A Brief Guide on Designing a Comprehensive Cell and Gene Therapy Bioanalytical Strategy

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Introduction

The field of cell and gene therapy (CGT) is rapidly developing, with the number of therapies in development greatly increasing over the last five years. The application of CGT provides the promise of significant long-term health benefits to people suffering from a wide range of diseases, from ophthalmological disorders to cancer.

In cell therapies, cells from a donor (allogeneic therapies) or from the patient (autologous therapies) are propagated outside the body and then introduced into the patient.[1] In gene therapies, modifications to the genetic material of somatic cells can be conducted inside (*in vivo*) or outside (ex vivo) the body.[1] Functional copies of a mutated gene can be inserted or new proteins related to the therapeutic approach can be introduced to the patient's genome. For example, an inherited disease may involve a genetic mutation in a gene for a key metabolic enzyme, resulting in a nonfunctional enzyme and toxic accumulation of the enzyme's substrate in the body. A gene therapy would introduce a functional gene into the body with the use of a viral vector or lipid nanoparticle. Genetic modifications can allow for constitutive production of the replacement enzymes, providing significant advantages over alternative approaches that require regular dosing of exogenous enzyme replacement therapies. Current research is also aiming to develop therapeutics that perform finer-scale editing of genetic mutations inside cells ("gene editing").[1]

The transduction of host cells with viral vectors has a relatively high efficiency compared to non-viral methods. However, challenges of immunogenicity and cytotoxicity have led to an increase in the use of non-viral gene delivery vectors for *ex vivo* and *in vivo* processes. In addition to safety benefits, non-viral methods also offer the advantages of easier preparation, the ability to transfer larger genes and considerably improved transfection efficiencies. Non-viral genetic modifications are being explored as potential therapeutics for HIV, beta-thalassemia and other diseases.

Case Example: CGT in Oncology

The oncology field provides a case example of the utility of CGT. Historically, cancer treatments were based on surgery, chemotherapy and radiation interventions. Over the past two decades, targeted therapies – primarily monoclonal antibodies – have become the gold standard for many cancer treatments. More recently, immunotherapies that attack tumors by harnessing the patient's immune system have emerged as a powerful tool in cancer treatment regimens.[2] One emerging immunotherapeutic approach is cell-based therapy. Cell-based therapy can involve the transplantation of stem cells or adoptive cell transfer (ACT), which consists of collecting and using patients' own immune cells to treat their cancer.[2]

The advantages of autologous therapies include low immunogenicity and avoidance of graft-versus-host disease. Conversely, the main drawbacks are the length of time required for cell expansion, the variability of the donor's starting material and the logistical challenges of scaling production of individualized treatments.

Still, challenges such as source material shortage, invasive retrieval methods, cell heterogeneity and purity have driven the search for alternatives to allogeneic cell and gene therapies. In response to these challenges, new methods for creating cells suited for allogeneic approaches are emerging. Mesenchymal stem cells (MSCs) are derived from induced pluripotent stem cells (iPSCs), offering

significant improvements over tissue-derived cells. For cancer therapies, scientists are trying to turn autologous chimeric antigen receptor T cell (CAR-T) approaches into allogeneic treatments by silencing proteins that mediate host-vs-graft immune responses. Some examples of these adaptations are using CAR-T cells with silenced T cell receptors[3] or switching from T cells to less immunogenic natural killer (NK) cells.[4]

Owing to its targeted and personalized nature, *ex vivo* gene therapy generally has an improved safety profile. However, cell retrieval and *in vitro* manipulation can be a costly and intricate process. *In vivo* gene therapy is potentially more straightforward; but faces challenges such as toxicity or the induction of immune responses. Clinically the most frequently observed toxicities for *in vivo* gene therapies are hepatic toxicity and cytokine release syndrome (CRS).[5,6] In addition, genome-editing technology is being explored as a strategy to advance the scientific engineering of the CAR. Compared with conventional CAR-T cells, CRISPR/Cas9-edited CAR-T cells have shown an enhanced potency as well as delayed differentiation and exhaustion.[7,8]

Conceptualizing a Bioanalytical Package for CGT

Due to the unique delivery and therapeutic mechanisms employed in cell and gene therapeutic products, nontraditional and comprehensive bioanalytical testing strategies must be developed to demonstrate the CGT's safety and efficacy. The bioanalytical package for a CGT must be able to determine that the therapeutic protein is present and functional at the site of action, sometimes systemically. Because many gene therapies are delivered by a vector, usually a virus, the existence and emergence of anti-vector antibodies also should be monitored. Special consideration also should be given to pre-existing and emergent immunogenicity to the therapeutic protein, particularly in heavily pretreated disease populations. For example, patients with enzymatic deficiencies that have been treated with enzyme replacement therapy for long periods of time may have pre-existing antibodies to the therapeutic enzyme[9] Additionally, the host immune system may interpret the newly expressed protein as foreign in deficient patients and mount an immune response.

Approaches for Measuring Therapeutic Protein Levels

Creating a pharmacokinetic (PK) profile of a CGT is a complicated undertaking. The bioanalytical strategy for therapeutic protein pharmacokinetics or exposure does not follow the normal pharmacokinetic model that is used for traditional small and large molecule drugs. For example, the translated products of transgenes only may be expressed at the site of action, so the bioanalytical strategy must be able to detect the expressed protein at the site of action and/or systemically, depending on the specific therapeutic. Additionally, since the protein is constitutively expressed, exposure does not follow the typical elimination phase. Instead, exposure is monitored for persistent expression of the functional replacement protein or enzyme. Adsorption, distribution and metabolism also have unique profiles from conventional biologics and small molecules. There are additional considerations for the delivery vehicle in many cases, e.g., for viral delivery vectors the vector copy number, biodistribution and functional gene insertion must be monitored.

Determining the ideal matrix to use to identify changes in the level of the therapeutic protein is an important component of the bioanalytical program. This may require expressed protein monitoring in multiple matrices (e.g., serum and cerebrospinal fluid) and may even necessitate monitoring of protein levels in human tissues from biopsy of accessible sites such as skin or muscle. With the advent of precision medicine, these procedures also must be minimally invasive for the patient in question, particularly if a repeat biopsy is required. For gene therapies that result in expression of enzymes, the instability of proteins in collected matrices may require special considerations for sample handling, including rapid collection at the clinical site, reduction of freeze-thaws, addition of stabilizing excipients to collection tubes, minimization of thaw time and thawing on ice. Therefore, investigation of the impact of upstream processing steps prior to arrival of samples at the bioanalytical lab may increase in importance as compared to more standard pharmacokinetic testing of antibody therapeutics in patient serum samples.

In the case of gene therapies targeted to rare disease applications, the patients at enrollment may have little to no protein expression and after treatment the protein may be many orders of magnitude higher. This necessitates using a method that is highly sensitive, sometimes to the pg/mL range, and has a wide dynamic range to fully characterize the patient response to treatment. The preferred methodology for protein PK assays is the immunoassay format. Immunoassays are simple to design, have readily available reagents and can be easily adapted to be automated to reduce operational changes over time and to enable high-throughput analysis. Immunoassay design will need to consider whether the antibody pairs can properly discriminate between truncated or other nonfunctional proteins that may be present, for which options such as enzyme-linked immunosorbent assay (ELISA), meso scale discovery electrochemiluminescence (MSD-ECL) and Luminex assays are all available. Ultra-sensitive immunoassay platforms can be evaluated when designing a protein PK method to maximize sensitivity and dynamic range.

Many gene therapies involve dosing of the components to express proteins with similar endogenous counterparts, which adds complexity to protein PK immunoassay design. The presence of endogenous material can complicate assay design: the calibrator must be prepared in matrix that has been stripped of the endogenous protein, a laborious and variable process, or the calibrator must be prepared in buffer/assay diluent. One common tactic is to utilize a standard curve made in buffer/assay diluent with a corresponding recombinant protein, produced either in-house or obtained from a commercial source. To confirm the appropriateness of the recombinant protein as a calibration material, specific assay validation experiments are recommended. The assay design strategy also should ensure that the capture and detection antibodies used are able to suitably detect both the recombinant and endogenous proteins. An assessment of endogenous quality control sample (QC) precision should be performed to ensure that the assay has

acceptable precision for recombinant and endogenous material. To assess the parallelism of the recombinant and endogenous material, endogenous QCs are prepared from samples from healthy individuals and are analyzed at multiple dilutions to determine which dilutions return consistent (between dilution) results. When endogenous protein levels are low in comparison to the calibration range, calibrator protein can be added to endogenous QCs to assess the accuracy of the full quantitation range of the calibration curve in matrix. Alternatively, QC samples can be prepared by adding recombinant protein to the assay buffer to assess calibrator curve accuracy. An additional endogenous QC sample can be added in this case to represent actual study samples.

In addition to determining the presence of protein, the function of the protein also must be assessed in the case of gene therapy applications. The gold standard for determining drug efficacy is clinical outcome, but a comprehensive bioanalytical package also should inform on the mechanism of action of the therapeutic. In the case of many gene therapies, this is accomplished by using pseudo-PK/PD (pharmacodynamics) functional assays, e.g., enzyme activity assays or bioassays. Functional assays are most informative when paired with protein quantification assays because functional assays may have greater assay variability and reduced dynamic ranges. These factors can reduce the granularity of the data produced and may reduce the ability to detect subtle differences between treatment groups. Additionally, enzymatic activity is often more prone to instability in patient samples, as compared to protein levels. Enzymatic assays can be validated with a similar approach to the endogenous QC strategy described previously and should use a mixture of recombinant enzyme and/or endogenous QCs to monitor potential shifts of the assay performance from day to day due to environmental conditions.[10]

In the case of cell therapies like CAR-T, options for quantitating the number of therapeutic cells in the body include flow cytometry and quantitative polymerase chain reaction (qPCR). Recently, digital PCR (dPCR),[11] including droplet digital PCR (ddPCR),[12] has been used for patient samples to quantitatively assess CAR-T levels with high sensitivity and precision. These methodologies allow for assessment of persistence of CAR-T therapeutics over time. Although dPCR and ddPCR report absolute values and do not rely on calibrators or standards for quantitation, the lack of a standard CAR-T reference material has complicated analysis for cell-based therapies.[13]

Assays using qPCR- and ddPCR-based technologies have been successfully used to monitor both biodistribution and viral shedding in CGT therapies.[14] These assays allow for the detection and quantification of viral insertion and integration within the tissues of the target organism or patient, resulting in a vector copy number (VCN), which is the average number of integrated copies of the target gene within each diploid cell. These integration events risk aberrant inactivation of necessary genes or expression of unwanted genes. To safely monitor the integration of virus within a study, integration site analysis (ISA) is typically performed using an unbiased approach through next-generation sequencing (NGS).[15] ISA both confirms the presence of the target gene within the cell or tissue of interest and identifies the location of the integration event. Together, these strategies can monitor the quantity and location of a therapeutic driven by lentiviral and AAV vectors.

Additional Assessments: Safety and Immunogenicity

Detection of introduced or newly expressed proteins or cells is only one component of the overall bioanalytical package for CGTs. Depending on the specific product and its applications, safety and immunogenicity also will need to be assessed as part of the clinical bioanalytical package.

For CAR-T cell therapies, a biomarker-based approach is important to monitor safety risks. For example, patients receiving CAR-T cells are at risk of CRS (cytokine release syndrome), an overreaction of the immune system. The use of assays to detect C-reactive protein, as well as inflammatory cytokines, can monitor for CRS development. These assays also can determine the risk of CRS development in CAR-T patients.[16]

Immunogenicity assessments also are essential to CGT programs. In addition to the standard anti-drug antibody (ADA) immunogenicity screening, pre-existing antibodies to the vector and expressed protein also must be characterized before treatment initiation and throughout the study. The viral vectors commonly used for CGT are adeno-associated viruses (AAV), adenoviruses (AdV) and lentiviruses (LV). Due to the widespread natural occurrence of these viruses, patient immunogenicity to the viral vector must be assessed prior to patient enrollment to ensure the therapeutic can be delivered to the target site and to assure a therapeutic benefit.[17,18] Many CGT studies include viral vector immunogenicity screening as part of their inclusion screening process, so patients with high levels of pre-existing antibodies may be excluded from the study or have their dose adjusted or supplemented with empty capsid to ensure target delivery. For example, cell-based assays are regularly used for assessing the presence of neutralizing antibodies to AAV delivery vectors. Viral immunogenicity also must be monitored during the conduct of the study to determine if adverse events or low efficacy is due to emergent immunogenicity.

Viral immunogenicity assays, monitoring B-cell mediated immunogenicity, usually have a format similar to ADA immunogenicity assays; they generally are bridging or sandwich immunoassay formats. The challenges of designing a viral immunogenicity assay include reagent acquisition and viral strain cross-reactivity. Reagents usually can be commercially obtained, but nonstandard viral strains and a positive control antibody with sufficient sensitivity and specificity may need to be custom produced since they may be difficult to obtain. Additionally, viral vectors with a reporter gene insert (i.e., luciferase) will be required for Nab assays. It should be noted that poor transfection efficiency resulting in a high degree of empty capsids and subsequent poor reporter gene expression can have severe negative effects on the precision and sensitivity of AAV-based Nab assays. Sponsors should investigate the availability of reagents early in the drug development process and begin contracting or producing high-quality reagents early on. The presence of pre-existing AAV antibodies in the population caused by environmental exposure to related viruses can

complicates assay development.[18] These complications may require extensive prescreening of negative individuals for cut point determination and/or negative control pool preparation.

The second piece of B-cell mediated immunogenicity for CGT concerns antibodies against the expressed protein. Patients in CGT trials may be heavily pretreated, particularly in cases where there is an approved enzyme or protein replacement product on the market. Immunogenicity needs to be evaluated at enrollment and throughout the study. Populations with a high occurrence of pre-existing antibodies may need alternative statistical strategies to detect and characterize treatment-emergent immunogenicity such as determining fold-change in ADA titer after treatment.[19]

The analytical approach to protein immunogenicity usually consists of standard immunoassay bridging or sandwich assay formats. Some challenges to method development include obtaining adequate quantities of quality protein stocks, labeling non-antibody proteins and obtaining sufficiently sensitive positive control antibodies. Additionally, protein interference and protein binding partner interference may be more difficult to engineer out of the assay, so nontraditional methods of dissociation or target depletion may need to be employed, such as heat treatment or immunoprecipitation sample treatments.[20]

ELISPOT (Enzyme Linked Immunospot) assays, monitoring T-cell immune response, detect specific cytokines or antigen-specific antibodies as well as the frequency of secreting cells that occur at low frequencies and can best be detected using this sensitive assay technique. This assay can detect cytokine or effector molecule secretion at the single cell level and is more sensitive than the ELISA or intra-cellular staining techniques. While it requires isolation of PBMCs or other cell subsets, it is able to be automated, allowing for high throughput screening. This becomes useful in gene therapy when, for example, an AAV-based gene transfer anti-capsid T cell response could eliminate transduced cells and would need to be monitored.[21]

Flow cytometry monitors the cellular kinetics of infused cells in adoptive cell therapy, such as in CAR-T cells. This is important for evaluating *in vivo* post antigen exposure-related expansion and persistence of the infused cells. Flow cytometry can also immunophenotype panels of multiple cell types often monitored in cell therapies. One such example is the T-cell lymphocyte phenotypic panel, which can monitor regulatory markers, activation, memory or proliferation.

In the case of cell therapies, immunogenicity assessments consider the presence of ADAs, both pre-existing and treatment-emergent, against key expressed proteins on the surface of the CAR-T cells. In designing a bioanalytical approach, research teams must decide on the specific target proteins on the CAR-T cell for ADA development and consider whether expressed, soluble protein or cell-linked protein will be used for assay development and validation.

Summary

While many commonalities are present in assay types between CGT programs and other large-molecule bioanalysis, the distinct features of cell and gene therapies require attention to a variety of bioanalytical challenges, including assay sensitivity, dynamic range, analyte stability, pre-existing antibodies, performance of endogenous vs. recombinant material, cell product and vector (viral or LNP) heterogeneity, and the need to integrate multiple analytical platforms. Therefore, early assessment of analytical needs, as well as technical capabilities, is important to ensure that a bioanalytical package is produced that can accurately characterize the therapeutic product's exposure, mechanism of action, efficacy and safety.

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