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Original Article

Activation Testing of CAR T Cells: Methods, Biomarkers, Functional Assays and Clinical Relevance

Jasmine Jinugu
Independent Researcher, USA.

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Abstract *- Chimeric antigen receptor (CAR) T-cell therapy has revolutionized the management of relapsed and refractory hematologic malignancies [1,7,16,17]. Despite remarkable clinical success, substantial variability exists in manufacturing quality, in vivo expansion, persistence, toxicity, and therapeutic efficacy [2,3,5,9]. Activation testing represents a critical component of CAR T-cell characterization, enabling assessment of potency, functionality, safety, and clinical performance. Modern activation testing integrates phenotypic biomarkers, cytokine profiling, cytotoxicity measurements, proliferation assays, molecular analyses, and emerging multi-omics approaches [2,7,13,20]. Recent studies emphasize the importance of memory T-cell subsets, cytokine polyfunctionality, metabolic fitness, CAR T-cell persistence, and immune microenvironmental factors as predictors of clinical outcomes. Furthermore, increasing standardization efforts seek to harmonize analytical methods used from manufacturing through post-infusion monitoring. This review summarizes current methodologies for CAR T-cell activation testing, discusses key biomarkers and functional assays, examines clinical relevance, and highlights future opportunities for integrating advanced immunological and computational approaches into CAR T-cell quality assessment.*

Keywords *- CAR T Cells, Activation Testing, Biomarkers, Cytokines, Potency Assays, Flow Cytometry, Immunotherapy, Functional Assays.*

1. Introduction

CAR T-cell therapy combines cellular engineering with adaptive immunity by introducing synthetic receptors that redirect T lymphocytes toward tumor-associated antigens [1,4,12]. Since the approval of multiple CAR T-cell products targeting CD19 and BCMA, engineered T-cell therapies have become an essential treatment option for several hematologic cancers [16,17]. However, clinical responses remain heterogeneous, and the biological characteristics associated with successful treatment continue to be actively investigated [2,3,5].

Activation testing serves as a bridge between manufacturing quality control and clinical outcome assessment [2,13,20]. The ability of CAR T cells to recognize antigen, initiate signaling, proliferate, produce cytokines, and eliminate tumor targets determines therapeutic efficacy [1,6,15,18]. Consequently, analytical assays capable of measuring these functions have become indispensable throughout product development and clinical monitoring.

2. Biology of CAR T-Cell Activation

CAR T-cell activation begins when the extracellular antigen-recognition domain binds a target antigen on malignant cells [1,4,15].

Following antigen recognition, intracellular signaling domains initiate phosphorylation cascades that activate transcriptional programs responsible for cytokine production, proliferation, survival, and cytotoxic function [1,6,15]. Incorporation of co-stimulatory domains such as CD28 and 4-1BB significantly enhances expansion and persistence [6,15].

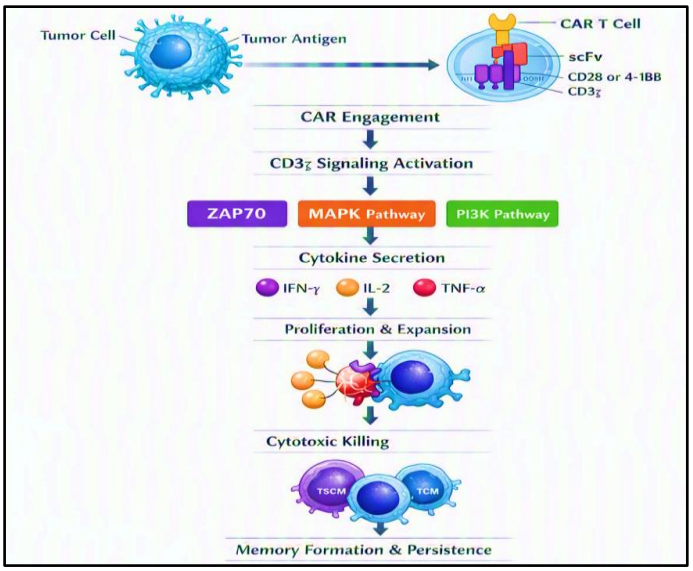

Fig 1: CAR T-Cell Activation Cascade

Important biological consequences of activation include [1,6,11,15]:

- Upregulation of activation markers.
- Cytokine secretion.
- Clonal expansion.
- Tumor-cell killing.

- Memory differentiation.
- Metabolic reprogramming.

Each of these processes can be quantified through dedicated activation-testing methodologies.

3. Role of Activation Testing

Activation testing addresses four major goals [2,7,13,20]:

- Potency Assessment: Verify functional activity
- Product Characterization: Define phenotype and quality
- Clinical Monitoring: Track expansion and persistence
- Safety Monitoring: Predict toxicity risks

Survey data from European CAR T-cell centers indicate significant variability in analytical approaches and support a need for assay harmonization and standardization [20].

4. Phenotypic Activation Biomarkers

Table 1: Major Biomarkers Used in CAR T-Cell Activation Testing

Biomarker	Function	Method of Detection	Clinical Significance
CD69	Early activation marker	Flow cytometry	Rapid assessment of antigen-specific stimulation [1,4]
CD25	IL-2 receptor α-chain	Flow cytometry	Indicates sustained activation [4,12]
HLA-DR	Late activation marker	Flow cytometry	Reflects persistent activation [13]
CD137 (4-1BB)	Co-stimulatory signaling	Flow cytometry	Sensitive marker of antigen engagement [15]
Ki-67	Cellular proliferation	Flow cytometry, IHC	Associated with expansion potential [2]
PD-1	Exhaustion marker	Flow cytometry	Chronic stimulation indicator [14]
TIM-3	Exhaustion marker	Flow cytometry	Functional impairment biomarker [14]
LAG-3	Inhibitory receptor	Flow cytometry	Associated with T-cell dysfunction [3]

Flow cytometry remains the most widely used platform for activation testing [7,13,20].

CD69

CD69 is an early activation marker appearing within hours after antigen engagement [4,12].

CD25

CD25 reflects IL-2 receptor upregulation and sustained activation [4,12].

HLA-DR

HLA-DR indicates prolonged activation and immune function [13].

CD137 (4-1BB)

CD137 serves as a sensitive marker of antigen-specific activation and is frequently incorporated into CAR T-cell characterization assays [15].

OX40

OX40 provides information regarding co-stimulation and survival potential [6,15].

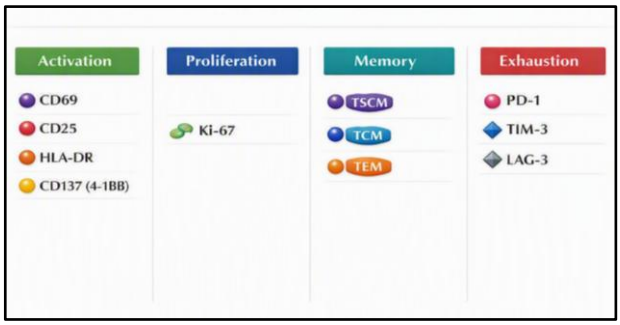

Fig 2: Flow-Cytometry Biomarkers

5. Memory and Differentiation Biomarkers

Recent evidence highlights the importance of memory T-cell subsets as predictors of therapeutic efficacy [2,3,5,10,11]. High proportions of central memory and stem-cell memory T cells are associated with favorable outcomes [5,10,11].

Important markers include:

- CD45RA
- CD45RO
- CCR7
- CD62L
- CD27
- CD28

Memory-associated phenotypes frequently demonstrate superior persistence and expansion following infusion [2,5,10,11].

6. Exhaustion Biomarkers

Persistent antigen stimulation may induce T-cell exhaustion [3,14].

Key markers:

Monitoring exhaustion-associated markers is increasingly incorporated into activation and potency-testing workflows [3,13,14].

7. Cytokine Biomarkers

Cytokine release is one of the most important manifestations of CAR T-cell activation [1,2,13].

Important cytokines include [2,5,8,13]:

- Interferon-γ (IFN-γ)
- Interleukin-2 (IL-2)
- Tumor Necrosis Factor-α (TNF-α)

- Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)
- IL-6
- IL-10

Table 2: Cytokine Biomarkers Used for Assessment of CAR T-Cell Function

Cytokine	Biological Role	Common Assay
IFN-γ	Effector cytokine promoting antitumor immunity	ELISA, ELISPOT, ICS
IL-2	T-cell proliferation and survival	ELISA, multiplex assay
TNF-α	Cytotoxic and inflammatory mediator	ELISA, ICS
GM-CSF	Myeloid-cell activation	Multiplex assay
IL-6	CRS-associated cytokine	ELISA
IL-10	Regulatory cytokine	Multiplex assay

Polyfunctional cytokine production involving simultaneous secretion of IFN-γ, IL-2, and TNF-α has been associated with enhanced CAR T-cell fitness and therapeutic efficacy [2,5,10].

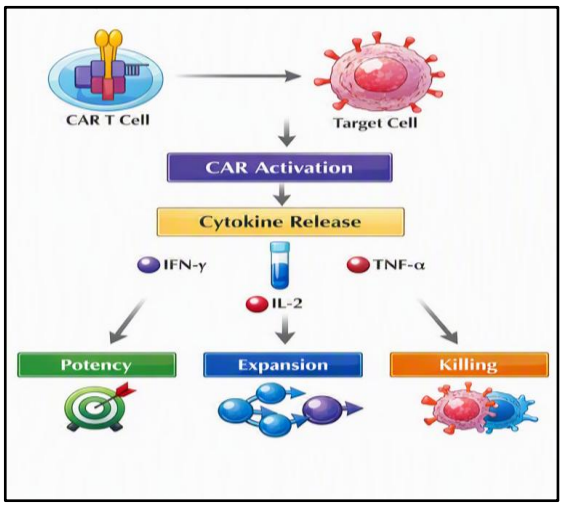

Fig 3: Cytokine-Response Biomarkers

8. Intracellular Cytokine Staining

Intracellular cytokine staining permits single-cell assessment of cytokine production [2,13].

Advantages:

- Multiparametric analysis.
- Single-cell resolution.
- Simultaneous phenotyping.
- Assessment of polyfunctionality.

ICS remains a cornerstone method for evaluating activation-related cytokine production [2,13,18].

9. Cytotoxicity Assays

Direct measurement of tumor-cell killing represents a critical potency endpoint [18,20].

9.1. Chromium-51 release assays

Historically considered the gold standard for measuring cytotoxicity [18].

9.2. Flow-Cytometric Killing Assays

Enable simultaneous evaluation of target-cell viability and CAR phenotype [18].

9.3. Luciferase-Based Assays

Provide sensitive and scalable measurements [18].

9.4. Real-Time Imaging Assays

Allow kinetic study of target-cell elimination.

9.5. Impedance-Based Platforms

Enable label-free monitoring of cytotoxicity. Standardization of functional potency assays remains an important unmet need across institutions [20].

10. Degranulation Assays

Degranulation reflects the release of cytotoxic granules [18].

CD107a Assay

CD107a mobilization serves as a surrogate marker of cytotoxic capability [18].

Additional markers include [18]:

- Granzyme B
- Perforin

Combined evaluation provides insight into killing competence.

11. Proliferation Assays

Expansion capacity strongly influences therapeutic success [5,10,11].

Common methods include:

CFSE Dilution

Measures cellular division through fluorescence dilution [13,18].

Cell Trace Violet

Provides improved long-term tracking.

Ki-67 Staining

Quantifies actively cycling cells [2,13].

Serial Restimulation Assays

Evaluate long-term proliferative potential. Persistence and expansion are among the most consistently reported correlates of therapeutic response [2,5,10,11]

Table 3: Functional Assays Used for CAR T-Cell Potency Testing

Assay	Principle	Advantages	Limitations
Chromium-51 Release	Radioactive target-cell lysis	Historical gold standard	Radioactivity requirements
Flow-Cytometric Killing	Direct viability measurement	Multiparametric	Complex data analysis
Luciferase Assay	Reporter-based killing	High throughput	Requires engineered targets
Impedance-Based Assay	Real-time cell monitoring	Label free	Specialized equipment
IncuCyte Imaging	Live-cell imaging	Kinetic information	Higher cost
CD107a Assay	Degranulation marker	Rapid functional assessment	Indirect cytotoxicity measurement

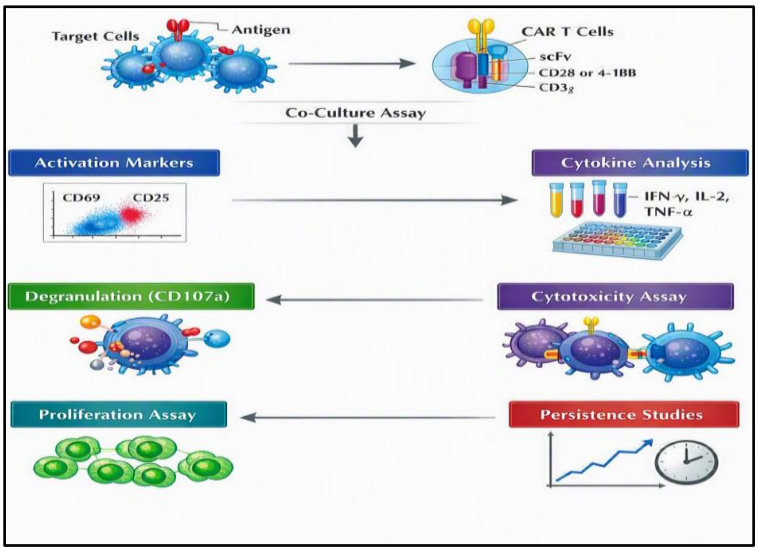

Fig 4: Functional Testing Framework

12. Molecular Activation Assays

Modern CAR T-cell characterization increasingly incorporates molecular technologies [2,3,5].

Methods include:

- Quantitative PCR
- Digital PCR
- RNA sequencing
- Single-cell RNA sequencing
- ATAC-seq
- Proteomics
- Metabolomics

Recent reviews highlight single-cell transcriptomics, proteomics, and high-dimensional immunophenotyping as promising approaches for identifying predictive biomarkers and improving manufacturing quality assessment [2,5].

13. Multi-Omics Biomarkers

Emerging multi-omics approaches integrate [2,3,5]:

- Genomics
- Transcriptomics
- Epigenomics
- Proteomics
- Metabolomics

These technologies provide a system-level understanding of activation and have enabled identification of favorable memory-associated and metabolically fit phenotypes [2,3,5,10,11].

Table 4: Emerging Omics-Based Activation Testing Technologies

Technology	Information Obtained	Application
Single-cell RNA sequencing	Transcriptional heterogeneity	Activation-state identification
Proteomics	Protein expression patterns	Potency biomarker discovery
Metabolomics	Cellular metabolism	Fitness evaluation
ATAC-seq	Chromatin accessibility	Epigenetic characterization
CyTOF	High-dimensional phenotyping	Immune profiling
Spatial transcriptomics	Tissue-level analysis	Tumor-microenvironment assessment

Recent reviews highlight single-cell transcriptomics, proteomic profiling, cytokine polyfunctionality analyses, and machine-learning-driven immune profiling as promising approaches for future biomarker discovery and CAR T-cell monitoring [2,5].

14. Clinical Relevance

Activation testing has direct implications for patient management [2,13].

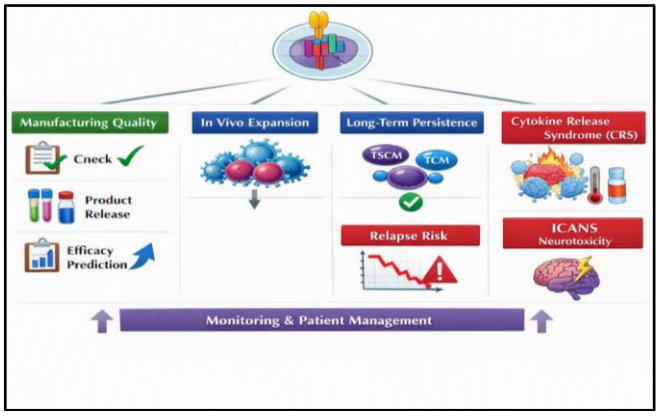

Fig 5: Clinical Relevance of CAR T -Cell Activation Testing

14.1. Efficacy Prediction

Biomarkers associated with positive outcomes include [2,3,5,10,11]:

- Early memory T-cell enrichment.
- Polyfunctional cytokine production.
- Strong expansion kinetics.
- Long-term persistence.

14.2. Relapse Monitoring

Reduced persistence and functional exhaustion may precede disease recurrence [5,10,14].

14.3. Manufacturing Optimization

Activation biomarkers help identify high-quality cellular products before infusion [2,5,11].

15. Toxicity Monitoring

Major toxicities include:

Cytokine Release Syndrome (CRS)

Associated with excessive inflammatory cytokine production [8,19].

ICANS

Immune Effector Cell-Associated Neurotoxicity Syndrome [8,19].

Predictive biomarkers continue to be actively investigated because severe toxicities remain a major limitation of CAR T-cell therapy [3,8,19].

16. Standardization Challenges

Despite rapid growth of the field, analytical methodologies remain highly variable across institutions [20]. The European multicenter survey identified substantial differences in characterization assays, potency testing, and post-infusion monitoring practices [20].

Key limitations include:

- Lack of assay harmonization.
- Variable reporting standards.
- Limited regulatory consensus.
- Inconsistent potency definitions.
- High costs of advanced technologies.

17. Future Directions

Future activation testing will likely incorporate [2,3,5]:

- Artificial intelligence.
- Machine learning.
- Digital biomarkers.
- Automated manufacturing analytics.
- Integrated multi-omics platforms.
- Advanced immunological synapse analysis.

High-dimensional immunophenotyping and machine-learning methodologies are increasingly being explored for automated quality control and biomarker discovery [2,5].

18. Conclusion

Activation testing is central to CAR T-cell development, manufacturing, clinical deployment, and long-term monitoring [2,7,20]. No single biomarker captures all aspects of CAR T-cell function [2,3,13]. Instead, comprehensive assessment requires integration of phenotypic markers, cytokine measurements, cytotoxicity assays, proliferation analyses, molecular profiling, and clinical monitoring [2,13,18,20]. Recent evidence points toward the importance of memory T-cell populations, cytokine polyfunctionality, persistence, and systems-level multi-omics biomarkers in predicting therapeutic efficacy [2,3,5,10,11]. Standardization of testing methodologies and incorporation of advanced computational approaches will likely define the next generation of CAR T-cell potency and activation assessment [2,5,20].

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