Innovative Laboratory Testing Methods for Clinical Monitoring of Cell Therapies Using Flow Cytometry ddPCR and qPCR Assays

Gene-modified cell therapy for the treatment of cancer and other diseases is a rapidly growing area of clinical research. The therapeutic use of engineered cells has necessitated the development of novel lab tests for assessing patient safety and efficacy. We'll explore how unique applications of PCR and flow cytometry can be used to provide critical and complementary data that is needed to bring new life saving treatment options to patients.

Cell therapy involves employing human cells to treat genetic diseases, cancer and infectious diseases; the manipulated material can either be from the patient's own cells (autologous) or from a donor (allogeneic). Gene-modified cell therapies involve engineering the donor cells using customised lentiviral or retroviral vectors that have been altered to remove most of the viral genes. The altered viral vectors allow a functioning human gene to be integrated into the genome of the donor cells.

What is CAR-T?

A common gene-modified cell therapy is chimeric antigen receptor T cell therapy (CAR-T), a progressive new immune cell therapy used to treat cancer. CAR-T cells are engineered *ex vivo* to express a functioning receptor that recognises tumour antigens, resulting in attack and destruction of the tumour cells. Following the gene modification and manufacturing process, the CAR-T cells are infused into the patient. Several CAR-T therapies have been approved for the treatment of haematological malignancies.

Biomarkers of CAR-T Response

Since its development, CAR-T therapy has transformed the treatment landscape for haematological malignancies. As with any novel treatment, CAR-T therapy isn't without its challenges. Not all patients respond to treatment. Moreover, the duration of response to CAR-T therapy can be variable. Various factors could be potential predictors of response and the duration of remission such as the patient age, tumour burden and CAR-T dose. More recently the cellular kinetics of the CAR-T cells during the first 3-6 months have been shown to be promising potential biomarkers of response. As a "living drug", CAR-T cells exhibit the phases of distribution, expansion, contraction, and persistence following administration. Measuring the cellular kinetics of a "living drug" requires novel assay designs.

Tools to Monitor CAR-T Expansion and Persistence – PCR and Flow Cytometry

Blood and/or bone marrow samples are taken at frequent timepoints following the infusion of the CAR-T therapy. These samples are used to assess the amount of CAR-T cells that are in circulation, i.e. whether the CAR-T cells are proliferating and persisting. One tool or methodology used to monitor the level of CAR-T cells in circulation is polymerase chain reaction (PCR). Here at ICON Laboratories, we have developed multiple custom PCR-based assays for various CAR-T and other gene-modified cell therapy programs, for use in baseline assessments and pharmacokinetic (PK) monitoring of the cell therapy product.

Using DNA extracted from the blood or bone marrow samples, both real time quantitative PCR (qPCR) and droplet digital PCR (ddPCR) can be used to amplify and quantify a specific segment of DNA. For the purposes of monitoring the kinetics of CAR-T cells, PCR-based vector copy number (VCN) assays are designed to detect, amplify and quantitate specific vector sequences that are unique to the cell therapy product.

qPCR requires the use of calibrators and a standard curve to calculate copy numbers of the targeted sequence. Unlike qPCR, ddPCR offers absolute quantitation without the need for calibrators and is ideal for VCN analysis. ddPCR is also more sensitive than qPCR, which improves the precision and reproducibility of quantification of lower levels of therapeutic cells. The custom ddPCR assays are a multiplex design, allowing for the detection of the target vector sequence and a reference gene sequence in the same reaction.

One challenge with the detection and quantification of therapeutic cells using VCN assays is especially notable when testing samples that are collected within the first 3 months post-infusion. Patients must undergo a short course of chemotherapeutic treatment prior to receiving the CAR-T cell infusion. The purpose of the conditioning, aka lymphodepletion, is to reduce the number of immune cells in the patient's body in order to increase the chances of survival of the therapeutic T cells.

The quantity and quality of the DNA extracted from blood and bone marrow samples that are collected in the first few months following conditioning is typically low. The overall DNA yield is low due to lower than normal cell counts and the DNA can be fragmented due to apoptosis caused by the chemotherapeutics. Therefore, our researchers have developed and implemented different strategies to overcome the sample quality issues that are seen at this early, critical timepoints. First, efficient and reliable extraction methods have been validated to maximise DNA yields from blood or bone marrow samples. Second, the DNA is quantified using Tapestation or Qubit methods rather than Nanodrop. These methods are better suited for samples with a lower DNA concentration, and they enable a more accurate calculation of expected reference gene copies in the sample. Third, guidelines for reporting results have been developed based on the limit of detection (LoD), lower limit of quantification (LLoQ) and the expected reference gene copies in the sample. This approach maximises the number of data points that can be reported, allowing the drug sponsor to gain a more complete picture of the therapy's cellular kinetics in each patient. Finally, experiments are performed during assay validation to determine the selectivity (recovery) of the target using samples that are created to

simulate the lymphodepleted timepoints. These experiments also allow the determination of the lowest possible sample DNA input that can be used without compromising the safety or the reliability of the data.

While PCR methods can be used to detect and quantify vector DNA sequences that are found in the therapeutic cells, flow cytometry can be used to detect, quantify and subtype the therapeutic T cells at a cellular level based on the presence of cell-specific proteins.

Flow cytometry is a laboratory tool that is used to identify and measure the physical and chemical characteristics of cells or particles. The flow cytometry platforms that we use allow for up to 25-colour multiplexing. This capability enables comprehensive profiling of the CAR cells; measurement of CAR expression as well as the expression of other T cell subset-specific protein markers such as CD4 and CD8. Detailed information about the CAR-T cell subsets that are proliferating and persisting in the first 3–6 months provides additional information that could be critical for predicting overall patient response that cannot be obtained using PCR methods. High parameter panels can also be designed to characterise the cancer cells as well as the patient's own immune cells, providing additional data related to efficacy and response.

The reagent of use in flow cytometry to detect cellular proteins is critical to the success of an assay. There are two reagent approaches: generic or specific. Generic reagents such as a protein L, enable the researcher to bind to the LG light chain and detect multiple CARs. CAR specific reagents are preferable because they give precise results and low background; however, the availability of these reagents is limited and more costly.

As with any flow cytometry assay, the panel design should be thoroughly evaluated to avoid spectral overlap with the CAR detection reagent, which should have a bright fluorophore. A panel designed to monitor the therapeutic CARs and the patient's own cell subsets cannot use CD19 protein for CAR detection and anti-CD19 antibodies on the same panel because they will bind and interfere with the study's results.

The selection and creation of an appropriate positive control must be discussed with the trial's sponsor. It is preferable to use stably expressing CAR cell lines and Jurkat cells in the case of a CAR-T cell, both of which take time; however, they are critical to the development of the assay. As a negative control, researchers may consider using a control cell line or a mock-transfected cell line.

Conclusion

The rapid and continued growth of gene-modified cell therapies shows great promise for those suffering from both haematological and solid tumours. To support that promise, biomarker assays will be needed to monitor cellular kinetics and safety, and patient selection assays may be needed for therapies designed to attack cancer cells with more unique targets or to identify other predictive biomarkers of response.

Dr. Chad Galderisi

Dr. Galderisi currently serves as Vice President and Global Medical Director for ICON Specialty Laboratories. He has over 15 years of experience in diagnostic surgical and molecular pathology including the oversight of laboratory medical device testing for the FDA submission and approval of multiple companion diagnostics. Dr. Galderisi completed his residency in Anatomic Pathology at Penn State/MS Hershey Medical Center and a fellowship in Molecular Genetic Pathology at Oregon Health and Science University. He is licensed to practice medicine in California, Washington, Oregon, Massachusetts, and New York states.