Bioanalytical strategy to support novel generation CAR-T therapies

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Overview

• Evolution of Therapeutics: from Aspirin to CAR-T therapies
• Brief introduction to CAR-T therapies
• Safety concerns and mitigation strategies regarding CAR-T therapies
• Bioanalytical strategies for support of CAR-T programs
  – Next-Generation CAR-T
Evolution of Biologicals

With the increasing complexity of therapeutic modalities comes a need for more sophisticated bioanalytical assays for pharmacokinetic and immunogenicity assessments.

<table>
<thead>
<tr>
<th>Level of Complexity</th>
<th>Modality</th>
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<tr>
<td>Aspirin</td>
<td>ADC</td>
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<td>Insulin</td>
<td>Bi-/Tri-specific</td>
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<td>EPO</td>
<td>Scaffolds</td>
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<td>mAb</td>
<td>Gene Therapy</td>
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What is **Cancer Immunotherapy?**

- Consists of multiple approaches that **focus on harnessing and enhancing the powers of the immune system** to destroy tumor cells and to provide a durable antitumor immune response.
  - Vaccines, checkpoint modulators, adoptive T cell transfer therapy, cytokines/immunomodulation agents, monoclonal antibodies, oncolytic viruses, **cell-based therapies**

- Class of patient-specific therapies involves **collecting immune cells from a cancer patient**, engineering them to **recognize and kill cancer cells**, growing large numbers of these, and reintroducing the into the same patient.
What is a Chimeric Antigen Receptor (CAR) T?

CAR-T-cell therapy takes its complicated name, in part, from a fire-breathing monster in an ancient Greek myth.

- In Greek mythology, the Chimera was a fire-breathing female monster comprising parts of different animals—a lion’s head, a goat’s body, and a serpent’s tail.
- The term "chimera" has come to describe anything composed of very disparate parts.
- A CAR-T cell is a T cell that has been genetically engineered to express an antigen-specific, non-MHC restricted receptor, composed of the scFv of an antibody fused to a transmembrane domain and an intracellular signaling domain.
Chimeric Antigen Receptor T Cells

- First generation contained only the CD3ζ signaling domain
- Second generation contain an additional costimulatory signaling molecule, such as 4-1BB, CD28, CD27, OX40, ICOS or RIAD
- Third- and fourth-generation contain two or more signaling domains
The application of CAR-T cells has achieved excellent clinical results in cancer patients, especially those with CD19-positive hematologic malignancies.

In August 2017, the FDA approved the use of CART19 (Kymriah) to treat pediatric relapsed or refractory acute lymphoblastic leukemia.

In October of the same year, another CD19-targeting CAR (Yescarta) was approved by the FDA for adult relapsed or refractory large B cell lymphoma.

The EMA also approved the use of both these drugs in June of 2018.
CAR-T Therapy Limitations & Toxicities

• Though CAR-T-cell therapy has demonstrated incredible success with certain hematologic cancers, **challenges still remain to successfully treat patients with solid tumors.**

• CAR-T cell therapies are challenged by **the complexity of their production** and by the adverse events related to their activity.
  – On-target Off-tumor, **cytokine release syndrome**, anaphylaxis, and neurotoxicity

• Patients treated with **tocilizumab (anti- IL-6R)** or corticosteroids to block CRS.

• Management of neurotoxicity remains difficult.
  – IL-1 produced by activated macrophages plays an **important role in the pathophysiology of CAR-T (IL-1 blockade)**

• Important to find a safe TAA, given the possibility that even low levels of the target antigen on normal tissues can result in significant toxicity.
Bioanalytical Assays to Support CAR-T Cell Therapies
Regulatory Landscape for CAR-T Therapies

• Guidelines / guidance's / recommendations on the conduct of studies supporting new therapeutics and on the parameters to be assessed:
  – Guideline: Minimum information for publication of quantitative real-time PCR experiments (2009)

• Opinion papers:
Bioanalytical Challenges

- Purification of T cells
- *Ex vivo* stability of T cells
- Successful gene transfer
- Lot to lot variability of CAR-T cells
- Measuring absolute dose
- Measurement of cellular kinetics
- Availability of CAR-specific and cell phenotyping antibodies
- Lack of patient and control samples for assay development
- Monitoring adverse effects
- Long time period for patient monitoring
- Lack of regulatory guidance
Technologies and Reagents

- Quantitative PCR and Droplet Digital™ PCR
  - DNA is stable, assays are sensitive, surrogate measurement for presence of CAR
- High-throughput, multi-parameter flow cytometry
  - Direct measurement, accurate quantification and phenotypic analysis of CAR-T cells, real-time immune cell monitoring in vivo, supports qPCR data for PK
- Customized anti-idiotypic antibodies
  - Highly specialized critical regent to determine the percentage of transfected T cells prior to administration, differentiate CART-T cells from other cells in patient samples
- Multiplex immunoassay analysis of cytokines and chemokines
  - Identification of severe responses, such as cytokine release syndrome, crucial for patient monitoring and treatment
Bioanalytical strategies: Cellular Kinetics for CAR-T

- Cellular kinetic **assays to assess CAR-T cell presence, frequency and persistence** in peripheral blood, bone marrow, additional tissues as deemed necessary

- **qPCR**
  - highly sensitive assay which **quantifies the number of integrated CAR transgenes** in a population of cells
  - Number of **CAR vector copies /µg of sample DNA** or vector copies/ ml of blood sample
Bioanalytical strategies: Cellular Kinetics for CAR-T

- Cellular kinetic assays to assess CAR-T cell presence, frequency and persistence in peripheral blood

- Flow cytometry
  - Measures CAR expression on T cells using anti-CAR antibody staining
  - Number of CAR positive T cells/mL of blood or matrix cells
Bioanalytical Strategies: **Humoral immune response**

- Methodologies to detect ADA have been well established and can be applied to the CAR protein or other components of the CAR-T construct

  - **Flow cytometry to detect antibodies directed against the scFv of the CAR in human serum**
    - Require transduced cells with a vector containing an anti-target CAR or mock transduced cells
    - Identify CAR transduced cells by the bound anti-CAR surrogate positive control antibody
    - Identify a screening cut point using individual disease specific human serum samples

- **Humoral Immunogenicity FACS**
  - FDA recommendation: **100 ng/ml**
  - This FACS assay: **15 ng/ml**
Cellular Immunogenicity Assessments

- ELISpot assay is a **very sensitive immunoassay**, allows for the detection of secreted analyte at the single cell level.
- Require sensitive tests for presence of CAR-specific T cell responses since these have the potential to lyse CAR-T cells.
- Isolate and stimulate PBMC using peptides pools derived from CAR construct.
- **Measure cytokine production** (ELISpot, Immuno-staining, cytokine release assay)
- Aim to **quantitate T-cell activation** (e.g. IFNγ producing CAR-specific CD4+ & CD8+ T cells increase)

- **Positive control stimulus**: PHA
- IFN gamma secretion by T cells: for T cell activation
- **Granzyme B**: correlates with CD8+ cell stimulation and cytotoxicity
  - Serine protease most commonly found in the granules of cytotoxic lymphocytes, natural killer cells, and cytotoxic T cells.
  - Secreted by these cells along with the pore-forming protein perforin to mediate apoptosis in target cells.
Example of a BA strategy for a next generation CAR-T
Next Generation CAR-T Therapies

IL-7 and CCL19 expression in CAR-T cells improves immune cell infiltration and CAR-T cell survival in the tumor

Keisai Aoiuchi, Yosuke Kano, Tomohiko Nagai, Numiko Okuyama, Yukimi Sakoda & Keji Tsumada

- The scFv was fused with the transmembrane domain of CD8, cytoplasmic regions of CD28, 4-1BB and CD3
- Constructing a third-generation CAR
- Which was then cloned into the retroviral vector MSGV1
**Critical Reagents Required**

<table>
<thead>
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<th>Proprietary Reagent</th>
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<tr>
<td>Human T cells transduced with the CAR construct or Jurkat cells transduced with the CAR construct</td>
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<tr>
<td>Antibody against CAR (polyclonal or various monoclonals against various parts of the protein)</td>
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<tr>
<td>Proprietary antibody against CAR</td>
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<tr>
<td>Purified CAR extracellular domain (or full protein)</td>
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<tr>
<td>Antibody against 2A peptide</td>
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<tr>
<td>Purified recombinant IL-7 and CCL19 (plus additional 2A sequence if used in construct)</td>
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<tr>
<td>Purified recombinant fusion (tandem) protein: IL-7+CCL19 + 2A peptide sequence between the genes</td>
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Exposure to IL-7 and CCL19

- In addition to the exposure assessments described previously

- Measure systemic (total) IL-7 and CCL19
  - Assays must detect both forms: endogenous and encoded by the CAR-T
  - MSD and ELISA commercial kits available

- Assays to specifically detect the CCL19 and IL-7 forms secreted by the CAR-T may be necessary, including peptide 2A
Humoral Immunogenicity: Anti-CAR Ab assay format: CBA or LBA?

CBA Format Analyzed

• Transduced and mock transduced human T cells
• CBA allows the detection of ADAs against scFv.
• Mock transfected as control, otherwise use non-transduced cells.

LBA Format Analyzed

• From informal discussions with scientists in the CAR-T field, this format could be acceptable by HAs
  – **Prerequisite 1**: the cellular drug must be autologous, i.e. the patient’s own T cells are collected, transduced and reinfused. In this case, the risk of immune reaction against all the T cells (except the CAR construct) is extremely limited.
  – **Prerequisite 2**: the purified recombinant CAR (extracellular domain and mb-anchoring structure) is available and soluble.
Anti-IL-7 and anti-CCL19 Antibody Assays

- IL-7 and CCL19 are produced from a tandem polypeptide containing a 10-20 AA-long 2A peptide sequence between the proteins.
- Anti-IL-7 and anti-CCL19 assessments are recommended to monitor patient’s ADA formation:
  - against endogenous cytokines
  - against the new epitope created by the additional 10-20AA 2A peptide
- Anti-IL-7 and anti-CCL19 commercial reagents could be used to start with to demonstrate feasibility
- Need of NAb assay if anti IL-7 and/or CCL19 Ab are detected
Cellular Immunogenicity Assessments
Cellular Immunogenicity Assessments

T cell response against CAR-T construct using ELISPOT

- Stimulate frozen PBMC with 2 or more peptides pools
- Potential to use CAR-T cell line
- One (or more) pools containing only extracellular sequence (most likely to induce a response)
- One (or more) pools containing the corresponding intracellular sequence (plus transmembrane part)

- Positive control stimulus: PHA
- IFN gamma secretion by T cells:
- Granzyme B: correlates with CD8+ cell stimulation and cytotoxicity
  - Serine protease most commonly found in the granules of cytotoxic lymphocytes, natural killer cells, and cytotoxic T cells.
  - Secreted by these cells along with the pore-forming protein perforin to mediate apoptosis in target cells.
Specific T Cell Response: **Immune Cell Phenotyping**

- Quantify if there is an **increase in certain immune cell populations after treatment** as compared to pre-dose
- Technique: **Flow cytometry on whole blood**
- Separate CD4+ and CD8+ cells by **MACS® Cell Separation**

**Immune cell populations:**
- CD3+T cells
- CD4+ T helper cells
- CD8+ T cytotoxic cells
- CD127+ (and possibly FOXP3+) T reg cells
- HLA-DR+ activated T cells

**Cytokine Release Assays**
- Full blood cytokine assay
- Fresh/frozen PBMCs
- Cultured PBMCs
- Cell Culture
Level of Validation of Assays

✓ Validated assays are always recommended.
  – Precision, sensitivity, robustness
  – Population-specific matrix effect
  – Cut-point assessments

✓ Even more for advanced therapies because if CAR-T has spectacular results, the sponsor may want to seek accelerated approval with FIH data.

✓ This is an additional advantage to use validated assays from the beginning.
Future of **CAR-T Therapies**

- The unique challenges posed to CAR T cell therapy by solid tumors can be described in three steps: **finding, entering, and surviving in the tumor.**
- Use of dual CAR designs that recognize **multiple antigens at once and local administration of CAR-T cells** are both strategies that have been used to overcome the hurdle of localization to the tumor.
- **Combining CARs with checkpoint blockade or depletion of other suppressive factors** in the microenvironment has shown very promising results to mitigate the phenomenon of T cell exhaustion.
- **Identifying and overcoming mechanisms associated with dysfunction in CAR-T cells** is of vital importance for generating CAR-T cells that can proliferate and successfully eliminate tumor cells.
- **TALEN and CRISPR/Cas systems** modify the T cell genome enabling the knock-out of negative T cell regulators and knock-in of transgenes.
• Significant research has been done with CAR T cells to identify target antigens, avoid toxicity, improve CAR T cell trafficking and entry into the tumor site, and promote better signaling, less exhaustion, and memory phenotypes in solid tumors.

• Early identification of patients at high risk of severe CRS is imperative.

• Improving the CAR-T production platform of CAR T-cell therapy is also an important issue to enable patients to access this treatment more easily.

• Modern genetic technologies such as CRISPR/Cas9 and synthesis of large custom gene constructs enables significant manipulation of CAR T cells to improve their cytotoxicity, persistence, and safety.

• With the development of more complex CAR-T therapies will come a need for more sophisticated bioanalytical methodologies to ensure the efficacy and safety of these promising cancer therapeutics.

• CAR T cells have revolutionized the field of tumor Immunotherapy.

• CAR-T are personalized advanced therapies that require personalized advanced bioanalytical packages tailored to the specifics of each CAR-T therapeutic.

• Off-the-shelf CAR-T bank to reduce the production waiting time; UCART19.
Thank You!

Merci

Grazie

Danke

お世話になりました

감사합니다

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Gracias

Efharisto

Tack

 obrigado

Спасибо
Back up slides
Future of **CAR-T Therapies**

- To target intracellular antigens with CAR-T cells, *investigators showed success in an* *in vivo* *myeloma study with a CAR / TCR hybrid* that recognized the antigen NY-ESO-1 in the context of HLA-A2.

- These TCR-CARs were *shown to effectively bind an HLA-A2+ T cell artificially engineered to express NY-ESO-1.*

- TCR-CARs that recognize antigen in combination with MHC can recognize both extra and intracellular antigens in the way that wild-type or modified TCRs can.

- TCR-CAR composed of a soluble TCR directed against either the melanoma-associated antigen MART1 or TGFβR2 (a neoantigen peptide) joined to a CAR signaling component resulted.

- The result was a *versatile receptor that bound antigen in an MHC-I restricted manner,* but with signaling and killing similar to that of a CAR.

- This construct was *shown to be transduced not only into T cells but also into an NK cell,* with successful *in vitro* killing.
Future of CAR-T

- CubiCAR, a tri-functional CAR architecture that enables CAR T-cell detection, purification and on-demand depletion by the FDA-approved antibody Rituximab.

- This novel architecture has the potential to streamline the manufacturing of CAR T-cells, allow their tracking and improve their overall safety.

- Different molecular safeguards have been developed to address safety concerns
  - enable efficient on-demand depletion of engineered T-cells
  - display specific drawbacks including their size, potential immunogenicity and reliance on unapproved small molecules as activating agent
FIGURE 1: A representative figure of an armored 3rd generation CAR in a T cell and a schematic of the transgene, which includes the extracellular scFv, two intracellular costimulatory domains (4-1BB and CD28), the t-chain, a 2A linker, and the gene of interest to be coexpressed (61, 62). Examples of “armor” added to the CAR T cell are the CD28 receptor (63), which has been shown to increase T cell migration and homing to the tumor site (64, 65) or constitutive secretion of the cytokine IL-7 and chemokine CCL19, which are important to memory differentiation and T cell migration, respectively (66). CARs that constitutively secrete IL-12 have also been used in several studies to boost survival and cytokotoxicity (67). Also depicted is an example of an inducible suicide gene, tEGFR, which consists of the truncated transmembrane and extracellular portion of the EGFR protein. When targeted by the antibody Cetuximab, the receptor triggers apoptosis in the cell, providing a safety switch to protect against potential toxicity (68). Inducible caspase 9 (CS9) and HSV-TK are other common suicide genes that have been coexpressed with CARs.