic, including LNP and mRNA technology. Several challenges remain, but many groups are trying different approaches for the *in vivo* reprograming of cells, some of whom are quickly approaching the clinic. These first clinical trials are likely to be very informative regarding the long-term feasibility of this approach.

The next steps for Capstan Therapeutics are to find out which indications are conducive to this type of therapy, and the doses and dose regimens that are required. We aim to optimize the platform technology and product candidates to different conditions for reprograming specific cells and subsets of cells, and work out the payloads and the clinical outcomes we want to see.

After validating the platform and showing that it is effective, we want to find where the greatest medical need is. Ultimately, we are interested in bringing these life-saving medications and therapies to all the people who need them, and to then broaden treatment to even wider indications.

Lymphodepletion and other types of conditioning regimens can be extremely harsh on patients. With the transient approach of mRNA without lymphodepletion, we may be able to avoid some of the more toxic side-effects, such as bone marrow toxicities, cytopenia, neurotoxicity and cytokine release syndrome. This could enable the therapies to be given in an outpatient setting.

From the preclinical models, we have seen that the number of cells that we reprogram in the body is much greater than what is seen with *ex vivo* adoptively transferred CAR T

cells, even after a full *in vivo* expansion. Upon confirming the translatability of these findings, we hope to effectively reprogram the immune system to go after the pathogenic cell of interest - either an activated pathogenic fibroblast, a malignant cell, or neoplastic cell - and quickly and efficiently ablate these cells. This could have the same type of effect that an *ex vivo* CAR T cell could have, but potentially in a shorter amount of time. In addition, we aim to test and optimize methods to co-opt the endogenous immune system during treatment – utilizing one of the advantages with *in vivo* therapy having an intact immune system vs *ex vivo* which requires lymphodepletion conditioning.

In terms of the clinic, questions surrounding pharmacokinetic (PK) and pharmacodynamic (PD) profiles, as well as dosing, are yet to be answered. Ultimately, success in reprograming immune cells in the body will hopefully be sufficient to treat these clinical indications that come with a high medical burden.

How far away is *in vivo* CAR T cell therapy? We at Capstan Therapeutics believe it is closer than many people think.

BIOGRAPHY

HAIG AGHAJANIAN is Co-Founder, VP and Head of Research at Capstan Therapeutics, and Adjunct Assistant Professor of Medicine at the Perelman School of Medicine at the University of Pennsylvania. At the University of Pennsylvania, he authored several impactful studies including a First-author publication in Nature using CAR T cells for the treatment of heart disease and a Senior-author publication in Science using targeted lipid nanoparticles to reprogram immune cells in vivo. His work has been featured in The New England Journal of Medicine, New York Times, Scientific American, and The Journal of the American Medical Association. He received his PhD from the Perelman School of Medicine at the University of Pennsylvania.

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY

SPOTLIGHT

INTERVIEW

Advancing genome edited allogeneic cell therapies into the clinic

David McCall, Editor, Cell & Gene Therapy Insights, talks to **Steve Kanner** PhD, CSO, Caribou Biosciences

STEVE KANNER is the CSO at Caribou Biosciences responsible for the company's therapeutic discovery, research, and development activities. Before joining Caribou in 2017, Steve was Vice President, Head of Biology, at Arrowhead Pharmaceuticals, leading a department in discovery of RNAi therapeutics for oncology, genetic diseases, and other indications. Prior to Arrowhead Pharmaceuticals, he served in various positions of increasing responsibility in both oncology and inflammation drug discovery at Bristol-Myers Squibb, Agensys/Astellas, and Astex Pharmaceuticals. Steve has authored over 85 publications in both peer-reviewed journals and books, and he is an inventor on numerous U.S. and foreign patents and patent applications. Steve received his undergraduate degree in Genetics from the University of California, Berkeley and his PhD in Immunology and Microbiology from the University of Miami's Miller School of Medicine. His post-doctoral fellowship was funded by the National Institutes of Health and was conducted at the University of Virginia.

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Q What are you working on right now?

SK: At Caribou Biosciences, we are implementing a next-generation genome editing technology with high precision and high specificity to develop innovative and transformative therapies for patients with cancer. We are developing two cell therapy platforms, allogeneic chimeric antigen receptor (CAR)-T cell therapies for hematologic malignancies and allogeneic CAR natural killer (NK) cells derived from induced pluripotent stem cells (iPSCs) for solid tumors.

We are pleased to have presented positive initial clinical data recently from our first clinical program with CB-010. CB-010 is an allogeneic anti-CD19 CAR-T cell product candidate with a PD-1 knockout which is being investigated in the ANTLER Phase 1 trial for the treatment of patients with relapsed or refractory B-cell non-Hodgkin lymphoma (r/r B cell NHL).

We have two other allogeneic CAR-T cell programs in development: CB-011 targeting BCMA, which is an immune cloaked product candidate that we plan to investigate for the treatment of relapsed or refractory multiple myeloma (r/r MM), and CB-012, a CD371-targeted product candidate with potentially multiple armoring strategies that we plan to investigate for the treatment of relapsed or refractory acute myeloid leukemia (r/r AML). We also have two CAR-T cell programs under development with AbbVie.

In addition, our genome-edited iPSC-derived NK cell therapy platform is being used to develop CB-020, which is in preclinical development for the treatment of solid tumors.

Q What's your assessment of recent progress in the allogeneic cellular immunotherapy area in terms of improving safety, both by Caribou and the field in general?

SK: For any allogeneic CAR-T or CAR-NK cell product, ensuring that the edits you are making are as precise and specific as possible is of utmost importance to reduce unintended genomic errors or potential cancer-causing properties that could be inadvertently administered to the patient. In addition, having an optimized lymphodepletion regimen has been shown to improve safety. We have been working with both approaches to enhance Caribou's product candidates.

At Caribou, the genome editing technology we use is called clustered regularly interspaced short palindromic repeats (CRISPR) hybrid RNA-DNA (chRDNA), or "chardonnay." This enables us to introduce double-stranded breaks, using Cas9 or Cas12a, with specificity designed to eliminate detectable off-target edits. This reduces the likelihood of translocations that could occur in the cells during development, even when making a single edit.

In our process, we generate cells with multiple edits - for instance, our first product candidate in the clinic, CB-010, has three edits. We knock out the TRAC locus to eliminate expression of the T cell receptor (TCR) while inserting the anti-CD19 CAR into the same location. The third edit is a knockout of the gene encoding the immune checkpoint receptor PD-1. To reduce the likelihood of translocations between these edits, we have developed a proprietary

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delivery technology that reduces the level of chromosomal rearrangements. So, from a safety point of view, we are reducing the likelihood that we could be interfering with important genes such as tumor suppressors.

In the generation of our product candidates, knockout of the TCRs is intended to improve safety by reducing the likelihood of graft-versus-host disease (GvHD) in the patient. Moreover, we take it one step further during our manufacturing process. Once we complete the genome editing, we use a method with antibodies targeting the TCR to deplete the residual population of TCR-positive T cells. This further reduces the possibility that the patient would experience GvHD.

> "ensuring that the edits you are making are as precise and specific as possible is of utmost importance to reduce unintended genomic errors or potential cancer-causing properties that could be inadvertently administered to the patient."

Beyond safety in the product candidates, we have been evaluating a lymphodepletion chemotherapy regimen to improve safety. In our phase 1 ANTLER trial, our protocol includes a well-established lymphodepletion regimen that enables easier monitoring of the patient's tolerance to the chemotherapies. Unlike many lymphodepletion protocols for CAR-T cell therapies, the ANTLER protocol separates administration of two commonly used chemotherapies, so they are administered independently over a longer period. This enables the physician to follow the tolerance to those agents more carefully than if they were combined. Thus, we have included flexibility for patient safety.

Clinically, the readouts of safety to date in the allogeneic CAR-T field are consistent with what has been observed in the autologous CAR-T cell field. The types of adverse events monitored and observed are similar to what has been seen in the autologous space, such as cytokine release syndrome (CRS), immune effector cell–associated neurotoxicity syndrome (ICANS), and neutropenia. We have not observed adverse events that were surprising or outside of what would be expected based on the autologous CAR-T cell experience.

Q On the efficacy side, there has been some encouraging data coming through in the past several months, too. What's your view of progress there?

SK: For most allogeneic cell products, ensuring the cells persist sufficiently to destroy tumor cells is key. Advances across the field to improve persistence of antitumor activity, retention in circulation, or both should help improve efficacy.

At Caribou, our pipeline has been designed to increase the persistence of our allogeneic CAR-T cells. For CB-010, we implemented a modification that is distinct in the allogeneic field. We knock out the PD-1 checkpoint receptor from T cells, which prevents PD-L1 engagement and subsequent downregulation and exhaustion of the CAR-T cells. To our knowledge,

CB-010 is the first allogeneic CAR-T cell therapy with a PD-1 knockout in clinical studies, and, as I mentioned before, it is being evaluated in the ongoing ANTLER Phase 1 clinical trial in adults with r/r B-NHL.

In the preclinical setting, the PD-1 knockout leads to more durable antitumor activity in animal models with high tumor burden and metastatic disease, similar to the disease burden that may be observed in the clinical setting. Comparing CB-010 head-to-head to identical cells without the PD-1 knockout, we observed increased duration of antitumor activity and reduced tumor recurrence in a xenograft model, which we believe is translating clinically given our recent initial clinical data from the ANTLER trial.

As we look at our clinical data, we are observing something unique in the field. The standard assumption of other players in the allogeneic space is that a low dose should not yield significant efficacy, but as one increases the dose, efficacy may emerge. That is typical of what one would expect in a cancer-targeted trial.

However, with our first dose of CB-010, which was 40 million CAR-T cells, we observed that all of our patients experienced a complete response (CR). A single dose of CB-010 at the first dose level led to a 100% CR rate, which is unprecedented in CAR-T cell clinical trials. It was thrilling to see that play out.

Given these results and the green light from our study steering committee, we are now enrolling patients at dose level two, which is 80 million CAR-T cells. Generally, dose escalation is done to evaluate safety and find the dose that is safe and efficacious for the expansion and Phase 2 studies. But with a 100% CR rate, we will dose escalate to gain a better understanding of the durability of a single higher dose of CB-010.

Q As one of the pioneers in translating gene edited allogeneic therapies into the clinic, what are some important learnings that you will take from the experience of preparing successfully for an Investigational New Drug Application (IND) and first-in-human trials in this novel area?

SK: When we started out, the FDA was still building consensus about allogeneic CAR-T cells that were modified with different genome editing technologies. They were learning alongside our developmental process in a collaborative manner.

This collaboration helped us navigate a complex set of expectations by the Agency and internalize guidance that would allow us to apply learnings to our future programs. We became sophisticated quite quickly after going through the CB-010 IND, which enabled us to build upon this experience for our

"One advantage of developing next-generation CAR-T cell therapies in a highly competitive space is learning from what others have done and using that collective knowledge to navigate our own programs."

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second product candidate, CB-011. CB-011 is an allogeneic CAR-T cell product designed to persist by cloaking it from the patient's immune system. We plan to submit the IND for this product candidate in the second half of 2022.

One advantage of developing next-generation CAR-T cell therapies in a highly competitive space is learning from what others have done and using that collective knowledge to navigate our own programs. That helped us with respect to the approaches that we took when preparing for and strategizing our clinical plans.

Q The recent FDA guidance relating specifically to gene therapy was generally welcomed by the field as an important first step. What might be the key next steps for regulatory evolution?

SK: As sophisticated cell therapies are evolving quickly, the FDA must evolve with them. As the technology becomes more complicated, the FDA must simultaneously respond to the ever-changing landscape of these cell therapies.

One of the things we are implementing at Caribou is introducing an induced pluripotent stem cell (iPSC) platform. This is a platform that allows us to make a multiplicity of genome edits to iPSCs, which can then be cloned. If we clone a cell that is 100% edited for all the different changes we want to implement, we'll generate a master cell bank from that harvested clone. T cells are somewhat limited in terms of the number of edits one can make, because genome editing isn't usually 100% efficient and the cell population that contains all the intended edits will be diminished once many edits are implemented. With iPSCs, one can make changes to a cell population and then clone out the one cell that has all the intended features for generating a cell bank to be differentiated and expanded for clinical evaluation.

Using the chRDNA genome editing platform, we plan to take an edited clone and differentiate it into an NK cell that has the features that we believe will be key for targeting solid tumors.

The development of such a product candidate goes hand-in-hand with the collaboration with the FDA to ensure that they have confidence in the genomic stability and integrity of the cells. They need to know that we fully understand the implications of these genetic manipulations after turning it into an immune cell to have antitumor activity, from a safety point of view. Safety is both the FDA and Caribou's number one concern as we begin implementing these novel product candidates in the clinic.

Q Can you tell us more about the manufacturing strategy Caribou is employing?

SK: At Caribou, we have invested in an in-house process development team. We believe that building this expertise directly is critical to the success of our programs. Our PD colleagues develop the process, scale it up, optimize it, and ultimately tech transfer it to the contract manufacturing organizations (CMOs) who makes the clinical materials for us.

Generating CB-010, or any other genome edited cell-based therapy, as a product candidate requires a multiplicity of drug substances to come together to make the drug product. Each one of those elements must be generated GMP. At Caribou we partner with expert CMOs to generate GMP materials that we use in manufacturing our product candidates.

These materials include genome editing components, Cas9 or Cas12a enzyme, the chRDNA guides, viruses, plasmids, and leukapheresis material from healthy donors. These different elements go into making these sophisticated products, and we retain the expertise of vetted CMOs as well as backup CMOs for the materials. This ensures we have the product candidate cells readily available for patients who enroll in our clinical trials, and once approved, to a broader patient population who need these therapies.

Q Where next for genome editing platform evolution? What might be some key directions for future innovation and clinical application, both with CRISPR and beyond?

SK: Genome editing technologies have only just begun to scratch the surface of their potential. For example, we have heard in the news about some of the advancements that have been made using different genome editing technologies, including CRISPR, in hematopoietic stem cells to target diseases like beta thalassemia and sickle cell disease.

Another possibility is that through genome editing, iPSCs could be converted into a myriad of different cell types to address a wide variety of diseases. For example, they could be converted into cardiomyocytes for heart disease, into neurons for neurological disorders, or into muscle cells. There are plenty of places where genome editing technology could be used to fortify or improve the activity of different types of cells to address various diseases.

A different approach could be direct delivery of the CRISPR technology to organs of interest. Recently, companies described implementing the CRISPR reagents into lipid nanoparticles for *in vivo* genome editing to address liver disease. One might also imagine this approach could target other organs to address different diseases, particularly for inborn errors of metabolism where one might want to make a base change or reintroduce a normal gene. I think that the technology is there, but it is a matter of putting the right components together. It needs more development work in terms of delivery to the organ of interest, but the machinery itself is ready. This is a direction that could have vast impact across the medical landscape.

Q What are some chief goals and priorities, both for yourself in your own role and for Caribou as a whole, over the next 12–24 months?

SK: In addition to progressing CB-010 through dose escalation, expansion, and into Phase 2 studies, we have several milestones planned. By the end of this year our goals include submitting an IND application for CB-011 to target r/r MM. Next

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year, we plan to submit an IND application for CB-012, which is in development to target r/r AML. We feel that CB-010 is paving the path for the regulatory submission of these product candidates, and we are excited to be moving into different areas beyond B cell lymphomas.

> "Genome editing technologies have only just begun to scratch the surface of their potential."

There is our upcoming iPSC-derived NK cell platform, which is designed to target solid tumors, and before the end of this year, we intend to disclose the target and some of the armoring strategies we are developing for our CAR-NK platform.

So much is happening at Caribou, and I am excited to see what will emerge over the next few years.

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AUTHORSHIP & CONFLICT OF INTEREST

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FASTFACTS

Robust low-volume production of lipid nanoparticles for nucleic acid drug discovery & screening

Reka Geczy, Scientist II, Product Development, Precision NanoSystems

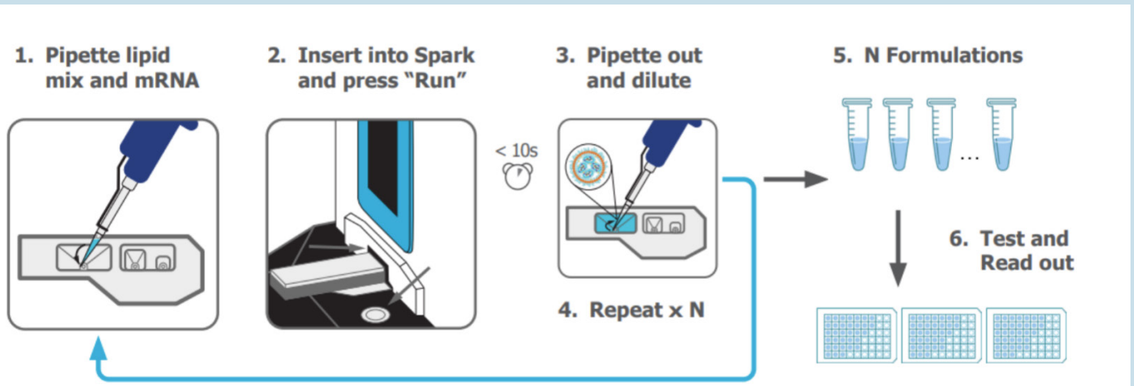
The discovery of genomic medicines requires rapid, reproducible, and small-scale nanoparticle formulations. Furthermore, novel genomic material can be costly or in limited supply. The NanoAssemblr® Spark™ encapsulates nucleic acids to create rapid and low-volume formulations to conserve limited and expensive ingredients during discovery and early preclinical development of lipid nanoparticles (LNPs).

THE SPARK PLATFORM

The NanoAssemblr Spark can be used for nucleic acid delivery and screening, nanoparticle design and screening, as well as drug discovery and target validation. It is well suited to developing nanomedicines that involve screening large libraries of potential candidates to identify leads.

The Spark instrument uses proprietary NxGen™ microfluidic technology, overcoming the challenges of traditional techniques for the controlled and reproducible manufacturing of lipid nanoparticles (LNPs) at volumes from 50 to 250 µL, encapsulating several µg of nucleic acid with minimal waste. Non-turbulent particle formation ensures reproducible results for a wide range of nanoparticle types. Formulations are created in less than 10 seconds, and the resulting LNPs can be diluted and applied immediately to cells in culture (Figure 1). Due to its space-saving design, the Spark instrument can be placed in a biosafety cabinet to integrate easily into a sterile cell culture workflow.

Figure 1. Spark workflow.



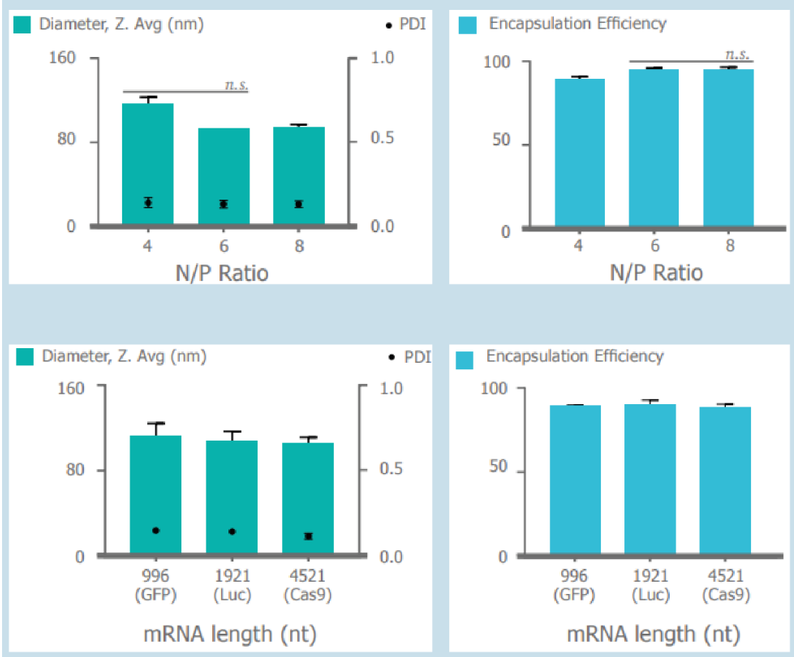
OPTIMIZING ENCAPSULATION EFFICIENCY

N/P ratio is a key performance factor. To determine the effective N/P ratio of mRNA LNPs, three ratios were tested using Spark (Figure 2). At N/P of 6:1 and 8:1, similar polydispersity index and encapsulation efficiencies were reached, suggesting that an asymptotic limit has been reached.

LNPs can be used as a platform to deliver different-sized nucleic acids. LNPs encapsulating three different mRNAs were made with different mRNA lengths on the Spark with no change in N/P size, as illustrated in Figure 2.

The Spark platform provides users with an easy-to-use system with the freedom to formulate nanoparticles rapidly and reproducibly for discovery and development. This low-volume solution for limited and costly materials provides a formulation workflow integrated with sterile cell culture processes to give uniform particles with controllable and fully scalable results. NxGen technology allows formulations to be scaled across the NanoAssemblr platform from mL/min to L/h with the same mixer design to accelerate future development.

Figure 2. Optimizing encapsulation efficiency with varying N/P ratios (top) and encapsulation with varying sized payloads (bottom).



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NEW HORIZONS IN CELLULAR IMMUNOTHERAPY

SPOTLIGHT

The power of combining iPSC-derived NK cell therapy & NK-cell engager antibodies

Daniel Teper & Wei Lin
Cytovia Therapeutics

"...the combination of gene editing and iNK platforms offers a powerful platform to unleash the great potential of immuno-oncology cell therapies."

VIEWPOINT

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Immuno-oncology therapy has evolved rapidly in the past decade with approval of a number of CAR-T therapies, checkpoint inhibitors, and T cell engagers. Although some remarkable efficacy has been achieved, many challenges need to be addressed. T cell-associated toxicities, such as cytokine release syndrome and neuro-toxicities, are commonly observed and can be severe. CAR-T therapies can also cause severe graft-versus-host disease. Conventional CAR-T is an autologous therapy where manufacturing logistics pose major hurdles, including undesirable wait times, high production failure rates and batch-to-batch variation, as well as prohibitory high costs. In addition, limited efficacy has been observed for all approved immuno-oncology therapies in solid tumor indications due to limited immune cell tumor penetration, suppressive tumor microenvironment, and the heterogeneous nature of these tumors. Reduction of immune cells in many patients with advanced disease also limits the efficacy of checkpoint inhibitors and immune cell engagers.

In recent years, people have begun to evaluate immune cell types other than T cells, among which, NK cells have shown great potential. NK cells are the first line of defense against cancer with their ability to recognize tumor cells through many activating receptors. NK cells' MHC-independent killing activity makes them a natural allogeneic therapy. Clinical proof-of-concept data (PoC) has been obtained for NK/CAR-NK cell therapies with efficacy similar to that of CAR-T therapies, whilst the safety profile is notably superior, not presenting cytokine release syndrome (CRS), neuro-toxicities, or graft-versus-host disease (GvHD) [1].

NK cells can be derived from donors or induced pluripotent stem cells (iPSC). With the unlimited self-renewing capability of iPSC, the iPSC-derived approach provides for a more consistent starting material and better scalability. However, the major advantage of the iPSC-derived approach comes when it is combined with gene editing. Small-scale gene editing will be sufficient for the iPSC-derived approach since a single-edited iPSC clone will be selected afterwards to serve as a new source of starting material. Given that a single cell clone will be screened, rigorous quality control can be implemented at this stage to ensure an optimally edited clone is selected. As NK cells will be derived from a master cell bank (MCB) of the single-edited iPSC clone, the gene editing step does not need to be repeated and the final NK cell product will be homogenous. This is not feasible with the donor-derived approach, especially when several rounds of gene editing are required. The safety and activity of iPSC-derived NK (iNK) cells have been demonstrated in phase 1 clinical trials by Fate Therapeutics [2].

While NK cells are a good choice for immuno-oncology cell therapy, non-edited NK cells have their limitations, such as short persistence. However, NK cells can be gene edited to improve their persistence, and they may also be edited to improve their tumor infiltration and functional activities, be more resistant to suppressive tumor microenvironment (TME), and better recognize tumor cells with addition of a CAR. Therefore, the combination of gene editing and iNK platforms offers a powerful platform to unleash the great potential of immuno-oncology cell therapies.

Another approach to harness the power of NK cells against cancer is the use of NK cell engagers. Compared to T cell engagers, NK cell engagers have outstanding clinical safety profiles and can be dosed at much higher level. However, patients with impaired immune systems may not have enough NK cells to support the activity of NK cell engagers. Affimed has pioneered in pre-complexing their NK cell engager, AFM13, with NK cells and have shown impressive clinical efficacy data, revealing the great potential of combining NK cell engagers with NK cell therapies [3].

Cytovia is the only biotech company with both iPSC CAR-NK and NK engager platforms. At Cytovia, we have fully integrated in-house iPSC CAR-NK process development as well as cGMP manufacturing capabilities for this cutting-edge technology. In addition, we partnered with Cellectis to use TALEN for gene editing. TALEN directs

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site-specific gene editing with higher specificity and better efficiency for heterochromatin region compared to CRISPR/Cas9 [4]. Besides aiming to introduce CARs into our iPSC-NK cells, the edits for our initial products are focused on improving the persistence of the iPSC-NK cells and reducing their sensitivity to the TME. Cytovia's NK cell engagers utilize a proprietary novel FLEX-NK™ multifunctional antibody scaffold, which presents a tetra-valent format that provides for better avidity and specificity. It also has a full fragment crystallizable (Fc) region that can increase its half-life, engage other cell types such as macrophages and monocytes, and allow the possibility to modulate the activity of the antibody. The scaffold contains proprietary specific mutations enabling consistent proper pairing of the heavy and light chains of the antibody for manufacturability. It also has a flexible linker to facilitate simultaneous binding to multiple antigens and allow plug-and-play for different target binders. FLEX-NK™ cell engagers present a NKp46 binder to engage NK cells. Unlike other NK activating receptors, such as NKG2D or CD16, NKp46 is expressed in tumor infiltrating NK cells and may be a better engaging target for NK cells in solid tumor indications [5]. NKp46 expression is also highly specific to NK cells [6], as opposed to NKG2D, which is also widely expressed in T cells, and thus may have a superior safety profile.

Cytovia's lead product candidate series are targeting GPC3, an oncofetal protein overexpressed in many solid tumors, including hepatocellular carcinoma (HCC), but not in adult normal tissues or liver with non-cancer diseases. The most advanced product candidate of the program is CYT-303, a FLEX-NK-™ engager targeting GPC3, for which IND is targeted later in 2022, and that will also be combined with universal iPSC-NK cells (CYT-100). Preclinical proof of concept data for CYT-303 as a monotherapy and in combination with CYT-100 (Cytovia's unedited iPSC-NK cell product) have already been obtained and published at AACR meetings in 2022 [7]. CYT-503 is an iPSC CAR-NK product targeting GPC3 with additional edits to improve its persistence and resistance to TME.

Besides the GPC3 series, Cytovia's pipeline includes another FLEX-NK™ cell engager targeting CD38 for multiple myeloma which has already demonstrated *in vitro* and *in vivo* activity, as presented at the European Hematology Associational (EHA) 2022 congress [8].

Whilst combining gene editing and iNK technologies can powerfully unleash the power of NK cell therapies, combining NK cell therapies with NK engager therapies can provide cancer patients with optionality based on their disease status.

BIOGRAPHIES

DR WEI LI is the Chief Scientific Officer for Cytovia Therapeutics. Wei Li is a founding member of 2 biotech companies and is an expert in all aspects of drug research & development, including preclinical development & pharmacology, clinical development and operations, regulatory, biomarker development and biomanufacturing. Wei was previously the Chief development Officer at OliX Pharmaceuticals, a leading public South Korean biotech company developing siRNA therapeutics for multiple indications including pulmonary diseases, ophthamology, cancer immunotherapy, liver diseases, CNS diseases and dermatology. She also served as a Member of Board of Directors for OliX US. As Executive Vice President, Product Development at Boston Biomedical, Inc (BBI) from 2007-2018, Dr Wei Li played a key role in the growth from a 10-person start up in 2007 to a 250-person global oncology leader. She led the development of napabucasin (BBI608), a first-in-class drug selected as one of the world's top ten cancer drugs in late stage clinical development by Fierce Biotech. BBI was ranked as the most capital efficient company of the year (2012) and deemed one of the most capital efficient biotech companies in history by Venture Capital Journal. BBI was acquired in 2012 by Sumitomo Dainippon for $2.63 Billion. Dr Wei Li started her biotech career at ArQule (NASDAQ:ARQL), a leading oncology company developing first in class therapeutics. She completed her Postdoctoral Training at Harvard Medical School | Brigham

& Women's Hospital in the Department of Rheumatology, Immunology, and Allergy in Boston, holds a PhD in Molecular Virology from Georgia State University and has a BS in Biochemistry from Sun Yat-sen University in China.

DR DANIEL TEPER is the principal founder of Cytovia Therapeutics. He established the strategic vision of the company and secured the key foundation product and technology acquisitions. Previously, Dr Teper was the founder and CEO of Immune Pharmaceuticals, which he listed on NASDAQ. He also served as New York-based Managing Partner (Head of North America) at Bionest Partners, where he advised companies on corporate strategy and business development. Dr Teper is a Strategic Advisor to Steba Biotech, an innovative drug-device oncology company affiliated with the Weizmann Institute and Memorial Sloan Kettering Cancer Center. He was previously a Partner at ISO Healthcare Group (now Deloitte Monitor) in New York. Dr Teper helped drive the accelerated growth of Softwatch, a pioneer digital health company, as senior vice president of sales and business development. He also served as global president of Havas Health, advising companies on global launches of major new drugs in multiple disease areas. Dr Teper started his career at Novartis in Basel and then in the US, where he held management responsibilities in sales and marketing and as head of cardiovascular, new product development. Dr Teper held general management positions in Europe at GlaxoSmithKline and Sanofi. He was the co-founder and CEO of Wintec Pharma, a European specialty pharmaceutical company focused on anti-infectives and dermatology, which he went on to sell. Dr Teper co-founded Novagali, an ophthalmology specialty pharma later listed on EuroNext Paris and acquired by Japan's Santen. He holds a Doctor of Pharmacy degree from Paris XI University and an MBA from INSEAD, where he was the J Salmon scholar.

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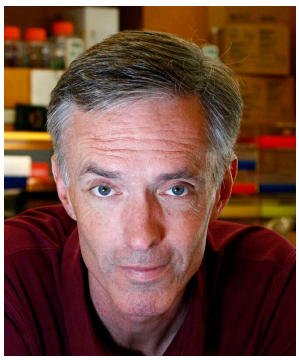
CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY

SPOTLIGHT

A journey in synthetic biology: using gene circuit technology in immuno-oncology

Timothy Lu, Senti Biosciences, **& James Collins,** Senti Biosciences and MIT



VIEWPOINT

"The clinical need for synthetic biology is urgent, and the technology has evolved... we can start designing and manufacturing gene circuit-engineered cell and gene therapies at clinical and eventually, commercial scale."

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Cell and Gene Therapy Insights spoke with Tim Lu, Co-Founder & CEO, Senti Biosciences and Jim Collins, Scientific Co-Founder, Senti Biosciences & Termeer Professor, MIT's Institute for Medical Engineering and Science

We founded Senti Biosciences, a synthetic biology company based in South San Francisco, in 2016 with the goal of turning cells in the body into programmable medicines that can sense disease, make decisions, and then treat disease in sophisticated ways. We developed the technology of gene circuits, which are snippets of DNA containing multiple genes that can give specific instructions to cells. Gene circuits can be used to program cell and gene therapies with sophisticated behaviors.

Using gene circuits, Senti Bio is engineering immune cells, particularly chimeric antigen receptor natural killer (CAR-NK) cells, to detect signatures of cancer and specifically target cancer cells with multi-pronged attacks. We are advancing multiple CAR-NK programs: SENTI-202 for acute myeloid leukemia (AML); SENTI-301 for hepatocellular carcinoma (HCC); and SENTI-401 for colorectal cancer (CRC). We are also collaborating with Spark Therapeutics and Bluerock Therapeutics on non-cancer applications of our technology.

THE TRAJECTORY OF SYNTHETIC BIOLOGY

Alongside several other researchers, we launched what became synthetic biology in the late 1990s, after recognizing that engineering principles could be applied to molecular biology. These principles could be used to model, design, and build synthetic gene circuits that could be used to reprogram living cells with novel functions for a variety of applications. In the 2000s, synthetic biology took off as a field, bringing additional engineers, computer scientists, mathematicians, and physicists into molecular biology, expanding the repertoire of control and logic circuits, and moving from bacteria to human cells. We began collaborating in the mid-2000s and showed that synthetic gene circuits could be created for a variety of biotech applications.

In 2013, we were invited to visit Atlas Ventures, an early-stage venture capital firm, to discuss opportunities in synthetic biology. The Atlas team wanted to launch a synthetic biology company to pursue the idea of engineering bacteria as living therapeutics. For the next year, we worked with our colleagues at Atlas and launched what became Synlogic, which is now a public company with multiple human clinical trials underway.

In early 2016, we met at Massachusetts Institute of Technology (MIT) to talk about extending synthetic biology into the mammalian therapeutic space, and to launch another startup using synthetic biology to engineer next-generation human cell and gene therapies. Engineering human cells added a significant layer of complexity compared to bacteria. Nevertheless, synthetic biology's ability to program human cells had accelerated with increasingly powerful DNA sequencing and synthesis technology, whilst groundbreaking clinical successes with CD19 CAR T cells and adeno-associated virus (AAV) gene therapies had demonstrated the transformative potential of cell and gene therapies.

However, these first-generation therapies were confined to a small number of diseases. First-generation CAR-T cells could cure cancer patients with B-cell cancers, but also could overreact in the body and become

uncontrollable, resulting in significant side effects such as cytokine release syndrome, and even death. First-generation AAVs could not be easily targeted to specific tissues, thus resulting in off-target effects. With the aim of solving these issues, we teamed up with Philip Lee, along with several scientific co-founders, including Dr Wilson Wong of Boston University, and started Senti Bio.

GENE CIRCUIT TECHNOLOGY

Gene circuits are designed to enable cell and gene therapies to act with significant autonomy, with the integration of smart sensors to respond to different disease signatures such as the overexpression of antigens or transcription factors, and logic gating circuits to integrate multiple sensors to decide when and where to trigger therapeutic activity. The killer cells then destroy the cancerous cell while sparing healthy cells that do not have that specific signature. Regulatory dial circuits have the potential to enable control over these products even after delivery into the body. One type of regulator dial responds to an FDA-approved oral drug.

Multi-arming circuits allow product candidates to attack multiple disease pathways at the same time to enhance therapeutic activity. Most existing drugs are designed to address a single target; but complex diseases like cancer can escape these treatments. One type of multi-arming we have engineered involves calibrated release cytokines. Using calibrated release (cr) IL-15, we could simultaneously create secreted IL-15 and membrane-bound IL-15, thus resulting in the ability to target both surrounding immune cells and natural killer (NK) cells.

THE ADVANTAGES OF CAR-NK CELLS

Gene circuits can be applied to various cell and gene therapy modalities. We have chosen to focus primarily on NK cells for several reasons. In the field generally, the first generation of approved CAR T cells were autologous. That approach is expensive, however, and cannot always produce a high-quality product. NK cells are relatively safe and do not typically generate graft-versus-host disease. Multiple studies have shown that NK cells can offer activity comparable to that of CAR T cells with fewer safety issues.

SENTI-202

SENTI-202 is a novel therapeutic approach currently under development for treating AML, a cancer with a 5-year survival rate of ~30%. SENTI-202 employs two types of logic gating, concurrently activating and inhibiting CARs to enable CAR-NK cells to better identify and kill cancer cells while sparing healthy cells.

OR gate: Bivalent FLT3 OR CD33 logic gated activating CAR (aCAR) is engineered to identify the FLT3 and/or CD33 antigens on a tumor cell and kill it, thereby increasing AML leukemic stem cell (LSC) and blast clearance, preventing single antigen tumor escape, and potentially providing deeper and longer remissions.

NOT gate: FLT3 can be found on healthy hematopoietic stem cells (HSCs). To prevent the OR gate from killing healthy cells, the Endomucin (EMCN) NOT logic gated inhibitory CAR (iCAR) is engineered to identify EMCN positive (EMCN+) healthy HSCs. The iCAR inhibits NK cell killing, protecting healthy cells from on-target, off-tumor toxicity, potentially increasing therapeutic specificity and improving post-treatment regeneration of the hematopoietic system.

SENTI-202 is also engineered to express crIL-15, which is designed to stimulate the patient's surrounding immune cells and to promote NK cell persistence to enhance tumor killing.

FUTURE DIRECTIONS & COLLABORATIONS

We want to apply our technology beyond NK cells, as we consider gene circuit technologies

an expansion beyond genome editing. We have initiated collaborations with induced pluripotent stem cell (iPSC)-based gene therapy companies with the goal to deploy this genetic software into many different cell and gene therapy modalities and disease areas.

To tackle the complexity of biology, Senti Bio is developing a broadly applicable synthetic biology platform that leverages high-throughput assays, automation, and computation to design, build, test, and optimize gene circuit designs for multiple applications. This involves integration with machine learning and computational methodologies. The clinical need for synthetic biology is urgent, and the technology has evolved to the point at which we can start designing and manufacturing gene circuit-engineered cell and gene therapies at clinical and eventually, commercial scale.

BIOGRAPHY

DR TIMOTHY LU has served as CEO since he co-founded Senti Bio in 2016. Since 2010, Dr Lu has been an MIT faculty member in the departments of Biological Engineering and Electrical Engineering and Computer Science. In addition, Dr Lu has been a co-founder and a Scientific Advisory Board member to a number of biotechnology and biopharmaceutical companies, including BiomX, Corvium, Eligo Bioscience, Engine Biosciences, Synlogic and Tango Therapeutics.

DR JAMES COLLINS is the Termeer Professor of Medical Engineering and Science in the Institute for Medical Engineering and Science as well as Professor of Biological Engineering at Massachusetts Institute of Technology. He is one of the founders of the field of synthetic biology, has been elected to all three national academies-the National Academy of Sciences, National Academy of Engineering and National Academy of Medicine and has received a number of awards recognizing his work, including the Dickson Prize in Medicine and the Sanofi-Institute Pasteur Award. Dr Collins has been a Rhodes Scholar, a MacArthur Fellow and a recipient of the NIH Pioneer Award. In addition to his role as a faculty member at MIT, Dr Collins is a Core Faculty member of the Wyss Institute at Harvard and an Institute Member of the Broad Institute of MIT and Harvard. Dr Collins is a co-founder of Synlogic, Sherlock Biosciences, Cellarity and Phare Bio, and serves on the SAB of multiple companies including Danaher and Shape Therapeutics. Dr Collins has a PhD in Medical Engineering from the University of Oxford, and an AB in Physics from the College of the Holy Cross.

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN
CELLULAR IMMUNOTHERAPY

SPOTLIGHT

EXPERT ROUNDTABLE ARTICLE

Autologous versus allogeneic: the future of manufacturing and standardization in cell therapies

Rupa Pike, Delara Motlagh & Patrick J Hanley

RUPA PIKE, PhD is the Senior Director of Technical Affairs for Advanced Therapies, Pharma Services Group at Thermo Fisher Scientific. The Office of Technical Affairs comprises scientific experts that serve as a strategic, innovational and educational leaders in the area of cell-based therapies, plasmids and mRNA therapeutics. In her prior role as the Director of Enterprise Science and Innovation Partnerships, she developed and managed strategic partnerships with global BioPharma, Biotech and Healthcare customers in the area of Cell and Gene Therapy. Prior to this, she was the Head of Technical Operations (Patheon/Thermo Fisher Scientific) where she worked closely with customers to conduct technology transfer and process optimization activities related to GMP manufacturing of cell-based therapies. She has over 15 years of expertise in GMP manufacturing and has successfully led GMP operations, Process Development and MSAT activities, infrastructure buildout, customer relations and business development.

DELARA MOTLAGH, PhD the General Manager of Cell Therapy Technologies at Terumo Blood and Cell Technologies, headquartered in Lakewood, Colorado. She is passionate about the cell & gene therapy market and the potential these innovative therapies hold to improve the lives of patients.She brings more than 18 years of experience in biotechnology and healthcare in various therapeutic areas including oncology, cardiology, orthopedics, hematology, and nephrology. Prior to joining Terumo Blood and Cell Therapies in 2017, Delara served in diverse leadership roles at Baxter Healthcare in marketing, research & development, and operations.

Her cross-functional background provides a unique perspective and deep understanding of development, cell manufacturing, and commercialization elements in the industry. Delara received a PhD in Physiology and Biophysics from the University of Illinois, fellowship in Vascular Tissue Engineering at Northwestern University, and Executive MBA from Kellogg School of Management.

PATRICK HANLEY, PhD is the Chief and Director of the Cellular Therapy Program and an associate professor of pediatrics at Children's National Hospital and the George Washington University, respectively. He oversees processing for standard of care stem cell transplantation as well as the development, manufacture, quality, and testing of novel cellular therapies and is responsible for seeking partnerships and commercialization of promising cell and gene therapies. Trained as an Immunologist, Dr Hanley has an extensive background and interest in cellular therapy and is passionate about improving regulations for cellular therapy, training the next generation of cell therapists, and facilitating the translation of new therapeutics. Over the past 15 years he has helped to translate more than 300 products on over 25 cell therapy protocols – ranging from mesenchymal stromal cells to cord blood virus-specific T cells and tumor-associated antigen specific T cells – into the clinic.

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Q Where is the market going? More specifically, where do you see the future of autologous versus allogeneic cell therapies heading?

RP: To set the stage, we do not believe that it is autologous 'versus' allogeneic cell therapies. We believe that both are important, and both will positively impact the cell therapy space for patients who have stopped responding to traditional treatments.

Autologous therapies, specifically chimeric antigen receptor T cell (CAR-T) therapies, have charged ahead with six commercial products now on the market. Some of these products are even pushing towards usage as a second-line treatment. The chemistry, manufacturing, and controls (CMC) requirements and the regulatory journey for autologous CAR-Ts is clear and well documented because of this progress. Autologous therapies have demonstrated an excellent safety profile, and a significant durability of response, as we have seen from the real-world data that has been published by multiple companies. The strong efforts of many companies are evidenced in the number of clinical trials in Phases 1, 2 and 3.

Since 2020, when the first clinical study was published on off-the-shelf CARs, there has been a huge investment and effort in developing off-the-shelf approaches. Many major pharma companies as well as small start-ups are putting large efforts into this field.

However, safety, efficacy, and durability for gene-modified allogeneic cell therapies is yet to be proven, as there are no commercial products on the market. Immune rejection is a concern. Haploidentical matching and other human leukocyte antigen-related concerns need to be addressed. Understanding and managing the risks associated with chromosomal aberrations and off-target effects is still a concern, too. As more therapies come to market, we will learn based on how patients respond. But ultimately, off-the-shelf allogeneic cell therapies will be the only way to democratize the cost and make these therapies available to patients in the remotest parts of the world.

"Both allogeneic and autologous products will continue to play a big role in the lives of patients who are in the refractory or relapsed cancer settings."
- Rupa Pike

Both allogeneic and autologous products will continue to play a big role in the lives of patients who are in the refractory or relapsed cancer settings.

DM: The science continues to outpace technology. Unlocking some of those scientific challenges will help us to advance the field.

PH: There has to be a need for using allogeneic therapies. It is not enough to just want to continue the traditional model of pharma in having off-the-shelf medicines. When going after the hardest-to-reach tumors, it does not make sense to start with an allogeneic approach. For example, hopefully, we will see the approval of tumor-infiltrating lymphocytes from Iovance Biotherapeutics, who are submitting their Biologics License Application (BLA) soon. Right now, it does not make sense to use this as an allogeneic therapy.

There are plenty of examples where it does make sense to use an allogeneic model, though. Zooming out, virus-specific T cells is a great area where there is demonstrated efficacy, need, and logic behind using an allogeneic approach. Hopefully, with Atara Biotherapeutics or AlloVir, we will start to see some licensed products coming soon in that space.

RP: Quality of cells also comes to mind. The starting material is very important, and for autologous therapies, sometimes the patient has gone through multiple rounds of chemotherapy and the cells can be fragile. Anecdotally, clinicians are often more inclined to go to autologous therapies first. In the case of a patient from whom it is not possible to obtain high-quality cells, would looking to allogeneic therapies first be beneficial?

PH: The manufacturing success rate of licensed CAR T cells is approaching 95%, so it is only a very small subset of patients who will lack cells of a high enough quality. However, in these cases, it could be beneficial to try allogeneic therapy.

In 2017, there was no infrastructure to deliver CAR T cells. Five years later, there are now 300+ centers across the world that can treat patients with CAR T therapies. In the last 6 months, 3000 patients have been treated with commercial CAR T cells. The curve is

growing exponentially. It would be foolish of us to neglect the infrastructure we have created that seems to be working. This is not to say there is not a need for allogeneic, but it should not be at the expense of autologous.

DM: It is important to note that the infrastructure for the care continuum is getting strained, though. As we treat more and more patients, the ecosystem we currently have will eventually fall apart. We need to build the plane as we are flying it, bearing in mind that the logistical aspects of allogeneic approaches will likely be less cumbersome and more conducive to treating a higher volume of patients. There is a lot of science and thinking that has gone into allogeneic therapies, and they are here to stay.

PH: There are also infrastructure constraints from the manufacturer. Companies have built large facilities to accommodate this. From the hospital perspective, apheresis collection is a massive bottleneck. Then, getting that product back, storing it, and scheduling the infusion is a more difficult process than chemotherapy. Thawing the product is logistically more difficult than giving a pill.

At the end of the day, we did not train 700 people on Risk Evaluation and Mitigation Strategy (REMS) to give only a handful of CAR T cells a year. We saw that this was the next generation of cancer treatment and built that infrastructure for all these different therapies.

Q What are the best practices for successful manufacturing in both autologous and allogeneic cell therapy spaces?

RP: Many of the best practices are going to apply to both autologous and allogeneic approaches, though there are some differences.

An important factor to consider is ensuring the availability of critical raw materials. No one was prepared for the COVID-19 pandemic, and we saw an acute shortage of raw materials. We learned a lot of lessons as a result of that experience, including the importance of choosing your vendors carefully. It is important to understand the benefits of established vendors versus younger, less experienced vendors, including the possibility of exit strategies that they may have in place.

At Thermo Fisher Scientific, we perform extensive vendor qualification, and for critical raw materials, we practice dual vendor sourcing, wheever possible. We are establishing more robust supply agreements and have put in place measures to allow us to monitor lead times and inventory levels in real-time. Interestingly, in a recent publication by McKinsey & Company, the idea of creating a digital twin was proposed. This means creating a simulation of your circular supply chain to have more control and understanding of chain of custody and chain of identity events.

Secondly, de-risking the manufacturing process is critical for success in both autologous and allogeneic therapies. This involves closing the open steps and reducing human touchpoints, which can be done using closed and automated instruments and/or platforms. Digitalization can also streamline good manufacturing practice (GMP) record keeping. Master

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batch records are critical, and process and quality oversight functions can be simplified by digitalization.

Another important aspect is having a meaningful in-process analytical assay portfolio. We focus on final release testing, which is if course absolutely critical, but having robust and meaningful in-line, in-process assays is also important. This allows us to track the phenotype and behavior of cells as they transition from one unit operation to another to give us the confidence that they are conforming to the critical quality attributes (CQAs) of the final product.

Lastly, establishing excellent training programs is important, not only for GMP operators, but also for process development scientists, quality control (QC) scientists, quality assurance (QA) staff, and warehousing staff.

So, regardless of whether a cell therapy product is autologous or allogeneic, ensuring the availability of raw materials, de-risking the process through various aspects, and having a highly trained workforce are all best practices for manufacturing.

DM: If we zoom out and think about raw materials in a broader context, whether you are producing an allogeneic or autologous product, you benefit from more consistent starting materials. To be able to define a raw material, it is important to firstly characterize the process. Understanding the process and the things that are impacting it will help to define what an ideal raw material looks like, whether it is a starting material for a cell product or reagents used in the process.

Given that we are manufacturing living therapies, person-to-person variability is always going to exist. That will be amplified in sicker patient populations where autologous therapies have comorbidities. With allogeneic therapies, you can at least define an ideal donor with eligibility and screening requirements.

Q From a manufacturing standpoint, what aspects and logistics are distinct for autologous and allogeneic cell therapies respectively?

DM: When moving into manufacturing, the most critical unit operation is the modification and expansion of these cells. Automated and closed systems will help to control and manage the process. As you look to treat more and more patients, that process must be scaled.

For autologous therapies, you need to scale the process out with multiple platforms and workstations, each making a single drug product for one patient. Whereas for allogeneic therapies, your lot size can be much larger. You are treating hundreds of patients. This is scaling up, to produce larger quantities that can be aliquoted into doses to treat many patients.

The manufacturing timeframe is also different. For autologous therapies, we are all envisioning a patient waiting for their therapy. Time-to-manufacture is important because there is a life waiting at the other end. Faster manufacturing allows you to treat more patients. With allogeneic therapies, there is less of a time constraint, because you produce a large batch in advance so treatment can be more readily available.

PH: It is important to firstly note that the manufacturing differences have an impact from the patient's perspective. For autologous therapies, patients may have to wait a while. This means maintenance or consolidation therapies are important to make sure they can receive those cells. The time between evaluating whether the patient is eligible, and the infusion can be months. That changes when looking at an allogeneic therapy, where you can infuse the patient in three days.

How do we do a conditioning regimen for an allogeneic therapy? For example, do we give patients one large dose of an allogeneic therapy with a conditioning regimen, then give smaller follow-up doses? Do the patients need an additional boosting regimen, or will one dose allow endogenous immune response? I do not think we are going to see long-term persistence of these cells, so we may need additional treatments.

DM: This does impact manufacturing. It is important to think of the clinic as both the starting point and the end destination, regardless of the therapy. I get excited when I see faster manufacturing, but I also wonder how it is going to play out in real-time. It is one thing to make a product in one to three days, but it is another thing to complete the release testing so the treatment can be infused.

PH: Right now, we have a 10–17-day manufacturing process. The new T-Charge next-generation CAR-T platform from Novartis, which offers 24–72-h manufacturing, could make a big difference. If you can get to a final cell therapy product in 4–5 days, that is close to the allogeneic therapy timeline. The logistics and supply chain will be different, but it will be similar in terms of delivery time. Currently, we do not have much data on this, though.

RP: Everyone is excited about the possibility of shorter expansion time outside the body. This is where a very small population of pristine naïve T cells – for example, in CAR-T therapies, they – will be infused and then expand inside of the body. We want the patient to be the bioreactor. It will be interesting to find out what the regulatory agencies are going to say about release testing in this setting, which is going to take longer than the manufacturing process. Many people are bringing most of this testing in-house, such as quantitative PCR assays for mycoplasma. I want to wait and see how that piece is going to come together with this short manufacturing process and expedition of the release testing. Will in-house release testing become standard practice? This will save time, but it will not involve the traditional 14- and 28-day assays.

PH: One of the inherent challenges of this field is that we need differently qualified people – Delara, Rupa and I may all need a differently trained technologist for example. It is an interesting conundrum we face in the field.

It is great to have the full force of big pharma behind us. I am confident that they can validate an assay that will allow rapid-release – for example, endotoxin testing that we can do in-house in three hours. There will also be assays to reliably detect mycoplasma within 24 h.

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Sterility testing may be more difficult. I have heard of companies that have seven-day tests validated with US food and drug administration (FDA) approval – but I think it needs to be shorter than this. The frequency of endotoxin and mycoplasma contamination is incredibly low, and we have systems to detect them. It will take some flexibility from the regulatory agencies to demonstrate that this can be done safely.

"Allogeneic therapies will have more flexibility than autologous both in terms of timing and sample volumes required."
- Delara Motlagh

Q The regulatory landscape is somewhat different for autologous and allogeneic cell therapies. but are the chemistry, manufacturing, and controls (CMC) requirements the same for the two different product types?

DM: Overall, CMC packages are probably one the biggest pain points for the industry right now. If you want to see a developer stricken with fear, talk about a CMC package! Half of all approval delays are related to CMC package difficulties. It is not a copy-paste exercise: you cannot copy and paste an autologous package for an allogeneic product, much as you cannot copy-paste from the traditional drug manufacturing of small molecules and biologics into cell and gene therapy. These are living therapies. There are going to be different cell characteristics, and the processes will vary.

The good news is that associations like Alliance for Regenerative Medicine (ARM) and National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) have recently partnered to publish A-Cell – a case study designed to assist developers as they are planning their CMC packages.

The process analytics are key to support a robust package. Depending on the modifications made to the cells for autologous and allogeneic products, there are different types of cell-based testing needed. This is a motivated industry, though, and we will be able to get these analytical tools faster and have them validated. Process testing in addition to release testing defines your process, and both require refinement over time. CQAs will also all be product-specific and need to be defined.

For an autologous process, the innate variability from patient to patient will lead to wider specifications. This is justified by looking at the data and being able to demonstrate comparability to support your manufacturing.

In product release testing, there is both timing and volume to consider. Every sample volume that you take, you are taking away from the precious final drug product. There is a desire and a need to maximize the cells for therapy versus using them for testing.

Allogeneic therapies will have more flexibility than autologous both in terms of timing and sample volumes required. However, these are still areas where developers need to lean in

early and understand their process, to be able to make those modifications as time goes on. This will allow more robust CMC packages for both autologous and allogeneic cell products.

RP: There are a lot of excited people in the field, and a lot of incentives to develop new things. One thing that has already helped the industry is the premise of non-invasive, non-destructive sampling for in-process testing. There are many companies coming out with instruments that can sit in the GMP setting, where cells can pass through these instruments and certain measurements can be made. The cells can then be returned to the process and are still useful to us. Investments in artificial intelligence will help with this effort.

There is always a friction between QC scientists who want more cells to do the assays, and GMP operators who do not want to give out those cells as they need to ensure they achieve the clinical dose.

PH: One key distinction between autologous and allogeneic is the criticality of the starting material. In the autologous setting, the material you have is the only material you are going to get. It can be unethical in some instances to not deliver that final drug product to the patient, as long as it can be done safely. There might be technical deviations, but for the most part, you want to try to deliver that product to the patient.

In the allogeneic space, it is the opposite. We might have to wait weeks to collect from the healthy donors a second time, but we can. There is less flexibility in terms of deviations because you have the ability to go back to the donor and do it again or choose a different donor. Those deviations happen in every product – that is why there are processes for deviations and an audit system; but with autologous it is more critical that we get it right.

Q Rupa, from a contract development manufacturing organization (CDMO) perspective, what preparations are needed to allow the manufacturing of allogeneic cell therapies?

RP: Everyone is thinking about this right now because it is only a matter of time before allogeneic therapies become as prevalent as autologous therapies. It is important for any CDMO that does manufacturing for multiple customers to understand the differences in the manufacturing processes and logistics. They then need to decide which of the existing facilities and infrastructure will work for both autologous and allogeneic cell products and make changes accordingly to accommodate partners and customers who want to scale manufacturing. It is possible that larger GMP suites will be needed for allogeneic therapies, because it is a scale-up process rather than scale-out process.

Allogeneic therapies are more similar in some ways to traditional bioprocessing with upstream and downstream processes. They use multiple-step bioreactors as they keep expanding the cells, they may have seed trains, and they may be manufacturing multiple GMP cell banks and working banks at a time. It therefore becomes important to have a suite that is reconfigurable, modular, and can accommodate large pieces of equipment that can be wheeled in and out as necessary.

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Staff training is also going to be different, as understanding and characterizing starting material for allogeneic products is going to be different. It is important to have the right training of QA and QC staff, and to have the right assays in place. It can also be good idea to have a dedicated suite available for cell banking.

Another factor is cryopreservation. Cryopreservation of large numbers of doses requires specialized equipment – the decision of whether this happens in the same suite, or an adjacent suite needs to be made.

Allogeneic therapies require a large storage capacity because there is a need for thousands of doses rather than a single dose per patient. For truly off-the-shelf therapies, your goal is for them to reach the global regions where is it currently difficult or impossible for autologous therapies to make an impact. We are putting all the best practices in place and making changes so we are ready to manufacture allogeneic to the same standard as we can manufacture autologous.

DM: With allogeneic cell therapies, you are storing much more in the way of samples, including starting material from the donors, the final drug product, and everything in between, including master cell banks. Carefully managing that to make sure that these products are viable and well taken care of is important.

Given that these are all different products that are being manufactured, there is a lot of innovation in the types of platforms that will process these cell materials. Having flexibility in these manufacturing environments to be able to accommodate different kinds of platforms depending on the process is also going to be key. Being modular supports a nimbler process and workflow, which will be important for success, especially given the variability of cell types and applications.

PH: Mesenchymal stromal cells have been used in an allogeneic way for 20–30 years. We need to build on that work and develop better systems with better expansion capabilities, as we want to be able to treat as many patients as possible to drive down cost.

A lot of these therapies could be derived from induced pluripotent stem cells; in which case we could make an infinite number of doses. We all hope to get there someday, but it seems far away right now.

Q How can standardization help for both types of therapies?

PH: It is important to frame standardization because everyone has a different take on it. Some people want to throw everything in the same piece of equipment, which I think is a bad idea. We want innovation in this field. We do not want every CAR T cell therapy to cost US$500,000.

The three key areas where we can perform standardization are:

1. The upstream collection of the apheresis product
2. The delivery of the cell therapy
3. Patient monitoring

In the upstream patient or donor collection, we could standardize the criteria for the selection, the collection volumes, and the desired total nucleated cell count. We should agree on the type of collection on the apheresis machine. Some people use different additives. That makes it hard for the collection centers because every company must have their own specific collection protocol. It means everyone must be trained for each individual product, and every time they mess it up there is a deviation. We should try to standardize that.

Let's also standardize the required testing. For autologous products, the FDA does not have criteria for donor eligibility, as they are exempt. For safety purposes, almost everyone still wants to do that same infectious disease testing. Because there are no guidelines, some people treat it as a stem cell product which is required 30 days prior to collection or 7 days after, and some people treat it as a GMP therapeutic product, required seven days before or after. If we could standardize that it would greatly help the collection centers.

Fast forward to the delivery – generally, people use the same vials and bags, but the cassettes can differ as can the liquid nitrogen storage. Other considerations include the expiration date and REMS training. These are other areas where we can standardize.

With allogeneic therapies, we can standardize to a greater degree, because we know certain outcomes such as dosages.

DM: As we look at standardization with the starting material, there is much variability in how we do the collection for apheresis, for example. Over 70% of cell therapy products manufactured today start with an apheresis product. It is a natural place to start.

When we look at autologous therapies, people consider standardization to be a tight, specific thing that takes away some of the flexibility. We need to be purposeful and intentional in how we define what a standardized product looks like. We must consider the variability of these patients. We want to prevent any bottlenecks in collections and ensure that the products meet the standards necessary to go into manufacturing.

The other piece is cell viability, which is such a basic thing, but everyone defines it a bit differently and uses different assays to do so. There can be huge variation. For example, Trypan blue is not the most robust viability measure. This is a place where the industry could come together and define a standard.

There is also a lot of intellectual property (IP) associated with the process. There is sometimes a reluctance, particularly for the biotech pharma companies, to share details of their process. However, it is important to remember that in the end, the opportunity to collaborate and have more standardization benefits everyone and need not compromise some of the concerns people have around IP.

In the hospital setting, there are bottlenecks in collection but also in the infusions, as there are so many different protocols. The major academic institutions are robust and have fantastic capabilities. It's also incumbent on us to determine how we can make this work in more rural centers, in order to truly expand access. Having guidelines and standards that can be rolled out globally is a way in which we can do this.

RP: There is already talk of commercial products being made available in different parts of the world. Regulatory agencies in the US, EU, and the UK have some

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similarities, but the Asia-Pacific region can be different. There are a few things that can be standardized, though, even when treatments are given in different countries – for example, labeling for chain of identity and chain of custody. There is FDA-approved software currently available and validated to do this. Even if you are manufacturing in one country and sending it to another, it can become easy to carefully monitor the chain of identity and chain of custody by creating unique donor identification numbers. The testing of the incoming material could also be standardized.

There are conversations happening between regulatory agencies, grassroots organizations, patient advocacy groups, and non-profit organizations. From a payer perspective, there will be relief when we have proof of better chain of custody and chain of identity.

"Keep an open mind with an eye to the future. I would also encourage us to think differently about how we deliver these cell and gene therapy products. Perhaps they can be delivered in a decentralized way, even if we cannot do that right now."
- Patrick J Hanley

Q Finally, what is your brief call to action for our industry?

DM: Firstly, as a piece of advice to the cell and gene therapy industry, it is important to have the end in mind. The end is not just getting a product out the door – it is treating a patient.

My call to action is, regardless of the role you play in the ecosystem, consider how you can enable the treatment of more patients, whether it is through logistics, manufacturing, or hospital management. These patients are counting on each and every one of us.

PH: Keep an open mind with an eye to the future. I would also encourage us to think differently about how we deliver these cell and gene therapy products. Perhaps they can be delivered in a decentralized way, even if we cannot do that right now. And I'll give you one very good reason why we should try to do what seems impossible: The patients. We might need to work with the agencies to create the regulatory framework in a safe and ethical way, but it would drive down costs and increase patient access.

RP: My call to action, or what CDMOs and everyone in this field should strive to do, is to always have the patient in mind. What was not possible in the blood transfusion and bone marrow transplant industry in the past is the standard of care now. We should all work together on solutions where experimental therapies can become the standard of care. I am excited – I think it is possible, but it may take a long time. We are already at the second line of treatment, though, and working towards these therapies becoming first-line treatments.

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CELL & GENE THERAPY INSIGHTS

NEW HORIZONS IN CELLULAR IMMUNOTHERAPY

SPOTLIGHT

INTERVIEW

Working together to safely advance cell & gene therapies




David McCall, Editor, *Cell and Gene Therapy Insights*, speaks to (from left to right), **John Maher,** Scientific Founder & Chief Scientific Officer, Leucid Bio Ltd, **Francisca Neethling**, Head of Cell & Gene Therapy, Eurofins Discovery, **Alastair J King,** Head of Biology, Eurofins Discovery & **Andrea Bisso**, Associate Director Pharmacology & Pre-Clinical Development, Gadeta B.V.

"Drug development is a costly and time-consuming process associated with a significant attrition rate in the clinic. Safety is an important factor in this, and is a particular issue for cell and gene therapies. This point is illustrated by the fact that around 40% of all clinical holds in 2021 were attributed to this particular class of pharmaceuticals."

– **John Maher**, Scientific Founder & Chief Scientific Officer, Leucid Bio

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Q What do the panel think are the primary safety considerations at the preclinical stage?

FN: Primary considerations for gene therapy are vector safety, target efficiency, and feasibility. Is the target efficiently druggable, with the least amount of concomitant damage?

The same can be said for cell therapy, but using different testing systems, and somewhat different discovery processes and preclinical testing. The key is to make the drug as specific as possible to treat the condition. There is a significant amount of *in vitro* investigation that can be done to ensure that.

The most important consideration for both approaches is whether the treatment would be toxic to the patient, and whether there may be unintended on- or off-target side effects. Unintended cytotoxicity is also a real concern for any drug treatment, and one that is not always predictable *in vivo* in animal studies.

AK: Some of the aspects of biologics drug discovery are already being dealt with in the form of things like immunogenicity. We are all familiar with assays that are traditionally designed to evaluate the potential immunogenicity or activation of the immune system upon administration of a foreign agent to the body. For cell and gene therapies, we need to think of these therapeutic modalities with the same mindset.

We do have simple assays in place for examining the effect of a variety of agents on peripheral blood mononuclear cells, whole blood, cell samples, etc. But, in the case of cell therapies, they can elicit their own effects and, in some cases, immune responses, especially when they are of T cell origin. Do we need more complex predictive *in vitro* models to accommodate the evaluation of these kinds of off-target activities?

Genotoxicity is another thing that could potentially be a concern on the gene therapy side. We have standard assays to look at genotoxicity in anti-cancer agents, but these may not be enough in the context of cell and gene therapies. This raises the question of looking at key tissues that could potentially be liabilities. We want to be able to target therapies to get to the tissues where they are designed to act, but do we need to put in place assays and platforms that can evaluate off-target activities within the context of those specific tissues? This may also be dependent on where the target is expressed.

JM: My own particular area is chimeric antigen receptor-T (CAR-T) cell immunotherapy, particularly focusing on solid tumors. In that setting, the three major toxicities we have concerns about are cytokine release syndrome, various forms of neurotoxicity, and on-target off-tumor toxicity.

At the early stage of development of a new CAR-T, it can be helpful to consider establishing a target product profile for that particular drug, thinking particularly about where you wish to position it in the clinic. It may raise issues such as regional delivery being a more appropriate route to get these cells to the site of disease, which may have an impact on the safety of that drug.

In the context of preclinical modeling in animal models, often a CAR-T that you have designed will not recognize the ortholog of the human target in the animal model you are using.

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You must think carefully about how to get around that problem, sometimes with the use of homologous modeling systems.

AB: We are all aligned with the importance of understanding the safety profile of the cell-based therapies that we are developing, particularly the importance of on-target or off-target effects.

We have not yet defined the minimal level of characterization–in terms of safety–that we should provide for a candidate product. We are still looking for a consensus between the leaders in the field, including scientists from industry, biotech, and academia, in addition to regulators. We have some general guidelines, but there are no established or unique assays that should be included in this characterization.

There is a boom of additional systems to consider, following great improvements in *in vitro* tissue engineering, including 2D versus 3D models, organoids, and organ(s)-on-a-chip. All these should provide new models that we can evaluate for their capability to predict product safety. We should also define the minimum dataset required to prove safety.

In March, the US food and drug administration (FDA) provided two updated guidelines for industry, for the development of human cell and gene therapies, in particular (for) CAR-T. These documents included some of the aspects that we need to address, such as the characterization of antigen recognition and the domain of the target antigen of cellular components. They also deliver a degree of flexibility in that they encourage continuous communication between sponsors and authorities. It is difficult to define the details required and the models that should be used to assess the safety, because cell-based products have unique biology.

JM: Some predictive assays for small molecule adverse impacts, such as the BioMAP® Toxicity Signature Analysis, drew from published literature and clinical findings. One of the critical safety issues for gene therapy is the occurrence of unpredicted death of subjects treated with these advanced therapies. We cannot wait for that body of literature to accrue on this matter. How do we create more predictive approaches for these types of biologic agents?

AK: The BioMAP Platform is a translationally relevant and predictable *in vitro* model platform. We have demonstrated the ability to use this platform in a highly predictive manner both for small molecules and more traditional biologic agents such as antibodies. These kinds of *in vitro* models can be set up in a way that is clinically relevant. The key here is to be able to learn from some of the existing data we have around clinical evaluations for cell and gene therapies, and build that into models that are more relevant to those particular therapeutics.

Many of the models contained within the BioMAP Platform are more related to specific tissue biology and biological effects. In some ways, that platform is agnostic of the therapeutic modality, because it focuses on the effect on the surrounding tissues in a clinical situation. Capitalizing on this kind of approach is the way to take this forward.

However, a key aspect would be to establish systems with 'the right biology'. This means modeling the kinds of biology where we see potential liabilities with cell and gene therapies, and modeling biologies that are relevant to the target tissue types for those therapies.

The platform and the approach have already been proven with other therapeutic modalities. We now need to pay attention to aspects that are more unique to cell and gene therapies, and couple that with the existing and growing body of data we have from clinical evaluations to put in place similar models for the cell and gene therapy field.

> "The BioMAP® Platform is a translationally relevant and predictable *in vitro* model platform. We have demonstrated the ability to use this platform in a highly predictive manner both for small molecules and more traditional biologic agents such as antibodies."
> - Alastair King

FN: There is a lot we can do *in vitro* for gene therapy. We can simulate the clinical setting and assess the effect of the therapy in these *in vitro* models, whether by functional assays or methods such as western blot, enzyme-linked immunosorbent assay (ELISA), or quantitative polymerase chain reaction (qPCR) to determine the amount of expression in cells.

The choice of viral vector depends on many factors, including the disease being targeted, the amount and type of genetic material to be delivered, and the location and characteristics of the cells being targeted. Viral vectors have been around for quite some time, so there is a significant amount of literature available, with more being published regularly. We cannot wait for new literature, but we can use the basis of this published literature to build on for safety and efficacy studies in gene therapy.

Humanized mouse models or xenografted models can be used for greater accuracy in assessing the efficacy of viral vectors in humans, but they do not always translate into the human setting. With 3D cultures of different tissues or organoids becoming more common, and the field growing fast, *in vitro* data is (*sic*) becoming more relevant.

AB: I would like to stress the importance of a better understanding of the physiology and behavior of an organ or tissue, both in normal conditions and in the alteration of disease. This will be key to directly recapitulating the complexity of an organ or tissue in an *in vitro* system that considers the microenvironment in a certain setting. This is the first step to predicting the safety of a candidate product. By learning from these data, we can then go back and redesign and optimize the product to reduce any possible toxicity, in a way that can increase patient safety.

JM: In the context of CAR-T, preclinically, one of our CARs exhibited unpredictable, on-target, off-tumor toxicity. We found that reconfiguring the CAR so it was co-expressed with a chemokine receptor, which preferentially trafficked the CAR-T cells into the tumor, not only boosted efficacy, but also enhanced the safety profile because there were fewer of these cells in organs where they could cause toxicity.

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AK: The key here is thinking outside the box. We have many standard safety pharmacology assays which traditionally have been used for things like small molecule drug discovery, and these outline toxicities we know and understand well.

As we move towards more complex aspects of cell and gene therapies, there is the potential for the occurrence of toxicities that we cannot currently predict. We need to be open-minded about that, and take new data to constantly refine our approach. This might provide a second-generation of safety pharmacology approaches, looking at the cellular level rather than the biochemical level.

JM: Animal models have had a somewhat inconsistent profile of success in predicting clinical toxicities of cell and gene therapies. What have been your experiences here? How do you think that non-animal alternatives could be employed for safety testing and how may these evolve over the next decade?

Q Considering the recent FDA Modernization Act, what do you think will be the greatest challenges to implementing non-animal alternatives in preclinical testing?

AB: We are faced with growing scientific knowledge and terrific technological development, with more and more sophisticated *in vitro* non-animal models that will be helpful in predicting the safety and efficacy of our cell and gene therapies.

From the perspective of cell-based therapy for tumors, we are also experiencing increased use of more complex *in vitro* models and assays to assess safety and efficacy. For example, culture organoids and spheroids can recapitulate the specific microenvironment of a tumor to allow the *in vitro* investigation of the efficacy of a cell-based therapy. We also use a lot of primary tissues or organoids to assess the safety and toxicity of products and even more complex pharmacology/toxicology models with a mix of normal healthy cells, which can then be cultured together with primary tumor cells: the effect of a product can then be simultaneously measured on both components, to better recapitulate the real situation of a patient.

These *in vitro* assays will gain more space in the next five to ten years, although we are still bound to the use of animal models for specific questions. We are indeed limited in the type of answers we can gain from *in vitro* models and, while there are huge limitations in animal models–such as the lack of human cytokines that support cell survival, homing, and expansion, and the lack of cross-selectivity for mouse targets–they have a value, though. For instance, the trafficking of a cellular product into an organ or tissue can only be studied using these models.

I envision that the development and application of more advanced techniques, such as omics and single-cell technologies, coupled with *in vitro*, multi-cellular cultures, may allow us to closely capitulate the physiology of a tissue and its microenvironment, and will probably represent the future to minimize testing in animal models.

AK: Especially as we aim to move away from animal models, we are reducing, refining, and improving the models that we do have. The Eurofins BioMAP® Platform is

an excellent way of illustrating how we can do this *in vitro* in a highly translational, relevant, and predictable manner. The key here is understanding what is happening in the complexity of an animal system.

Sometimes there are events that occur within the context of a disease that are not immediately obvious prior to using that therapeutic within the context of an *in vivo* model. Building that back into those *in vitro* models will give us a more accurate model of the biology, in a constant process of refinement. We often talk about initiatives to reduce and refine animal models; we should do the same thing for *in vitro* models, and use the data that we can generate from *in vivo* models to help boost their predictability.

We are at an exciting point in the development of models to support drug discovery, from the perspective of having a vast number of omics capabilities available, including proteomic modeling, metabolomics, and transcriptomics. We have a wealth of data that can potentially be generated. This, coupled with the fact that we are now more able to evaluate larger datasets, puts us in the unique position of being able to look at the complexity of these biological systems in response to cell and gene therapies. This will allow us to generate more safety or toxicology fingerprints. For any therapeutic, even for the same target, there may be different safety profiles generated.

Beyond that, machine learning and artificial intelligence can assist in evaluating the large datasets generated from *in vitro* models, and we could then move towards more in silico approaches in terms of safety and toxicity issues. We would still need to evaluate whether those safety and toxicity effects do occur, but I think this can capitalize on some of the technologies that we currently have at our fingertips.

FN: There is relatively little guidance from authorities for cell and gene therapy, considering that it has been around for quite some time now. We can help build the body of data to allow clients to have conversations with the authorities that can help to shape guidance in the future. Eurofins and other contract research organizations (CROs) have done so in the past for other therapeutic modalities, so there is no reason we cannot do that for cell and gene therapy.

We already have various capabilities up and running routinely, and we can deliver the type of data that clients will need to present to the authorities at a pre-Investigational New Drug (IND) application conversation, for instance, regarding reduced need for animal testing.

JM: From my own experience in the CAR-T space in the UK, the feedback I have received from regulators is that they want to see data surrounding the safety and efficacy of therapeutic drug products. For human CAR-T cells, the only way that can be done *in vivo* under existing guidelines is by using animal models. I am heartened by the recent moves the FDA and others have made to begin to broaden this, so that animal models are no longer a compulsory component of preclinical testing. We have a wealth of *in vitro* models to choose from, from organotypic cultures, induced pluripotent stem cell-derived differentiated cell types, and elegant organ-on-a-chip cultures. For example, a pulmonary organ-on-a-chip can have lung epithelial cells in an air channel, separated from endothelial cells in a blood channel using a semi-porous membrane. This gives all the components in the system

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to infuse CAR-T cells in through the blood channel and examine if they can be incited to traffic into the epithelial layer, and what kind of damage they could do. I can see great potential for some of these *in vitro* model systems in satisfying some of these questions in the not-too-distant future.

> "From my own experience in the CAR-T space in the UK, the feedback I have received from regulators is that they want to see data surrounding the safety and efficacy of therapeutic drug products" - John Maher

Q We are all agreed that the need for innovation is enormous in this space. How does the panel envisage that existing platforms could be modified, thinking not only about the technologies themselves, but also target-related issues?

AK: We need to further explore some of the learning that we have from clinical failures. From looking at previous clinical evaluations, failure comes from three sources. First is a lack of efficacy, which often is related to the target or the validation of that target. There is also the occurrence of off-target toxicity, which may not necessarily have been seen or predicted from existing assays that are put in place. Finally, there is target-related toxicity.

Off-target toxicities is where we need more complex, predictive platforms to help address whether there would be expected off-target activities. Because they are off-target, there is going to be some degree of not necessarily knowing what is going to happen–the effects are agnostic by nature. This is where having models in place becomes even more important. They can generate data that can be predictive in terms of how these toxicities may manifest themselves.

Traditionally, we have tended to look down certain avenues, such as conventional cytotoxicity of cell types and electrophysiology relating to hERG and targets. The existence of more complex platforms, such as omics, provides a way to be able to address the off-target toxicities that are not traditionally seen with some of the existing models. This will go a long way towards addressing those issues earlier in drug discovery, to mitigate the risks when declaring a candidate and going forward into clinical trials. The technological advances will undoubtedly enhance our ability in the off-target toxicities area.

Target-related toxicity advances will more than likely need to come from other avenues, such as in partnerships with academic research–for example, looking at how modulation of a given target can manifest not only in disease tissues or in target tissues, but in other tissues. Academic research where one is potentially looking at a whole range of different tissues and systems can provide tremendous value in terms of helping validate the target from the perspective of safety.

FN: As a CRO, we are well suited to being able to work with clients to adapt what already exists. We can optimize, customize, and innovate, to help clients meet their

goals. Some of that innovation can also lead to building guidance and aiding regulators in the industry. While addressing safety, we can work with clients to optimize their methods to build a more efficient process. Several of the gene therapy programs have had problems with producing enough viral material to be able to characterize it appropriately. There is room for improvement, and we can help with that by optimizing methods and expanding processes. All of this is done by monitoring new innovations and considering implementation, so that when a client comes to us with a request, we can do the innovation for them.

AB: The great technological development of these omics techniques, from transcriptomics to metabolomics, will allow us to gain unprecedented molecular characterization and understanding of the behavior of cell products, and a better understanding of the on-target and off-target unwanted effects. The combination of omics with single-cell technologies would allow us to make a further step. We will hopefully be able to investigate for instance the potential heterogeneity within our cellular products, and whether this is a potential issue in terms of safety or lack of efficacy.

All this would lead to maximizing the safety and efficacy of these products. For example, the identification of a specific sub-population of product cells that can cause unwanted toxicity can allow us to adopt strategies to reduce the impact of this population within the product. This could be achieved by tweaking the conditions of manufacturing to exclude this population or expanding a different, more effective population.

JM: This highlights one of the issues we have in many forms of cell therapy, which is the lack of standardization of these products.

When it comes to CAR-T, I struggle to keep up with all the innovation going on. There is so much activity in this space. An example of this is that we are seeing the advent of many forms of controllable CARs, such as CARs whose expression is dependent upon the presence of a second pharmaceutical, or CARs that are only expressed under conditions of hypoxia, so they are preferentially switched on in the hypoxic tumor microenvironment.

There is a tremendous amount of innovation around the development of so-called gated CARs, which are not just recognizing a single target but are instead recognizing a signature associated with the tumor microenvironment. There are many variations, including dual-sensing CAR systems and SynNotch systems. We are also seeing the advent of CARs which are being used as drug delivery devices in their own right. They can be armored either to produce cytokines with the capacity to modulate the tumor microenvironment, or to produce oncolytic viruses, for example.

Another area of enormous innovation is the refinement of the signaling properties of CARs. For example, calibrating the activation signal by mutating Immunoreceptor Tyrosine Activation Motif (ITAM) units in the activation module, and potentiating co-stimulation by the placement of co-stimulatory units in their natural location close to the plasma membrane. This is just the tip of the iceberg in terms of tweaking the CAR itself.

CARs need a cell within which to work, and modification of the cell host–for example, by selecting different subsets of long-lived T cells *in vivo*–to give the greatest therapeutic impact. The cells could also undergo manufacturing in the presence of chemicals, which can

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retard the differentiation of the cells, thereby ensuring they have a greater capacity to proliferate and persist in the patient.

We are seeing the advent of a huge toolbox of genome editing technologies, which also further the potential for innovation in this space. For example, the advent of base editing allows us to modify gene expression without the introduction of double-stranded DNA breaks, thereby potentially reducing the genotoxicity of the approach.

A final area of innovation that I am excited about is in manufacturing. For anyone in the cell therapy field, manufacturing is the core business. The advent of automated platforms to reduce the human factor in manufacturing has shortened the duration of the manufacturing process, and reduced the cost of goods, to achieve a fitter, less differentiated cell product. We are now seeing some manufacturing processes that are shorter than 24 hours, as well as *in vivo* delivery of vectors to transduce T cells in the patient rather than manufacturing *ex vivo*.

Q How do the panel members go about choosing an outsourcing partner, and what is the primary role of the CRO?

AB: I can speak from the perspective of a small biotech, but this may hold true for bigger organizations. Our main reason to go for an outsourcing partner is to get access to complex assays and services that do not make sense for us to set up in-house, due to a lack of resources, narrow capabilities, or access to material.

In other words, we look for a balance between costs and having access to high-quality, complex, translational assays, coupled with proper advanced bioinformatic analysis when needed. We also value the possibility to customize assays based on the goal or experimental needs of a specific study. For example, assays like the BioMAP® Platform, or high-throughput assays based on the use of advanced imaging or the use of primary material from patients, require a lot of setup, know-how, and access to patient material.

For a small biotech, having the possibility to perform these assays in collaboration with a CRO will allow to test what we need, whether that is a higher number of variables, multiple replicates, or a greater number of patients or donors. This will lead to the acquisition of valuable data to allow risk reduction for the company.

The final consideration is the importance of the timing of the experiments. We need to get the results as soon as possible, and as early as possible in the development of a product. This is another important parameter when selecting a CRO. The possibility of fast and agile communication and fast execution of the experiment are relevant parameters that we consider when we decide on a partner.

FN: As a CRO, we work in a highly consultative way with the client throughout the execution of a project. We need a clear understanding of what the needs are and then together with a client, we decide what the approach is going to be. We can either customize what we already have within our versatile catalog of capabilities, or we can develop new approaches and assays, if required. We communicate regularly and keep the client up to date on progress.

As Andrea said, quality, cost, and speed are of the utmost importance to our clients, and we are aware of that. While we do not focus on the cost of a project, we do try to increase efficiency both in terms of cost and time, so that we can deliver the right data to our clients to allow them to move their projects forward in an efficient manner. Reduction of risk in the execution of the project is also a key consideration, with a focus on risk reduction in the clinical setting.

> "As a CRO, we are well suited to being able to work with clients to adapt what already exists. We can optimize, customize, and innovate, to help clients meet their goals. Some of that innovation can also lead to building guidance and aiding regulators in the industry"
> - Francisca Neethling

AK: Thinking about the role of a CRO from a more global, overarching perspective, one of the fundamentals is to listen to our client's needs. From a strategic perspective, we are in the position of seeing trends in the industry in terms of safety and toxicology aspects. Understanding where the same kinds of toxicities or safety issues are coming up time and again becomes the need in the field. We not only listen to our individual clients on a project-by-project basis but listen to the industry and see where those needs are changing. As we develop newer therapeutic modalities, cell and gene therapy being a perfect example, we are uncovering new needs in the field, in a whole variety of different aspects of drug discovery.

The other aspect is listening to the regulatory authorities as we gain greater understanding of how cell and gene therapies work. We want to understand where some of the key issues that need to be resolved are. As with any therapeutic, regulatory agencies will eventually formulate their own framework for what they would like to see as part of the vetting of an agent before declaring a candidate or approving it for clinical trials.

From a CRO's perspective, listening to the client, the industry, and the regulatory requirements are some of the key aspects of being able to provide services that are relevant and appropriate for the needs of the industry.

One of the powers of CROs comes from having not only a wide range of technologies but platforms available. We are in the privileged position of seeing things from a 10000-foot viewpoint. We can see a broader picture of safety and toxicity issues that are becoming more recurrent. Every study that we conduct with each one of our clients is under the strictest confidentiality, but there are trends that one sees from the perspective of safety and toxicity that are of a non-proprietary nature. We have seen this before with some of the safety pharmacology consortia that have been set up, that have provided tremendous value in outlining practices and assays that are needed, in particular for small molecule drug discovery. Being able to bring sophisticated bioinformatics and analytical approaches to the table can then provide additional value and impact to the kinds of services we provide in addressing the potential liabilities of a particular agent with respect to safety, pharmacology, and toxicities.

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Q JM: What is the view of the panel on virtual companies? Can they succeed in this space as well as they might do for small molecules, for example? Do virtual companies have special needs regarding assay and safety considerations?

FN: As a CRO that can offer a full range of services, we see that the needs of the virtual company and small biotech are essentially the same. There could be different levels of input from either. Some small biotechs might have assays or materials they want to transfer to us, while a virtual company might have intellectual input based on previous experience that their team has had. They may have a larger need for assays to be built or established, and we can certainly do that for them.

We can customize assays that we already have up and running to meet client needs. We are all in this together and we can work together to accommodate the needs of both virtual companies and other partners that might need our services. We are their lab, so they do not need to establish or expand one of their own.

AK: We are not working in pursuit of new therapeutics in isolation. A key way to view the CRO dynamic, which pertains to virtual companies, is that we are effectively part of the client's drug discovery team. The question of what differences there might be between the needs of virtual companies versus more traditional drug discovery companies with internal assets and lab space is an interesting one.

When one considers the needs of drug discovery, they are generally very similar, only differing in terms of the therapeutic modality being developed. Cell and gene therapies do however have different things that need to be explored compared with more traditional biologics.

As the drug discovery paradigm is the same within a certain area – for example, in the development of a specific cell therapy – it remains the same whether one is functioning with a virtual company, a small biotech, an academic institution, or even big pharma. As a result, the same needs exist within that program regardless of where it is situated. Although the challenges are the same, the real difference between a virtual company and other companies is simply the existence of labs.

Being able to contribute both intellectually from an advisory perspective and leveraging the experience that we have is important. People within the CRO world are experts in the context of the assays and services that they provide. Many also have pharma industry experience, myself included. That helps to get rid of some barriers in some of those areas where there might be less opportunity for cross-pollination between the companies.

Q JM: How can we all work together to safely advance cell and gene therapies? What are your closing thoughts on this?

FN: CROs work with clients not only to assess and ensure safety for cell and gene therapies but to optimize their processes with the goal of a faster and safer

path to the clinic. The CRO industry is there to work with any client that needs us to execute complex assays that they do not routinely run in-house, freeing up their scientists to do other aspects of research and development, or allowing them access to technology that they may not have available. We can work collaboratively to get their therapeutics to patients as quickly and as safely as possible.

"Working together as academia, CROs, and industry with the final goal of creating novel products and better characterizing the features and safety profile will be the key to success. "
- Andrea Bisso

AB: Working together as academia, CROs, and industry with the final goal of creating novel products and better characterizing the features and safety profile will be the key to success. We should never forget that we started from biology and pathology. A better understanding of the context in which a cell should work will allow us to develop better models and knowledge around the product itself, allowing movement toward better safety and efficacy *in vivo*.

AK: I am looking forward to seeing how some of these aspects play out with the continuing success of the cell and gene therapy field. It all starts with the biology, and there are processes that need to be followed as part of drug discovery that can differ from one therapeutic modality to another.

Everybody has something to bring to the table with respect to drug discovery, whether they are present in a pharma company, an academic organization, a CRO, or a biotech. The fundamental nature of any drug discovery team is that everybody has different areas of expertise, whether it's located within one organization or matrixed across multiple organizations.

The shared goal of everyone working in the field is to see the successes and ultimately, to get therapeutics to patients who are in need. We truly are all in it together, and I am looking forward to seeing how we can develop newer, more efficacious, and safer medicines in this interesting therapeutic field.

BIOGRAPHIES

JOHN MAHER, MD & PhD is the Scientific Founder and Chief Scientific Officer of Leucid Bio. He is also a clinical immunologist who leads the "CAR Mechanics" research group within King's College London. Dr Maher played a key role in the early development of second generation (CD28) CAR technology while a visiting fellow at Memorial Sloan Kettering Cancer Center, an approach that has achieved clinical impact in haematological malignancies. His research group is focused on the development of adoptive immunotherapy using CAR engineered and gamma delta T-cells, with a primary emphasis on solid tumour types. In addition, he is a consultant immunologist at Eastbourne Hospital.

FRANCISCA NEETHLING, PhD is the Head of Biotherapeutics Discovery at Eurofins Discovery. She brings over 20 years of academic, start-up and pharmaceutical Industry experience to support portfolio and program strategy for large molecule drug discovery. Dr Neethling holds a BSc

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in Microbiology and Genetics and a MSc and PhD in Transplantation Immunology. She was a Postdoctoral Fellow at the Transplantation Biology Research Center at Harvard Medical School and at Oklahoma State University. Prior to joining Eurofins Discovery in 2021, Dr Neethling spent almost 10 years as a Sr. Principal Scientist in the antibody discovery group at Boehringer Ingelheim, developing novel biologics across a broad range of therapeutic areas.

ALASTAIR KING, PhD is the Head of Biology at Eurofins Panlabs, Inc., a part of Eurofins Discovery. He has over 20 years of scientific research experience, with a specific focus on drug discovery in the oncology and inflammation therapeutic areas, and use of cellular assays to profile and advance drug leads. Dr King holds a BSc (honors) and a PhD in Biochemistry, and completed his postdoctoral training in the field of mitogen signaling at the Indiana University School of Medicine/Walther Oncology Center in Indianapolis. Dr King has deep experience in all stages of drug discovery, gained from previous positions he has held at SmithKline Beecham, GlaxoSmithKline, and Johnson & Johnson, where he has led drug discovery programs, international teams, and strategic initiatives in the pursuit of both small molecule and biologic therapeutic agents.

ANDREA BISSO, PhD is Associate Director at Gadeta BV. There he oversees the investigation of the efficacy, safety and mechanism of action of the new proprietary candidate cellular products, by leading the Preclinical Pharmacology team and managing the collaborations with external partners and CROs. He joined Gadeta at the end of 2020, after a nearly 15 years career in academia, during which he gained extensive experience in the cellular and molecular mechanisms at the basis of cancer. Working as Scientist at the European Institute of Oncology (Milan, Italy), he contributed to the understanding of the role of the MYC, WNT and Hippo pathways in tumorigenesis, by performing functional genetic screenings and by developing new preclinical mouse models of B-cell lymphomas and liver tumors. Dr Bisso received his PhD in Molecular Medicine from the University of Trieste (Italy), focusing on the role of microRNAs regulating the activity of the p53 pathway and on novel potential therapeutic approaches to block the oncogenic functions of p53 tumor-associated mutants. He holds a patent covering the application of peptides and aptamers as specific modulators of mutant p53.

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*ADCP=antibody-dependent cellular phagocytosis, ADCC= antibody-dependent cellular cytotoxicity, CDC= complement-dependent cytotoxicity

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

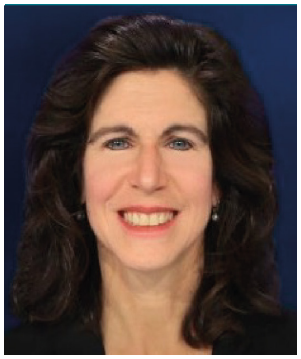
INTERVIEW

Ensuring commercial readiness in the cellular cancer immunotherapy space

David McCall, Editor, *Cell & Gene Therapy Insights*, talks to three experts from Gamida Cell's leadership team – **Julian Adams PhD**, CEO, **Michele Korfin PhD**, COO & CCO & **Ronit Simantov MD**, CMO & CSO

JULIAN ADAMS joined Gamida Cell's leadership team as CEO in November 2017, bringing more than 35 years of drug discovery and development experience to his role. Prior to his CEO appointment, Julian served as President and Chief Scientific Officer at Clal Biotechnology Industries (CBI), where he oversaw the Boston office, evaluating investment opportunities and supporting portfolio companies, including Gamida Cell. Before joining CBI, he served as president of research and development at Infinity Pharmaceuticals and as senior vice president of drug discovery and development at Millennium Pharmaceuticals, now part of Takeda Oncology. At Millennium, he played a key role in the discovery of Velcade (bortezomib), a therapy widely used for treatment of multiple myeloma. He was also instrumental at developing an oral inhibitor of phosphoinositide 3-kinase (PI3K), also known as duvelisib. In 2018, the FDA approved COPIKTRA (duvelisib, Verastem, Inc.) for adult patients with relapsed or refractory chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL). Earlier in his career, while at Boehringer Ingelheim, he was credited with discovering Viramune (nevirapine) for HIV. He has also worked in research and development leadership roles at LeukoSite and at ProScript. Julian serves as Chairman at Elicio Therapeutics since 2017. He also serves on the Stand Up to Cancer Scientific Advisory Committee, AACR Council of Scientific Advisors, and MGH Center for Cancer Research Scientific Advisory Board.Julian has won several awards for his drug development efforts throughout his career, holds more than 40 patents from the United States Patent

and Trademark Office and has authored more than 100 papers and book chapters in peer-reviewed journals. Julian holds a BSc from McGill University and a Ph.D. from the Massachusetts Institute of Technology. He also holds a ScD, *honoris causa*, from McGill University.



MICHELE KORFIN joined Gamida Cell's leadership team as Chief Operating and Chief Commercial Officer in July 2020. Prior to Gamida Cell, Michele served as Chief Operating Officer at TYME Technologies. From 2016–2018, she was Vice President of Market Access at Kite Pharma, where she oversaw market access strategy, including payer relations, reimbursement and government affairs for YESCARTA®, the first approved CAR-T therapy in lymphoma. She also worked closely with the manufacturing and supply chain teams at Kite to prepare for FDA approval and commercialization. Before joining Kite, Michele spent more than a decade at Celgene in a variety of key strategic and operational roles, including in commercial leadership and oversight of the global development programs for REVLIMID®, a foundation therapy for multiple myeloma and MDS del 5Q. She also led the Celgene oncology sales force of over 120 representatives who were responsible for $650 million in revenue for ABRAXANE®, which is now a standard of care in pancreatic cancer. Michele has also held positions at Merck & Co. as a manufacturing scientist, Bain & Company as a consultant and Schering-Plough in sales and marketing. Michele holds an MBA from Harvard Business School and a BSc in pharmacy from Rutgers University. She is also on the Board of Trustees of BioNJ, the organization that represents the biotechnology industry for New Jersey.

RONIT SIMANTOV joined Gamida Cell's leadership team as Chief Medical Officer in July 2017, bringing more than 20 years of experience in hematology and oncology research, development, registration and product launch. Prior to joining Gamida Cell, Ronit served as Head of Oncology Global Medical Affairs at Pfizer, where she was responsible for multiple programs including Sutent® (sunitinib), Inlyta ® (axitinib), Ibrance® (palbociclib), Bosulif® (bosutinib) and Xalkori ® (crizotinib). Ronit previously led phase 1-3 studies as Vice President of Clinical Research at OSI Pharmaceuticals. She also served as Chief Medical Officer at CuraGen Corporation (acquired by Celldex), where she led development of small molecules and antibody-drug conjugates. At Bayer HealthCare Pharmaceuticals, Ronit led the phase 3 study of Nexavar® (sorafenib) resulting in the first approval of a tyrosine kinase inhibitor in renal cell carcinoma. Prior to joining industry, Ronit spent seven years on the academic faculty at Weill Medical College of Cornell University, where she directed the fellowship program and conducted angiogenesis and vascular biology research. She has authored over 40 peer-reviewed manuscripts.Ronit holds an MD from New York University School of Medicine and a BSc. from Johns Hopkins University. She completed a residency in internal medicine at New York Hospital Cornell Medical Center, and a fellowship in hematology and oncology at Weill Cornell Medicine.

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Q What are you working on right now?

> "Omidubicel is the first stem cell transplant donor source to receive Breakthrough Therapy designation and Orphan Drug Status."
> - Julian Adams

JA: Gamida Cell is dedicated to the next generation of cellular therapies to treat life-threatening hematologic malignancies and other diseases that are difficult to treat by traditional methods.

We have a proprietary cell expansion technology called nicotinamide (NAM) that helps expand the number and functionality of allogeneic donor cells, irrespective of their source, and can be applied to any cell type. All our cell therapies are enabled by NAM technologies. The technology allows cell rejuvenation and enhances their function and potency. We have applied it to CD34+ stem cells for omidubicel, as well as our next program entering Phase 1/2 clinical trials, which is a cryopreserved expanded natural killer (NK) cell program: GDA-201.

Our most advanced program is omidubicel, a new approach with stem cell therapy for bone marrow transplants. This will be potentially the first allogeneic advanced stem cell therapy candidate to reach U.S. Food and Drug Administration (FDA) approval after having completed an international, multi-center, Phase 3 randomized study. Omidubicel is the first stem cell transplant donor source to receive Breakthrough Therapy designation and Orphan Drug Status. We are the first sponsor to ever perform a randomized Phase 3 study using allogeneic stem cells to treat hematologic malignancies. The FDA has recently accepted for filing the Biologics License Application (BLA) for omidubicel for the treatment of patients with blood cancers in need of an allogenic hematopoietic stem cell transplant. The FDA granted Priority Review for the BLA and has set a Prescription Drug User Fee Act (PDUFA) target action date of January 30, 2023.

For GDA-201, our pilot Phase 1 study was completed by Dr Jeffrey Miller's group at the University of Minnesota, with Dr Veronika Bachanova as the Principal Investigator (PI). This study generated an encouraging dataset in lymphoma with fresh cells and based on the data we have seen so far, with high levels of durable, complete response rates, we have developed a cryopreserved off-the-shelf therapeutic candidate that is ready to be evaluated in a multi-center Phase 1/2 study.

The third pillar of our efforts, and the youngest part of our portfolio, is engineered NK cells. We have learned how to introduce gene editing through clustered regularly interspaced short palindromic repeats (CRISPR/Cas9), as well as how to introduce chimeric antigen receptors (CARs) and other membrane-bound manipulations, in order to enhance the potency and targeting of those NK cells for specific malignancies, including both hematologic and solid tumors. We have four different Investigational New Drug Application (IND) candidates from which we plan to select one to progress to IND-enabling studies, with the IND planned for filing next year.

MK: I joined Gamida Cell two years ago, with dual responsibility as COO and CCO.

We have made some exciting advances in our in-house manufacturing capabilities over the last couple of years. Our facility will be the one utilized for omidubicel upon potential FDA approval. Eventually, we plan to bring our other pipeline candidates into that facility, as they advance in their clinical studies.

This facility was built from the ground up and designed to be state-of-the-art. It is modular, giving us the ability to add additional capacity as demand requires. We have brought in a subject matter expert, Vladimir Melnikov, who has 30 years of experience in manufacturing and aseptic processing, to lead the facility. We have completed all BLA-filing requirements for omidubicel, and more importantly, we are ready for commercialization, we have mapped out the commercial processes from a manufacturing and supply chain standpoint. (Omidubicel has less stringent matching criteria than other donor sources for patients in need of a transplant, although the chain of identity and chain of custody are still critical).

Now that we have completed the rolling BLA, we are diligently working to assure we fully understand the unmet need for patients and how omidubicel could potentially address those unmet needs for patients upon potential FDA approval, with our first potential market being the United States (US). We believe that, upon approval, there is an unmet need that omidubicel will be able to address. We are fortunate to have strong leaders throughout the organization to help us move forward with the launch upon FDA approval.

RS: As CMO and CSO, my teams are focused on advancing the clinical programs and scientific pipeline.

The clinical team is focused on initiating the Phase 1/2 study with our NAM NK platform candidate, GDA-201. Our preclinical group is busy interrogating and advancing the pipeline products of engineered NK cells through proof of concept and beyond.Meanwhile, our medical affairs team is busy engaging with transplant centers and moving forward with our plans to prepare for the launch of omidubicel as we work through the regulatory process.

Q Gamida Cell has been seen as a pioneer for a long time in the cell and gene therapy space. How has the current focus of the company evolved?

JA: Gamida Cell as a company is over 20 years old–it was initially an Israeli start-up company, which invented the technology and executed the early-phase clinical trials. The foundations of discovery for the current pipeline are over a decade old.

RS: The company was first founded to research the ability to expand stem cells and administer them to patients who need them for a bone marrow transplant. These are patients who have hematologic malignancies, in desperate need of a transplant for a potential cure.

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Those patients may not have a match in the system that would provide them the appropriate cells to transplant. Omidubicel starts with a source of cells from umbilical cord blood. Cord blood is a great source for transplant, but the number of cells is too small for many patients. Finding a way to increase this number of stem cells in culture has long been the holy grail in the field. Stem cells can repopulate the entire hematopoietic system, but they had not been cultured successfully outside of the body until Gamida Cell applied its NAM technology. Our proprietary NAM technology not only increases the number of cells but also enhances cell function, including homing to the bone marrow.

> "The main aspect is the strength of the clinical data. Time to neutrophil engraftmennt is encouraging becasue that is a clear metric these transplaters are looking for."
> - Michele Korfin

After establishing that mechanism and showing that we can produce an increased number of stem cells that can still repopulate bone marrow, we initiated a randomized phase 3 clinical trial, using standard umbilical cord blood and omidubicel, in blood cancer patients. We followed those patients after transplant.

The primary endpoint of the Phase 3 trial was the time it took for the neutrophils to recover, or in other words, time to neutrophil engraftment. The neutrophil engraftment in patients treated with omidubicel was about 10 days, compared to about 20 days in the patients treated with standard cord blood. The outcome was statistically significant and clinically meaningful, and the primary endpoint was met. Every day that patients wait for their bone marrow to recover is a day spent in the hospital with intensive care required and risk of infection.

The secondary endpoints described the effect of that recovery on the immune system. This included the number of infections that patients had, the recovery of other blood cells, and days spent in the hospital. All of those secondary endpoints were also met in a statistically significant way. Overall, the study was successful in demonstrating that omidubicel was able to treat patients for transplant effectively and was an improvement over the standard umbilical cord blood transplantation. Beyond that, the neutrophil engraftment, rate of infections, and days in the hospital were improved when compared to any graft source or other sources of transplant for patients. Omidubicel could be an important option for patients who need a transplant.

MK: Based on the clinical data that Ronit described, we have conducted several market insight studies initially targeted in the US.

We have seen good consistency in these qualitative and quantitative insights. If approved, the unmet need that omidubicel may help address for patients in need of a transplant falls into two key categories: the ability to improve outcomes as compared to current donor sources, and the ability to increase access to patients–especially those who are deemed eligible for a transplant but who cannot find a transplant donor.

In terms of expanding the ability to improve outcomes, in the US, there is no clear standard for a donor source. It comes down to the best available donor source at the time for that patient. In blinded market research studies and conversations with transplanters, we found that the reason omidubicel could potentially improve outcomes comes down to a few things. The main aspect is the strength of the clinical data. Time to neutrophil engraftment is encouraging because that is a clear metric these transplanters are looking for. The days alive and out of the hospital are beneficial for patients, the transplant center, and the payer alike. The reduction in infections and time to platelet engraftment are also compelling.

Another key area in terms of omidubicel's ability to improve outcome is the time it takes to reach the patient. The majority of patients in the US still receive their donor source from an unrelated donor. Data from our market insights indicate it can take on average two to three months to align an unrelated donor to the patient. Our clinical trial showed the time from cord identification for manufacturing to the return of omidubicel to the clinical center to be consistently around one month.

The last piece is that the older the donor is, the more we see statistically significant detriment to patient outcomes. As we get older our cells become impaired, and we may also have co-morbidities or other challenges that impair our cells as a donor source. With omidubicel, we remove the donor age issue, as the cord blood is coming from a newborn.

The other critical opportunity is to increase patient access. In the US, if you are non-Caucasian, it is incredibly difficult to find a match in the public database. If you are a black patient in the US, your chance of finding a match in the public database is less than 20%. Unfortunately, these patients often succumb to their illness. In our clinical trial, 40% of the patients were non-Caucasian. Most oncology clinical trials are probably in the five percent range. In our clinical trial, we demonstrated our ability to help address this unmet need for patients.

If omidubicel receives FDA approval, these opportunities to improve outcomes and access in the US alone may equate to approximately 20–25% market share of addressable patients at the time of peak sales. That equates to 2,000–2,500 patients per year who could potentially benefit from omidubicel.

Q What differentiates Gamida Cell's platform?

JA: The NAM technology can accomplish amazing things.

At a technical level, NAM is an allosteric inhibitor of all NAD-dependent enzymes. There are hundreds of these enzymes in the cell, in multiple pathways, governing how cells mature and evolve. When trying to expand these cells with growth factors, particularly stem cells derived from umbilical cord blood, the stress of the cell culture allows the cells to expand but they differentiate, and they lose their stemness.

NAM creates a mimicry of the bone marrow niche. This is a hypoxic region of our anatomy, typically one percent oxygen. By preserving that microenvironment in the cell culture, it allows us to expand the cells massively so that we can achieve those requisite doses to see the rapid neutrophil engraftment beneficial effects for omidubicel.

DOI: 10.18609/cgti.2022.133

It also turns out that we can apply this to any cell type. We have successfully applied it to subsets of T cells, dendritic cells, and mesenchymal cells, for instance. It is this robust, universal expansion technology that differentiates us from any other approach in cell expansion.

Q Tell us about your combination therapy development strategy, both currently and in the future

RS: We first tested GDA-201 in the clinic in combination with two different antibodies in two different cancer populations.

It was in combination with rituximab, an anti-CD20 antibody, in patients with lymphoma, and in combination with elotuzumab in patients with multiple myeloma.

In the first-in-human study, we were able to deliver those therapies with all three escalating doses of the cells successfully and safely, with no dose limiting toxicities. We observed dramatic and interesting responses in patients with lymphoma who were treated with GDA-201, in the presence of that antibody. This informed the further development of GDA-201, beyond a fresh product that must be manufactured onsite to a cryopreserved formulation that we manufacture centrally at our facility and can deliver anywhere in the world.

Gamida Cell has substantial experience in developing and operationalizing cellular therapies to be delivered to patients around the world. As Michele mentioned, we conducted a global clinical study using stem cells that had been cryopreserved. Now, we have taken that knowledge to NK cells, and we are initiating a multi-center study to look at the safety and efficacy of those cells in combination with rituximab in patients with non-Hodgkin lymphoma.

JA: This is a deliberate strategy.

NK cells displace the CD16 receptor, which binds the Fc-gamma portion of antibodies, and creates a synergistic killing effect by the antibody-dependent cellular cytotoxicity (ADCC) process.

We have laboratory data in combination with Herceptin for HER2-positive tumors. We have also combined with other antibodies for targeting different malignancies. It is notable that lymphoma was a sensitive cohort of patients because the NAM-enabled NK cells naturally display another ligand, CD62L, which allows for efficient homing to the lymphoid tissues where lymphoma resides. We have both preclinical and clinical evidence for this activity.

NK cells are innate cells, so there is no matching requirement. They can trigger an adaptive immune response to recruit CD4 and CD8 cells to create a much broader based anti-tumor effect with great durability. For example, in the cohort of lymphoma patients treated at University of Minnesota, the median duration of response is 16 months. In the 19 patients treated with different doses, we had an overall response rate of 74%. 13 of the 14 responsive patients achieved complete response.

When we think about engineering NK cells, we must think about different anatomical sites, such as cells in other solid tumors that have immunosuppressive microenvironments. How can we develop other ligands on the surface of those cells to allow for both homing and

better efficacy? We have learned how to introduce different constructs through mRNA and electroporation. We transiently transfect these cells with other agents, including the ability to introduce CARs for specific targeting that occurs for both hematologic malignancies and solid tumors.

> "My...goal is to continue to drive the clinical program forward to bring the next-generation therapies through their clinical trials and to patients."
> - Ronit Simantov

Q Can you expand on how you are approaching the challenge of preparing for eventual commercialization in an area that is increasingly competitive, and where the successful market and patient access models are yet to be clearly defined?

MK: One of the most important hires we brought in was Rocio Manghani, our Senior Vice President of Market Access, who has over 20 years of experience in market access in hematology and cell therapy.

Rocio has hired an excellent team focused on assuring appropriate education with payers, in partnership with the medical and clinical teams. You need to ensure that stakeholders such as payers understand early on what the clinical data looks like for a one-time therapy with curative intent like omidubicel.

In the US, although we expect the majority of omidubicel patients to fall under commercial payers, the importance of interacting appropriately with Centers for Medicare & Medicaid Services (CMS) is also critical.

The feedback on the clinical data from the payers has been very encouraging. Our value proposition surrounds both clinical data, health economics and health equity–for health economics, what resonates is the reduction in healthcare resource utilization as an endpoint within our clinical trial. On the commercial payer side, we are interfacing with medical directors who have expertise in stem cell transplant. They understand the challenges associated with patient care, and the importance of both the clinical data and the reduction of healthcare utilization from omidubicel.

We have a clear understanding of the path to coverage and to reimbursement. In the US, commercial payers have said that for one-time therapies with curative intent, they will cover those therapies upon FDA approval.

Payers in the US have identified the potential pathways for reimbursement for a therapy like omidubicel. They have had the experience of other cell therapies launching in the US. One other important aspect for omidubicel for both the commercial and government sides is that there are established mechanisms to reimburse transplant and reimburse donor sources.

JA: It is notable that in the US, the top 70 transplant centers perform around 80% of the procedures.

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We need a relatively small footprint of account managers, coupled with medical affairs. The commercial team is under 50 people, which is tractable for an emerging company like us for our first cell therapy treatment.

Q Finally, can you sum up some key goals and priorities that you each have for your work over the next few years?

JA: I would like to see Gamida Cell become more recognized.

We want to deliver a successful BLA and meet the pre-approval inspection requirements. Then, I would love to see us launch successfully, match our marketing forecast, and deliver this breakthrough treatment for patients immediately.

We have positioned Gamida Cell for short and long-term success, so we have expanded and optimized our proprietary NAM technology platform. We have an existing pipeline of multiple cell therapy candidates for NK cells and are continuing to evaluate other cell types that may offer potential clinical benefit for cancer patients. The team at Gamida Cell are working tirelessly to become real innovators in the field of cellular therapy and to redefine the future of cancer care.

RS: My first priority is to bring omidubicel to patients–my team and I are focused on successful regulatory interaction and launch.

My second goal is to continue to drive the clinical program forward to bring the next-generation therapies through their clinical trials and to patients.

MK: Gamida Cell is a patient-focused organization, and we recognize that omidubicel will help address great unmet needs for patients with hematologic malignancies in need of a stem cell transplant.

Our primary focus on both the commercial and operations side is ensuring a positive patient experience upon FDA approval. We are confident that we are prepared for both our BLA and overall commercial readiness. Upon FDA approval, we will be ready for that first patient.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

FASTFACTS

POROS™ CaptureSelect™ AAVX elution optimization study for optimized recovery of AAV6 capsids

Jenny England, Thermo Fisher Scientific

In this study, the optimum elution conditions to maximize recovery of AAV6 capsids using POROS CaptureSelect AAVX were explored. Various elution buffers were tested to determine the impact of pH (2.0, 2.5, and 3.0) and excipients (Arginine, MgCl2, and Propylene Glycol) on AAV recovery.

Cell & Gene Therapy Insights 2022; 8(7), 809; DOI: 10.18609/cgti.2022.124

AAVX ELUTION OPTIMIZATION STUDY

High-throughput screening (HTS) experiments were performed to determine the effect of excipient concentration and pH on recovery. Purified AAV6 was tested in batch binding in 96-well plates using an automated liquid handler instrument. AAV6 was added to 20 µL of resin at a load density of 114 total capsids/ml of resin. Equilibration buffer (50 mM Tris, 150 mM NaCl, 0.01% Pluronic™ F (PF)-68 pH 7.5) was applied and the sample was incubated for 1 h at 1,000 rpm and room temperature. A high salt buffer wash was used (50 mM Tris, 1.5 M NaCl, 0.01% PF-68 pH 7.5), before adding the various elution buffers. The resin was stripped using 100 mM phosphoric acid and the absorbance at 280 nm was used to quantify the capsid recovery.

The results are detailed in **Table 1**. It was found that 0.5 M Arginine had the greatest positive impact, independent of pH.

ROBOCOLUMN™ ELUTION

The effect of excipients on AAV recovery was confirmed using 0.2 mL POROS™ AAVX RoboColumns, with similar experimental conditions to the HTS. Elution buffer conditions consisted of 0.1 M Glycine, varied pH (2.0, 2.5, 3.0), and the following excipient concentrations:

- 0.5 M arginine
- 0.5 M arginine, 0.5 M $MgCl_2$
- 0.5 M arginine, 40% (v/v) propylene glycol
- 0.5 M arginine, 0.5 M $MgCl_2$, 40% (v/v) propylene glycol

The results, presented in **Table 2**, demonstrate the addition of 0.5 M Arginine had an additive effect on recovery independent of pH, while the addition of 0.5 M $MgCl_2$ had a negative impact on recovery.

LAB-SCALE COLUMN ELUTION

The results from the HTS and RoboColumn runs were further confirmed using clarified sample and additional elution conditions using 1 mL column runs. The results shown in **Figure 1** demonstrate that the highest recoveries were obtained at pH 2.0 and 2.5, and the addition of Arginine allowed similar recoveries at pH 3.0. In contrast, the addition of Propylene Glycol and $MgCl_2$ in the elution buffer did not improve recovery.

Table 1. Summary of HTS screening results.

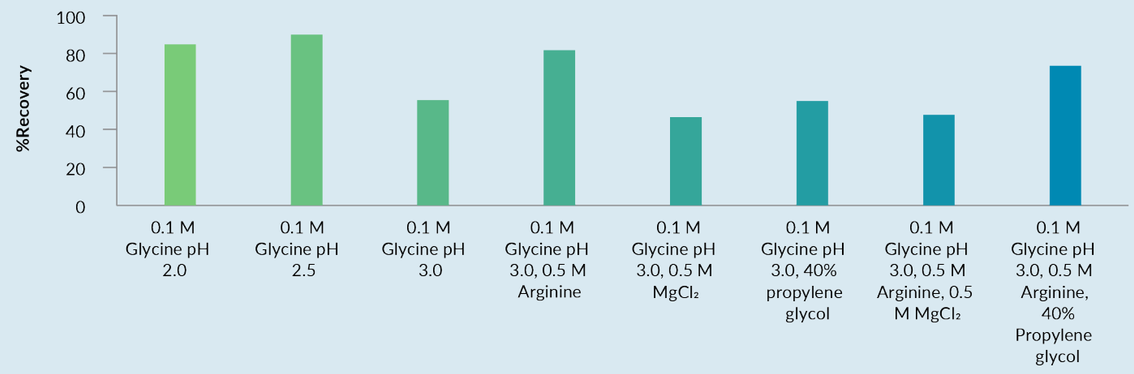
pH	Arginine	$MgCl_2$	Propylene Glycol
2.0	Greatest impact on recovery Increasing concentration improves recovery. Highest recovery with 0.5 M	No impact as a function of concentration	No impact as a function of concentration
2.5			
3.0		Negative impact on recovery Increasing concentration reduces recovery	

Table 2. Summary of RoboColumn screening results.

Excipient	2.0	2.5	3.0
0.5 M Arginine	(++)	(++)	(++)
0.5 M Arginine 0.5 M $MgCl_2$	(-)	(=)	(--)
0.5 M Arginine 40% Propylene Glycol	(+)	(-)	(+)
0.5 M Arginine 0.5 M $MgCl_2$ 40% Propylene Glycol	(=)	(=)	(--)

Change in recovery relative to in the absence of the excipient: (++) >25% increase; (+) 10-25% increase; (=) no difference in recovery; (-) 10-25% decrease; (--) >25% decrease.

Figure 1. Comparison of elution buffers on AAV6 recovery.



CELL & GENE THERAPY INSIGHTS

In partnership with: ThermoFisher SCIENTIFIC

FASTFACTS

Tips and tricks for transfection, adaptation, and scale-up for AAV production

Amanda Zunic, Thermo Fisher Scientific

With limited biological understanding of recombinant AAV vector production, and the limited amount of time developers have to optimize and scale-up their manufacturing processes, flexible solutions that can adapt to several different vector types, cell lines, or facilities are needed. Thermo Fisher Scientific has developed a panel of media to support high-titer AAV production by helper-free triple transfection using HEK293 cells, which is agnostic of specific manufacturing process or cell lineage.

Cell & Gene Therapy Insights 2022; 8(7), 797; DOI: 10.18609/cgti.2022.123

VIRAL VECTOR HEK MEDIA PANEL

The Gibco™ Viral Vector HEK Media Panel is a media library designed for HEK293 cells. The panel includes five chemically defined formulations and allows rapid customization for improved AAV titer. With triple transfection compatibility, this ready-to-go library of unique media can be optimized with the expert support of Thermo Fisher Scientific's field application scientists and R&D team, with a clear path toward customer large-scale manufacturing, operating according to a GMP quality management system.

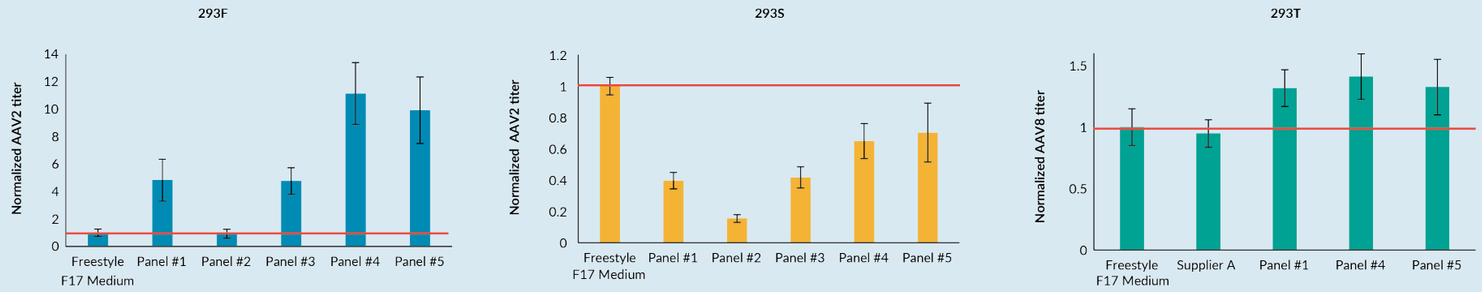
Transfection parameters may require optimization based on media, cells, and the gene of interest being used. Examples of different processes tested in shake flasks are shown in Figure 1.

OPTIMIZING SCALING OF BIOREACTOR

Implementation of HEK293 DynaDrive S.U.B. protocol results in tight controls and good aeration, although greater optimization can be beneficial (Figure 2). The medium can be tailored for bioreactor use by using Gibco™ Pluronic™ surfactant and carbon source optimization. The transfection process needs to be controlled at scale. Aeration and agitation are key parameters to control cell health and aggregation, and parameters for lysis and DNase treatment in bioreactor also need to be set. Analytical assays and the transfection process are major factors in process variability and should be addressed early on during development.

This configurable approach to high-performing media can be adapted and optimized to your viral vector manufacturing platform. Gibco has the capability and network to allow any required changes to chemicals, containers, and formats, to accompany you from small scale to clinical manufacturing.

Figure 1. Media screening across HEK cells 293F/293S/293T



OPTIMIZATION CHECKLIST

- Leverage robust and high-throughput analytics to rapidly assess quality parameters in upstream processes
- Select, adapt, and subclone the right HEK293 derivatives
- Optimize transfection ratios and adjust Pluronic surfactant and
- GlutaMAX Supplement concentrations
- Select high-performing media that can scale to dry format
- Optimize seed train for large-scale efficiency

Future work, beyond media development:

- Optimize bioreactor parameters to allow linear specific productivity increase across vessel platform
- Assess repeatability of transfection complex mixing and addition at scale

Figure 2. Thermo Scientific™ DynaDrive Single-Use Bioreactor (S.U.B.).

In partnership with: ThermoFisher SCIENTIFIC

CELL & GENE THERAPY INSIGHTS

SUMMARY

Simplifying residual DNA analysis in viral vector production: focus on E1A

Srinath Kashi Ranganath, Field Applications, Pharma Analytics Group, Thermo Fisher Scientific

Cell & Gene Therapy Insights 2022; 8(7), 837–840

DOI: 10.18609/cgti.2022.129

RESIDUAL DNA ANALYSIS

Residual DNA left in a product can impact quality, efficacy, and safety. Regulators worldwide, therefore, require limitations on the amount of residual DNA in the final dose. The WHO recommends that the amount of residual DNA per dose is kept below 10 ng. It is suggested by the FDA that a method with a sensitivity of 10 pg be used to determine DNA levels. Residual fragment length analysis is expected to demonstrate <200 base pairs (bp). There is increased concern that encapsidation is leading to viral vector products with larger amounts and longer sequences of residual DNA. Oncogenic sequences are of particular concern and must not be present in the final product.

Thermo Fisher Scientific's resDNASEQ™ system offers end-to-end solutions consisting of all-inclusive kits with well-characterized standards and reagents. These assays have been designed to meet regulatory guidance with high sensitivity, reproducibility, and lot-to-lot consistency over several years.

ADENOVIRUS EARLY REGION 1A (E1A)

E1A is an oncogene integrated in chromosome 19 of HEK293 cells, providing essential genetic regulatory modulation for viral vector manufacture (Figure 1). This gene allows HEK293 and various related cell lines to be used to produce recombinant adenovirus, recombinant

FIGURE 1

Introduction to E1A.

What is E1A?

- Oncogene integrated in HEK293 cells
- Essential for transcription of viral genes; essential in production of recombinant virus

Gene therapy applications

- Recombinant adenovirus, adeno-associated virus, and lentivirus are produced using HEK293 cells containing E1a

adeno-associated virus (AAV), and recombinant lentivirus.

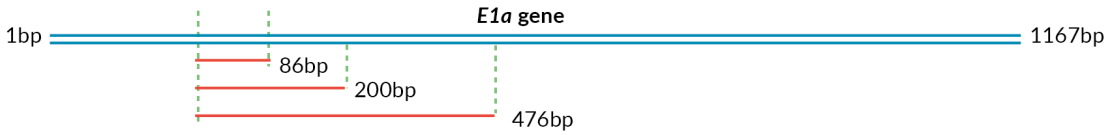
One current challenge in viral vector manufacturing is co-packaging of the host cell DNA within recombinant viral vector capsules. As E1A is both part of the HEK293 host cell genome and a known oncogene, any potential residual E1A requires detection and quantification as a harmful process-related impurity. Regulatory guidance requires the method used to demonstrate the effectiveness of the DNA reduction process to <200 bp fragments.

E1A FRAGMENT LENGTH ANALYSIS

resDNASEQ™ quantitative E1A DNA fragment length kit is the newest assay developed specifically for HEK293 processes. This kit can simultaneously detect and quantify E1A DNA of different fragment sizes. All of these assays can be used throughout the downstream process to support the characterization and optimization of your process and for routine quality control (QC).

FIGURE 2

E1A fragment length assay concept.



Assay design

- Three single-plex assays for known fragment sizes: short, medium and long
- Three standard curves for quantitation of each fragment size
- Sample Prep with PrepSEQ Chemistry, KingFisher Flex, qPCR with 7500 Fast or QuantStudio 5
- Readout directly in AccuSEQ software in copy number for unknown fragments

Example

Quantification

Short Frag assay (86bp)	110+100+90 = 300 copies
Med Frag assay (200bp)	100+90 = 190 copies
Long Frag assay (476bp)	90 copies

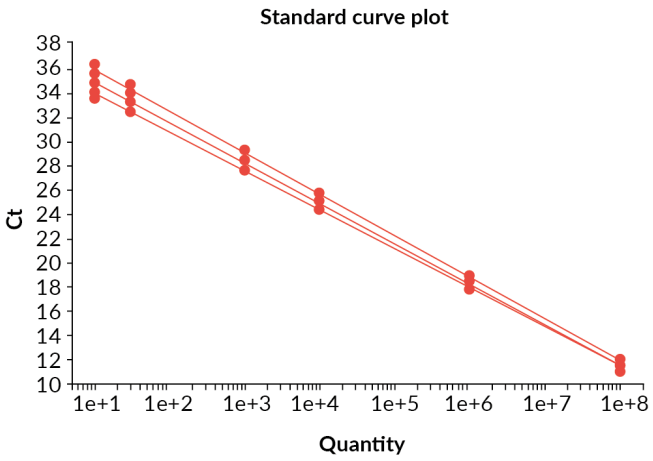
Ratio

<200bp	110/300
200–476bp	100/300
>476bp	90/300

DOI: 10.18609/cgti.2022.129

FIGURE 3

Standard curve performance.



	Short	Medium	Long
Fragment size	86–200 bp	200–476 bp	>476bp
Slope	3.284	3.363	3.433
R^2	0.999	0.999	0.999
Eff:	101%	98%	95%

As shown in Figure 2, the E1A assay design involves three single-plex assays targeting known overlapping fragment sizes of short, medium, and long fragments. The assay requires three standard curves, one for each fragment size, to quantitate E1A fragments of unknown samples.

The standard curve performance of the kit, as shown in Figure 3, demonstrates high linearity and efficiency to enable quantitative results across a broad range of DNA concentrations.

SUMMARY

The resDNASEQ™ system is a robust residual DNA quantitation solution for therapeutic-grade AAV production. The newest assay in the range is the resDNASEQ™ quantitative E1A DNA fragment length kit, which quantitates the fragment lengths of residual host-cell DNA by targeting the E1A gene in HEK 293 cell lines, often used in the development of cell and gene therapy.

Watch the webinar here

Read the full transcript here

ThermoFisher SCIENTIFIC

AUTHORSHIP & CONFLICT OF INTEREST

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How can we maximize the efficiency of large-scale LV vector production?

Scott Jeffers, GenSight Biologics, **Emily Jackson-Holmes,** Thermo Fisher Scientific, **Rakel Lopez de Maturana,** VIVEbiotech,
Steve Milian, Thermo Fisher Scientific, **Margherita Neri,** AGC Biologics

***Cell & Gene Therapy Insights* brought together a panel of industry experts to discuss the technological barriers to scaling up LV vector production and how they can be overcome. Here are some of the highlights...**

Cell & Gene Therapy Insights 2022; 8(7), 795. DOI: 10.18609/cgti.2022.122

How does your choice of bioreactor and upstream production platform affect LV titers, speed, and cost?

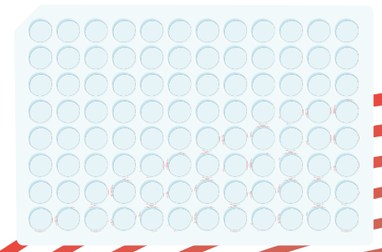
When developing products for upstream LV production, we use suspension-based systems, because they are advantageous in terms of scale-up, in addition to reducing variability and cost. To increase titer within the suspension system, we have used design of experiment (DoE) to optimize concentrations and timings of each component in the process, including the mammalian cell transfection process, cell line, transfection reagent, plasmid DNA, and any enhancers or supplements. This has resulted in a successful, optimized system that significantly increases titer and reduces cost.

Emily Jackson-Holmes,
Associate Product Manager, Cell Biology, Life Sciences Solutions Group, Thermo Fisher Scientific

What technological innovations are having the greatest impact on downstream LV processing?

The downstream side for LV is challenging, particularly because of the ~0.1µ dimensions of the LV. The most critical step is the final sterile filtration, where a large part of vector preparation is often lost. Clients frequently ask for more concentrated vectors. From a CDMO perspective, we must balance the concentration with the yield of the final sterile filtration. The more concentrated the vector, the more aggregation in the vector preparation, and the greater difficulty in balancing sterile filtration. Improvement in the analytical possibility to evaluate the vector aggregation will be important to solve the downstream challenge. Recently, many new membranes and resins for purification have become available on the market, and we are testing these to improve LV purification.

Margherita Neri, Vector Process Development Manager, AGC Biologics



How can we ensure robustness in assay selection and evaluation?

The big question is, 'do we have the assay under control'? We want low variance and high repeatability. One of the most important attributes of the assay is the ability to have different people do it – on different days, using different instruments – and still get the same answer. When assays are performed incorrectly, we should be investigating the impact of those changes on the assay itself to build a better understanding of how robust the methods are. If we notice that small changes are dramatically impacting the assays, it hints that the assay is not robust. We need to start building a library of investigations, to determine what are the critical parts of an assay and how they can be negatively impacted.

Steve Milian, Senior Staff Scientist, Science & Technology Pharma Services, Thermo Fisher Scientific

What bioprocess and analytical innovations will drive further scalability and quality/consistency improvements?

There are three key components for bioprocessing. One is the development of more producer cell lines to increase productivity. Second, automation is key, both in process and analytics. The third key point is the development of new serotyping strategies that better target the cell to be transfused, and the development of transduction enhancers. This is key to developing more cost-effective processes so that more patients can be treated.

Rakel Lopez de Maturana,
Quality Control Director, VIVEbiotec

How might the evolving regulatory landscape impact the picture?

The landscape has changed tremendously over the last 5 years. Regulators have placed great importance on quality and ensuring that we are monitoring our processes. Even from the early stages of process development, it is recommended to think about the final stages of commercialization. Understanding your process, with quality in mind from the beginning, is important to ensure you can get through the regulatory pathways. This ensures patients are safe, which is of primary importance.

Scott Jeffers, Chief Technology Officer, Gensight Biologics

Watch the webinar here

Read the full transcript here

CELL & GENE THERAPY INSIGHTS

LIVE30 TRANSCRIPT

Optimizing mRNA purification conditions by using a high-throughput screening approach

Jenny England

To support the development of mRNA-based therapies, Thermo Fisher Scientific has developed a platform chromatography solution for the purification of mRNA. The POROS™ Oligo (dT)25 Affinity Resin helps to address the selectivity and capacity requirements for the large-scale manufacturing of mRNA used in vaccine and gene therapy applications. Typically, mRNA binds to the Oligo (dT)25 affinity resin using high ionic strength conditions and neutral pH and is eluted from the column using low ionic strength solutions such as water. Although water works well for most mRNA constructs, a need to identify alternative elution buffers to optimize mRNA recovery exists. This article describes experiments conducted to optimize mRNA purification using high-throughput screening.

Cell & Gene Therapy Insights 2022; 8(7), 925–933

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RNA THERAPEUTICS & PURIFICATION

Synthetic mRNA has diverse applications, including cancer immunotherapy, vaccines, allergy tolerization, protein replacement, gene editing, and genetic reprogramming. Methods for mRNA delivery include direct injection, *ex vivo* injection of the transfected cells, and transfection of genome editing enzymes. These diverse applications and delivery

methods require a robust and easy-to-use purification platform.

The approval of an mRNA COVID vaccine in 2021 – the first approved vaccine against the virus – accelerated research into mRNA therapeutics for all applications. Despite the ever-growing list of applications for mRNA therapeutics, many challenges remain in mRNA purification. Current chromatography methods for RNA purification are detailed in Table 1.

mRNA capture is the critical first step in the downstream process before moving on to the polish, formulation, and fill and finish steps. The POROS Oligo (dT)25 affinity resin can be used as the capture step in the purification of mRNA in the downstream workflow to remove process-related impurities and some product-related species.

POROS OLIGO (dT)25 AFFINITY RESIN

The POROS Oligo (dT)25 resin has been specifically designed for the purification and isolation of mRNA from the in vitro transcription (IVT) reaction. The mRNA is captured through AT base pairing and contains a dT-25 (poly-deoxythymidine) ligand with a propriety linker attached to a 50 µm POROS™ bead. The resin can achieve a dynamic binding capacity of up to 5 mg/ml for a 4000 nucleotide (nt) mRNA with greater than 90% recovery. The resin also has excellent scalability and does not contain animal-derived components.

The POROS Oligo (dT)25 resin has three key attributes that differentiate it from other chromatography resins. First, the bead is made of poly(styrene-divinylbenzene), a rigid material that provides a linear relationship between pressure and flow on packed columns. This permeability is independent of column diameter, which facilitates scalability and enables the use of high flow rates with moderate pressure drops. Additionally, due to the polymeric nature of the backbone and the robust covalent chemistries of the beads, the resin shows physical and chemical stability from pH 1 to 14. This enables the use of standard cleaning solutions like 0.5 M NaOH, to meet resin lifetime targets.

The second attribute is the large pore structure that results in reduced mass transfer resistance. This is particularly important for large biomolecules like mRNA which diffuse into the bead at longer residence times.

The third attribute is the 50 µm average particle size. The relatively small beads allow for band broadening in packed beds which translates into smaller elution pool volumes.

TABLE 1

Chromatography methods of RNA purification.

Method	Advantages	Disadvantages
Reversed phase	▶ Native purification possible ▶ High resolution ▶ Some selectivity for product impurities	▶ Limited column capacity ▶ Use of expensive/ flammable/toxic chemicals
Ion exchange chromatography (IEX)	▶ Native purification possible ▶ Scalable ▶ Some selectivity for product impurities	▶ High pH may be needed for elution ▶ May need toxic chemicals for denaturation
Hydrophobic interaction chromatography (HIC)	▶ Native purification possible ▶ Scalable ▶ Potential replacement for reversed phase	▶ High salt concentration needed for binding may compromise stability ▶ Unproven approach for mRNA purification
Affinity chromatography	▶ Native purification possible ▶ Scalable ▶ Platform solution for a wide range of mRNA molecule sizes – selective to poly(A)	▶ Requires additional polishing step to remove product-related impurities

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TABLE 2

Experimental summary.

Resin volume	20 µL
Column load density	1 mg/mL of resin
Equilibrium buffer	10 mM Tris, pH 7.4, 1.0 mM EDTA, 0.8 M NaCl
Wash buffer	10 mM Tris, pH 7.4, 1.0 mM EDTA, 0.1 M NaCl
Elution buffer	Variable

FINDING AN ALTERNATIVE ELUTION BUFFER

Experimental summary

The objective of this study was to determine an alternative elution buffer for the POROS Oligo (dT)25 resin that would have similar or better recovery than water. Traditional methods have shown that mRNA binds to the affinity resin using high ionic strength conditions and is eluted from the column using low ionic strength solutions like water. Although water works well for most mRNA constructs, alternative elution buffers are necessary when water does not result in sufficient recovery or stability of the RNA.

A high-throughput screening (HTS) approach was implemented to test various elution buffer conditions on a 96-well plate format. An automated liquid handler instrument was used to execute the experiments. The purified mRNA sample was diluted with the equilibration buffer before loading onto the resin. The purified, 1000 nt mRNA sample was incubated on the resin for 1 hour at room temperature while shaking at 1000 rpm. After incubation, the resin was washed with equilibration buffer and a low-salt buffer. The elution buffer was varied to study the effect on recovery. The absorbance at 260 nm was used to quantify the eluted sample. A summary of the experimental conditions used in this study is shown in Table 2.

The buffers used in the study were chosen to evaluate various pH levels and ionic strengths to determine an alternative elution buffer to RNase-free water and included:

- 1 and 5 mM citrate with and without EDTA at pH 5 and 6
- 5, 10, and 25 mM Tris with 1 mM EDTA at pH 7 and 8
- 1 and 5 mM citrate in combination with 5, 10, and 25 mM Tris

Results

Comparing citrate-only elution buffers to RNase-free water, lower recoveries were observed for citrate buffer at pH 5, independent of the ionic strength or presence of EDTA. Comparable recoveries to water were observed for citrate at pH 6, with similar recoveries for 1 mM and 5 mM citrate. Interestingly, the addition of EDTA to 5 mM citrate pH 6.0 buffer resulted in a 25% decrease in recovery. The superior elution recovery of citrate at pH 6 could be explained by the charge of the citrate molecule, which has a pKa of ~6.

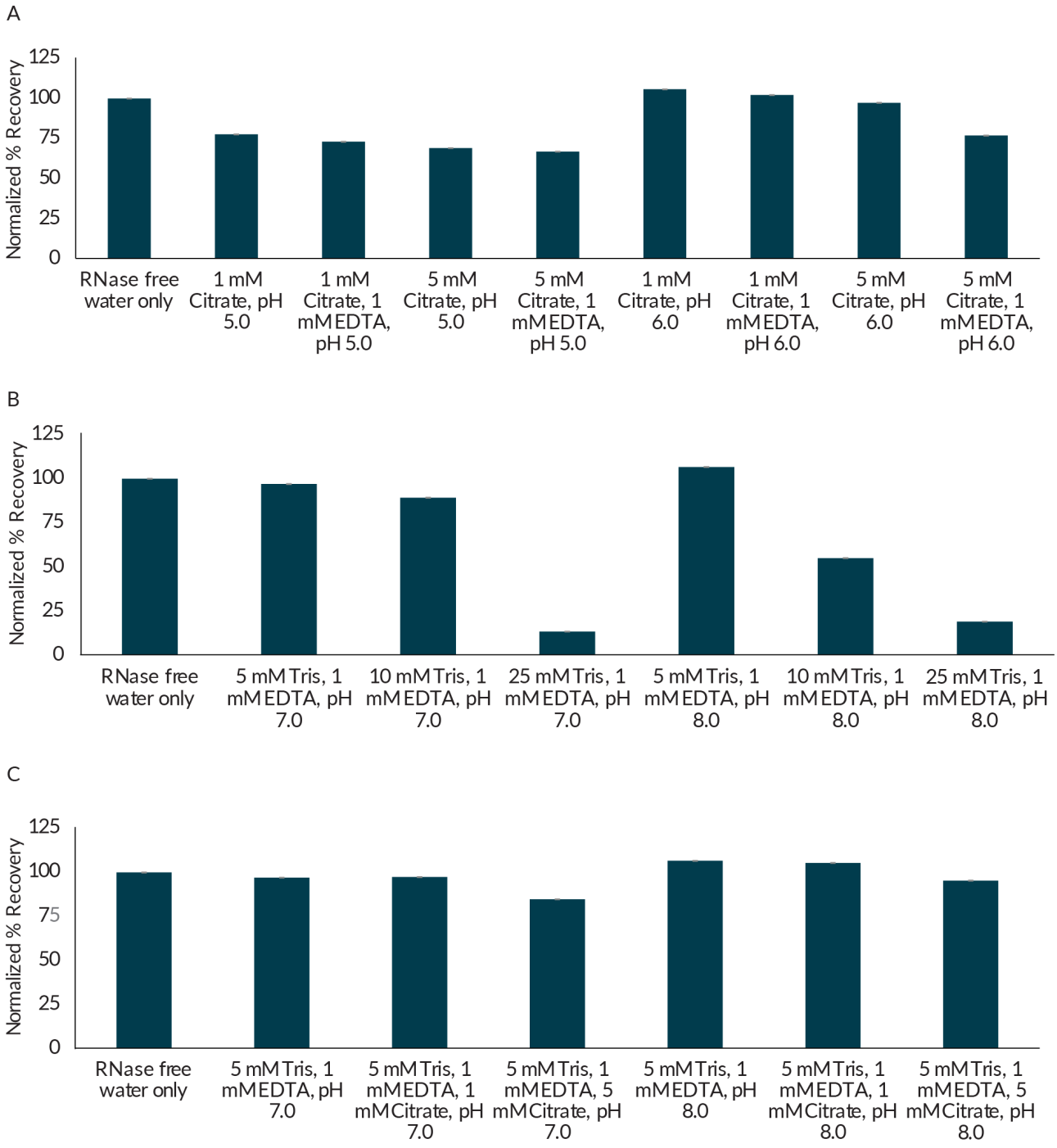
In observing the recoveries from Tris elution buffers only, Tris buffer at pH 7 showed similar recovery for 5 and 10 mM Tris, though a large decrease in recovery was observed for 25 mM Tris. At pH 8, 5 mM Tris resulted in the greatest recovery. An increase in ionic strength from 5 to 10 mM Tris resulted in a 50% loss in recovery. Even poorer recovery was observed for 25 mM Tris.

When citrate buffer was added to 5 mM Tris (Figure 1A), it was found that the combination of 5 mM Tris with 1 mM citrate does not have a significant impact on recovery, while the combination of 5 mM Tris with 5 mM citrate at pH 7.0 has the lowest recovery in comparison to RNase-free water.

In contrast, the addition of citrate to 10 mM Tris buffer had a notable impact on the elution recovery (Figure 1B). The addition of citrate to 10 mM Tris at pH 7.0 resulted in

FIGURE 1

Recovery results for Tris and citrate elution buffers in combination.



(A) Comparison of 5 mM Tris with citrate elution buffers. (B) Comparison of 10 mM Tris with citrate elution buffers. (C) Comparison of 25 mM Tris with citrate elution buffers.

a decrease in recovery, while the addition of citrate to 10 mM at pH 8.0 resulted in increased recovery, with 1 mM citrate having the best recovery relative to RNase-free water.

Tris buffers with a concentration of 25 mM showed the poorest recovery relative to RNase-free water (Figure 1C). In this case, the addition of citrate to the Tris buffer

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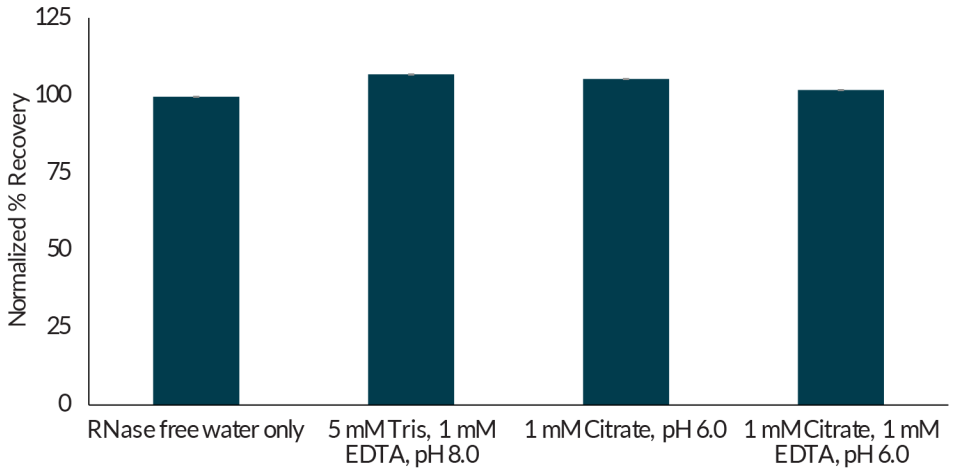
FIGURE 2

Best performing alternative elution buffer candidates from HTS.



did not show significant improvements in recovery.

The best performing alternative elution buffers from the HTS experiments are shown in Figure 2. These data show that the presence of EDTA does not have an impact on the elution recovery for citrate buffers. However, some improvement in recovery could be made with the addition of 1 mM citrate to a low-recovery elution buffer.

Column verification

The results from the HTS experiments were verified by testing the alternative elution buffers with column runs. Pre-purified, 1000 nt mRNA was diluted in equilibration buffer before loading onto a 1 mL pre-packed PO-ROS™ Oligo (dT)25 column. The column load density was 2 mg of RNA per mL of resin, and the sample load concentration was 0.25 mg/ml. The conditions of the experiment are shown in Table 3.

In the mL column verification runs, water showed the best recovery at 100%. This was followed by 1 mM citrate at pH 6, and 1 mM citrate plus 1 mM EDTA at pH 6 with an 89 and 88% recovery, respectively. The results confirm that no difference in recovery was observed with the addition of EDTA to the 1 mM citrate buffer. The lowest recovery of 84% was observed for 5 mM Tris plus 1 mM EDTA at pH 8.0.

To test if the recovery could be improved for the alternative elution buffers,

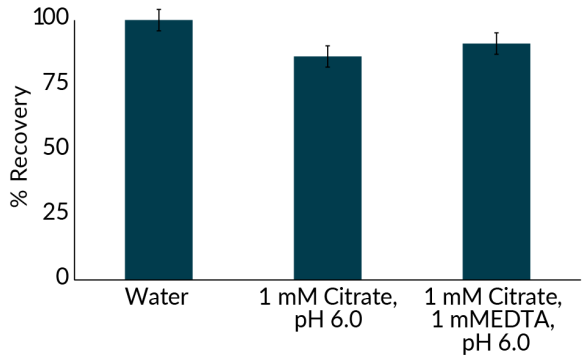
TABLE 3

Column experimental summary.

Step	Buffer	Column volumes	Residence time (min)
Equilibration	10 mM Tris pH 7.4, 1mM EDTA, 0.8 M NaCl	10	1.0
Load	1000 nt RNA	–	5.0
Wash 1	10 mM Tris pH 7.4, 1 mM EDTA, 0.8 M NaCl	15	1.0
Wash 2	10 mM Tris pH 7.4, 1 mM EDTA, 0.1 M NaCl	15	1.0
Elution	Variable	15	1.0
CIP	0.1 M NaOH	5	1.0
Re-equilibrate	10 mM Tris, 1mM EDTA, 0.8 M NaCl	5	1.0

FIGURE 3

Impact of elution buffer on recovery of a 2500 nt mRNA.



the residence time was increased from 1 to 2 min for the low-salt wash and elution steps. The same conditions as the previous runs were used. Comparable recoveries were observed for alternative buffers independent of residence time. Therefore, all three alternative elution buffers can be used to purify a 1,000 nt molecule with a 90% or greater recovery.

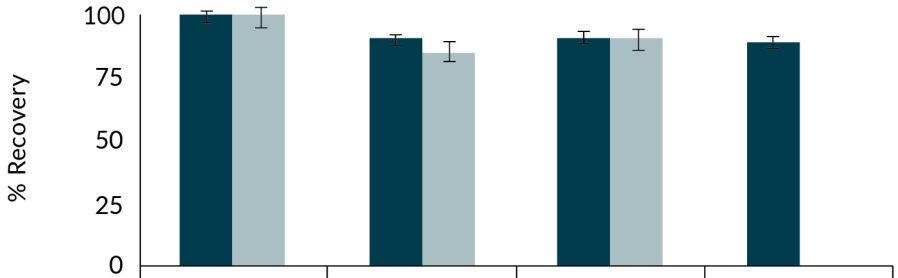
To further assess the alternative elution buffers, the recoveries of a 2,500 nt mRNA construct using these eluents were tested. The same sample preparation was carried out for these experiments, in which a purified 2,500 nt mRNA molecule was diluted to 0.25 mg/ml in equilibration buffer before loading onto a 1 mL prepacked column at a 2 mg/ml column density. After the sample load, no peak was observed in the equilibration wash step, but a small peak was observed in the low-salt wash. After washing with low salt, a narrow elution peak was observed with high absorbance, followed by a small peak in the CIP fraction.

When comparing the recovery of a 2,500 nt mRNA molecule for the POROS Oligo (dT)25 resin with various elution buffers, the broadest elution peak was observed in the presence of EDTA for the citrate buffer. It is hypothesized that EDTA causes a conformational change in the mRNA molecule that may result in entrapment in the pores of the resin and would require a greater elution volume to remove it from the resin. However, more studies would need to be done to investigate this phenomenon further.

A summary of the recoveries of a 2,500 nt mRNA molecule as a function of elution buffer from the POROS Oligo (dT)25 resin is shown in Figure 3. Citrate buffers can serve as alternative elution buffers with comparable recoveries in the absence and presence of EDTA for a 2,500 nt mRNA construct.

FIGURE 4

Most successful alternative elution buffers to RNase-free water for various mRNA sizes.



	Water	1 mM Citrate, pH 6.0	1 mM Citrate, 1 mM EDTA pH 6.0	5 mM Tris, 1 mM EDTA pH 8.0
1000nt	100	91	92	90
2500nt	100	86	91	Not tested

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CONCLUSIONS

In conclusion, low concentration citrate can serve as an alternative elution buffer to RNase-free water for various mRNA constructs. **Figure 4** shows there are comparable recoveries with 1 mM citrate pH 6 elution buffer for 1,000- and 2,500 nt mRNA molecules. Similar recoveries are observed for both size constructs for 1 mM citrate, 1 mM EDTA, pH 6. The elution buffer consisting of 5 mM Tris, 1 mM EDTA, pH 8 also showed good recovery for the 1,000 nt mRNA molecule and can be another alternative.

Based on this work 1 mM Citrate, pH 6.0 would be the recommended buffer as an alternative elution buffer to RNase-free water for various mRNA sizes. The addition of EDTA to the citrate buffer resulted in a larger elution pool with similar recoveries.

Q&A with Jenny England

Jenny England (Thermo Fisher Scientific) answers your questions on mRNA purification with POROS Oligo (dT)25

JENNY ENGLAND is a Staff Scientist in the Applications and Innovation group in Purification and Pharma Analytics at Thermo Fisher Scientific. Jenny is a biophysicist by training and earned her PhD from Georgetown University. After graduate school, she did a post doc at the National Cancer Institute that focused on structure-based drug design for protein kinase complexes. Jenny currently leads the application group for process development of the POROS resin products for antibody, mRNA, plasmid, and viral vector purification. Additionally, Jenny evaluates new and emerging technologies that can be applied to solve unmet customer needs in the bioproduction workflow.

Q What impurities remain after the Oligo (dT) purification, and what would you recommend as a polishing step for further removal of these impurities?

JE: Although the POROS Oligo (dT)25 resin works well in removing process-related impurities from the IVT reaction, it does not separate single-strand (ss) RNA from double-stranded (ds) RNA, if present in the load sample. dsRNA is the major impurity after affinity capture, and we would suggest including an additional polishing step such as ion exchange or hydrophobic interaction chromatography for further purification.

Q Why wasn't the 5mM Tris, 1mM EDTA, pH 8 elution buffer tested for the larger mRNA construct? Would you recommend this as an alternative elution buffer?

JE: We tested it for the 1,000 nt mRNA, but we did not move it into the 2,500 nt due to the peak broadening effect that we observed in the elution step, and therefore we recommended 1 mM citrate as an alternative elution buffer to water. However, this could be specific to your mRNA molecule, and it could be tested if citrate does not work well for your recovery needs.

Q Would you say that pH or ionic strength has a stronger impact on mRNA elution and recovery?

JE: This is dependent on the elution buffer used. For citrate, we saw that there was a greater effect of citrate between pH 5 and 6. For Tris, we saw a greater effect on ionic strength, where the 25 mM Tris had the lowest recovery.

It is important to keep in mind that these results may be dependent on the specific mRNA construct to be tested and are intended only as a guide.

Q Is there a minimum or maximum length of mRNA that the POROS Oligo (dT)25 resin can work with?

JE: The POROS Oligo (dT)25 resin works across a variety of mRNA lengths. We are always looking to work with customers to generate data with large mRNA constructs and have worked with customers on the purification of large self-amplifying mRNA molecules.

However, keep in mind that you will optimize the POROS™ Oligo (dT)25 purification to your specific mRNA molecule, and you may observe differences in binding capacity as a function of size.

AFFILIATION

Jenny England
R&D Manager, Thermo Fisher Scientific

ThermoFisher SCIENTIFIC

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AUTHORSHIP & CONFLICT OF INTEREST

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

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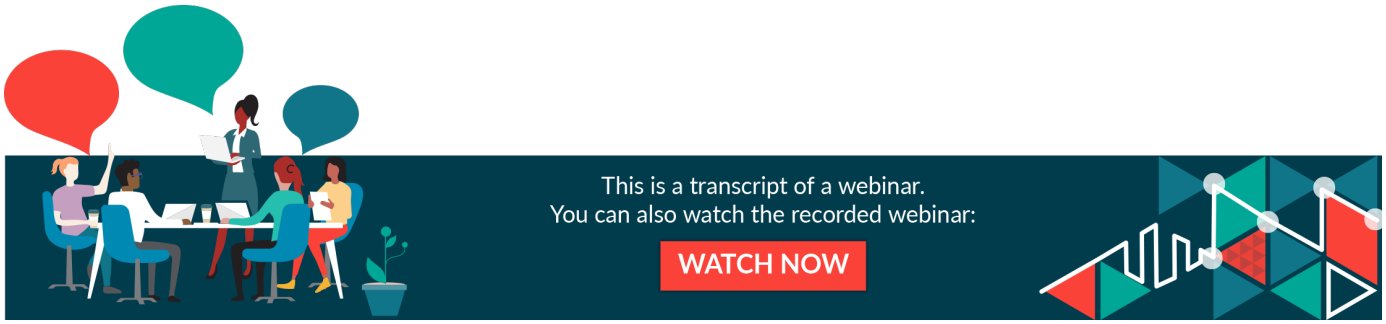

Scalable solutions for cell isolation and expansion

Evan Zynda, PhD, Staff Scientist, Cell Biology, Thermo Fisher Scientific

To realize the potential of revolutionary cell therapy treatments, challenges of safety and cost must be addressed. Scale-up of cell manufacture is a key approach to mitigating these issues. Gibco™ CTS™ DynaCellect™ Magnetic Separation System for the isolation and bead removal of cells and stirred tank bioreactors for cell culture are two scalable building blocks for cell therapy manufacturing processes that can help combat common industry challenges.

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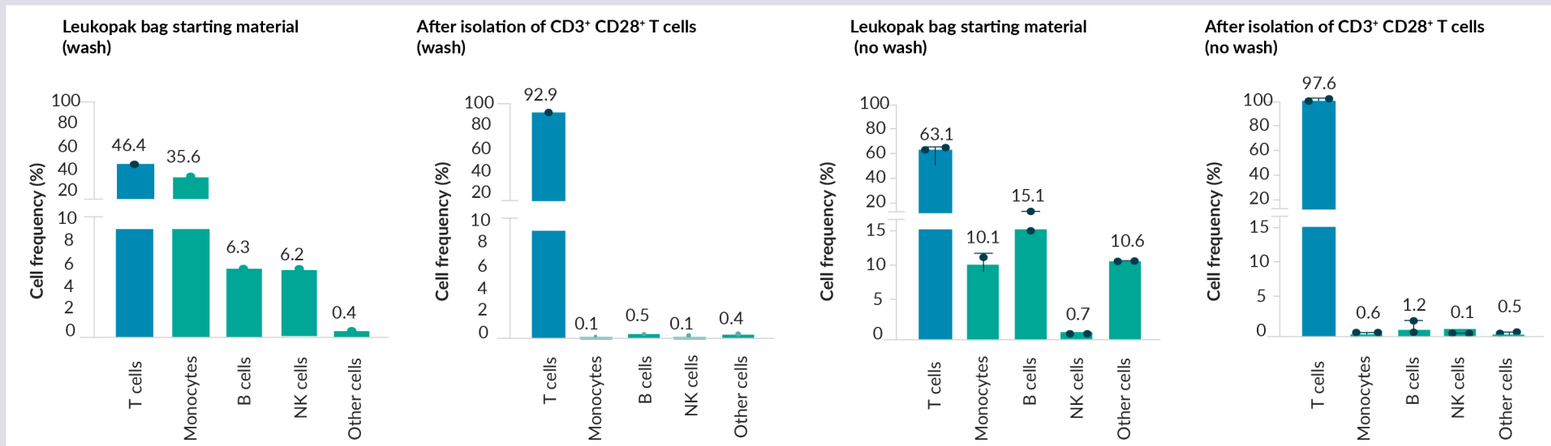
CTS DYNACELLECT MAGNETIC SEPARATION SYSTEM

Designed for use with Gibco™ CTS Dynabeads technology, the highly scalable, closed and automated CTS DynaCellect can be used for both cell isolation and bead removal.

The isolation process achieves >90% isolation efficiency of target cells with ~95% purity and no impact on cell viability, with or without a wash step (Figure 1). The CTS DynaCellect process is scalable up to 1 L or 10 billion target cells per isolation reaction, with a throughput time of ≤100 minutes.

The automated bead removal process is fast and efficient, and currently results in >85% target cell recovery. Bead removal is achieved through a continuous flow to ensure rapid processing of volumes that are both characteristic of autologous and allogenic workflows (Figure 2).

Figure 1. Leukopak bags were used in a one-step isolation and activation using the CTS DynaCellect, with and without a wash step prior to isolation.



STIRRED TANK BIOREACTORS

Following upstream cellular processing, stirred tank bioreactors have been identified as the most effective, scalable, and flexible closed and automated vessels for expansion of cell therapies. They possess superior control over culture characteristics, including mixing, gassing, and liquid exchange. These capabilities have improved yields compared to other dynamic and static bioreactors, whilst still maintaining the same levels of early memory cells. Ramped agitation sustains growth and viability and can support higher viable cell density (Figures 3 & 4). Moreover, stirred tank bioreactors are associated with a relatively small footprint and can be readily assimilated, both physically and digitally, into closed and automated workflows.

CTS DynaCellect and stirred tank bioreactors represent scalable, closed and automated building blocks for a cell therapy manufacturing process, which can mitigate overarching current and future challenges relating to safety and cost.

Figure 2. Process scalability and flexibility of bead removal.

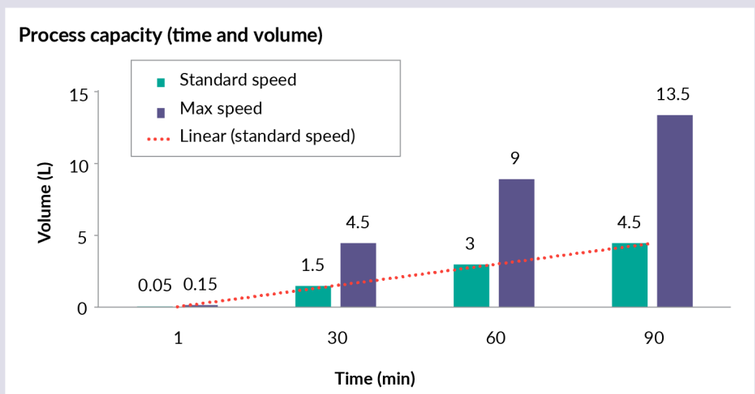


Figure 3. Dynamic reactors show improved expansion. Results are representative of more than five independent experiments.

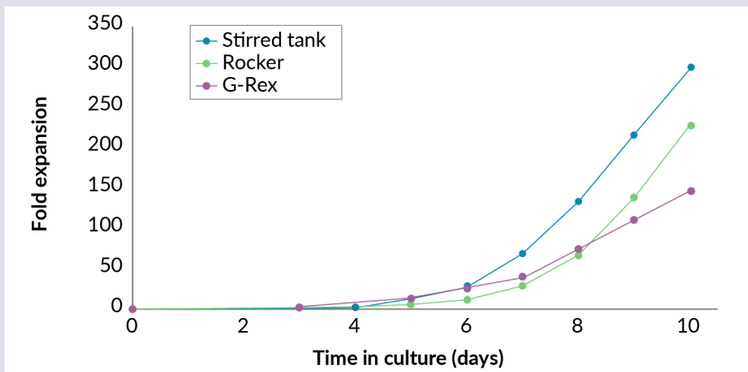
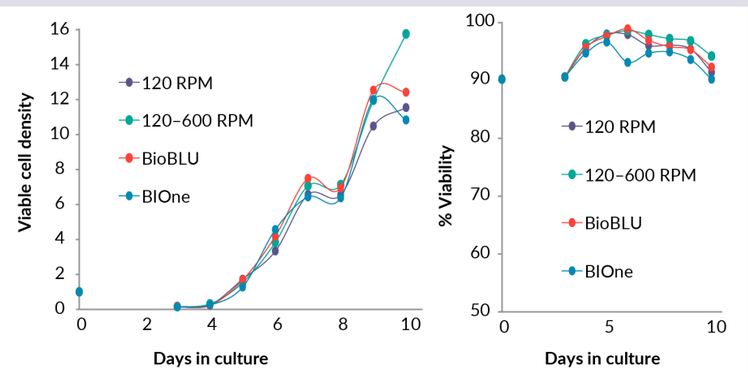


Figure 4. Increased agitation enhances T cell expansion and supports higher cell densities.



CELL & GENE THERAPY INSIGHTS

VIEWPOINT

Lessons in scientific endeavor and innovation

Irving Weissman
Stanford University

"What science needs is more people who can observe what happens when a physiological system is perturbed."

VIEWPOINT

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DOI: 10.18609/cgti.2022.130

On April 21st, 2022, David McCall, Commissioning Editor, *Cell & Gene Therapy Insights*, spoke to Irving Weissman about his storied career in stem cell R&D. This article has been written based on that interview.

Today, I am focused on some discoveries from earlier in my career – discoveries about which I still harbor some regrets that they stopped upon technology transfer, but a review of the clinical trials showed they were effective. I feel it is urgent that I now get this work done. Here, I will try to explain why.

PURIFIED HEMATOPOIETIC STEM CELLS IN THE TREATMENT OF CANCER

My lab first isolated mouse blood-forming stem cells in 1988 and in the same year, Mike McCune and I implanted human blood-forming organs into an immune deficient mouse, forming a blood forming and immune system in these SCID-Hu mice. We used human fetal tissue, including human fetal bone for bone marrow, fetal liver also as an hematopoietic stem cell (HSC) source, thymus to make T cells, and spleen and lymph nodes, in order to see the immune response. The results were spectacular: by giving low doses of radiation to that mouse, we could then test human cells in human organs for regenerating an entire blood forming and immune system. Within two years we had isolated the human HSC.

That mouse was infectable with human immunodeficiency virus (HIV). The HIV virus that Gallo and Montagnier discovered, which was grown on $CD4^+$ T cells, wouldn't cause acquired immunodeficiency syndrome (AIDS) when injected into the mouse, and yet every sample we got from an AIDS patient did cause AIDS. Therefore, we realized that growing a virus in a tissue culture without selecting for its important pathological property can lead to rapid loss of that property. Mutants that can grow faster have to replicate less nucleic acid.

We were unable to pursue this work further at Stanford at the time, so we formed a company called SyStemix. At SyStemix we did the final experiments to find stem cells. When we purified the HSCs, they had no T cells contaminating them. If you took blood forming cells from a breast cancer patient with widespread bone marrow metastasis, the mobilized blood of the bone marrow were contaminated with many cancer cells. Purification of HSCs from these same tissues had a 250,000-fold depletion of breast cancer cells.

We performed a clinical trial giving high-dose chemotherapy to reduce tumors in the body and rescued their bone marrow with mobilized blood transplants. However, the contaminating cancer cells infused with mobilized blood spread in the body. When we rescued the patients not with the mobilized blood, but with the purified, cancer-free stem cells, they restored blood and immune systems as well as did mobilized blood. We had shown in mice that you could return to normal blood formation with T cell-free and cancer-free stem cells (HSC). The restoration of blood formation in the patients transplant ed only with their own HSC proved that the HSC were the most important cells in a bone marrow transplant, as in mice.

With purified HSC lacking T cells used in donor to recipient transplants, there is not at the same risk of graft versus host disease (GvHD). Current transplants by bone marrow transplant teams are mainly hindered by GvHD, which if untreated, can be fatal. This could allow T cell depleted HSC to be used to treat patients with sickle cell anemia, severe combined immune deficiency (SCID), diabetes, lupus, or multiple sclerosis with a pure stem cell that will not cause GvHD. It could be applied throughout regenerative medicine.

As we were doing these studies, a large pharmaceutical company bought SyStemix. They told us that they were going to do everything that I thought we should do. We could not afford the clinical trials, but they could. However, nine years later, they made the business decision that they were not going to pursue development further.

It happened that the metastatic breast cancer trial was performed at Stanford. A few years later, I became the founding head of Stanford's Cancer Institute. A member of our group looked at all the data on our patients who received cancer-free stem cells versus those who received mobilized blood (the

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standard of care). The findings were amazing. The mobilized blood (contaminated as it was with cancer cells) used to rescue women after high-dose chemotherapy, showed a median survival in patients of two years. The overall survival at 12 years was zero. Meanwhile, the patients who received cancer-free stem cells had a median survival rate of 10 years. One-third of them are alive without disease today, some 25 years later.

I went to the bone marrow transplanters and the breast cancer doctors and proposed that I start this study up again in an academic setting. The bone marrow transplanters held the initial belief that stem cells did not work for breast cancer, but they were mistaking mobilized peripheral blood (including contaminating cancer cells) with cancer-free stem cells. And the oncologists weren't interested either, this time partially on financial grounds. However, we are now moving forward with our stem cell research.

We are going to pursue this right across the board: we plan to perform autologous stem cell transplants for cancer. We have chosen allogeneic stem cell therapy approach in place of gene therapy to try to cure genetic diseases in the blood-forming system, which can be caused either by a lack of healthy blood cells or a surplus of autoimmune cells.

I made the decision to step down as Head of the Stanford Institute of Stem Cell Biology and Regenerative Medicine about a year ago, effective this coming September, but to remain as head of the Stanford Ludwig Cancer Center in order to focus on this work. This research is going to happen, though it's going to be tough – the cell sorter companies involved will want to know if they can make money out of it, for instance.

One thing I realized when the large pharma shut our research down, is that they made a perfectly logical financial business decision in doing so. The function of a company is to make a profit, and there are stockholder issues if they don't maximize that function. Businesses are not going to take chances like we do in academia to sacrifice profits for potential advances for patients. Due to this fact, I helped found and develop Proposition 71 (the California Stem Cell Research and Cures Act), as I deemed it necessary to have a governmental agency that can extend discoveries past the translational 'valley of death'.

IDENTIFYING CD47 IN LEUKEMIAS

In the early-2000s, I was working in the field of blood-forming stem cells. We had isolated pure human acute leukemia stem cells at the stage of non-HSC multipotent cells; this allowed us to compare the genes leukemia stem cells expressed with normal HSC.

Ravi Majeti, a former trainee and now chief of Stanford hematology and I first identified a gene overexpressed by leukemia cells was a cell-surface molecule called CD47. I had no idea at the time what it was or what it did. A Swedish group led by Oldenberg and Lindberg published that if you make a CD47-knockout mouse, the red blood cells from that mouse when transfused into a genetically identical wild-type mouse have a half-life of only two or three hours. They correctly made the assumption that macrophages were destroying the unfit red blood cells. They showed that the macrophage has a receptor for CD47 and when that receptor is engaged, the macrophage is paralyzed and so cannot phagocytose and destroy the cell that it is attached to.

We had shown CD47 to be on all mouse and all human acute myelogenous leukemia (AML) stem cells. We then showed that we could make a monoclonal antibody that blocked the CD47 inhibitory signal to cognate receptors on macrophages. Following this work, we applied for and received a competitive grant from the California Institute for Regenerative Medicine (CIRM) to move the research forward. We could act as if we were a company, translating this discovery, but not have to worry about profits. We found that the "eat me" signal is a protein called calreticulin. It does not have what it takes to reach the cell surface, and it is retained inside the cell

via the KDEL receptor. It was shocking when we found calreticulin was the cell surface "eat me" signal on the tumor cells, revealed when we blocked the "don't eat me" signal. The cell surface calreticulin can be made by the cancer cells, or made and secreted by nearby activated macrophages; we are still trying to figure out how calreticulin gets to the cell surface, and how is it's binding site generated on these dangerous cells.

We showed that anti-CD47 plus azacitidine could cure human acute leukemias or myeloid dysplastic syndrome when the diseased bone marrow from patients was transplanted into our immunodeficient mice.

We then moved into a Phase 1 trial, filing Investigational New Drug (IND) applications to both the US and the UK. The UK was intriguing because of their National Health Service (NHS). I knew from my days at SyStemix that we had to hire a building full of people to get approval from US health insurance companies to let a patient be in a clinical trial. However, in the UK, the NHS were the only people we had to convince.

We showed that in elderly patients with acute leukemia or high risk myelodysplastic syndrome, treatment with our CD47 blocking antibody and azacitidine, most went into remission, and in many the disease was cleared. Azacitidine induces the "eat me" signal that we had discovered.

We also blocked the "don't eat me" signal on lymphoma cells in combination with the therapeutic antibody rituximab. The kind of antibody type that is rituximab has a part (Fc) that binds to a receptor on macrophages. If it is bound, the macrophages eat and destroy the target cell, so in a sense, we added a new "eat me" signal. In patients who did not respond the last time they were treated with rituximab plus or minus chemotherapy, when given this new treatment of anti-CD47 plus rituximab, half of the therapy resistant lymphoma patients responded. In the first year, 50% of these responses were complete, and I don't know the long-term results now.

Faced with the possibility that the university would license this research to groups unfamiliar with our discoveries, we formed a company called Forty Seven, Inc. Within four years of formation, Gilead Sciences bought Forty Seven, Inc. They are focused on bringing the CD47 based immunotherapy to many other cancers.

PRELEUKEMIC CLONES OF HSC COLLECT SUCCESSIVE MUTATIONS TO GENERATE BLOOD DISEASES & BLOOD CANCERS

Independent of the CD47 studies, we had shown that when leukemias start to form, mutational events trigger more and more danger signals, including the intrinsic danger signals like p53. Analyzing single HSC from AML patients, Majeti, Jan, and I found that the initial mutations were usually in genes whose expression opens or closes chromatin in preparation for changes in gene expression – genes such as tet2, idh1/2, dnmt3a, and each of these loss or change of function driver mutations led to HSC clonal expansion, as we had shown earlier for aml1-eto, bcr-abl, and in myelodysplastic syndrome, in which most or all HSC in the body derived from a single cell. In AML, the final mutations were of 'classic' oncogenes such as N or Kras, flt3itd, and activation of beta-catenin. These early driver mutations led to chronic phase CML, MPN, and as Jaiswal and Ebert showed, clonal hematopoiesis of indeterminate potential, or CHIP. One or more of these early signals triggers the receptor for calreticulin – the "eat me" signal. In myelodysplastic syndrome, hematopoietic progenitors in the clone have the "eat me" signal but not the "don't eat me" signal leading to anemia or thrombocytopenia or neutropenia. Most of the patients with myeloid dysplastic syndrome go on to suffer from AML, and concurrently upregulate CD47 so they have a "don't eat me" signal to counter the "eat me" signal.

It is likely that these pre-cancer and cancer mutations are not limited to HSC or hematopoiesis, and in other tissue stem cells

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can cause adult onset diseases from clonal competitions.

APPLICATIONS BEYOND CANCER

Nicholas Leeper had performed work on the gene expression of human atherosclerotic plaques and observed CD47 and calreticulin. We decided to work on this together.

We took mice that are designed to get atherosclerosis fast when we put them on a high-fat diet, simultaneously putting in our anti-CD47. They did not develop atherosclerotic plaques that were lethal – they developed smaller ones.

I had a set of 'rainbow' mice that we developed in the early 2000s. Provision of a signal to these mice caused each cell and their clonal progeny to express a set color or color combination encoded by genes taken from fluorescent sea creatures. As each cell makes a unique color combination, we could test whether the mice susceptible to atherosclerosis had a precursor of smooth muscle cells that started to divide to make clonal atherosclerotic 'plaque'. Each plaque was clonal.

By treating the mice with anti-CD47 we were able to prevent atherosclerosis by preventing the clonal expansion of pathologic smooth muscle clones by macrophages that removed dead cells by efferocytosis, and by programmed cell removal destroyed plaque cells.

We noticed that atherosclerotic plaques contain macrophages that produced tumor necrosis factor (TNF). We showed directly that the expanding smooth muscle cells with TNF receptors activate upregulation of the CD47 gene. We treated animals with anti-TNF and anti-CD47 after they had plaques, and the plaques regressed.

Gerlinde Wernig and I also did work in fibrotic diseases. Yuval Rinkevich and I had shown the mesothelium on the lung is a stem cell that makes more of itself every time it divides, but it also makes daughter cells that become either fibroblasts or smooth muscle cells. In her model of IPF, subpleural fibroblasts expressing c-jun undergo proliferation, moving inward, and upregulate CD47 and express calreticulin, as well as mesothelin. These pathogenic fibroblasts express IL6, and the combination of anti-IL6+anti-CD47 is therapeutic for even late stage mice.

REPLACING CHEMORADIATION FOR HSC TRANSPLANT CONDITIONING

We first discovered HSCs by being able to irradiate mice with lethal doses. That process was a holdover from using hematopoietic transplants to enable high dose radiation and chemotherapy for cancer patients. However, transplanting HSC into non-cancer patients to enable regenerative medicine does not require the anti-cancer effects of chemoradiotherapy, and so it limits the kinds of patients who could be treated. For example, with a patient with type 1 diabetes, we would previously have needed to give a dose of radiation sufficient to kill the patient's T cell population, then transplant diabetes resistant bone marrow. This would lead to graft-versus-host disease (GvHD). But purified HSC cannot cause GvHD, lacking T cells. So we sought a way to engraft allogeneic HSC without chemotherapy or radiation.

We started a series of experiments in 2007 to remove HSCs in a recipient. We found an antibody that blocked an essential receptor on the surface of HSC stem cells called c-Kit. We had shown through other experiments that if you do not get a signal to c-Kit by kit ligand (SCF), that cell dies. The blocking antibody removed HSC in SCID mice with a single treatment, and allowed curative syngeneic but non-SCID HSC to restore the immune system.

However, when we tried it in immune-competent mice, the T cells were making factors that blunted the effect. We needed the anti-CD47 to let macrophages eat the stem cells decorated with the anti-c-Kit antibody. It worked. To do a donor-to-host transplant, we added anti-T cell antibodies

to anti-c-Kit and anti-CD47, which allowed MHC matched but unrelated transplants. Finally, adding anti-NK antibodies to the mix allowed haplo-MHC transplants, and even unmatched HSC transplants to engraft in unirradiated recipients.

The adaptation of this approach to humans could enable regenerative medicine with HSC to replace diseased blood and immune systems. Over 25 years ago we showed that HSC engraftment induced tissue transplant tolerance of skin, or beta islets, or heart transplants specifically from the HSC donor. That is our objective for the future in humans.

BRAIN FORMING-STEM CELLS

Nobuko Uchida, Ann Tsukamoto-Weissman, Fred Gage and I used the general method we developed to isolate mouse HSC to isolate human fetal brain-forming stem cells. Stem Cells Inc was established in 1997, transplanting these stem cells to treat thoracic spinal cord injuries, cervical spinal cord injury, dry age-related macular regeneration, and two congenital neurodegenerative diseases. Both preclinical and phase I/II trials showed regenerative repair at some level, but the company did not martial the resources needed, and was sold in 2016. All donors of these CNS stem cells were fully allogeneic to the recipients, and were retained in trials using organ transplant immunosuppressive regimes. Cessation of immunosuppression could lead to gradual loss of therapeutic improvements dependent on the stem cells.

DISCOVERING GRAFT REJECTION & STEM CELL COMPETITION

How did stem cells arise and why is their engraftment limited by histocompatibility genes? While co-authoring an immunology textbook I sought a biologically robust naturally occurring histocompatibility system in more primitive species. I wanted an explanation from the life history of the species, not an experimental intervention that depended on artefacts. Such an example was observed by Oka and Watanabe in 1957, describing the colonial protochordate Botryllus, cited and discussed by FM Burnet in his discussion of self and non-self-recognition in 1971. Burnet quoted the amazing observational experiment of Ray Owen in 1945 that freemartin male and female cattle twins sharing a placenta were blood cell chimeras for life. Burnet considered the vertebrate immune system essentially as a protection against pathogens, usually microbial species that are infectious, but foreign to the host. Allorecognition came from the T cell system and its recognition of non-self or self MHC, later shown to be the carrier of non-self-infectious organism peptides.

Burnet wrote about an animal – a tunicate –that starts off as a vertebrate from sexual reproduction. As Oka described, and we confirmed, the zygote grows via vertebrate-like embryo and fetal stages to a tadpole chordate within the body of the mother. Hundreds of offspring tadpoles hatch into the ocean nearly simultaneously and eventually settle next to each other on subtidal surfaces, where they undergo metamorphosis and lose all vertebrate structures (notochord, somatic musculature in a tail, a photolith, and the chordate stage brain). Nevertheless, this invertebrate has a complex body, with a gelatinous tunic, a two-chambered heart which provides blood to the body and the tunic, and one of the two brains made in the tadpole stage. The newborn invertebrate stage starts budding a collection of cells from its body wall into the tunic, which, over a week, makes a organs that are arranged in a body plan like the individual it budded from, without any embryonic intermediate stages. In the gelatinous tunic, they send out blood vessels to connect with each other to form a common extracorporeal circulation that connects to the intracorporeal vessels of all individuals sharing the tunic. The organism they budded from dies with programmed cell death and macrophage mediated programmed viable and dead cell removal, at the end of three weeks. When

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making new clones, by this budding process, adjacent multi-individual colonies project terminal extracorporeal blood vessel ampullae into the adjacent colony to touch its tunic blood vessels. They commence a rapid immune-like rejection reaction, or they anastomose blood vessels and become living parabionts or chimeras, sharing a blood circulation.

We showed that the genetics of rejection was controlled by a single, highly polymorphic gene locus as predicted by Oka, which we named bhf. Rejection occurred if there was no shared allele at the locus, but anastomosis and chimera formation resulted from sharing one or both alleles, reminiscent of natural killer recognition. This could set up the situation wherein germline and somatic stem cells , if they existed, could be the pathogenic invaders Burnet sought.

In the mid-90s, we observed that when compatible tunicates became chimeras, if you subsequently separated them and waited until the time, they usually produce sperm and eggs, all of the sperm and eggs would come from just one of the pair, no matter which organism's body it was in. The pathogens were predatory germline stem cells! Without the bhf massive allelic polymorphism, the most competitive of the germline stem cells could have spread across large areas, bringing the dangers of homozygosity to the species. The stem cell competition and mutation could give an organism an advantage. We also showed that Botryllus had an HSC based histocompatibility system, and that the killer NK-like cells were produced from HSC.

We wondered if germline stem cell competitions existed in a vertebrate species; Ueno and I produced mouse blastocyst (embryo) chimeras using endogenous inner cell mouse cells and introduced blue, red, or green mouse ES cells to make chimeras. We were surprised to find in adult mouse blastocyst chimeras that large sectors of testicular spermatogenic cells in adjacent seminiferous tubules were only one of the possible colors. This was contrary to the usual derivation of the germline from a few (we determined 4) germline committed stem cells that migrated to the allantois, expanded massively, then migrated to the genital ridges to form the germline progenitors. The mystery was deepened when we showed that the ES derived and ICM cells migrated randomly into the incipient genital ridge seminiferous tubules, all colors found in the same seminiferous tubule. The mystery was solved when we found masses of apoptotic germline cells just premeiotic; the residual germline cells, probably 0 or 1 per tubule, expanded to include dozens of adjacent seminiferous tubules. So clonal germline lineage competitions are also found in mice, presumably to eliminate defective or dangerous cells.

We used these findings in Botryllus and mice to look for clonal stem cell competitive expansions in the preleukemic phases of human acute myelogenous leukemia development.

As described above, in the early 2000s, we had already identified human leukemia stem cells. By that time, we could look at leukemia stem cells and HSCs from the same person. We sequenced the DNA to identify the mutations on the leukemia stem cells that are not present in T cells, and we made DNA primers for each of the mutated nucleotides. We used all of those primers and analyzed one blood-forming stem cell at a time from the same patient. We worked out the order of mutations in these leukemias.

So, from observing natural biology of the tunicates, I got the idea of stem cell competition and we showed it in the germline, we showed it in the development of leukemia, and we have now shown every adult-onset (myeloid) blood disease comes from a mutant stem cell that is expanding.

Like Ray Owen and his chimeric fraternal cattle twins, most often we made observations that were hard to fit with what we were taught. From looking at how stem cells work, by unexpectedly doing the right experiment, we noticed things that led us to experiments. We are constantly told (by Study Sections or by our teachers) that one needs a hypothesis in order to carry out good science – that's often nonsense. Observations of normal or pathological events can lead the individual

scientist to see inconsistencies in the data; and now we are overwhelmed with accurate, single cell 'omics data. What science needs is more people who can observe what happens when a physiological system is perturbed. You can then go back and see what is wrong and see if that teaches you something.

BIOGRAPHY

DR IRVING WEISSMAN is the Founding Director of the Institute for Stem-Cell Biology and Regenerative Medicine at Stanford University (ISCBRM), Director of the Stanford Ludwig Center for Cancer Stem Cell Research, and former Director of the Stanford Cancer Center as well as the Immunology Program. His research on hematopoiesis, hematologic malignancies and solid tumors has led to several discoveries and the development of new therapies. These include the isolation and transplantation of pure hematopoietic stem-cells (HSCs), isolation of hematopoietic progenitors and the development of non-toxic Antibody-based conditioning for HSC transplantation. In AML he proposed and proved preleukemic development in clones of HSC accumulating one mutation at a time with the last mutation giving rise to leukemic stem cells (LSC). Next, by comparing LSC to HSC Weissman discovered on leukemic stem-cells of CD47 as a 'don't eat me' signal used by leukemias and all human cancers tested to evade innate immunity. He showed that anti-CD47 blockade unleashes phagocytosis of cancer cells by macrophages, and developed cancer immunotherapy, achieving remarkable results in phase-I clinical trials. Weissman is a member of the National Academy of Sciences, the Institute of Medicine at the National Academy, and the American Association of Arts and Sciences.

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Accelerating cures: funding stem cell innovation & ideas in an evolving cell therapy space

David McCall, Commisioning Editor, *BioInsights,* talks to **Amritha Jaishankar** PhD, Executive Director, Maryland Stem Cell Research Fund (MSCRF)

AMRITHA JAISHANKAR serves as the Executive Director, Maryland Stem Cell Research Fund (MSCRF). In this role, Amritha is responsible for oversight of all MSCRF activities, programs, scientific management and oversight of the portfolio, as well as MSCRF business development. She works with stem cell scientists and a variety of key stakeholders to develop a collaborative research program and promote commercialization of stem cell technologies. MSCRF has invested over US$175 million in identifying, supporting, and accelerating cutting-edge research, innovation, clinical trials and commercialization of human stem cell-based technologies in Maryland. Amritha is scientist by training who has devoted her career to developing cures for debilitating and life-threatening conditions of our time and to advancing this field, through various roles in federal, university, and industry settings. She is passionate about using stem cell technologies to accelerate cures for patients in need. Her experience in and outside of the laboratory has made her a proven leader and champion for stem cell research and commercialization. Since 2016, she has led an Accelerating Cures initiative at MSCRF and has helped create a vibrant and growing stem cell community in the region. She was recently recognized for her leadership in life sciences and contributions to the community by Women in Bio Capital Region and as a 2020 Leading Women honoree by The Daily Record, Maryland. Amritha serves on a number of advisory boards and committees in the region.

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Q What are you doing right now?

AJ: I'm building and growing MSCRF – our programs, our community, our collaborations and empowering our portfolio to reach their unique successful outcomes. Every day, we try to figure out how to better identify the next promising technology and help them reach the finish line.

One of the most fun parts of my job is interacting with our portfolio and identifying the gaps in the industry, in our region, or in the field and bringing together key stakeholders to figure out how we can address and fill them. Developing creative solutions is a lot of what I'm doing right now. I'm also building a strategy and creating a vision to ensure the program's success in the years to come.

Q Why and how did the MSCRF initially form?

AJ: MSCRF was established by the Governor and the Maryland General Assembly through the Maryland Stem Cell Research Act of 2006, during our 2006 General Assembly session.

The purpose of the fund is to promote state-funded, scientifically meritorious stem cell research and cures through grants and loans to public and private entities in the state of Maryland. We were one of the few states in the U.S. that made a visionary investment in regenerative medicine approaches that are potentially curative. The mission was and is to develop new medical strategies for the prevention, diagnosis, treatment, and cure of human diseases, injuries, and conditions using human stem cells.

Our goal is to accelerate stem cell-based research, commercialization, and cures. We do this through various funding programs, but also by serving as the connective tissue to the regenerative medicine industry in our state. After 15 years, while our programs have evolved to meet the needs of the industry, we have stayed true to our mission and continuously strive to improve human health and advance innovative cell-based research, treatments, and cures for patients with unmet medical needs.

Q Can you expand on how the MSCRF's specific areas of activity have evolved alongside the advancements in the advanced therapy field, particularly in recent times?

AJ: When we started, we had more traditional grants as the field was still in its infancy and we were supporting a lot of basic research, which is still key to creating those future cures. However, over the last five years, we have been able to put in place an 'Accelerating Cures Initiative', which consists of six programs modeled around what it takes to move a regenerative medicine discovery from the lab to the clinic, where it can reach patients.

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We have put initiatives in place that enable us to support new or new-to-the-field faculty to tackle emerging challenges in the field with orthogonal ideas and technologies, as well as programs to support high-risk, high-reward innovative ideas. We have been able to support the validation of these technologies through milestone-based payments to create value and incentivize progress towards follow-on funding.

> "...over the last five years, we have been able to put in place an 'Accelerating Cures Initiative', which consists of six programs modeled around what it takes to move a regenerative medicine discovery from the lab to the clinic, where it can reach patients."

We have been able to support both start-ups and established companies developing stem cell products, as well as clinical trials for companies that could be located anywhere in the U.S. but have a clinical trial site in Maryland. In addition, we support and train the next generation of industry leaders through our post-doctoral fellowship program. Our work will continue to evolve as we identify new gaps and how best to solve them.

Broadly, what we do falls under three pillars that we continuously improve upon. I take a much more hands-on approach to working with our whole portfolio. Through the first pillar, we foster research and innovation through our university-based programs. We get involved at an early stage, where we help shape the research and create the teams. The programs under this pillar enable us to support high-risk, high-reward ideas that will lead to the next medical breakthroughs. The second pillar allows us to create value through milestone-based grants and de-risk technologies so we can help build and grow strong cell therapy companies. With the third pillar, we serve as a resource and the connective tissue to Maryland's regenerative medicine ecosystem – facilitating scientific collaboration, driving academic and industry partnerships and building alliances with various local and global stakeholders. I serve in advisory functions and roles not just for our portfolio but for various organizations across the state to help drive innovation and grow the community. We also partner with colleagues and organizations across the globe to ensure our portfolio companies have the resources they need to be successful. We've now invested over US$170M in over 500 projects to move this field forward and we continue to innovate and evolve.

The type of work we support is also constantly evolving as the field evolves. When we first started, we supported a lot of embryonic stem cell (eSC)-based work, which moved into more of an induced pluripotent stem cell (iPSC) and broader cell-based therapy space. Today, we see and support more biomedical engineering combination approaches, computational approaches, and imaging approaches as well as other enabling technologies applied to advance research in the field.

I'm also delighted to announce that we are putting in place an additional program in our coming cycle to provide manufacturing assistance to cell therapy companies in MD. We've

moved over 104 technologies toward validation, into companies and clinical trials during my tenure here and we've supported the creation and/or growth of companies through 26 research/product development and clinical trial grants in the past 5 years (compared with 12 in the 10 years prior). It's been incredibly important to me to support our companies on their journey and in this industry, manufacturing remains a key challenge for early-stage companies. This program will provide initial resources to enable GMP production of cell therapy products in Maryland. This will help our companies advance their therapies to patients sooner and in a more cost-effective way, whilst simultaneously creating and retaining an advanced therapy manufacturing workforce in our region.

Q You are a stem cell scientist by training – what drew you to your current role?

AJ: I have been in the stem cell/regenerative medicine space for about 18 years now. I first started working with stem cells in graduate school, and then through my post-doc and career as a scientist. I was lucky to have been trained in a broad range of stem cells, across various indications, and with every imaginable technology in this space.

I was also involved in building a research institute from the ground up, which gave me the opportunity to learn every aspect of building a business. That was the shift for me. Whilst I enjoyed doing the science and had a strong pedigree in the stem cell space, I learned that I exceled at building collaborations and partnerships and in identifying the next best technologies or players. I have always been passionate about advancing therapies for patients in need, and I found myself at the intersection of science and business, but still drawn to patient-oriented and mission-focused work. That is what drew me to this role.

I was looking for ways to have a broader impact on the field and wanted to help empower scientists and move cell-based therapies to the clinic. When I heard that the Maryland Stem Cell Research Commission was looking for someone to spearhead an accelerating cures initiative, I knew I wanted to take on that role to lead this effort and grow the regenerative medicine community in Maryland. That was six years ago. I have been fortunate to be able to make an impact and help accelerate cures in a field that I am passionate about. I am proud of the community we have built at MSCRF, including the faculty and companies in our portfolio. I am thankful to my colleagues around the world for their support of our efforts.

Q How can organizations like MSCRF help to keep academic and start-up biotech innovation continuing to move forward to the clinic and avoid the translational 'valley of death'? What do innovators need to do to secure your support?

AJ: Today, post-pandemic, we are seeing unprecedented levels of scientific collaboration and unparalleled visibility of the work we do. This momentum has not stopped. We have increased our funding levels and the amount of funds we can provide to

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companies and clinical trials. In addition, we are working hands-on with academic institutions and partner with various universities to help move their research forward. We are also building in mechanisms where they can access regulatory and commercialization assistance through our networks. I work hard to get our portfolio the resources they need, and this means developing many creative collaborations with my colleagues and organizations around the world, leveraging our scientific and industry knowledge to create value and stay ahead. Building open innovation, knowledge-sharing, and collaborative models to advance the field is another way to keep us moving forward.

Our validation and commercialization programs are designed to help academics and early-stage companies navigate that precarious transitional period. Most of our companies have now raised follow-on funds and are progressing toward clinical trials.

To get our support, all you need is to do great science, build a good team, and have operations in Maryland. However, there are many ways to collaborate with us or even our portfolio, even if you are not located here. Science and innovation have no boundaries, and I am an example of that.

Q What are some of the exciting stem cell therapy modalities, indications, and platforms, which might continue to advance the field forward over the short-to-mid-term?

AJ: While we have seen tremendous progress in cell engineering and gene editing approaches, combination technologies, and innovative tissue engineering advances, we have also seen development of cutting-edge enabling technologies that are necessary to advance this field.

While we are focused on stem cells and cell therapy, we are disease-agnostic. We have supported everything from rare diseases to the top ten leading causes of death in Maryland, including cardiac disease, Alzheimer's disease, and diabetes. To me, it has been great to see advances made across a broad range of indications that go beyond tumors, and into some of the most devastating diseases and conditions of our time, like diabetes and neurodegenerative diseases. Personally, I started working with iPSCs in graduate school, so it has been a full-circle moment to watch and support these entering the clinic now.

We support various stem cell-based platform technologies, many projects advancing 3D culture systems and tissue-engineered products and biomaterials. As I mentioned earlier, we see innovations across many indications, predominantly neurological, cardiovascular, and orthopedic/musculoskeletal. But I'm also excited about emerging work we've supported to address ocular disease, digestive system disorders, infectious diseases including ARDS as well as work in other tissue repair and regeneration.

We recently also launched a YouTube channel to showcase some of the regenerative medicine work we support, such as a skin regeneration approach that could help the two million amputees and wounded warriors across the country.

We fund exciting work in the cardiovascular space, where we have seen great improvements in disease modeling, in the maturation of cells, and new stem cell and tissue engineering

approaches to address vascularization and other key issues in the field. We also fund clinical trials addressing congenital heart defects and heart failure. As an estimated 2,200 Americans die of cardiovascular disease every day, we strive to support research towards reducing this disease burden on individuals, their families, and society as a whole.

We have also funded work to improve stem cell transplants to impact and save the lives of many children and adults, whether they have severe combined immunodeficiency (SCID), primary immune deficiencies, aplastic anemia, or sickle cell anemia. Some of these approaches have also been applied to treating cancer.

Stem cell disease modeling and resulting drug development is leading to potential treatment for Parkinson's disease, which affects over six million people globally. We have also been able to apply some of the disease modeling we have developed, including organoid-based approaches, to understand COVID-19.

As I mentioned, we have supported work for prevalent diseases like diabetes as well as rare diseases like progeria which are also featured in this channel. This is an exciting time to be in this industry, when the progress is evident and rapid. We are, seeing cell and gene therapies deliver cures for some of these diseases for the first time in history.

Q Can you sum up some key goals and priorities, both for yourself in your own role and MSCRF as a whole, over the next 12–24 months?

AJ: At MSCRF, we are committed to enabling the next medical breakthrough by identifying and fostering cutting-edge cell-based research, commercialization, and cures. We are committed to translating scientific excellence and supporting the development of cures for these devastating diseases of our time. That will not change, but how we do so will continually evolve. I would like to further scale MSCRF and broaden the technologies we fund and be more able to support the manufacturing needs of the companies we have helped create.

We have always been proactive about identifying the next technology or company, and my priority is to enable their success and ensure the incredible work of our scientists and companies is seen and heard. We seek to continuously innovate, improve, and deliver excellence, and we have set internal goals for the coming years to achieve this.

For me personally, my goal is to grow the cell and gene therapy industry, broaden the impact I have, empower more scientists and companies, and further accelerate the translation and commercialization of these technologies in every way I can. I would like to build sustainable public-private partnerships to move the needle in this space. I'm invested in leading efforts to further cell and gene therapy research, commercialization and infrastructure not just because this is the future of medicine but also because its good business to do so. At MSCRF I do the same—I try to

> "Ultimately, we do great work that can have a global impact, and my goal is to keep doing just that."

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build this ecosystem and bring us all together with a sense of urgency and a shared purpose to advance science and improve human health. Ultimately, we do great work that can have a global impact, and my goal is to keep doing just that.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Possibilities for continuous closed-system processing of cell therapies

Sean Werner, Steven Thompson, Richard Day, Brian Hawkins & Joseph Petrosky

Cell and gene therapies have the potential to facilitate disease-modifying treatment of both rare and chronic conditions. Whilst approved treatments are now available, several challenges remain before these therapeutic modalities become first in line therapies. The supply and qualification of starting materials such as peripheral blood and bone marrow is one dilemma that must be overcome. For allogeneic programs, starting materials generally have a defined limit of productivity and carry requirements of substantial risk-mitigation testing. Autologous manufacturing processes, with lower attendant safety risks, are highly variable and may be compromised based on individual patient treatment programs or the target disease itself. An ideal starting material has a high capacity for expansion, providing consistent manufacturing, reduced qualification burden, and a high output of individual doses. However, even if a developer is working with an ideal starting material, manufacturing processes may limit the ability to capitalize on beneficial characteristics. One possible solution to improving manufacturing capacity is incorporation of continuous batch manufacturing.

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Cell and cell-based gene therapies have the potential to facilitate disease-modifying treatment of numerous serious conditions. Whilst approved treatments are now available, several challenges remain before these therapeutic modalities become first-in-line therapies. Robust manufacturing at scale to treat the relevant patient populations remains one of the most vexing challenges [1]. One possible solution to improving manufacturing capacity is incorporation of continuous, closed-system

manufacturing processes. Whereas continuous manufacturing processes are widely used in numerous industries, only now are cell and cell-based gene therapy developers beginning to explore these concepts. In this white paper, we outline a concept, utilizing existing commercial tools, that could potentially accomplish such a manufacturing process [2].

Modern development pathways benefit from a Quality by Design approach (QbD) at an early stage of development. Often however, the reality is that many groups are faced by significant pressures from multiple stakeholders to attain clinical and commercial development milestones. Thus, the identification and confirmation of Critical Quality Attributes (CQAs), and the Critical Process Parameters (CPPs) that allow these to be met, may be sub-optimal at early and mid-stage clinical phases [3]. Pressures for commercial success can lead to the direct transfer of research products and processes into early-stage clinical development platforms, with a retrospective, rather than proactive approach to defining CPP. Process or scale changes can lead to significant differences in analyzed parameters. Where those parameters are based on experience rather than empirical evidence of the boundary conditions, interpreting the implication can be daunting. To date, this has resulted in serious challenges in process improvement, modification, or even scale changes [2].

Continuous manufacturing processes provide a number of key benefits, if they can be properly implemented. Traditional batch manufacturing requires an entire process to be complete before a product can be released and a new batch can be started. As it relates to biopharmaceutical manufacturing, this can mean very long manufacturing cycles before a new batch is initiated. Furthermore, changeover procedures mean that expensive manufacturing infrastructure is not utilized every time a batch finishes until it can be re-set. With current manufacturing systems and the anticipated manufacturing scale for early allogeneic cell-based therapies, this could severely limit the manufacturing capacity without massive build out of infrastructure [4]. By contrast, establishing a continuous manufacturing process would mean release of products earlier, and fewer changeover cycles. One intriguing possibility of continuous process implementation is the use of smaller scale systems to achieve the same manufacturing capacity of larger systems over a given period. In this way, a developer might be able to use the same manufacturing systems applied during clinical development as they use at commercial scale; higher annual throughput a function of longer cycles rather than larger systems.

As part of a concept demonstration, Sexton Biotechnologies (part of BioLife Solutions) worked alongside PBS Bioreactors, Pluristyx, and Luna Therapeutics, to present how a closed-system, continuous manufacturing process might be established. In this example, we establish a small-scale process appropriate for production of MSC or iPSC seed cells.

MATERIALS & METHODS

Here we describe a potential workflow that outlines a continuous batch manufacturing cycle. If optimized, this process could reduce time to cell number targets, overall media requirements, and, by reducing the downtime and change-over burden, may increase manufacturing efficiency for a given manufacturing footprint. As with any manufacturing system, the ability for a process flow to accomplish a set of manufacturing parameters must be established on a per-product basis. Critical elements of process development such as determination of exhaustion limits, phenotypes based on changes in cell density, potential for cellular aggregation and the impact thereof, and many other aspects a developer would need to consider. These aspects were beyond the scope of the current project.

The Signata™ CT-5 (Sexton Biotechnologies) fluid management system allows weldable fluid line connections to close out many manual fluid management tasks. At the most basic level, it provides reproducible fluid movement with electronic records for traceability. However, it also functions as

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an independent unit operation platform for formulation and fill, closing processes such as media formulation, biopreservation media addition, and fill into bags or vials. The system is designed to embed flexible automation across cell therapy processes and allow integration of other standalone technologies within a single manufacturing platform.

PBS Bioreactors represent single use culture systems which can be scaled from 0.1 L to 80 L, with the vertical wheel technology enabling homogeneous particle suspension and low sheer stress. The integration of system design and function from very small scale to manufacturing scale limits challenges of platform migration throughout development.

Thermally induced phase separation (TIPS) microcarriers were originally developed at University College London and are now produced by Luna Therapeutics. Their material design incorporates hydrolytic resorption meaning cell detachment and particle separation may not be required (dependent on the requirements of any further downstream use). This offers the potential that they could be an ideal manufacturing and, potentially, delivery tool for cell-based therapies.

Pluristyx provides human iPSC for research and development applications, as well as offering custom manufacturing options for GMP cell banks for use in clinical manufacturing processes. The off-the-shelf nature of the cells offers developers a well characterized starting material for process modeling or development.

The project was divided across two workflows:

- The primary goal was to establish the ability of the PBS bioreactor to:
  - Propagate adipose-derived MSC (AD-MSC) adherence and expansion on TIPS microcarriers (Figure 1).
  - Propagate iPSCs in suspension culture (Figure 2).
- Once expansion was demonstrated, we sought to conceptualize how these commercially available tools could be adopted for a theoretical closed-system continuous process.

OUTCOMES

PBS Bioreactor enables adipose-derived MSC adherence & expansion on TIPS

A sterile, closed PVC tube was added to the access line of a PBS (Cartridge) in a biosafety cabinet to ensure sterile connection. Downstream additions or removal from the PBS system were made by sterile welding lines from the Signata CT-5 transfer sets or output sets. Samples were removed for analysis at D4 and D8. Figure 1 shows the expansion of AD-MSCs under these conditions as well as micrographs demonstration adherence to the TIPS microparticles.

PBS Bioreactor supports expansion & maintains pluripotency of iPSCs

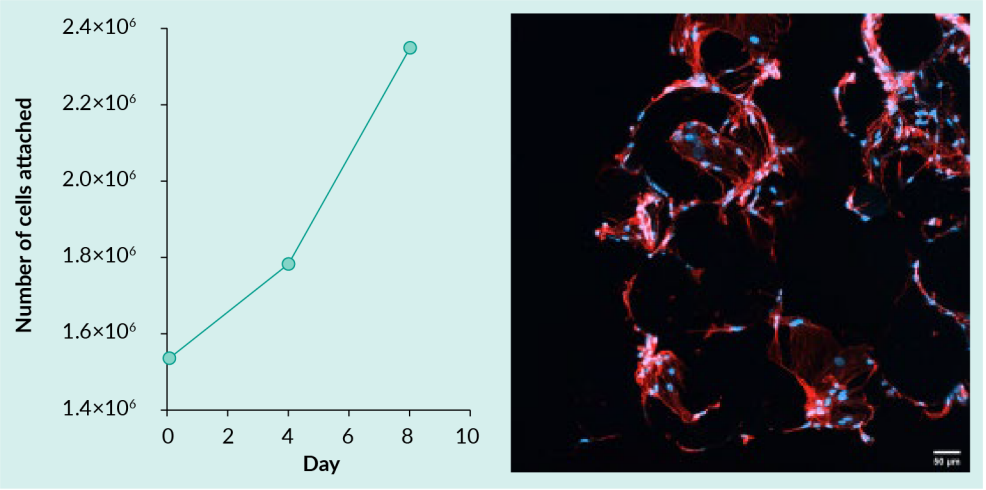
For the iPSC expansion, the PBS system was loaded with media and inoculated with cells. Samples were removed at D3, D6, and D9. The photomicrograph demonstrates iPSC clusters.

Continuous closed-system bioprocessing using the Signata CT-5

The ability to perform manufacturing in a closed system with minimal labor input can allow for an economical process that reproducibly generates products meeting quality expectations. In this example, the Signata CT-5 was used to implement closed system bioprocessing of both the MSC and iPSC processes described above. Several preparation steps are needed to complete this workflow. Firstly, all reagents needed to be packaged in containers suitable for sterile welding. Suppliers are

FIGURE 1

Adipose-derived MSC attached to TIPS microcarriers pre-treated with nLiven human platelet lysate (Sexton) and incubated in a PBS 0.1 MAG



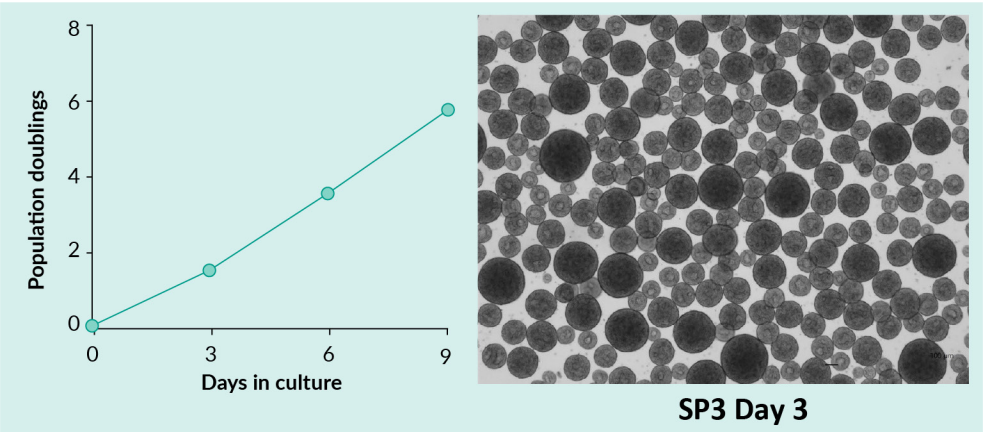
Initial seeding density 1.5 x 10^6 cells and 50 mg TIPS microcarriers. Cells imaged with confocal microscopy after staining nuclei with Hoechst 33342 (blue) and cytoskeleton with phalloidin (red). (Image courtesy of Haowei Wang).

moving toward appropriate packaging, however, in some cases accessing materials from vials or bottles is still needed. For bottles, caps with weldable tubing are available. For vials, transfer into a closed system may need to be carried out in a Biological Safety Cabinet (BSC). New packaging options such as vials with weldable connections are improving in this part of the process, but availability is limited at this point.

Second, in some cases, unit operation tubing sets may not be compatible with the downstream unit operation. In the case of interacting with the Signata CT-5, Sexton has used weldable lines with standard Luer connections. Tube size stepdown can be accomplished with Luer connected fittings if needed. Once these steps are completed, all processes can be accomplished with closed sterile welding.

FIGURE 2

Expansion of induced Pluripotent Stem Cells in suspension



A vial of Pluristyx's Ready-to-Differentiate® iPSCs were thawed directly into a PBS 0.1 MAG and cultured over 3 suspension passages. Population doubling was calculated (left panel) and iPSCs were imaged at suspension passage 3 (right panel) to demonstrate the morphology of iPSC clusters.
Data and images courtesy of Dr Raluca Marcu.

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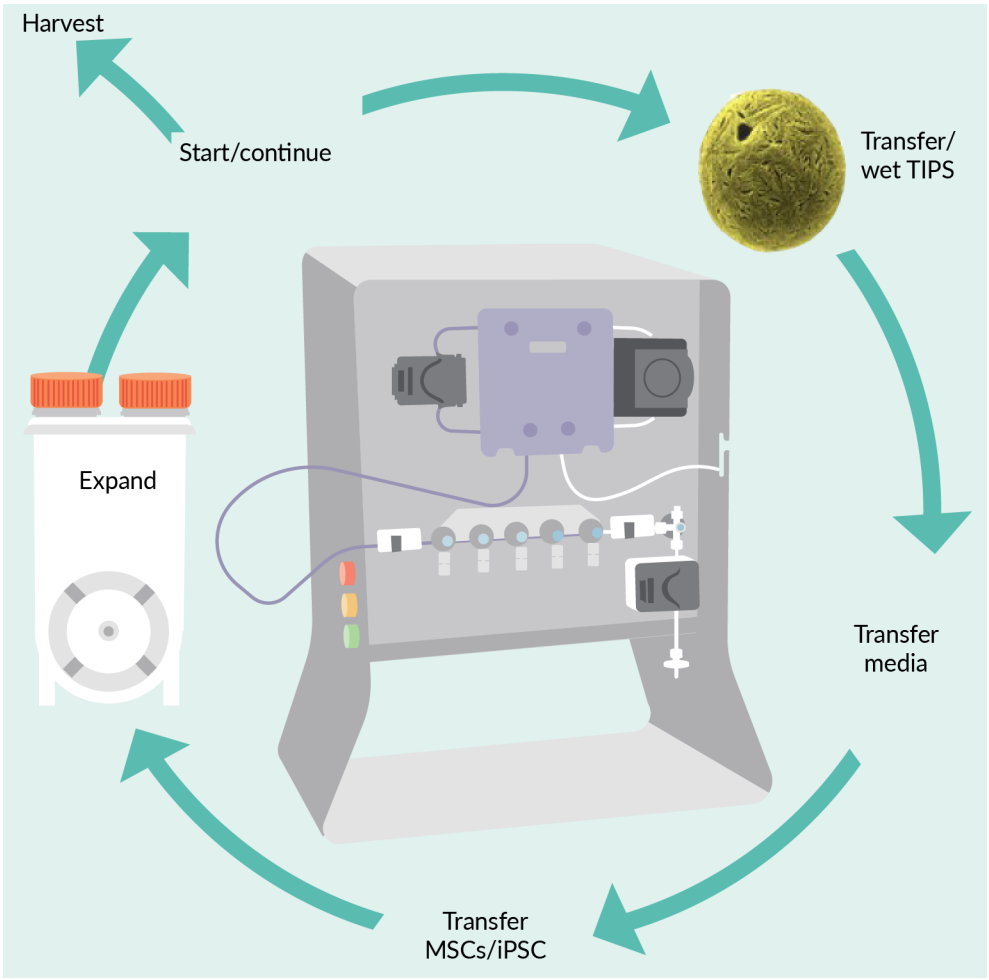
The conceptual continuous manufacturing workflow is described in Figure 3. In the case of manufacturing adherent cell lines such as MSCs, the microparticles must be prepared as per user instructions. Here, we used a vented vial adapter (connected in a BSC) to access the lyophilized TIPS particles. The wetting media (in this case nLiven™ hPL, Sexton Biotechnologies) was then added to the TIPS container using the CT-5. For non-adherent cell lines, this step would not be required.

Once fully wetted, the particles are transferred to the PBS bioreactor, without the need to disconnect or open the process. Because the CT-5 has multiple available fluid lines, several processes can be completed without the need to re-weld onto the same line. In this case, different source positions were used for the TIPS particles, expansion media, and seed cells. Once the PBS bioreactor was filled, the fill line can be flushed. After preparation, the weldable fill line can be sealed and separated if needed (i.e., transfer into an incubator) or left connected if environmental conditions are supportive. The CT-5 can be used to draw QC samples during incubation if needed using the same weldable fill line.

At the established times or when samples reach specified criteria, cells are withdrawn. Because the system is established with weldable connections, after withdrawal of a portion of the expanded cells, additional media with or without wetted beads can easily be introduced to the reaction chamber. In this way, a single batch of cells from a working cell bank can be used continuously until the cells reach any pre-determined end-of-life, such as phenotype changes, exhaustion, etc.

FIGURE 3

Concept for continuous bioprocessing of cell therapy.



The process steps are described in the section below and illustrated in Figure 4.

PROCESS STEPS

Pre-condition TIPS microcarriers with platelet lysate

TIPS microcarriers were incubated in 100% nLiven platelet lysate until the particles settle to the bottom of the vial. Using a vented vial adapter connected to the output line on the Signata, platelet lysate is transferred in a functionally closed manner to the microparticle storage vial. Wetted microparticles are then transferred to a PBS Bioreactor connected to an output line on the Signata CT-5 DIY Output set. Alternatively, multiple microcarrier aliquots can be prepared from an initial conditioned suspension by connecting the source bottles to the Signata CT-5 CellSeal vial output set.

Load expansion vessel

Expansion media and seed cells prepared in source bags are welded onto the Signata CT-5 system as needed with the PBS Bioreactor connected as an output location.

Expansion

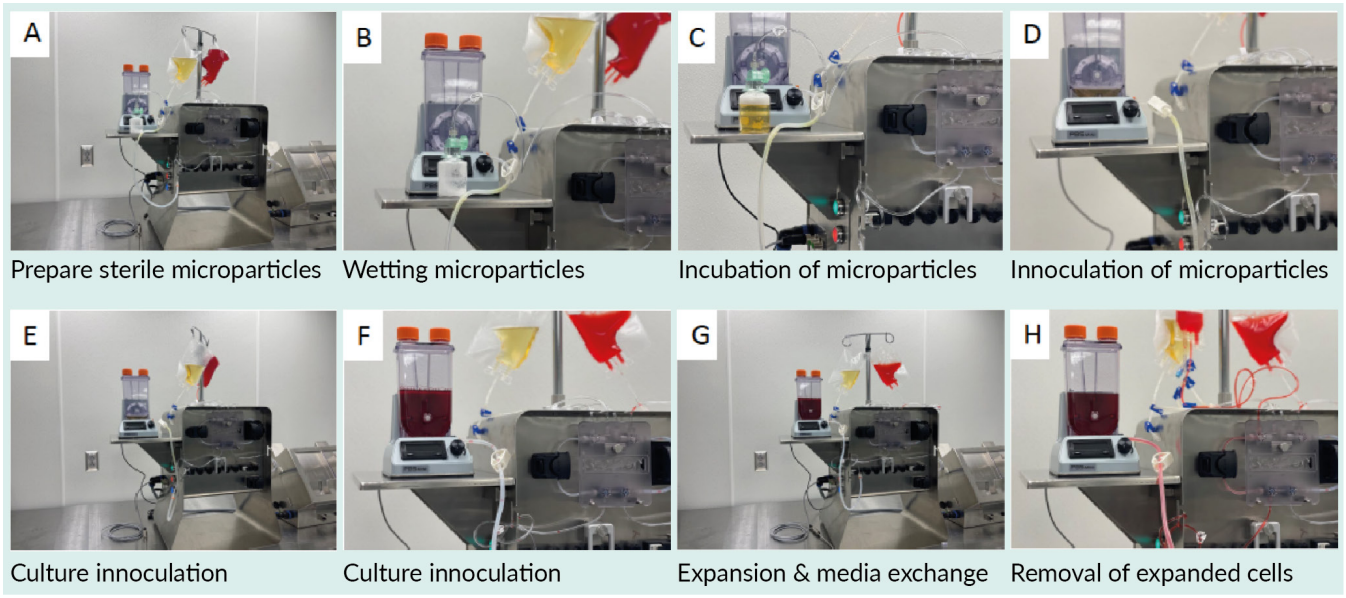
Culture expansion is carried out as per product specific optimized parameters. User guides and support for the PBS Bioreactors can be found at https://www.pbsbiotech.com/. Expand cells to desired density. Samples may be drawn using the connection to the Signata as needed.

Collection & re-seeding with microparticles

When cells reach desired density, a fraction of the bioreactor liquid may be drawn off and transferred to the desired wash/concentration system for further downstream processing (not shown). Formulation and fill can be completed using the Signata Formulation and Fill process into either cryobags or CellSeal® cryovials (Sexton Biotechnologies) for small volumes (up to 5 ml). To continue manufacturing, additional wetted particles and fresh media can be added to the Bioreactor. Expansion of cells

FIGURE 4

Set up of continuous processing concept.



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can continue in this manner to limits defined by the user's cell performance.

Continuous closed-system bioprocessing: impact on the field

In this short paper, we brought together technology from several tools providers to develop a process which was closed, definable and scalable. Through welding to the PBS bioreactor lines as well as all of the source and output lines, the Signata CT-5 integrates disparate tools and reagents. This enables closed introduction of source materials, media sampling and replenishment, collection, formulation, and fill. While the concept as shown does not include a wash/concentration step, compatibility with other systems is an inherent aspect of the built-in flexibility of the Signata CT-5. The range of PBS bioreactor vessels allows for small scale experiments during process development to be readily scaled once CPPs have been ascertained.

This presents several manufacturing possibilities.

- The ability to continually propagate cells in a closed and reproducible manner within appropriately sized PBS culture systems. These can be harvested into CellSeal vials or bags using the Signata CT-5 and cryopreserved.

- Continuous manufacturing enables higher output of cell product in smaller reactor vessels. Smaller, continuous outputs that can be formulated into final products. This suggests significantly faster production of final product, as well as reduced downtime due to changeover processes. In addition, the anticipated smaller scale of a reactor is a valuable risk management strategy: Implementation of multiple smaller scale reactors reduces the overall loss potential should a contamination or other manufacturing error occur. A scaled out continuous throughput system may have a higher initial financial investment (or, smaller sequential investments) but increases in efficiency can ultimately balance this out [5].

- As successful cell-based products expand into new indications, microcarrier expansion systems are understood to be one method of greatly expanding manufacturing capacity [6]. Should microcarriers be compatible with final formulation (i.e., without the requirement to separate from the cell-based product), downstream formulation and delivery may also become more efficient.

- This proposed strategy for continuous bioprocessing also maintains the flexibility to be scaled up or out. Larger PBS bioreactors with related volumes of iPSCs/TIPS microcarriers can allow up to 80 L scale up, whilst the ability to weld on and off multiple PBS Mini units can facilitate scale out, with both approaches utilizing the Signata CT-5 to maintain closed-system processing.

Further work is required to optimize processing parameters, but by harnessing the expertise of several technology providers, we have demonstrated how continuous closed-system processing can address several unmet needs within the cell therapy space.

Indeed, implementation of large-scale batch manufacturing systems early in development still represents a significant risk [7]. If scale up cannot be optimized, the infrastructure will be redundant, and as such smaller, scale-out models will be required. On the other hand, continuous, closed-system manufacturing has the potential to allow implementation of standardized processes early in development, with higher throughput in the same systems through longer batch cycles. As demonstrated, such a process could involve relatively small reaction vessels incorporated much earlier in development to limit the risk of scale-up failures. Incorporation of these systems minimizes human intervention, simplifies batch records, and increases the probability of success when higher throughput is needed.

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INNOVATOR INSIGHT

Why Pall's Allegro™ Stirred-Tank Bioreactor is ideal for viral vector cell culture

Emmanuelle Cameau, Ernest Asilonu, Sheriff Bah, Pauline Nicholson & Timothy Barrett

Viral vectors facilitate the delivery of genetic material to living cells for the potential treatment of multiple genetic diseases. With recent regulatory approvals, the rapid growth in demand for viral vector-based products highlights the need for proven, scalable manufacturing solutions that can fully meet this demand and ultimately increase the availability of viral vector-based treatments. Pall's Allegro™ STR stirred-tank bioreactor addresses the need for scalability, as it can be scaled up to 2000 L to enable the manufacture of viral vectors. In this article, we discuss the key attributes of the Allegro STR bioreactor such as the design, scalability, agitation and sparging which make it ideal for viral vector manufacture at a larger scale. Also, we show process scalability under controlled key parameters from Allegro STR 50 L to 500 L based on cell growth, metabolic profile, and viral vector production.

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Gene therapy has made significant advances over the past two decades. Gene transfer therapy involves the administration of specific genetic material (i.e., DNA or RNA) via a carrier, known as a 'vector'. Viral vectors offer a new class of biologics which facilitates gene transfer and modification in living cells, potentially treating many conditions with genetic causes. Currently, the most used viral vectors for gene transfer therapy include gamma retrovirus (RV), adenovirus (AV), adeno-associated virus (AAV), and lentivirus (LV) [1].

Previously, gene therapy mainly addressed rare or very rare diseases and therefore the manufacture of gene therapy viral vectors were only set out to meet the market demands of a relatively small group of patients within the orphan disease market space, where meeting demand has not always been a big problem. Advancement in gene transfer therapy-based treatments and its inevitable extension to common indications such as cancer, Parkinson's and Alzheimer's means that gene therapy viral vectors must be manufactured in larger scales. This production gap is one of the main challenges in the gene transfer therapy field.

According to Precedence Research, the global gene therapy market is expected to be valued at over US$15 billion by 2030 [2]. This expected growth has generated a huge pressure on biomanufacturing companies to develop new technologies to be able to satisfy the high demand of gene therapy products / technologies.

This trend is also driving a greater need for the scalable production of viral vectors for gene therapy. Traditionally, viral vector production is mostly based on adherent cell lines using systems such as multi-trays, that can only be scaled out. Adherent bioreactors such as Pall's iCELLis® bioreactor have been developed during the past decade allowing to scale up of such processes up to a certain surface (500 m^2 for the iCELLis 500+ bioreactor). Over recent years, more manufacturers have investigated adapting their cells for viral vector manufacturing to suspension culture to reach higher volume vector-producing batches, that can be required for large dose/large population applications. Pall developed the Allegro Stirred-Tank (STR) Bioreactor for suspension cells which can be easily scaled up to 2000 L (Figure 1). Pall's expertise and understanding of process scaling technology has enabled large scale manufacturing of gene therapy products to meet the ever-increasing demand.

In Pall's Allegro STR bioreactor, cell growth is substrate independent, hence high viable cell densities can be achieved and more importantly, these cells can produce high titers of viral vectors, including AAV, LV and AV. The Allegro STR bioreactors provide optimal environment for various cell types to reach their full growth and viral productivity potential.

FIGURE 1

Pall Allegro STR 50, 200, 500, 1000 and 2000 L bioreactors.

DESIGN OF PALL ALLEGRO STR BIOREACTOR

The success of meeting the increasing demand of gene therapy heavily depends on the provision of more bioreactor manufacturing capacity. The COVID-19 pandemic has added to the strained capacity as some of the vaccine's programs are also using viral vectors. Pall's Allegro STR bioreactors are perfect candidates to reduce this capacity crunch in producing viral vectors for gene transfer therapy.

The Allegro STR bioreactor family combines Pall's bioprocess engineering expertise, cell culture know-how and our drive for quality into a series of single-use bioreactors that deliver consistent and scalable cell culture performance for cell culture and viral vector production across the Allegro STR bioreactor range. From the outset of the design, Pall placed strong emphasis on providing compact, ergonomic, and intuitive turnkey bioreactor design concepts to maximize usability and process assurance, while maintaining optimal performance and reliability needed in a cell culture and viral

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vector production environment through several easy and intuitive operation features such as [3]:

- A bottom mounted pitched blade 'elephant ear' impeller with three 45-degree angle blades to promote efficient axial and radial mixing in a cuboid-shaped bioreactor (unique to Pall's Allegro STR bioreactors), while supporting options for both upward and downward flow depending on the application required. This type is common in bioreactors used for animal-cell culture because it is considered less likely to cause shear damage with optimal blade diameters and agitation speeds while ensuring effective mixing and oxygen mass transfer for high cell density cell culture [3,7];

- Wide range of agitation power inputs (W/m^3) for efficient mixing and gas dispersion;

- Headspace volume at ~25%, providing adequate allowance for high hold-up (and possible foaming) associated with high specific power and aeration rates;

- Three baffles that eliminate the need for customized shaping and welding of flexible side walls during manufacture and maximize biocontainer strength, integrity, and robustness;

- A cubical biocontainer with aspect ratio H/T = 1 has a similar volume to the cylindrical format with a ratio >1 (Figure 2). Because aspect ratios >1 can lead to poor homogeneity at the top surface, the cubical format's lower aspect ratio with its reduced fluid height provides for improved mixing and a greater headspace mass transfer capacity. This can allow for minimal sparging and enhanced CO_2 stripping;

- Use of computation fluid dynamics modeling studies to ensure that cuboid shaped bio-container matches those of conventional cylindrical stainless-steel bioreactors [3], performance further verified by empirical studies;

- Installation and inflation of the biocontainer is achieved in <30 minutes through a guided sequence via the Human Machine Interface (HMI) for ease-of-use;

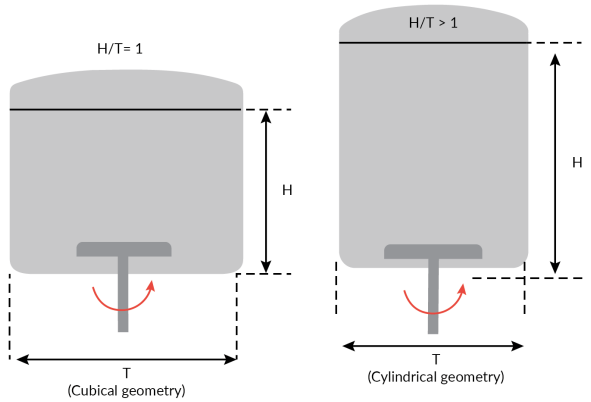
- All product contact surfaces in the Allegro STR bioreactors are single-use components that are cell culture compatible, thus reducing the demands and cost of maintenance, cleaning, and cleaning validation to a minimum;

- Addressing footprint restrictions in cleanrooms: With a maximum height of 2.9 meters for the 2000 L unit-scale STR, Pall's Allegro STR bioreactors are compact and are easily accommodated and installed into laboratories and manufacturing sites, negating the need for extensive re-fitting and installation associated costs such as hoists and ladders;

- See Nienow, Isalovic and Barret, 2016 [3] for further details on the bioreactor design considerations that were optimized during the design of the Pall's Allegro STR bioreactors.

FIGURE 2

Aspect ratio of a square cross-section Allegro STR bioreactor compared with a cylindrical bioreactor of similar volume



SCALABILITY

Scalable manufacturing is one of the critical processes required to be able to provide the quantities of vector needed to bring these potentially life-saving treatments to waiting patient populations. Many gene therapy manufacturing processes rely on culturing HEK293 cell lines (or derivative AAV293 cell lines), and several early and current forms of production culture these cells on an adherent substrate [4].

Pall's expert knowledge of process scaling, and critical scaling parameters ensures that processes are easily scaled up or down across all sizes of the Allegro STR bioreactors with working volumes ranging from 10 to 2000 L with focus on critical scaling parameters such as specific power input, kLa (volumetric oxygen mass transfer coefficient), mixing time, and aspect ratio so that cell culture environment and conditions are as similar as possible regardless of size [4].

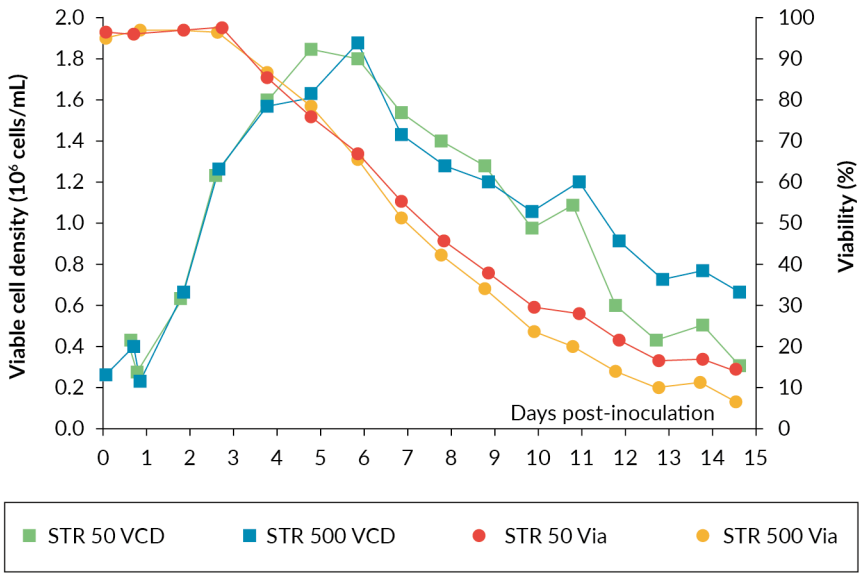
In a scaling study using Allegro STR 50 and 500 bioreactors for production of rAAV viral vector published by Sanderson *et al.*, productivity between the two scales was compared [4]. Both STRs were inoculated from the same cell culture bolus at half capacity and expanded to the full working volume after 24 hours. The operational parameters were matched throughout the process. The results showed near identical cell growth and viability between both the Allegro STR 50 and STR 500 bioreactor cultures up until transfection on day 3. After transfection, there was a drop in viability in the two STRs while the viable cell density continued to increase. Both cultures reached a maximum viable cell density of ~1.8 x 10^6 cells/mL (Figure 3). The data shows rAAV titer increases throughout the culture with maximum titer being observed at harvest. The final rAAV titers were 4.3 x 10^{10} gc/mL and 4.8 x 10^{10} gc/mL for the Allegro STR 500 and STR 50 bioreactors respectively (Figure 4), comparable in range to those reported in the literature [5,6].

The nutrient and metabolites were also analyzed daily throughout the production run, and they were comparable. Figure 5 shows a comparison of the glucose and lactate measurements.

The result from this comparative study demonstrates that the Allegro STR 50 and STR 500 bioreactors are appropriate for rAAV production and that they provide similar bioreactor cell culture conditions at both the 50 and 500 L scales. This scalability is realized

FIGURE 3

Cell growth and viability profiles in Allegro STR 50 and STR 500 bioreactors.



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when utilizing Pall's recommended scale up strategy across the Allegro STR family [4].

AGITATION

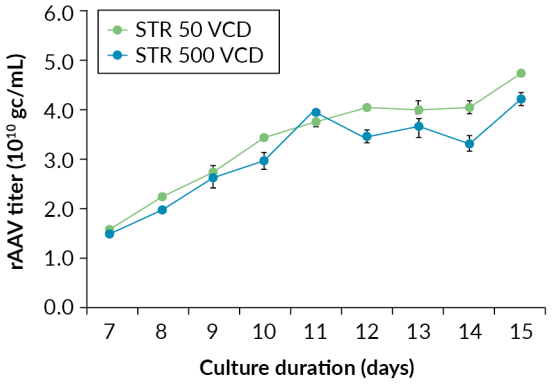
Cell damage caused by agitation is a topic that is commonly discussed in the industry but the design features of the Allegro STR bioreactor are such that they mitigate damage from shear. As discussed previously [3], a modern theory for damage to a range of cell types, including those on microcarriers, suggests that it occurs when cells are larger than the Kolmogorov eddy size, λ_K:

$$\lambda_K = (\nu^3 / \Phi\varepsilon_T)^{1/4}$$

where ν is the kinematic viscosity, Φ is the ratio of the maximum local energy dissipation rate compared to the average, and ε_T is the specific power input in W/kg (1 W/kg = ~10^3 W/m^3 for fluids of a density similar to cell culture media). In the case of the Allegro STR bioreactors, Φ is ~15 [3]. To avoid cell damage, clearly λ_K must be >~20 µm (average HEK293 cell size). However, at the maximum speed available, ε_T = 0.4 W/kg and λ_K = ~35 µm. Thus, cell damage should not occur [7].

FIGURE 4

rAAV titers in gc/mL in Allegro STR 50 and STR 500 bioreactors.



For most animal cell cultures, the Allegro STR bioreactors would be programmed for up-flow pumping, even if the system can do both directions. The Allegro STR 200 bioreactor impeller drive system is designed for agitation speeds of up to 150 rpm. In the qualification studies, this bioreactor achieved a specific power output of 0.31 W/kg at 150 rpm in the up-flow mode. This specific power level is significantly higher than what is used generally to meet the mass-transfer requirements of currently achievable cell densities [8]. The ratio of impeller diameter

FIGURE 5

Glucose and lactate profiles in Allegro STR 50 and STR 500 bioreactors.

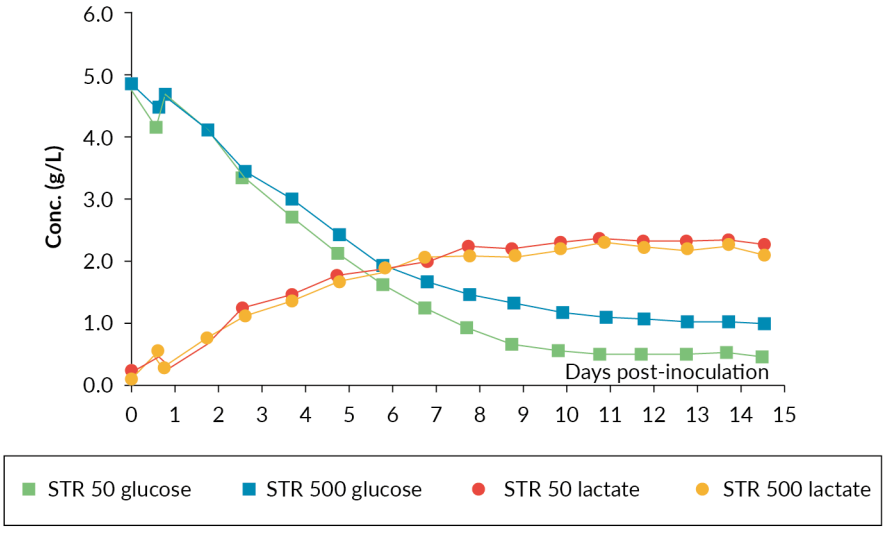
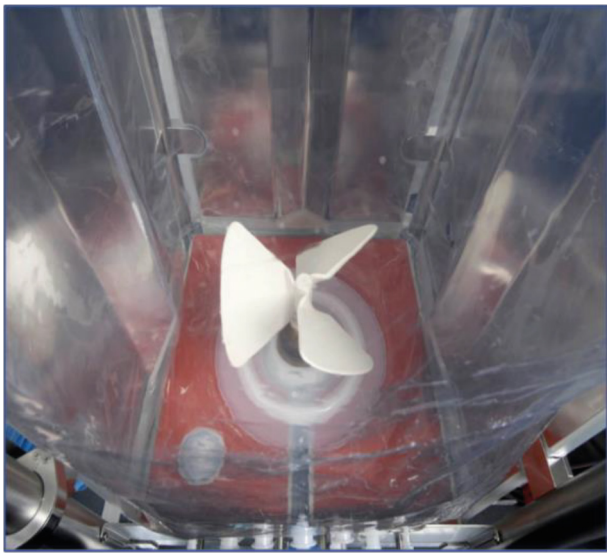


FIGURE 6

Allegro STR Impeller.



to bioreactor side length (D/T) is an important parameter (Figures 6 & 7) that affects both flow pattern and power input. For the 200 L Allegro STR systems, the D/T ratio was set to 0.5, which for a given specific power input (W/kg) ensures that mixing times are shorter than for smaller impellers [7,9].

Shear can often be perceived as being a potential cause of damage to the viral vectors once produced. Indeed, once the cells are transfected (or induced in the case of stable cell lines), they start expressing the viral genes and produce and package the vector [4]. In some cases, the vector remains mostly intracellular (AAV2, AAV5 for example), but it can also be completely or partially excreted by the cells into the cell culture media, either through exocytosis or cell lysis caused by the vector production cycle.

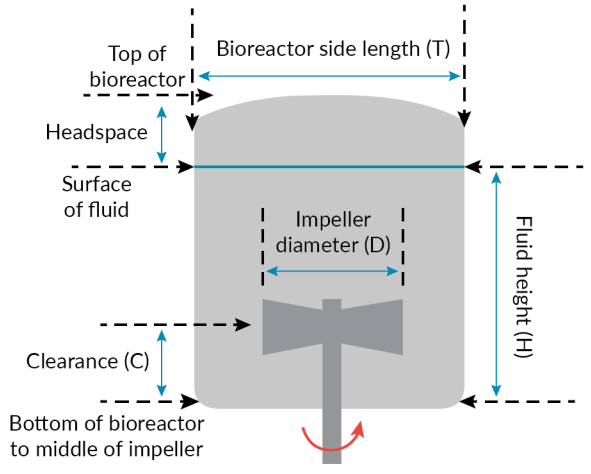
Viruses are smaller than cells, and the size difference can have an impact on how these cells or viruses are subject to turbulence in the bioreactor. Lentiviruses are traditionally the most sensitive of the viruses used for gene therapy applications, due to their enveloped nature. They are known to be sensitive to not only shear, but also pH, temperature, salt, and foam generation [10].

Through the data illustrated above and a numerous amounts of case studies comparing the Allegro STR bioreactor to other types of STRs, it has been shown there is no damage to AAV integrity [4,11,12].

In a recent Pall study performed with a customer, LV have also been cultured successfully in the Allegro STR bioreactor at 50 L scale, without any specific damage to vector integrity, suggesting the system to be gentle enough to successfully produce these very delicate vectors at any scale. Process performance in the Allegro STR 50 bioreactor was compared to a validated 5 L scale down model. Through replicate runs, metabolite profiles and product physical titer and quality (functional titer and impurities) were reproducible between the two scales [13].

FIGURE 7

Dimensional representation – Allegro STR bioreactor.



SPARGING

A constant and adequate supply of oxygen is crucial in cell culture. Allegro STR bioreactor spargers have been designed for optimal gas bubbles generation and distribution allowing good oxygen mass transfer (kLa) with reduced foaming. The most common efficient method for oxygen transfer and carbon dioxide (CO_2) stripping across all bioreactor scales is sparging through a ring sparger which was designed with holes of suitable size and number to achieve high flow rates (0.2 vvm) without excessive linear velocities. The system produces relatively large bubbles, which

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are less likely to damage cells than are small bubbles [7] while maintaining high oxygen transfer through adequate specific power and sparge rate. Pall Corporation document reference USD3381 outlines scalable gas transfer coefficients (kLa) and scalable CO_2 stripping rates for all bioreactors scales [14].

Overall, the Allegro STR spargers have been designed and aligned with the 'elephant ear' impeller for optimal gas bubbles generation and distribution allowing good oxygen mass transfer and carbon dioxide strip rates across all STR bioreactor scales.

CONCLUSIONS

Pall's Allegro STR range of single use bioreactors are designed for biotechnology manufacturing. The Allegro STR bioreactors are tested and proven bioreactors in mAb manufacturing [15]. With Pall's excellent customer support and bioprocess expertise the effective and successful transfer of any gene therapy processes into Pall's Allegro STR bioreactors is assured. The ability to effectively scale enables speed to clinic in the gene therapy space.

Attention to system design for excellent scalable manufacturing, and usability makes Pall's range of Allegro STR bioreactors a good choice for viral vector production. Successful testing and adoption by several companies have shown its effectiveness in the gene therapy space.

Collignon *et al.* transferred an r-AAV process from a competitor 50 L SUB to Pall's Allegro STR 50 bioreactor, with the objective to scale up to 1000 L. The user friendliness of the system and software, with close support from Pall scientific teams, was noted along with a favorable increase in production yields obtained through a change in dissolved oxygen strategy, thus reducing the overall gas consumption and foam formation [11].

In another study published by Mainwaring *et al.*, a stable AAV producer cell line was successfully transferred from bench scale BioBLU® 10c to the 50 L Allegro STR bioreactor, and further scaled up to the Allegro STR 200 bioreactor. They also demonstrated good capacity, yield, and scalability for the initial unit operations of the downstream process. As a result, processes developed with other manufacturers' bioreactor can readily be transferred to Allegro STR bioreactors based off known scaling process parameters [12].

As part of the Covid vaccine consortium in 2020, Pall supported the rapid development and scale up of the ChAdOx1 vaccine, an adenovirus-based vaccine. The process was scaled up to the Pall Allegro STR 50 and Allegro STR 200 bioreactors in record time. Pall Allegro STR bioreactors up to 2000 L are consequently being used at various manufacturing sites to successfully produce the vaccine [16].

Ultimately, these case studies show that the Allegro STR bioreactor portfolio leverages decades of process engineering expertise to support successful cell culture in the gene therapy space. The availability of cost-effective gene therapies is critical for wider success of these novel medicines. Platform processes can contribute to that, especially where fully disposable or hybrid manufacturing is adopted.

The Allegro STR's proven delivery of consistent and scalable cell culture performance can easily be part of the solution.

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The Allegro™ STR bioreactor system is a stirred-tank, single-use bioreactor for suspension cells that is small in size and big on performance.

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FASTFACTS

Leveraging a scalable, standardized platform process for suspension-based AAV and LV vector manufacture to accelerate time to clinic

Samira Shore, Director, Technical Program Design, Viral Vector Services, Thermo Fisher Scientific

Cell and gene therapy developers are looking for opportunities to accelerate their path to the clinic while still meeting critical regulatory and quality specifications. A platform process approach for viral vector product manufacturing can help to address these goals by minimizing process validation and tech transfer scope, standardizing process components, reducing manufacturing time and release, and ultimately de-risking the regulatory pathway.

Cell & Gene Therapy Insights 2022; 8(7), 791; DOI: 10.18609/cgti.2022.119

PATHEON™ QUICK TO CLINIC™ VIRAL VECTOR PROGRAM

The Patheon Quick to Clinic viral vector program is a standardized, all-inclusive platform process for 200 L suspension-based adeno-associated viral (AAV) and lentiviral (LV) vector manufacture, which offers a range of benefits compared to a standard process development (PD) program and other platform process solutions on the market, including:

- Phase-appropriate regulatory support throughout the product lifecycle
- Inclusion of relevant license rights to Thermo Fisher assets
- Serotype agnostic (minor fine-tuning for GOI changes)
- Next-gen analytics: chemistry-based, enhanced precision and accuracy, absolute quantification
- Raw & starting materials vetted for GMP-compatible packaging, regulatory risks, reagent quality, and appropriate identity testing
- In-stock raw materials optimized to work together
- Robust process control and consistent CQAs

The combination of optimized, IND-ready processes with platform qualified analytics supports robust suitability assessment in early development as well as subsequent scale-up. Moreover, the program timeline includes expedited plasmid manufacture, suitability, and scale up, providing an overall time savings of more than 6 months compared to standard process development **(Figure 1)**.

Figure 1. Quick to Clinic™ viral vector program timeline. Standardized, all-inclusive platform process for 200 L suspension-based AAV and LV vector manufacture. Timeline for Quick to Clinic includes plasmid manufacturing and process optimization time.

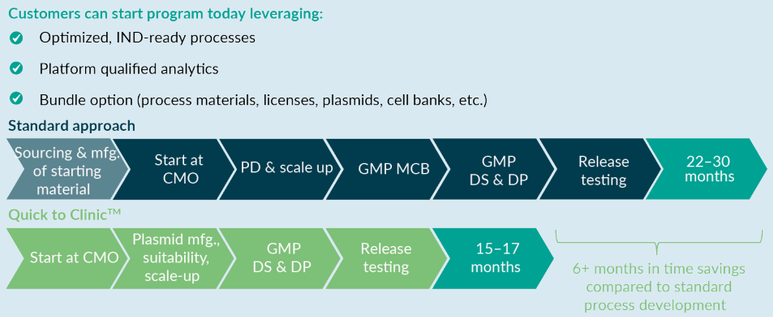
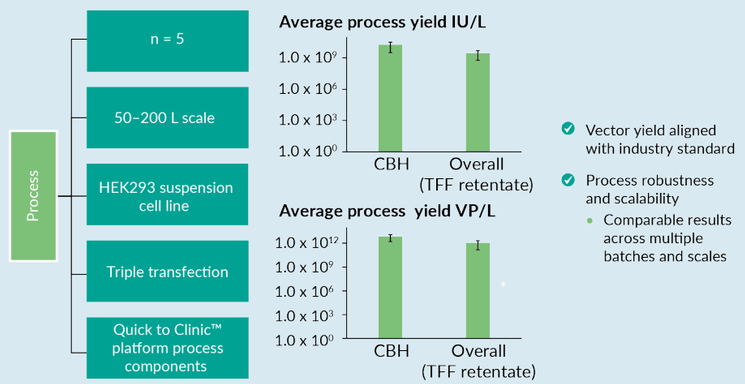


Figure 2. Quick to Clinic™ LV platform data: process performance.



PLATFORM OPTIMIZATION: LV PROCESS

Multiple batches of the LV vector process have been executed, which demonstrated that Quick to Clinic at minimum matches the industry standard process performance in terms of yield across multiple scales and batches, whilst supporting process robustness and consistency in process performance **(Figure 2)**. However, significant work is ongoing to continue process optimization in order to meet the demands of the evolving marketplace.

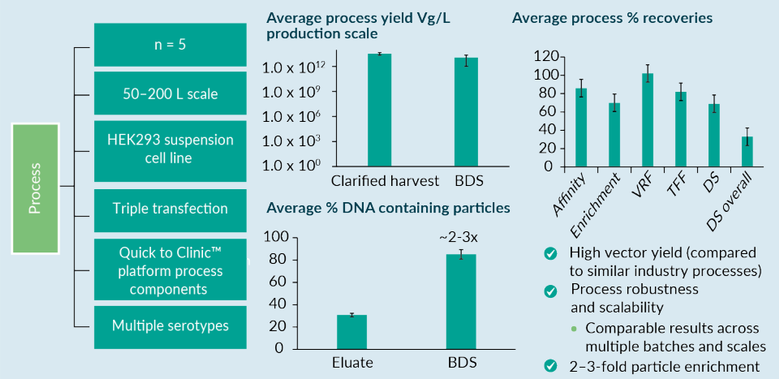
PLATFORM OPTIMIZATION: AAV PROCESS

Similarly, the AAV process has been tested through multiple batches of up to 200 L scale across several serotypes. **Figure 3** demonstrates the Quick to Clinic platform's ability to generate high Vg/L production yield, as well as high recoveries across the various purification steps. In addition, 2–3-fold particle enrichment was observed across multiple batches, indicating strong performance in terms of empty-full capsid ratio – a key critical quality attribute for AAV vector.

A DIFFERENTIATED PLATFORM

While there are a number of platform processes for GMP viral vector production available on the market today, the Quick to Clinic viral vector program is unique in providing an all-inclusive, end-to-end platform for both AAV and LV vector manufacture. Additionally, the availability of robust and comprehensive regulatory and raw materials support packages further assist end users in building for commercial success.

Figure 3. Quick to Clinic™ AAV platform data: process performance.



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CELL & GENE THERAPY INSIGHTS

UPSTREAM BIOPROCESSING

INTERVIEW

Driving significant AAV process yield and quality improvements for systemic delivery

David McCall, Editor, *Cell & Gene Therapy Insights*, talks to **Rahul Chelikani**, Director of AAV Upstream Process Development, Vertex Pharmaceutica

RAHUL CHELIKANI obtained a bachelor's degree in chemical engineering from Andhra University in Visakhapatnam, India and a PhD in chemical engineering from The University of Toledo, Toledo, Ohio, USA. After his PhD program, he started his career as an upstream process development scientist at Novavax, USA, working on the development of a bioreactor process for virus like particle (VLP) based vaccines. Then he moved onto a role in the bioprocess development division at Shire (Now Takeda) and worked extensively on development and scale up of batch and perfusion processes for different drug modalities (recombinant enzymes, monoclonal antibodies, fusion proteins, and AAV vectors). In his current role at Vertex Cell and Gene Therapies, he leads a team of scientists and engineers to develop a platform process to produce AAV vectors with significantly higher productivity by evaluating new basal media, transfection conditions & reagents, bioreactor parameters and novel cell lines. He has extensive experience in mammalian cell culture, media formulation development, high throughput platform development, scale up/scale down and tech transfer of single use bioreactor processes.

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Q What are you working on right now?

RC: In my current role, I lead a team of scientists and engineers involved in developing a high productivity, scalable platform bioreactor process for the production of viral vectors.

There are two aspects to my role. One is to support a manufacturing process to produce viral vectors for early clinical trials. The other is to develop a process with significantly higher productivity and scalability up to 2,000 L scale.

Q What challenges are Vertex and other developers of gene therapies in the neuromuscular disease space facing related to upstream processing of adeno–associated virus (AAV)?

RC: The challenge with neuromuscular versus other disease targets is the amount of dose required. In neuromuscular disease, because of the requirement for systemic treatment of the patient, the dose could be 104-times greater relative to that for an ocular disease indication, for example.

Dosing patients with such high titers requires high purity vectors. However, the more you try to achieve in terms of purity, the greater the loss of yield. There is a lot of innovation required here to produce vectors from the bioreactor at a high titer, whilst retaining the ability to purify them to a higher standard. Overall, we need to improve current productivity at least tenfold or more to improve the suitability of the process.

Q Where might the required improvements in productivity/yield come from? What are you seeing that is promising in terms of technological innovation?

RC: The approach we use to increase viral vector productivity and yield is not that different to the one taken by the monoclonal antibody (MAb) or protein therapeutics field. Even though cell lines used here are different from the monoclonal antibody or protein therapeutics industry, the unit operations (seed train, bioreactor) leading to the production of vectors are the same. The key difference between viral vectors and MAbs/proteins is the need for a transfection or an infection process to induce production of viral vectors.

Improvements in viral vector productivity will necessarily include optimization of cell lines and optimization of media. Depending on whether a transfection or infection process is used for AAV production, those parameters need to be carefully optimized. Adjustments to the bioreactor parameters could also lead to significant improvements in productivity.

Recently in the field, there have been many papers around the use of modified cell lines and use of new and improved transfection reagents. In the case of cell lines, researchers are engineering cell lines using clustered regularly interspaced short palindromic repeats–associated

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protein 9 (CRISPR/Cas9) by knocking out genes or overexpressing certain genes that could be useful for increasing the productivity viral vectors. Transfection reagents could significantly improve the productivity and scalability of transfection-based production processes. It is very important to choose the right transfection reagent for your cell line and continue to optimize the transfection process.

"Transfection reagents could significantly improve the productivity and scalability of transfection-based production processes."

Q More specifically, how and where is emerging innovation having an impact in AAV upstream processing in the area of automation?

RC: Automation could be applied in a couple of areas, including in early–phase process development. Implementing automation with high–throughput analytics early on could allow scientists to screen many parameters in a short period of time. One example of automation is the Ambr bioreactor system from Sartorius, which can run 48 reactors at once, meaning we can produce 48 different conditions to be evaluated in a short period of time. Automation can also improve batch–to–batch consistency.

The second aspect is automation within manufacturing. In the MAb space, automation is already being implemented, of course – processes are monitored in real–time and can be controlled within operating parameter specifications. However, the challenge with viral vectors is we have a limited number of batches or datapoints to enable and implement this automation. There is a strong need for better analytical methods and technologies that can measure things more accurately within the bioreactor.

As we grow as an industry in terms of numbers of programs in the commercial space, we will see more automation being implemented. Again, the data points are currently limited to fully implement automation, but I believe this situation will improve soon.

Q Many analytical tools from the MAbs space are currently being repurposed for AAV production. Where are you seeing these tools having an impact today, and which ones will have an impact moving forward?

RC: Many tools have been repurposed from antibodies to viral vectors. However, there are some unique aspects to viral vectors for which new, bespoke techniques need to come in.

I think the techniques are already there, in the mainstream, but most of these techniques require samples to be purified to a certain extent. This means the upstream process must be followed by a chromatography process in order for us to apply these techniques. One

key aspect is how we can bring these techniques further upstream, into the bioreactor. This would allow us to better analyze the data from the bioreactor.

There have also been tremendous advances made in the field of omics. That could be a powerful tool to understand the key metabolic pathways involved in the production of viral vectors, for instance. If we understand those key metabolic pathways and the related bottlenecks in production, we could significantly improve yield and quality. Omics has been applied in other cell culture processes for antibody and protein production and is starting to be implemented in some areas in the industry in viral vectors. I'm sure we will see more of that in the future.

Q What are the downstream challenges thrown up by the efforts to boost upstream productivity, and how can they be addressed?

RC: When upstream titers increase by 10- to 20-times, it causes significant challenges for downstream processing.

One aspect that we could borrow from the MAb industry is continuous processing, including of the downstream. Technologies such as the simulated moving bed (SMB) could be implemented here, rather than having a scale–up approach to columns. There are only so many column sizes you can scale–up to, but with continuous processing, the processes can be more scalable.

Q Over your 15 years specializing in upstream processing of virus–like particles (VLPs) and viral vectors, what are the key learnings you have gleaned that shape your current approach to process development?

RC: The fundamental approach to developing upstream processes for modalities such as MAbs, viral vectors, and VLPs are all somewhat similar: we must grow cells to a certain density and induce production through an infection or a transfection process to start producing viral vectors in place of MAbs; the recombinant cell lines have all the machinery required to ensure that when we get into a bioreactor, we can change a certain parameter and they will start producing either antibodies or viral vectors.

One key thing I have learned over the years is the importance of understanding the

> "If we understand more about the biology and metabolic pathways involved in producing these molecules, we can develop the optimal process conditions. This leads to significant improvements in the productivity and quality of these modalities."

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biology of the production of these molecules. If we understand more about the biology and metabolic pathways involved in producing these molecules, we can develop the optimal process conditions. This leads to significant improvements in the productivity and quality of these modalities.

Another key learning is around scaling up the process. Many people do not think about large–scale reactors when developing a small–scale process in their lab. It is important to get a grip on the final bioreactor scale and process. If you understand the commercial process early on and scale it down appropriately, you can solve many of the future issues you would have in scaling up the process.

Q What are some key goals and priorities for your work over the next few years?

RC: One of the key aspects that my team will be actively involved in is the improvement of productivity by a significant margin. We are looking at achieving that goal through the optimization of cell lines, media, and bioreactor parameters, as well as looking at new and upcoming technologies in the field.

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Achieving better AAV vector productivity and product quality

Diane Golebiowski and Marissa Stanvick

In a recent episode of The BioInsights Podcast, we spoke to two experts from Oxford Biomedica Solutions about their experiences in optimizing transient transfection of plasmids into suspension HEK293 cells for the production of recombinant adeno-associated virus (rAAV) gene therapies. Here, we sum up some of their key thoughts.

PODCAST
PERSPECTIVES

Listen to the full podcast here.

HOW IMPORTANT IS PLASMID DESIGN TO VECTOR PRODUCTIVITY AND QUALITY?

"Our team has worked very hard in the last few years to come up with an ideal arrangement of sequences that is critical to AAV production, and we have generated a novel dual plasmid design which results in a significant increase in AAV productivity and, more importantly, increases the percentage of full capsids upstream. This has been a huge innovation for our AAV manufacturing platform process, leading to significant process gain while still maintaining the same flexibility as triple plasmid transfection."

– **Diane Golebiowski,** Head of Vector Engineering Group

"... AND WHAT ABOUT PAYLOAD SEQUENCE?

"This is just as critical, if not more. We advocate for biology being the main driver for the decision-making process when nominating a lead for development, but you have to consider the whole package. If you have two candidates that are comparable expression-wise and efficacy-wise, it is crucial to look at how it manufactures, so that doesn't lead to problems later. We offer end-to-end services to support construct design, and look for red flags early that could cause problems with drug quality."

– **Diane Golebiowski,** Head of Vector Engineering Group

WHAT WERE THE CRITICAL ASPECTS YOU FOCUSED ON FOR SUCCESSFUL SCALE-UP?

"First, we designed a cell expansion strategy. Considering how unpredictable supply chain is we also needed to design a flexible process. The next area we focused on was ensuring the volume and process timing would be appropriate and consistent among scales. We came up with strategies to evaluate in a scaled down bioreactor model, before ultimately deciding on our two thousand litre scale recommendation.

Transient transfection traditionally has a reputation for poor productivity and scalability. We set out to prove that wrong and create a high-performing system that offers the flexibility of transient transfection with the productivity you might see with other systems. We've shown that we can scale our process from two to two thousand litres for three programs."

– **Marissa Stanvick**, Director of Upstream Process Development

WERE YOU ABLE TO INCREASE BIOREACTOR PRODUCTIVITY?

"Ultimately, we were able to optimise our platform conditions around the novel dual-plasmid design, and find a set of parameters that were ideal for multiple constructs and AAV serotypes. Once we combined these parameters, we increased our productivity by over a log to over 1E15 vg/L. We also found nearly 50% full capsids in the affinity product as a result of the dual plasmid design. Our purification sciences team designed a downstream platform where the final products achieved over 90% fully intact vectors in the drug substance, with high product quality."

– **Marissa Stanvick**, Director of Upstream Process Development

CELL & GENE THERAPY INSIGHTS

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In collaboration with: Oxford Biomedica Solutions

CELL & GENE THERAPY INSIGHTS

UPSTREAM BIOPROCESSING

LIVE30 TRANSCRIPT

Adapting the power of density gradient separations for characterizing viral vector fullness

Shawn Sternisha

A prominent hurdle in gene therapy is the inefficient packaging of nucleic acids into delivery vehicles. Depending on the type of viral vector used and the size of the transgene, the result is often a heterogenous population of capsids including empty, partially full, and full capsids. Only full capsids produce the intended therapeutic effect, with empty and partial capsids possibly causing adverse reactions and contributing to undesirable safety profiles. Capsid loading is therefore scrutinized rigorously by regulatory agencies such as the Food and Drug Administration (FDA), European Medicines Agency (EMA), or National Medical Products Administration NMPA (NMPA).
There is a need within the industry for robust, serotype-independent purification and characterization schemes. Two such methods are density gradient ultracentrifugation (DGUC) and analytical ultracentrifugation (AUC).

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INTRODUCTION TO DGE–AUC

DGUC is a high-resolution purification technique that separates on the basis of buoyant density. A density gradient is formed, and the particles migrate to the position where their density is equal to that of the surrounding media. As such, band position directly correlates to particle fullness and denser (fuller) particles sit lower in a density gradient.

Sedimentation velocity AUC (SV–AUC) is an analytical technique used for characterization based on sedimentation rate, which also correlates with fullness.

Density gradient equilibrium AUC (DGE–AUC) is a characterization technique that leverages the benefits of a density gradient, analogous to DGUC, to directly and quantitatively analyze the equilibrium positions of all species in a sample.

Comparing SV and DGE–AUC (Figure 1), DGE–AUC is far less complex with greater sensitivity, as off-density species often sediment or float away. Raw data from DGE–AUC can be analyzed from a simple viewing without any other plotting or processing required, unlike in SV–AUC, and deeper analysis is not dependent upon known material properties nor data fitting to predicted models.

The implementation and optimization of DGE–AUC is simple and intuitive. The workflow is described in Figure 2, alongside the best practices to optimize results.

SIMPLE EXCEL ANALYSIS OF ADENOVIRUS USING DGE–AUC

Once the data is acquired, analysis is straightforward and can be performed on easily accessible software such as Excel. First, data is exported from the instrument through remote access using a laptop. Data files can be directly imported in Excel; the data can then be plotted, and peaks can be identified and integrated using the area by splicing approach.

As the data are simple peaks and not moving boundaries, there is the opportunity to dramatically streamline, automate, and enhance this workflow using more advanced software, such as Chemstation, Empower, and Origin. By using a more advanced software, you can achieve automated import and plotting; automated peak detection, fitting, and deconvolution; fixed integration ranges and automated signal ratio comparisons; as well as baseline correction. A complete analysis and integration in Origin for a single dataset can be performed in under a minute with a basic level of understanding of the software.

DGE–AUC IN ACTION: EXAMPLES WITH ADENO-ASSOCIATED VIRUS, ADENOVIRUS, & PLASMID

Figure 3A illustrates how DGE–AUC can be used for semi-purified Adeno-associated virus (AAV) samples containing Pluronic, which are often problematic in SV–AUC or require buffer exchange. SV raw data demonstrates a moving baseline and a low titer. In the same sample using DGE, it is easier to correct the baseline in software such as Origin, resulting in sharp peaks that can be easily and quickly integrated.

One major advantage of DGE–AUC is that it can be used to detect low abundance species. Figure 3B shows two plasmid dilutions that were run in the same experiment. In the 250-fold dilution (blue), we see a single peak with an absorbance of about 1.2. In

FIGURE 1

Comparison of SV-AUC with DGE-AUC.

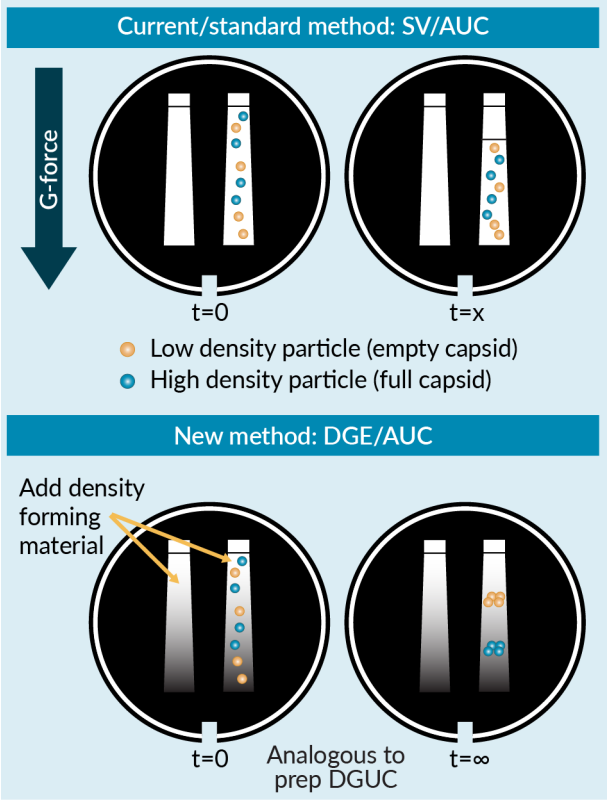

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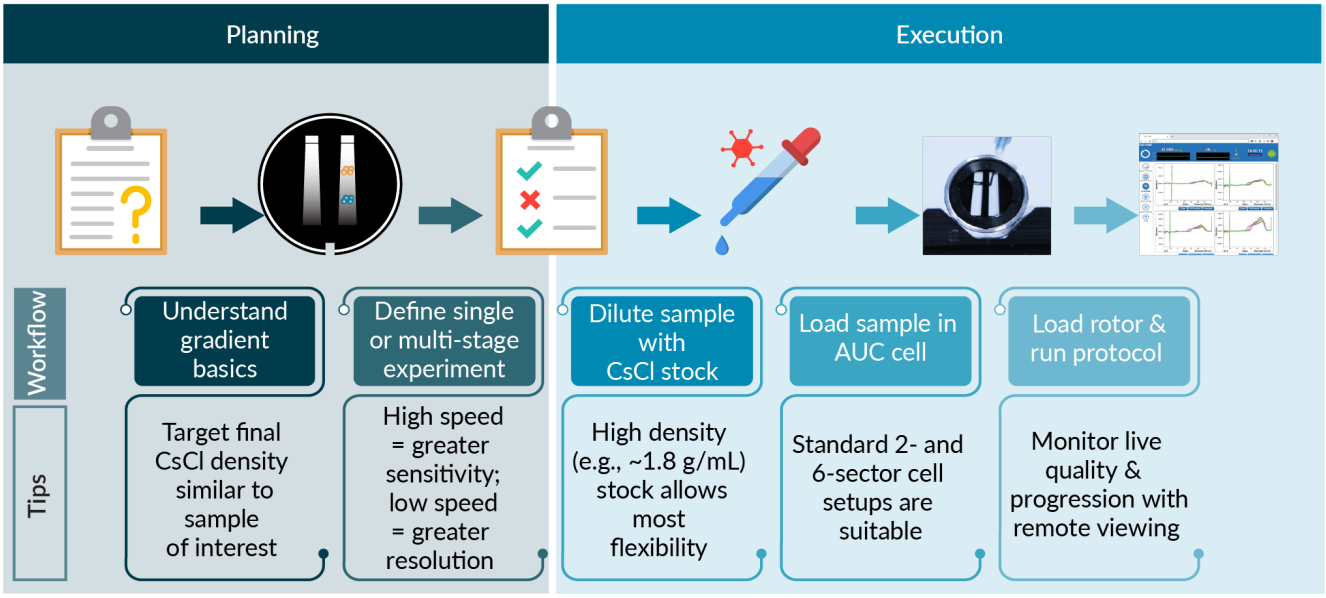
FIGURE 2

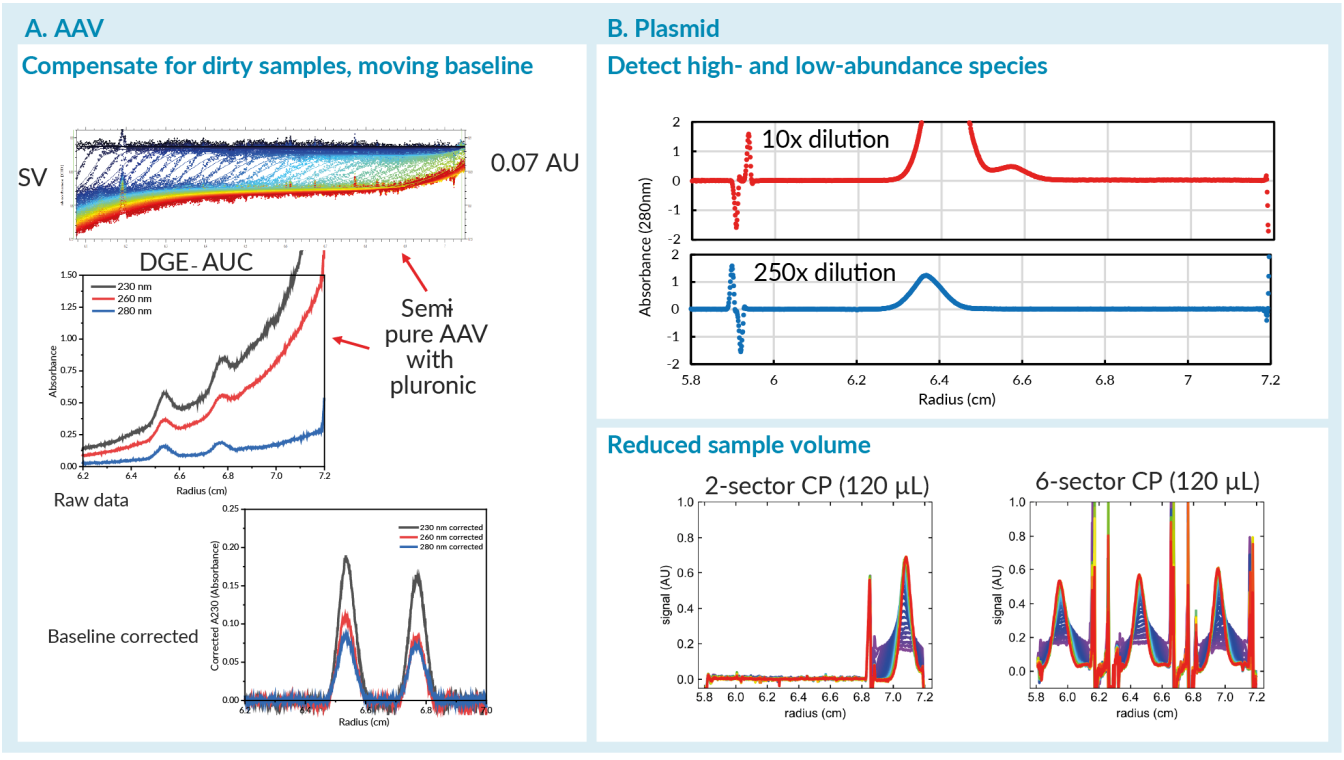
DGE-AUC implementation workflow, from planning to execution.



the 10-fold dilution (red), despite the primary peak signal being well past the dynamic range of the absorbance detector, we see a smaller shoulder peak to the right emerge, providing informative sample information. Since DGE–AUC overloaded sample and still give reliable data, you can tweak the loading concentration depending on your needs.

FIGURE 3

(A) DGE-AUC case study with AAV. (B) DGE-AUC case study with plasmid.



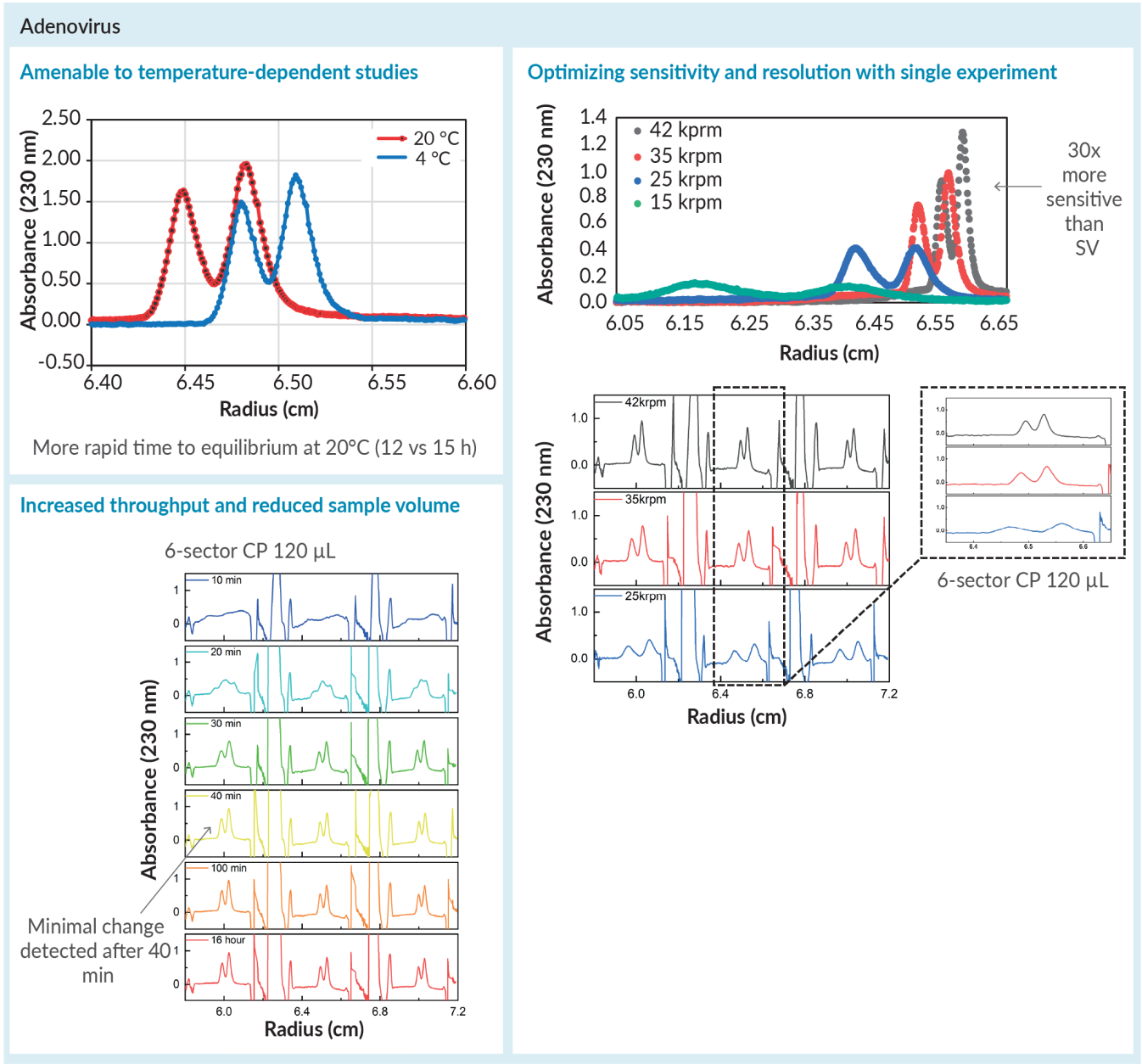
DGE–AUC also allows analysis of your sample using low volumes. As seen in the bottom panel of Figure 3B, 120 µL of plasmid sample were loaded into a two-sector cell and a single sharp, quantifiable peak is observed. Moreover, six-sector centerpieces (CPs) can also be used to significantly increase the throughput without sacrificing quality.

Large particles like adenovirus do not work well on SV–AUC, as they sediment too quickly to reach the required number of scans for analysis, especially when multiwavelength analysis is desired. As DGE–AUC is not size-limited, it works well with adenovirus, as shown in Figure 4. In fact, the larger the particles, the faster they move, and the faster equilibrium can be attained. Therefore, as you move towards larger particles, DGE–AUC becomes even more efficient.

Temperature is a key factor that impacts the time required to reach equilibrium. As temperature was dropped from 20 – 4oC,

FIGURE 4

DGE-AUC case study in adenovirus.



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a roughly 25% increase in time to equilibrium was observed. The impact of temperature on the gradient that forms can also be seen here, as evidenced by a shift in peak position.

Speed ramp studies allow one to quickly find the optimal balance of sensitivity and resolution with a minimal amount of sample and hands-on time. Running at a high speed (rpm) gives the greatest sensitivity and broadest range of densities to assess, while lower speed gives a narrower range and greater resolution.

Six-sector CPs provide much higher throughput and much lower sample requirements. The shorter path length in these cells allows equilibrium to be reached faster and at 42k, it is possible to run 21 adenovirus samples in as little as 40 minutes.

SUMMARY

AUC as a technology offers several techniques (such as SV- and DGE–AUC) to be conducted with a single instrument for acquiring complementary, orthogonal information. DGE–AUC is a versatile, thermodynamics-driven analytical method based on a well-known foundational understanding of CsCl density gradients. The method is orthogonal to well-established SV–AUC methods and analogous to industry-standard CsCl prep gradients with intuitive interpretation. DGE–AUC is serotype agnostic, not size-limited, and can achieve more than thirty times greater sensitivity. Multi-parameter optimization screen is achievable in a single experiment. It is high throughput, high resolution, and more tolerant of common buffer components, including stabilizers like Pluronic's and sucrose.

Q&A with Shawn Sternisha

Q Are there any new hardware or consumables that are needed for DGE AUC?

SS: I am pleased to say that the answer is no. Virtually all the existing AUC hardware in our catalog can be used for DGE–AUC – nothing new is required if already using the standard SV–AUC method. If you already own an AUC, you already have all the hardware, you simply need to add cesium chloride.

The two-sector and the six-sector are by far the most common CPs that are used and are the same cell assemblies that have been used historically. Aluminum CPs can also be used to allow you to reach slightly higher speeds. Both quartz and sapphire windows are useful for this technique as well.

Q Are there primary differences between the analysis of DGE–AUC and SV–AUC data?

SS: To analyze SV–AUC data, you need to fit the data to numerical solutions of the Lamm equation. This is a very computationally demanding process, which requires specialized software. There is a level of expertise required to be able to correctly model that data and derive accurate insights from it.

On the other hand, with DGE–AUC, what you see is what you get. It is simple XY data with peaks where the species are. It is an intuitive way to read data. Due to that simplicity, there is no need for specialized software or an AUC expert.

Q How do you determine initial run conditions, and how long does a typical run take?

SS: This depends on several factors. To start, you would choose a starting density that is about equal to your particles of interest. You would also select wavelengths early on. There are standard ones that are typically used for viral particles: 230, 260, and 280, but you can run more wavelengths than that. Since it is largely an endpoint measurement, you can access the entire UV/Vis spectrum without sacrificing resolution.

Instead of just selecting a single speed, I recommend trying out a speed ramp experiment to allow you to try out several different speeds in a single run to optimize sensitivity and resolution.

Another big consideration for AUC is the scan gap or frequency. I recommend trying to monitor that approach to the equilibrium process, doing a scan every five to ten minutes. Another advantage of DGE–AUC is the ability to scan average. If you reach equilibrium then you collect 50 more scans, you can average all those together to increase the signal-to-noise ratio.

Run duration takes some trial and error and depends on the speed. As goalposts for adenovirus, we have seen about 12 hours or more for the two-sector CP, and about one – two hours for the six-sector.

Q Can I use the DGE–AUC method in the Proteome Lab instrument?

SS: The answer is yes, but it is less desirable.

In the Proteome Lab, three wavelengths are possible in theory. In practice, however, it is not nearly as good as the wavelength accuracy after shifting is less than ideal. Wavelength precision is much better in the Optima. It is truly multi-wavelength and we have demonstrated 90 wavelengths at once. In addition, the radial resolution is better for absorbance and interference detection systems, directly correlating to more data points under each peak. The Proteome Lab also does not have remote monitoring, which is a useful tool to have for observing gradient formation, and the splash screen setup is much less user friendly.

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CELL & GENE THERAPY INSIGHTS

UPSTREAM BIOPROCESSING

INNOVATOR INSIGHT

Complete system for AAV production for clinical production: part 1 – upstream

Jonathan Zmuda & Jenny England

Gibco™ Cell Therapy system (CTS) products are designed to enable clinical and commercial cell and gene therapy manufacturing. All reagents are manufactured under cGMP guidance, with cell and gene therapy specific intended use statements, extensive safety testing, and proactive regulatory documentation. These measures enable the provision of quality materials to help minimize risk, ease the burden on quality systems, and support regulatory submissions. This article will explore the scalable, high titer production of adeno-associated virus (AAV) within the Gibco CTS AAV-MAX production system.

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INTRODUCING THE AAV-MAX SYSTEM

Today, the most common method for producing adeno-associated virus (AAV) is by triple transfection in the HEK293 cell line, using a RepCap plasmid, a helper plasmid, and the recombinant AAV expression plasmid containing the gene of interest. The AAV-MAX system has been developed as an optimized and fully integrated system with all the components required to streamline production of high-titer AAV, including Viral Production Cells 2.0, and a Viral Production Medium that is an animal origin-free, chemically defined, protein-free formulation.

The AAV-MAX Transfection Reagent and Booster enables highly efficient transfection of high-density HEK293. The Viral-Plex™ Complexation Buffer is also animal origin-free and chemically defined, as well as protein-free. The AAV-MAX Enhancer serves

to significantly increase the AAV titers produced in the system. Finally, the AAV-MAX lysis buffer is a polysorbate 20-based Lysis Buffer for the extraction of AAV from cells.

One key highlight of the AAV-MAX production system is the high titer that can be generated. Across various AAV serotypes, the system has been shown to generate greater than 5×10^{10} vg/mL of viral vector, meaning that the system is highly cost-effective. There are also currently research-use only options available for AAV-MAX.

VIRAL PRODUCTION CELLS 2.0

The VPC 2.0 cells are a clonal, 293F-derived suspension cell line for superior AAV production at multiple scales. Key attributes of this cell line include the lack of the SV40 large T antigen, which can be of regulatory concern. VPC 2.0 cells allow for high-titer viral vector production across different AAV serotypes, as well as high-density cell growth (typically greater than 12 million cells/mL) in routine shake flask cultures. It has a non-clumping phenotype with robust scalability, fast recovery post-thaw, and rapid growth rates (<24 hrs doubling time). The VPC 2.0 cells will be available cGMP banked cells in late 2022, coinciding with the launch of AAV-MAX CTS.

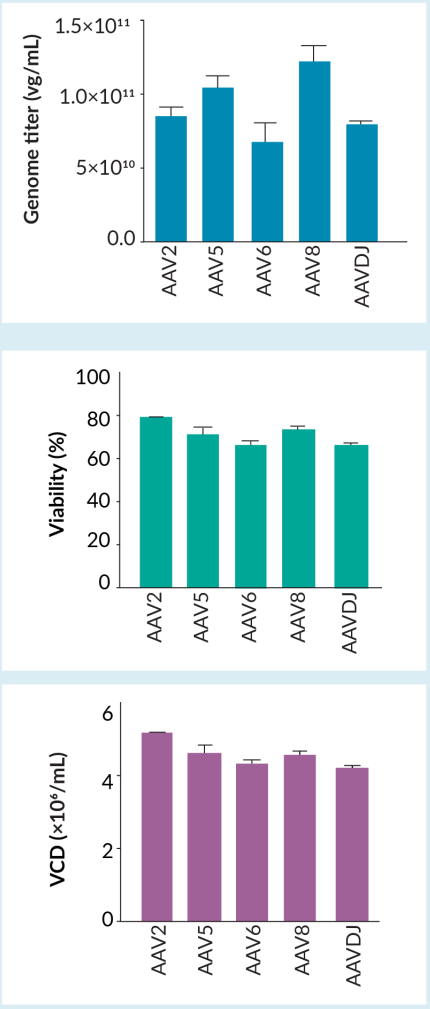
A COMPLETE SYSTEM FOR MAXIMAL PERFORMANCE

Figure 1 demonstrates how the AAV-MAX system can maximize AAV titers of multiple serotypes, with high AAV titers of $>5\times10^{10}$ vg/mL, the ability to obtain more viral particles in less volume, and the flexibility to use one system to produce multiple AAV serotypes.

The AAV-MAX system is a complete system for optimal performance allowing superior titers with optimized reagents in a simplified workflow. Multiple reagent formulations were tested to arrive at a complete system with integrated components that work together to deliver maximum AAV titers. Figure 2 shows

FIGURE 1

AAV-MAX system titers, viability, and viable cell density of five serotypes.



5-to-20-fold improvements when using the full AAV-MAX system, showing the synergistic effect of using the entire system rather than swapping out individual components.

SCALE UP OF THE AAV-MAX SYSTEM

Case study 1: AAV protocol optimization in 3 L stirred-tank bioreactors

The AAV-MAX system allows scalable production of AAV from multiwell plates to stirred-tank bioreactors. The first case study is centered around optimizing the AAV-MAX protocol at 2 L volume in 3 L stirred-tank

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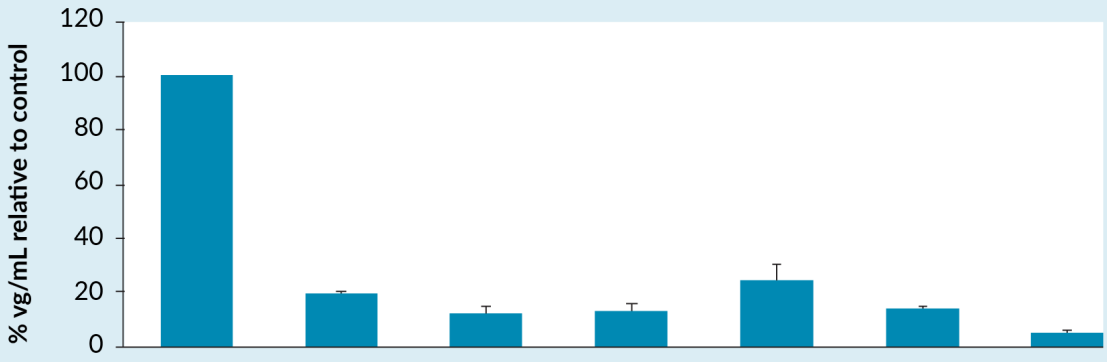
FIGURE 2

Optimal titers relative to control found using AAV-MAX, VPC 2.0 cell line, and viral production medium.



Cell line	VPCs 2.0					VPCs	VPCs
Medium	Viral Production Medium			Gibro™ FreeStyle™ F17 Medium	BalanCD™ HEK293 Medium	Viral Production Medium	LV-MAX™
Transfection reagent	AAV_MAX	PEIpro™	PEI MAX	PEIpro™	PEIpro™	AAV-MAX	LV-MAX™
Enhancer	+	–	–	–	–	+	+

HyPerforma™ Glass bioreactors, using AAV2 and AAV6. Two different inoculation strategies were evaluated to provide maximum flexibility and protocol robustness, before cell growth and AAV titer were assessed.

The experimental design for this case study is shown in Figure 3.

In the production of AAV2 using two inoculation strategies, similar growth profiles and titers were observed, demonstrating the flexibility of the system. Strategy 1 was chosen for additional testing based on its streamlined process and ease of scheduling experiments.

In the production of both serotypes, AAV2 and AAV6, similar cell growth and metabolite trends were observed in 3 L stirred-tank bioreactors and shake flask cultures. Both AAV2 and AAV6 titers trended higher in bioreactors. This demonstrates the easy transition of the AAV-MAX system from shake flask to stirred-tank bioreactors at the bench scale.

Case study 2: AAV production in 50 L HyPerforma DynaDrive™ single-use bioreactor

This study aimed to demonstrate AAV6 production using the CTS AAV-Max system in 50 L HyPerforma DynaDrive single-use bioreactors, and to compare production in the Thermo Scientific HyPerforma DynaDrive single-use bioreactor, the HyPerforma single-use bioreactor, and 250 mL shake flakes. The aim of this study was to evaluate scalability of AAV6 production with regards to cell growth and transfection.

Seed train expansions up to the 50 L scale at the time of transfection were all performed in the 50 L HyPerforma single-use bioreactor, which has a 10:1 turndown ratio. Comparable growth profiles from the shake flasks to the stirred-tank bioreactors were seen in each of the expansions, from 5 L to 45 L, giving confidence that the VPC 2.0 cell line can be grown easily and robustly up to the 50 L reactor transfection scale, comparable to shake flask cultures.

The VPC 2.0 cells grown in the 50 L single-use bioreactors after transfection showed comparable viability and VCD across all conditions.

As seen in Figure 4, even with minimal optimization of the 50 L process, the HyPerforma DynaDrive single-use bioreactor performed well for AAV6, with titers greater than 1.5×10^{11} vg/mL. With optimization, it

FIGURE 3

Eight-step protocol outline for case study 1.

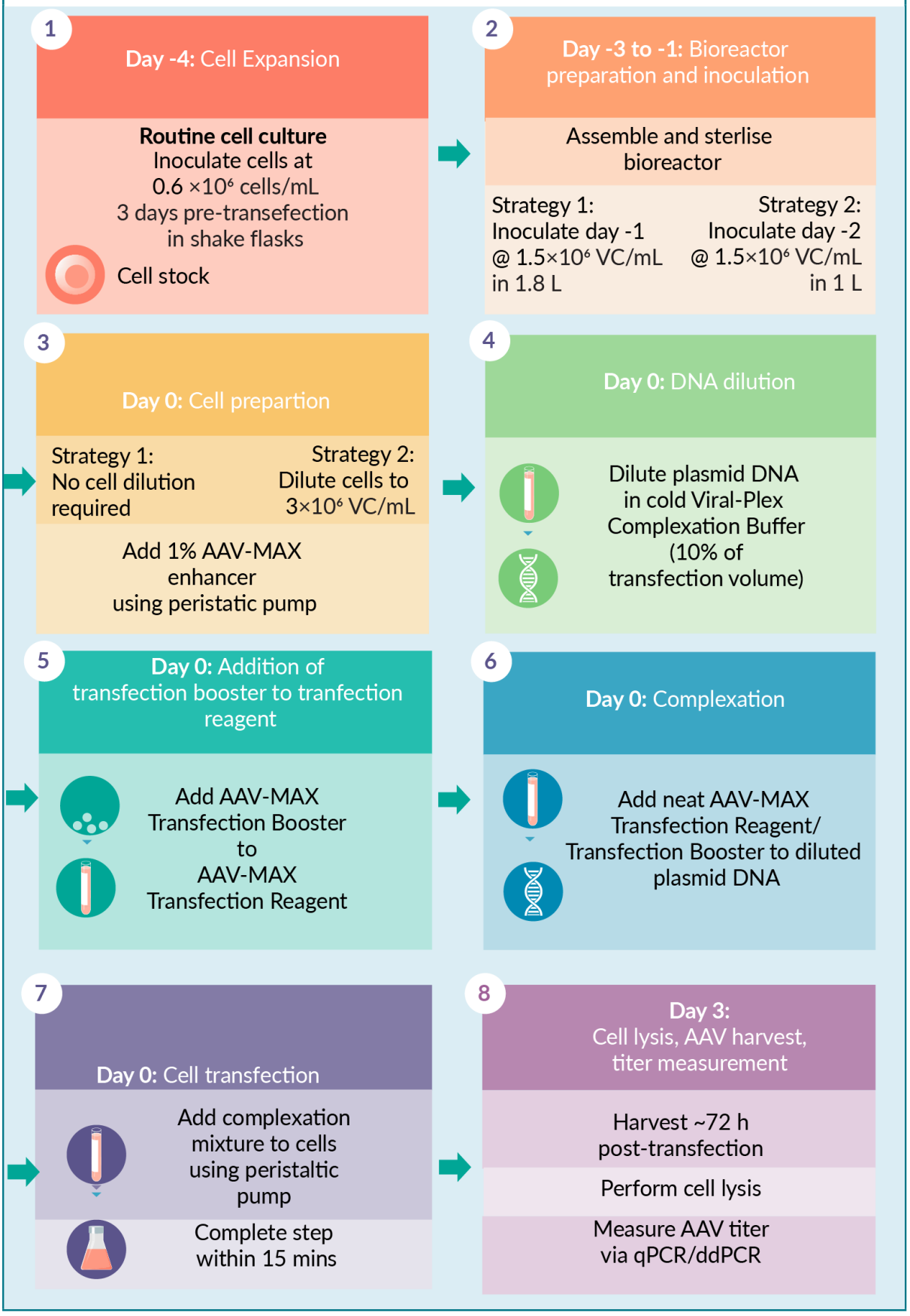


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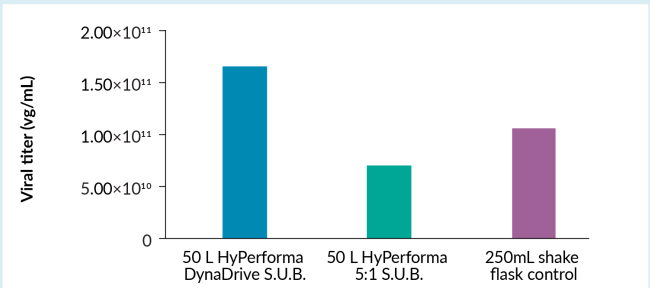
FIGURE 4

AAV6 viral titers for three different production conditions



is expected that these titers could be further enhanced.

Case study 3: VPC 2.0 growth to 3,000 L in HyPerforma single-use bioreactors

In this study, VPC 2.0 cells were scaled to 30 L within the 50 L HyPerforma DynaDrives bioreactor and used to seed the 3000 L HyPerforma DynaDrive single-use bioreactor.

The VPC 2.0 growth results up to 3000 L demonstrated the successful scalability of the cell line. Within the 3000 L bioreactor, comparable if not slightly improved cell growth was seen up to a maximum VCD of 14 million cells/mL.

PURIFICATION OF AAV: FROM BENCH SCALE TO BIOREACTOR

Simple process overview for downstream purification of AAV is shown in Figure 5. This process is for bench scale purification of AAV6 that does not require a large amount of specialized equipment or instrumentation.

For clinical manufacturing of AAV6 at much larger scales, the clarification step by centrifugation would not provide the necessary scalability. A truly scalable process for the AAV-MAX downstream process, which is currently in development, is outlined in Figure 6.

The clarification step is performed using depth filtration. The three-filter train consisting of the C0SP, F0HC, and Sartopore 2 filters, provides excellent clarification with extremely low pressure observed on the filters. Yields over clarification typically range from 70 to 80% or greater in this process step.

FIGURE 5

Process overview for AAV downstream purification.

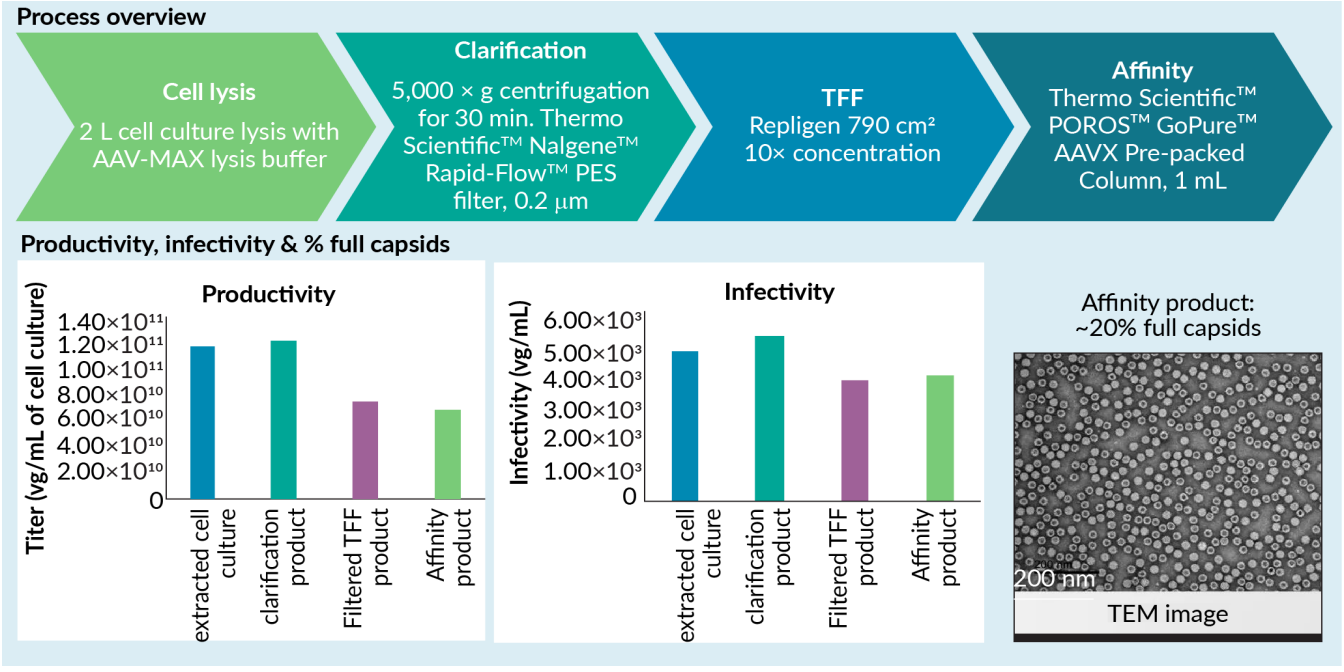
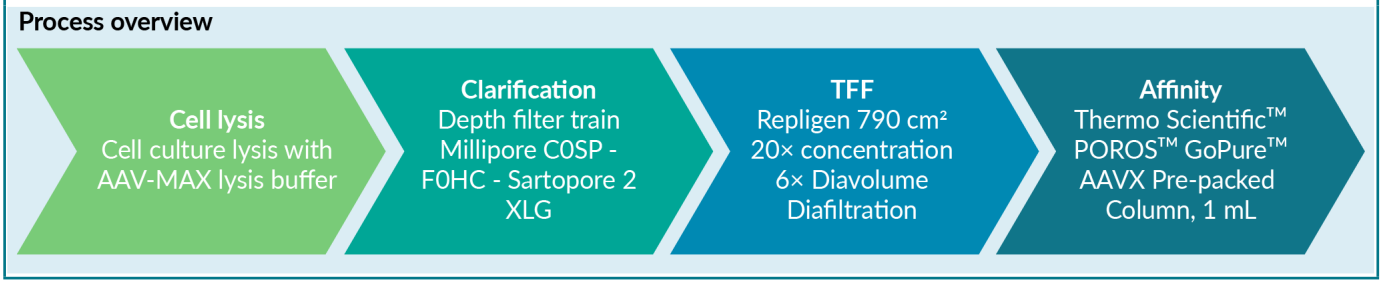


FIGURE 6

A scalable process overview for AAV downstream purification, with altered clarification and TFF steps



In TFF concentration, a 100KD hollow fiber TFF keeps the AAV retained while the cell media is removed through the permeate. Step yield recovery of approximately 90% is seen.

The POROS CaptureSelect AAVX affinity resin selectively binds to AAV6, allowing all of the contaminating proteins to flow through the column, and leading to a good percentage of full AAV capsids present in the elution. Typical step yields of approximately 80% or greater are seen.

Utilizing this more scalable approach, similar results for productivity, infectivity, and percent full capsids have been achieved. The next steps would be to further polish the resultant affinity elution to increase the percent full capsids.

OPTIMIZING THE AAV AFFINITY PURIFICATION

Despite its importance to the field of gene therapy, AAV purification remains a bottleneck in manufacturing. The downstream process involves a capture step to separate the AAV from the cell culture, followed by a polish step to separate full from empty capsids before the final fill-finish step. These steps have purification challenges such as an increased impurity burden due to cell lysis. There are also recovery challenges that result from cumulative yield losses with each unit operation. Additionally, the purification platform must ideally be able to serve a wide variety of AAV serotypes.

The POROS CaptureSelect AAVX affinity resin was designed to address several challenges in the downstream process of AAV manufacturing. The AAVX resin can bind a broad variety of serotypes and can therefore serve as a platform solution for AAV purification. In addition, the AAVX resin has a high capacity and elution recovery with excellent scalability, offering >80% vector recovery at 200 L scale.

Guidance from regulatory agencies recommends the determination of the viral clearance capability in each unit operation in an AAV process. In addition to host cell and process-related impurity clearance, the AAVX resin has been shown to provide effective viral clearance.

In a viral clearance study from REGENXBIO, the AAVX capture step was tested using a six-virus panel of enveloped and non-enveloped RNA and DNA containing viruses of various sizes. AAVX was shown to achieve greater than four log reductions of four out of the six viruses tested, and between one and three log reductions of the remaining two viruses. Other biopharma companies have also published their viral clearance data, further suggesting that AAVX can be a powerful viral clearance tool for the downstream process.

The performance of the AAVX resin is highly dependent on process development used to maximize yield. The elution and intermediate washes can be optimized with different buffer conditions such as pH or excipients. The quality of the load sample can be improved with optimized clarification and nuclease treatments, and the concentration of the load sample can also play a role in the binding capacity and purity. It is important to note that there can be significant variability in AAV analytics, so in some cases, relatively low recoveries may be attributed to the interference of the buffer with the assay.

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