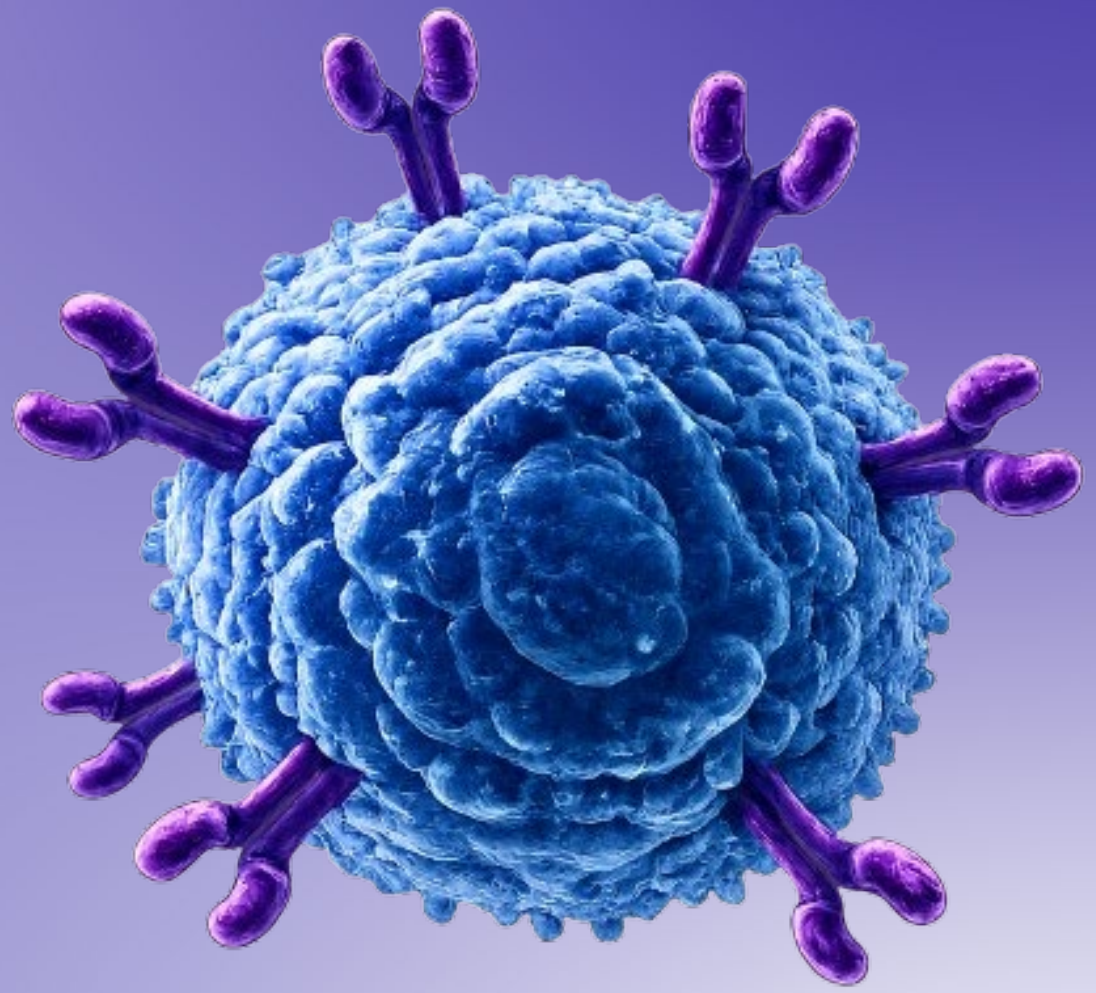
Decoding CAR-T Dynamics: Flow Cytometry-Driven Insights from Autologous and Allogeneic Trials

The “Living Drug” Revolution



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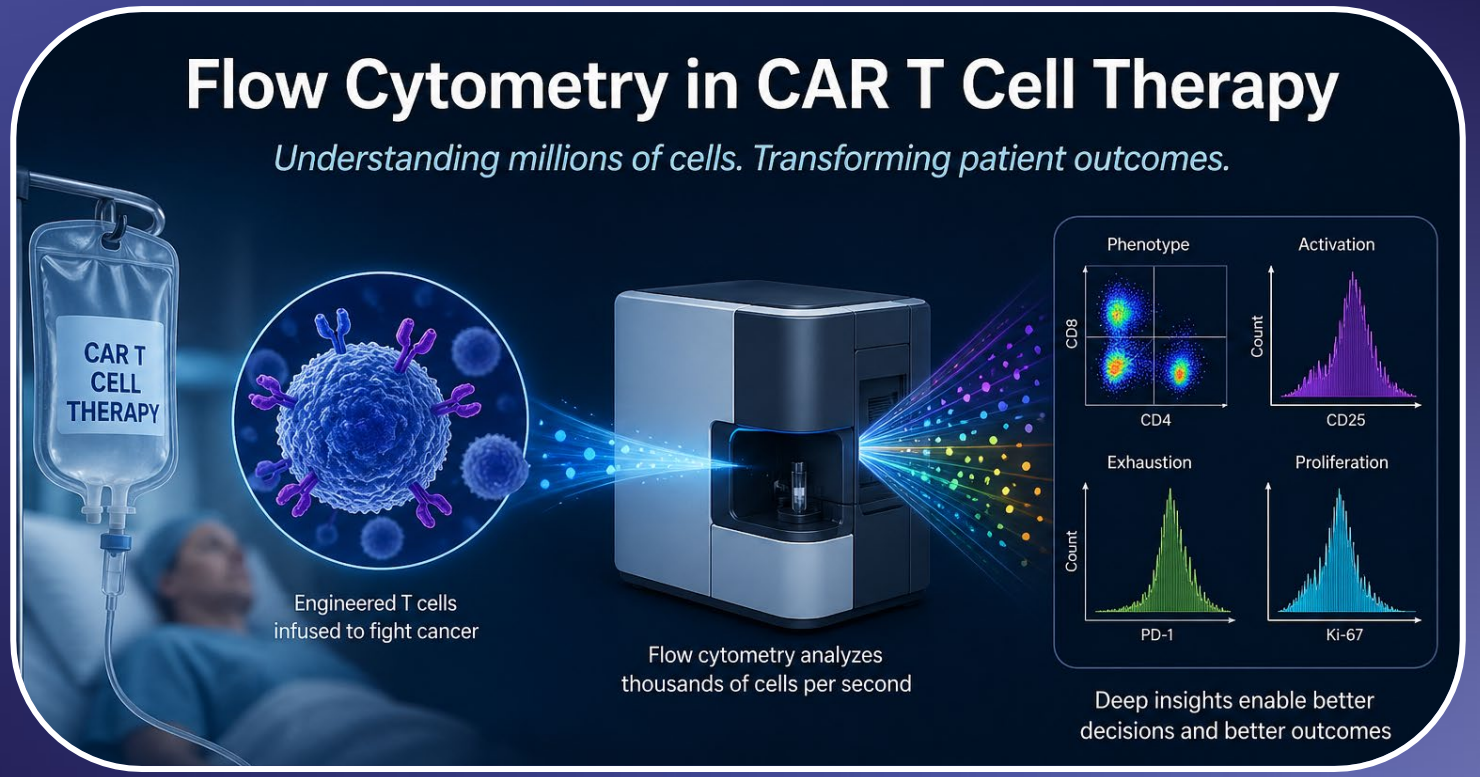
A patient with relapsed leukemia has just received CAR-T cells.

How do we know if those CAR-T cells are expanding in patients and killing cancer cells?



AI generated picture

One of the technologies that can help to answer this is **Flow cytometry!**

Learning objectives

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By the end of this tutorial, you will be able to:

1. **Differentiate between autologous and allogeneic CAR-T cell products** and how to monitor with Flow cytometry.
2. **Design effective Flow cytometry panels** for the CAR-T cell detection.
3. **Apply robust gating strategies** for accurate CAR-T cell identification.
4. **Interpret flow cytometry data** to assess expansion, persistence, activation, and exhaustion phenotypes in CAR-T cells.
5. **Identify and overcome challenges in standard immunophenotyping assays** (e.g., TBNK panels) for use in allogeneic CAR-T cell therapy.

Agenda

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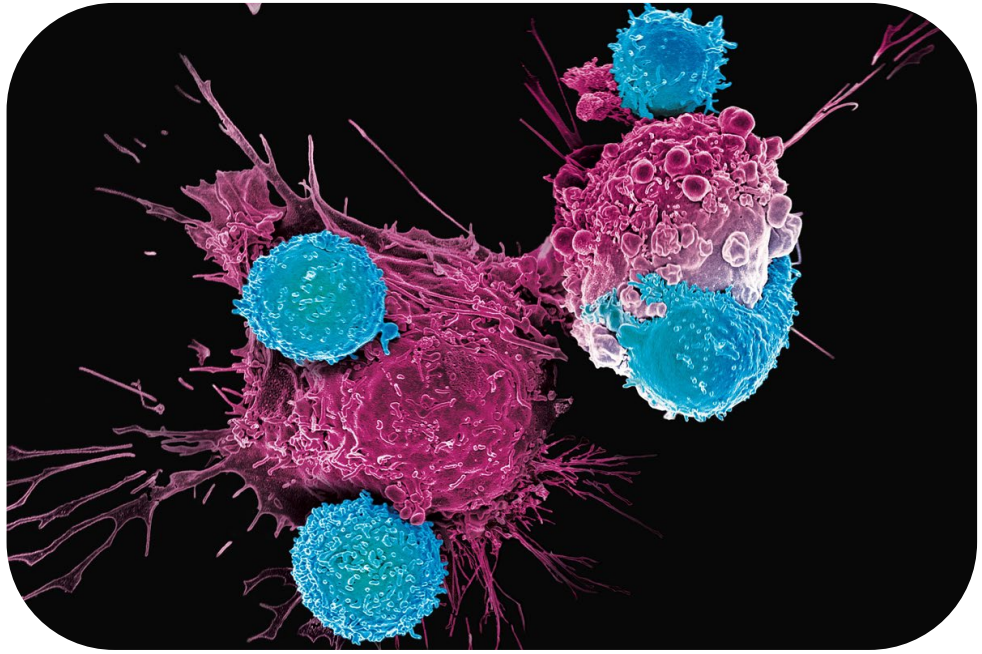
- Introduction – what is CAR?
- CAR-T cell therapy
  - Autologous CAR-T cells
  - Allogenic CAR-T cells
- Role for flow cytometry in CAR-T cell clinical trials
  - Detection of CAR-T cells
  - Assay development and validation – brief overview
  - CAR-T cell assay with lymphodepleted patient samples
- Case studies:
  1. CAR-T cell enumeration and characterization
  2. TBNK assay in allogenic CAR-T cell treated patients
- Future Frontiers in CAR-T cell therapy



This electron microscopic image shows CAR-T cells (blue) attacking cancer cells (pink).
Reference: Dr. Prasad Adusumilli, www.mskcc.org/news

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Engineered CAR-T cell (blue) attacking a leukemia cell (red)

Introduction

What is CAR?

Chimeric Antigen Receptor (CAR)

What is CAR?

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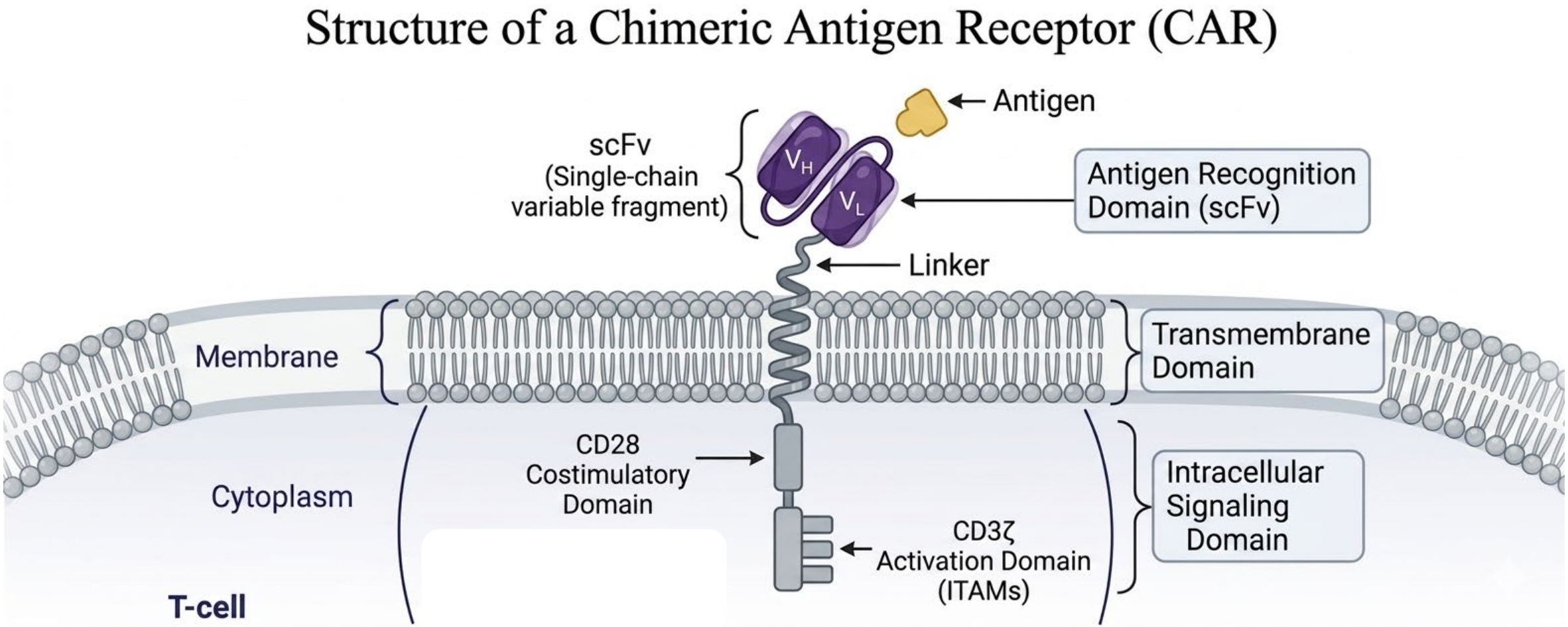
CAR: **C**himeric **A**ntigen **R**eceptor



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Advancement in CAR

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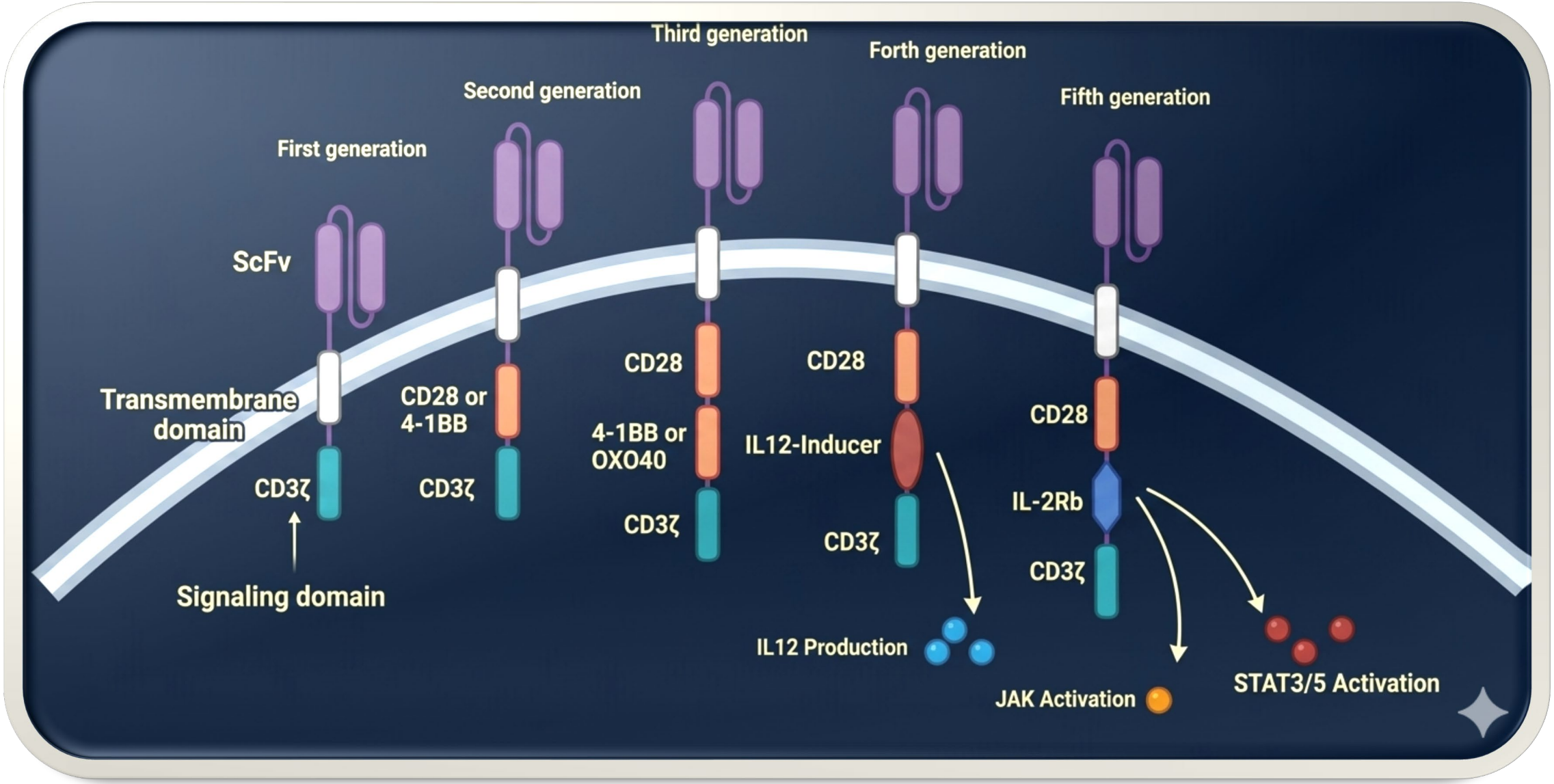

Reference: Shokati et.al. Frontiers in Immunology. 2025:1557157
Picture contrast adjusted by Gemini

Early Cancer Therapy vs CAR-T cell Therapy

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Surgery

- Best for solid tumors
- Not effective if cancer has spread
- Not effective for blood cancers

Chemotherapy

- Used for primarily blood cancers, but also for solid tumors
- Non-specific
- Toxicity – damages healthy dividing cells
- Side effects – hair loss, nausea and infections

Radiation Therapy

- Best for local control of tumors
- Limited to targeted area
- Can damage nearby healthy tissue

Targeted therapy

- Targets specific molecular pathways in cancer cells
- More selective than chemotherapy
- Requires identifiable targets

Hormonal (Endocrine) Therapy

- Blocks hormones that drive certain cancers
- Only works in hormone-dependent cancers

Hematopoietic stem cell transplant (bone marrow transplant)

- Replaces damaged bone marrow after high-dose of chemotherapy/radiation
- Autologous (patient's own cells) or Allogeneic (donor cells)
- Significance: One of the earliest "cell-based" therapies
- Risk of graft-versus-host disease (GVHD)

Early Cancer Therapy vs CAR-T cell Therapy

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Early immunotherapy

- Cytokine therapy (Interleukin-2 etc)
- Monoclonal antibodies (anti-CD20 (Rituximab) etc)
- Immune checkpoint inhibitors (CTLA-4, PD-1/PD-L1)
- Variable response
- Not highly personalized at the cellular level

- This inspired the development of CAR-T cell therapy

Key Limitation Before CAR-T cell therapy

- Lack of **precision targeting (especially of immune cells)**
- Limited ability to **reprogram the immune system**
- Relapses common in hematologic malignancies
- Toxicity was often high

CAR-T cell therapy introduced:

- **Personalized treatment** using the patient's own T cells
- **Genetic engineering** to target specific tumor antigens (e.g., CD19)
- High remission rates in certain blood cancers (like B-ALL and lymphoma)

Evolution of CAR-T cell Therapy

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Era of Discovery (1989-2002)

1989

Dr. Zelig Eshhar creates the first synthetic "T-body" receptor.

Early 1990s

First proof that CAR-modified cells can kill human cancer cells in vitro.

Early 1990s

First in-vivo mouse models demonstrate receptor functionality.

Early 2000s

Development of 2nd gen CARs with costimulatory domains for persistence.

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Clinical Proof (2010-2016)

2010

Dr. Carl June reports first major success in CLL clinical trials.

2012

Emily Whitehead becomes the first child cured of ALL with CAR-T.

2014

FDA grants "Breakthrough Therapy" designation for the first time.

2016

Pivotal trials for CD19-targeting therapies reach primary endpoints.

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Clinical Proof (2010-2016)

In 2010, Emily, then 5 years old, went from a being a healthy youngster one day, to a child diagnosed with ALL. Chemotherapy typically works well in pediatric ALL patients; Emily was one of the exceptions. After 2 years of intermittent chemotherapy, she continued to relapse. And when a

On March 1, 2012, Emily was transferred to CHOP and a few days later an apheresis catheter was placed in her neck; her T cells were extracted and sent to a lab. Emily received more chemotherapy, which knocked out her existing immune system, and she was kept in isolation for 6 weeks. Waiting.

Stephan Grupp, MD, PhD: "Since we treated Emily, we have treated more than 420 patients with CAR T cells at CHOP. She launched a whole group to be treated with this therapy; thousands have been treated around the world."—Stephan Grupp, MD, PhD

Oncology Times

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The Approval Era (2017-2026)

2017

Kymriah and Yescarta receive historic FDA approvals.

2021

First BCMA CAR-T (Abecma) approved for Multiple Myeloma.

2024

FDA approves Aucatzyl; Satri-cel reaches pivotal data in China.

2026

First CAR-T launches for solid tumors (gastric/pancreatic cancers).

FDA Approved Autologous CAR-T cell Therapies

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Target CD19:

- **Kymriah** (tisagenlecleucel): The first FDA-approved CAR-T (2017)
- Aucatzyl (obecabtagene autoleucel)
- Breyanzi (lisocabtagene maraleucel):
- Tecartus (brexucabtagene autoleucel)
- Yescarta (axicabtagene ciloleucel)

Pediatric/young adult B-ALL
Adult LBCL
Follicular lymphoma

Target BCMA:

- Abecma (idecabtagene vicleucel) - **now approved after ≥2 prior lines** (depending on label evolution).
- Carvykti (ciltacabtagene autoleucel) - Now **expanded to ≥1 prior line (early relapse setting)**

Other Approved CAR-T cell Therapies

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European Union (EMA):

- **M**ost FDA-approved products: **Kymriah**, **Yescarta**, **Breyanzi**, **Abecma**, and **Carvykti**
- **Ebvallo (tabelecleucel)**, an **allogeneic T-cell therapy**, received European Commission approval (for relapsed or refractory EBV+ post-transplant lymphoproliferative disease (PTLD)).

China (NMPA):

- **Relma-cel** (relamitogene autoleucel) and **Yuanruida** (inaticabtagene autoleucel).
- **Satri-cel** (CT041) - Claudin18.2-targeting therapy for solid tumors is expected to launch in China in the first half of 2026.

India (CDSCO):

- **NexCAR19** and **Qartemi**.

Note on Solid Tumors: While CAR-T cell therapy has historically been limited to blood cancers, **satri-cel** in China marks a significant milestone as the first potential approval for solid tumors (gastric and pancreatic cancers).

What is the Status of CAR-T cell Therapy?

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Is CAR-T cell Therapy a first-line treatment for any disease?

Short answer: 👉 No!

- ✓ As of 2026, there is no widely FDA-approved CAR-T therapy used as true first-line (initial) treatment.
- ✓ CAR-T is **primarily approved for relapsed/refractory (R/R) disease** after prior therapies have failed.

🚫 Why NOT First-Line Yet?

Key reasons:

- Safety (CRS, ICANS),
- Manufacturing time/logistics,
- Cost
- lack of data

What is the Status of CAR-T cell Therapy?

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What has Changed Recently?

✅ Movement Earlier (but NOT first-line)

CAR-T cell therapy is now **2nd-line for aggressive lymphomas** (e.g., axi-cel, liso-cel)

🔬 **Current Status for Frontline CAR-T cell therapy:**

👉 **Still investigational**

There are promising trials, but no routine approvals yet.

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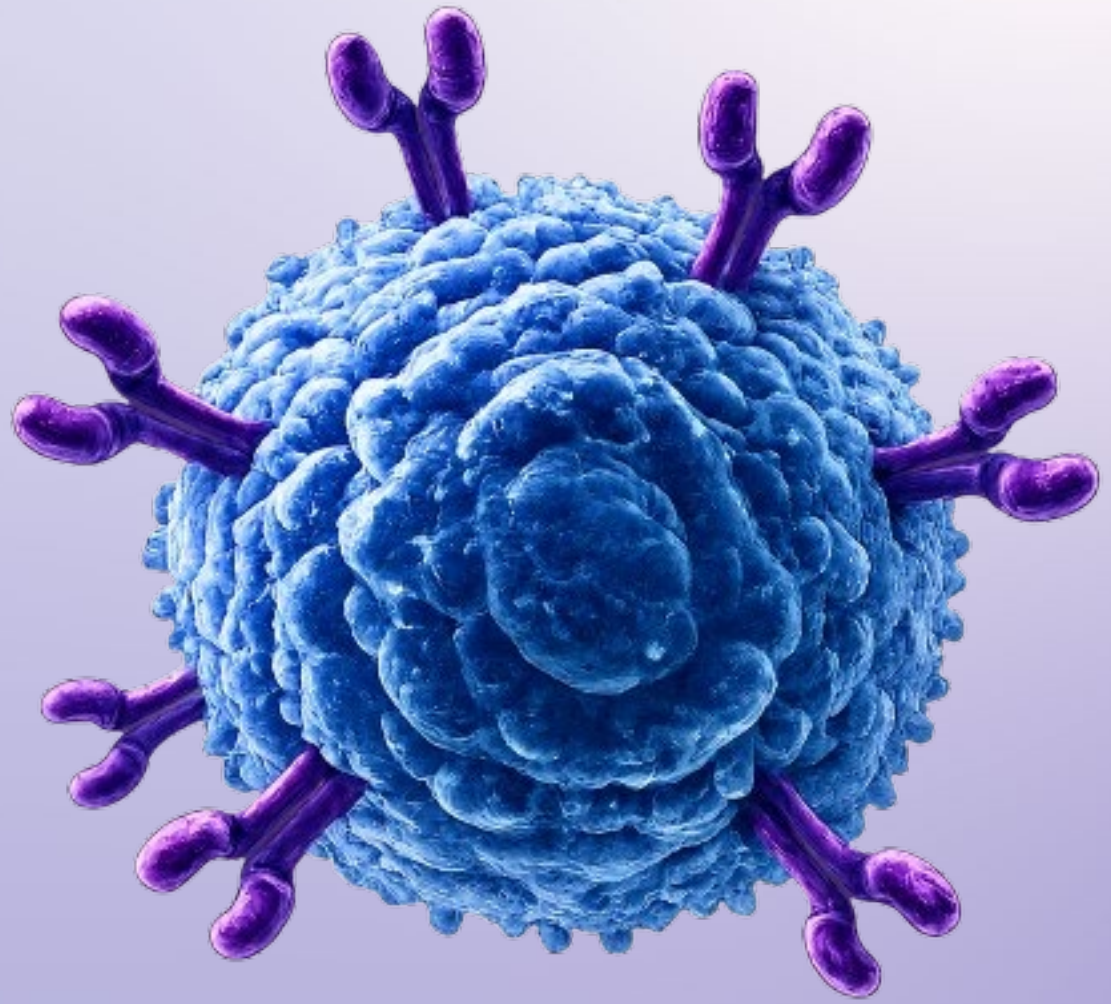
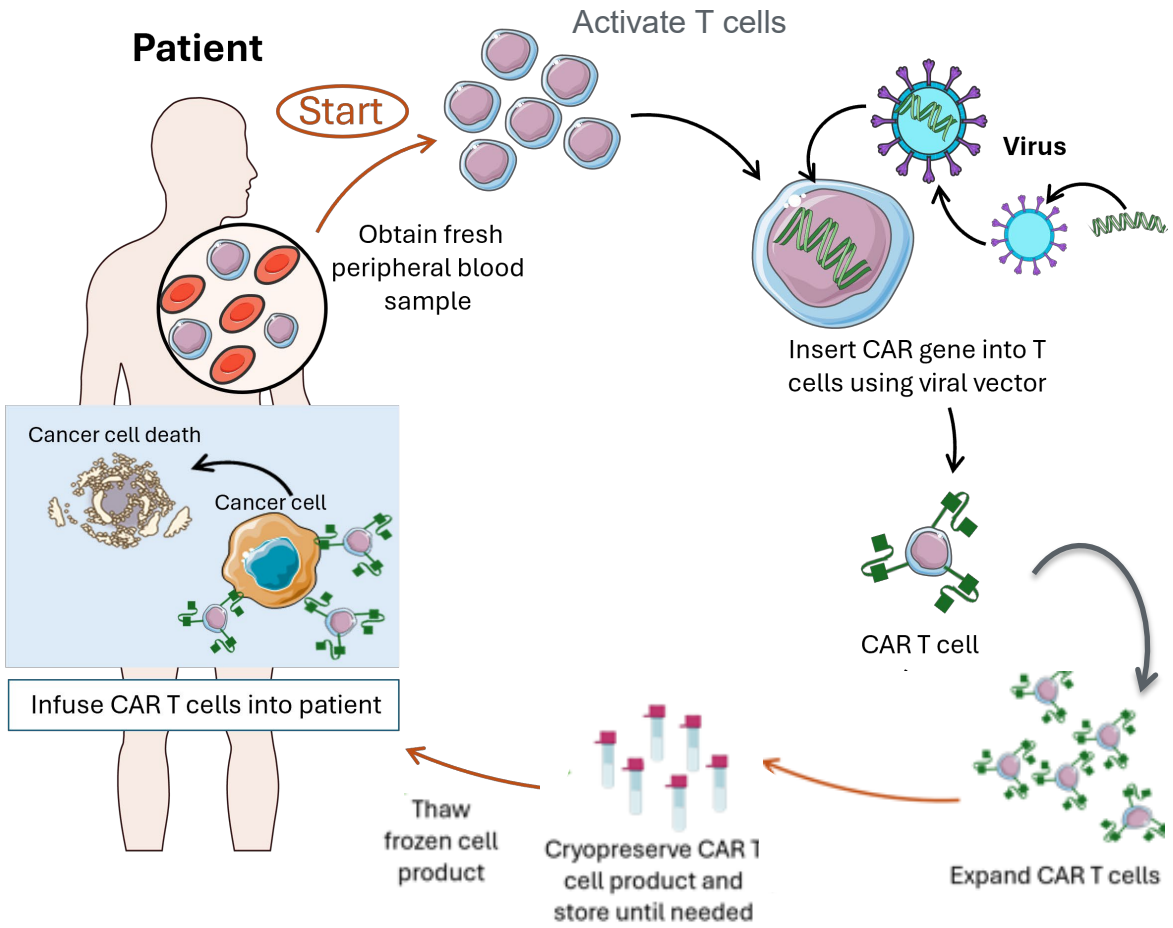


CAR-T cell Therapy

Autologous CAR-T therapy

What is Autologous CAR-T cell Therapy?

Personalized immune therapy where a patient's own T cells are genetically engineered to recognize and attack cancer cells.



Reference: Flow Matters Flow Cytometry Blog, Cerba Research, June 2025
https://www.cerbaresearch.com/insights/blog/flow-matters/

Vector Usage for CAR Expression

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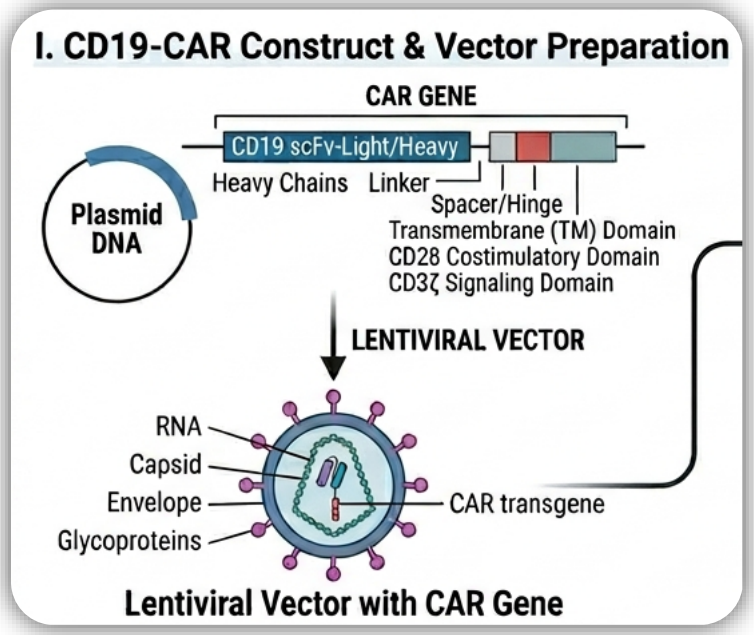
➢ Lentiviral transduction is currently the dominant and preferred method for CAR expression in clinical CAR-T cell therapies.

Lentiviral vectors

- Derived from HIV-1 (replication-incompetent)
- Integrate stably into the T-cell genome
- Enable **long-term CAR expression**
- Efficient in both dividing and non-dividing T cells



✅ **Advantages**

- Stable, long-term expression
- High efficiency
- Safety profile: Lower risk of insertional mutagenesis compared to older retroviruses

Overview of CAR-T cell Transduction in T-cells

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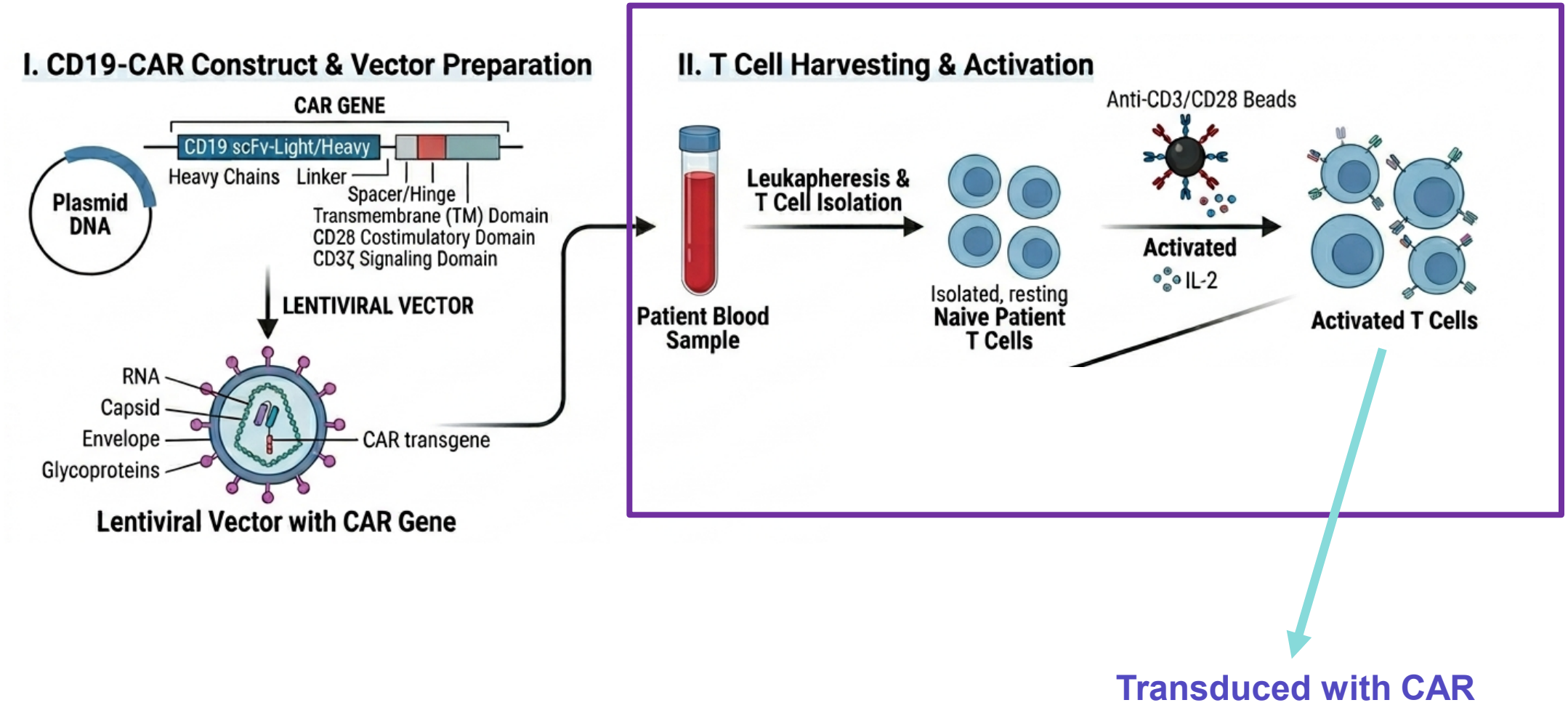

Transduction of T-cells

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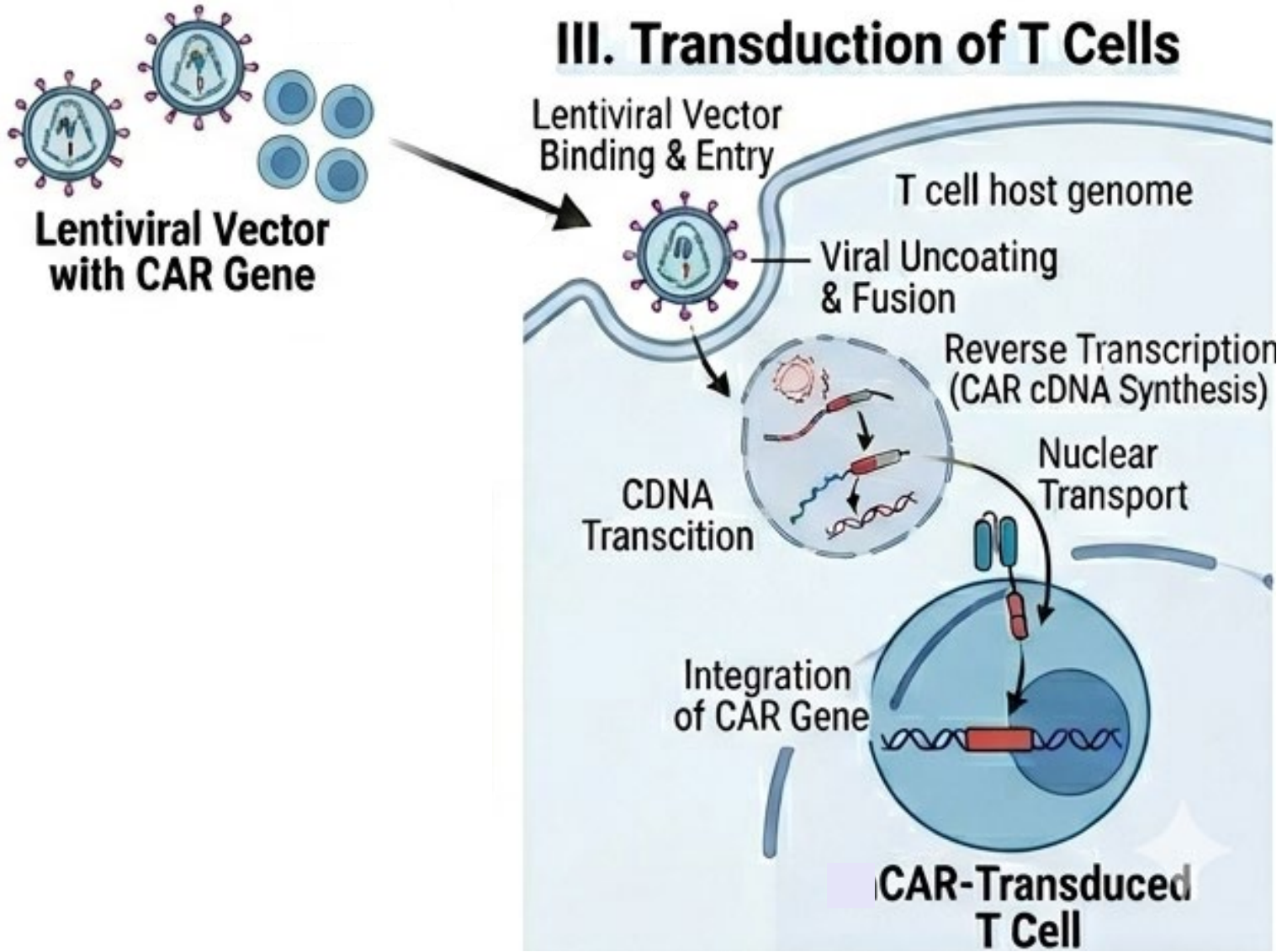

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Killing of Cancer Cells by CAR-T cells

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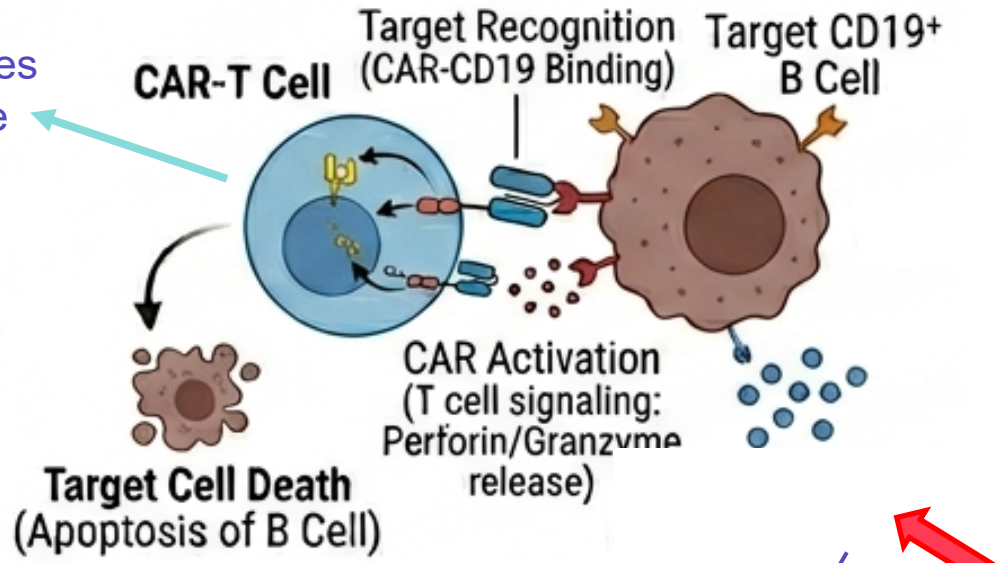
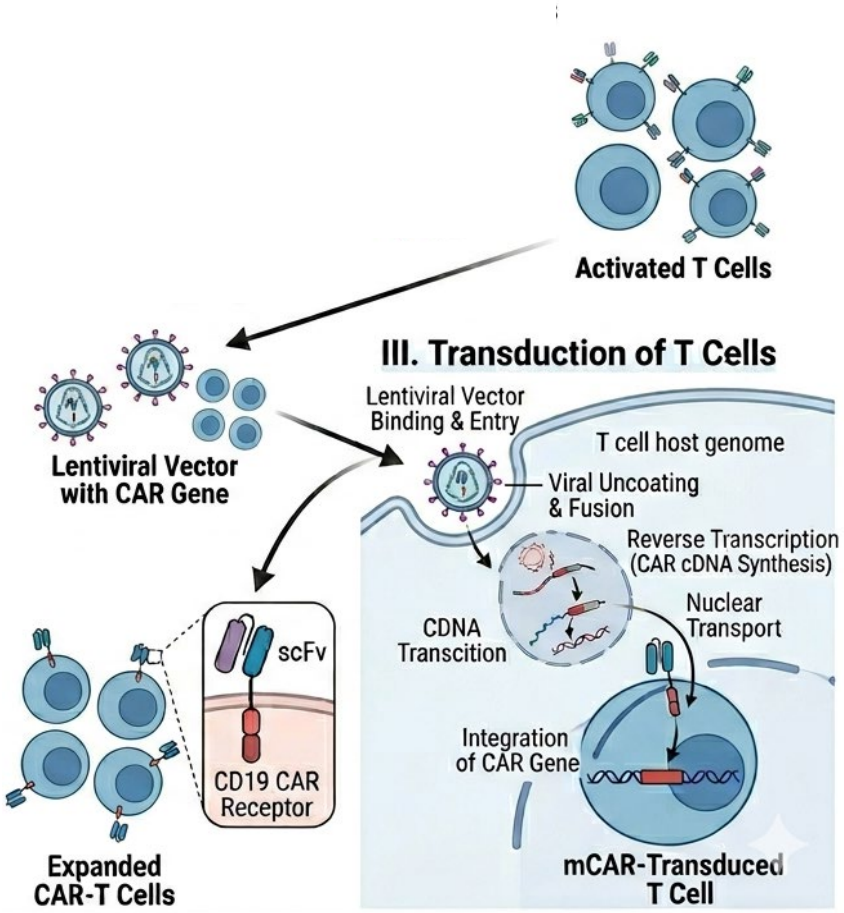
CAR- T cells produce cytokines and proliferate (expansion in patients)



Infusion into patient



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Overview of CAR-T cell Transduction in T-cells

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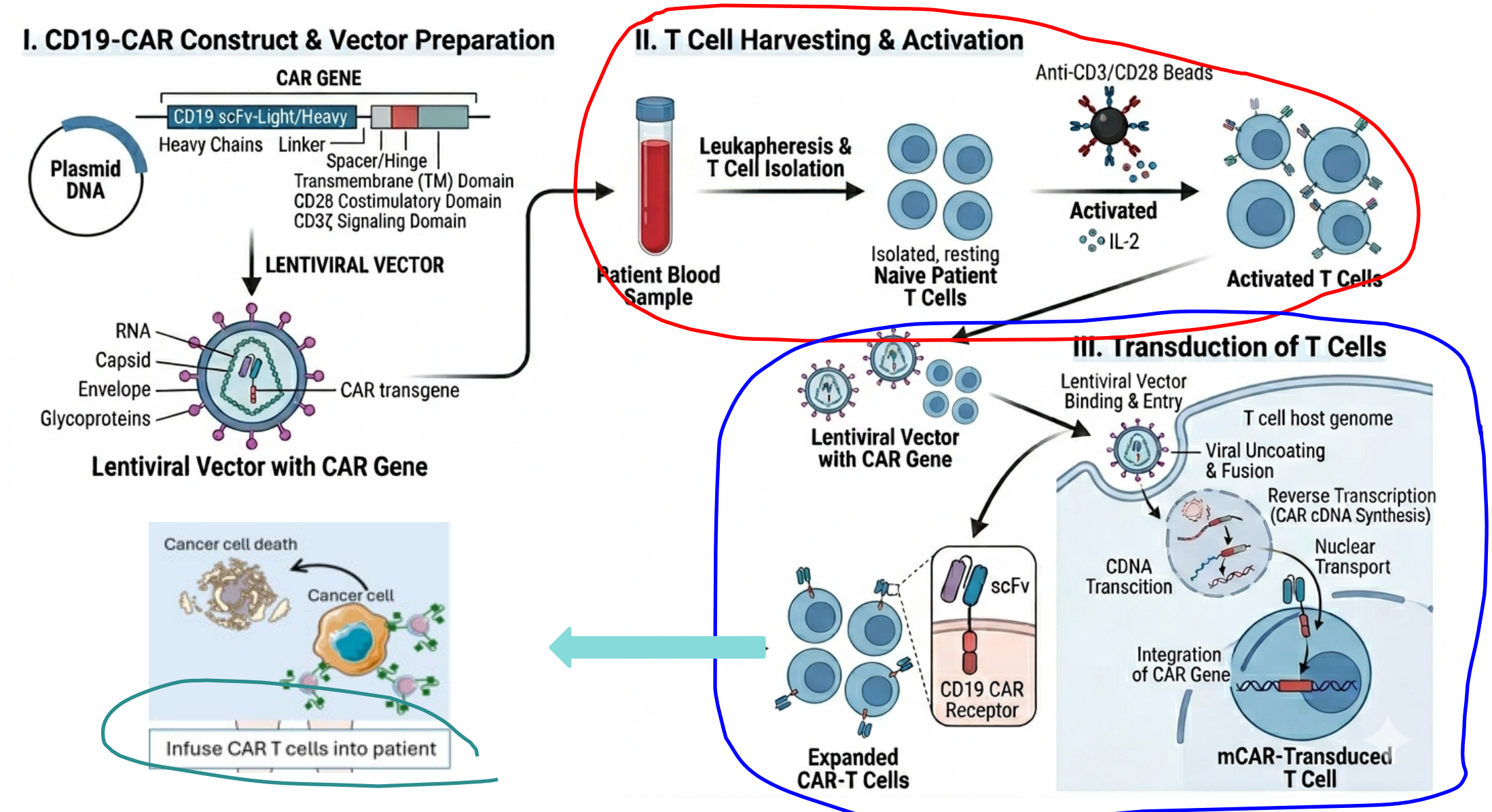

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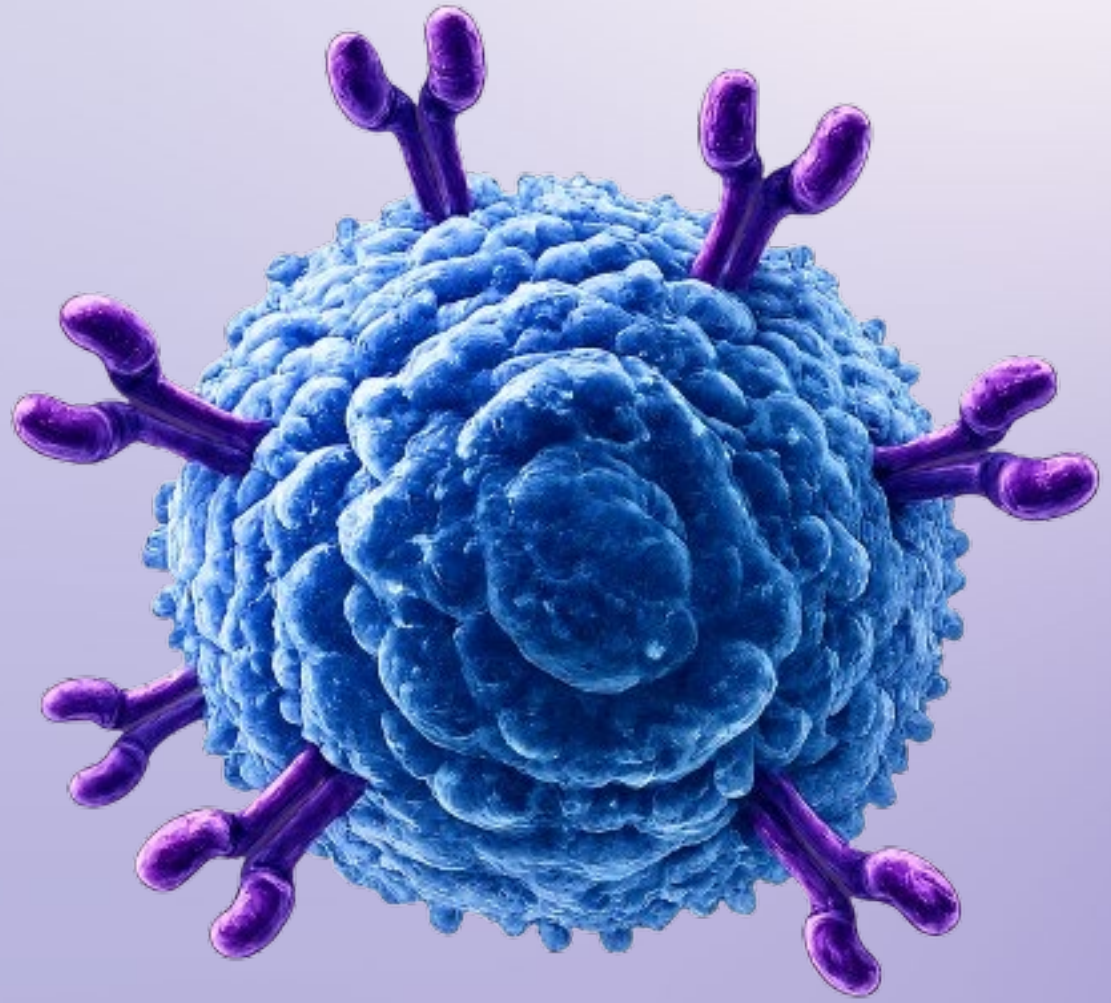

CAR-T cell Therapy

The Allogeneic Revolution

Allogenic CAR-T cell Therapy

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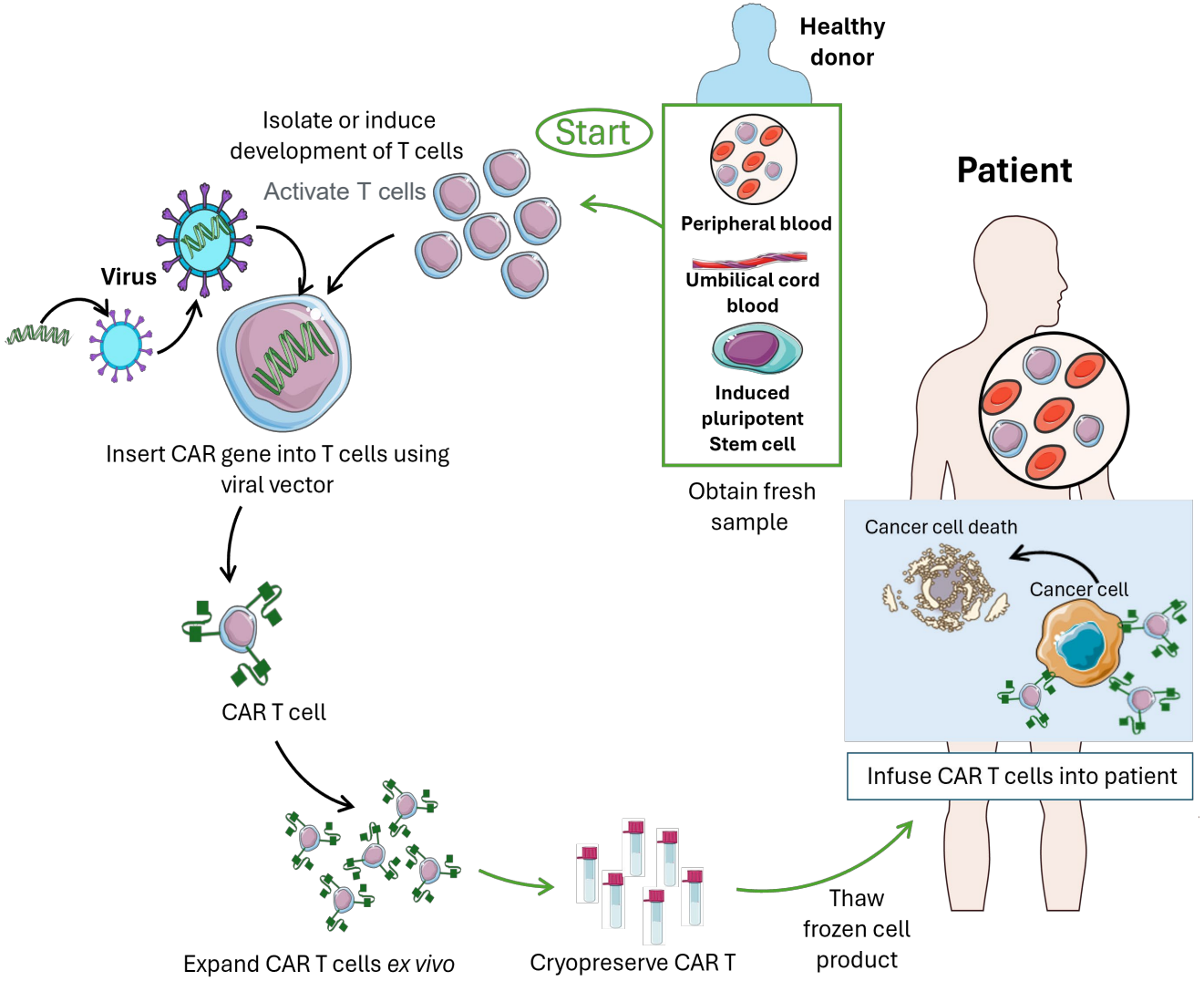

Reference: Flow Matters Flow Cytometry Blog, Cerba Research, June 2025
https://www.cerbaresearch.com/insights/blog/flow-matters/

Overview of Autologous vs Allogenic

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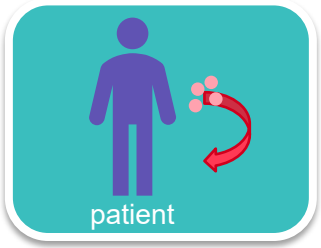

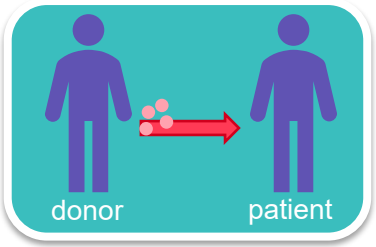

Feature	Autologous CAR-T	Allogeneic CAR-T
Source	The patient's own blood. T-cells might be affected by previous treatment.	A **healthy** third-party donor.
Manufacturing	Custom-made for each individual. Complex logistics.	**Mass-produced** in large batches. Industrialized and standardized.
Availability / Timelines	3 - 4 week wait (Vein-to-vein).	**Immediate** ("Off-the-shelf").
Persistence	Intermediate to long (months to years).	Short to intermediate (weeks to months). ⊖
Redosing	Limited by number of cells from the patient.	**Possible**, not limited by number of cells.
Challenges / Risks	Delay in treatment could lead to exacerbation of the disease.	Risk of GvHD and rejection. ⊖
Cost	Extremely high (labor intensive).	Potential for **lower cost** per dose.

Allogeneic CAR-T cell:
Graft versus Host Disease (GvHD) and Host Rejection

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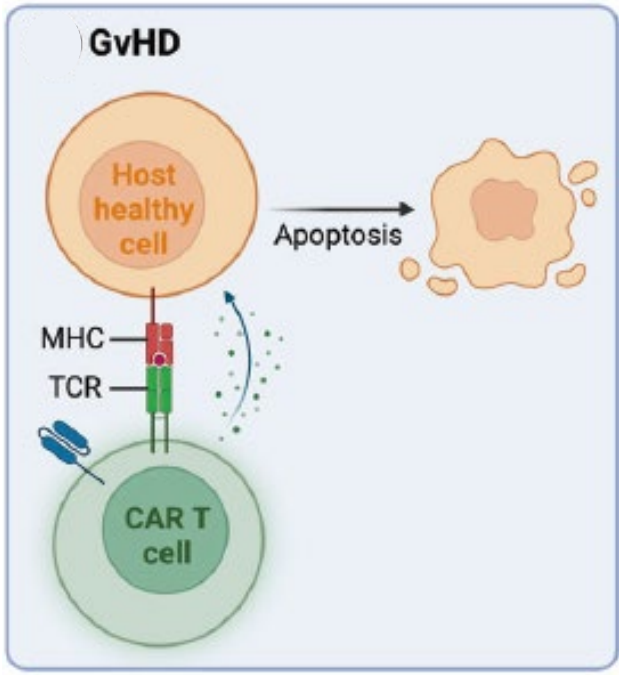

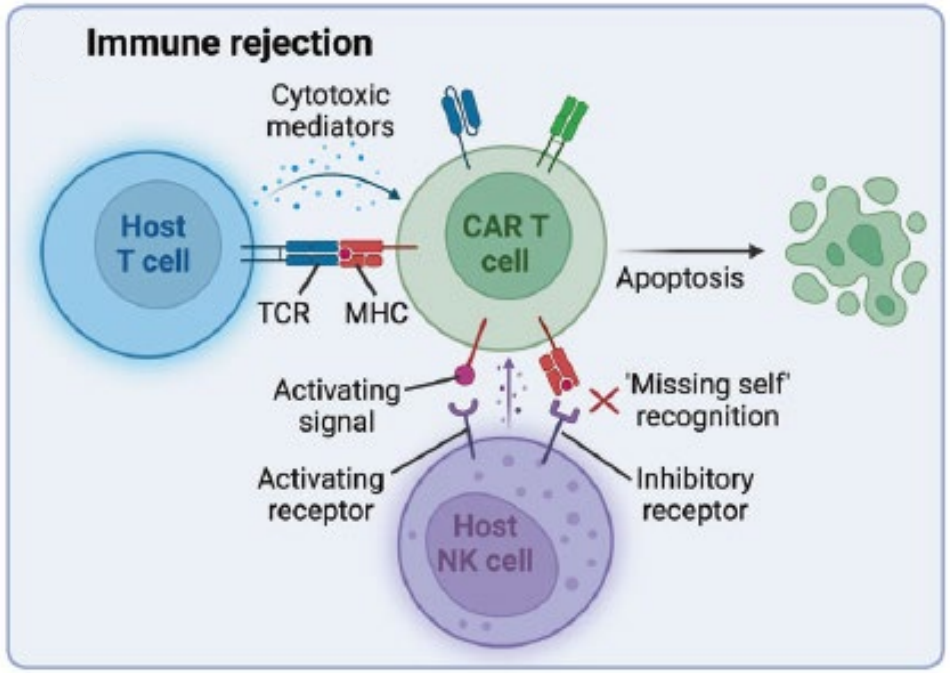

Reference: Castelli et. al. Cell Res. 2022; 32(12):1036-1037

Allogeneic CAR-T cell: Strategies to Overcome GvHD

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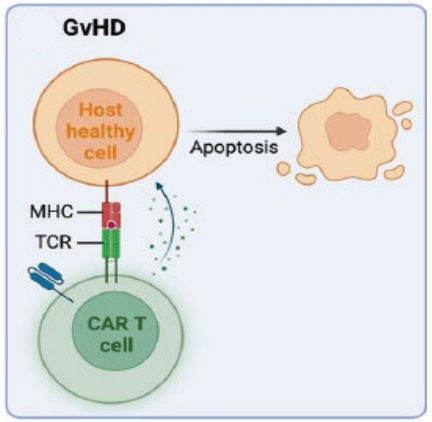

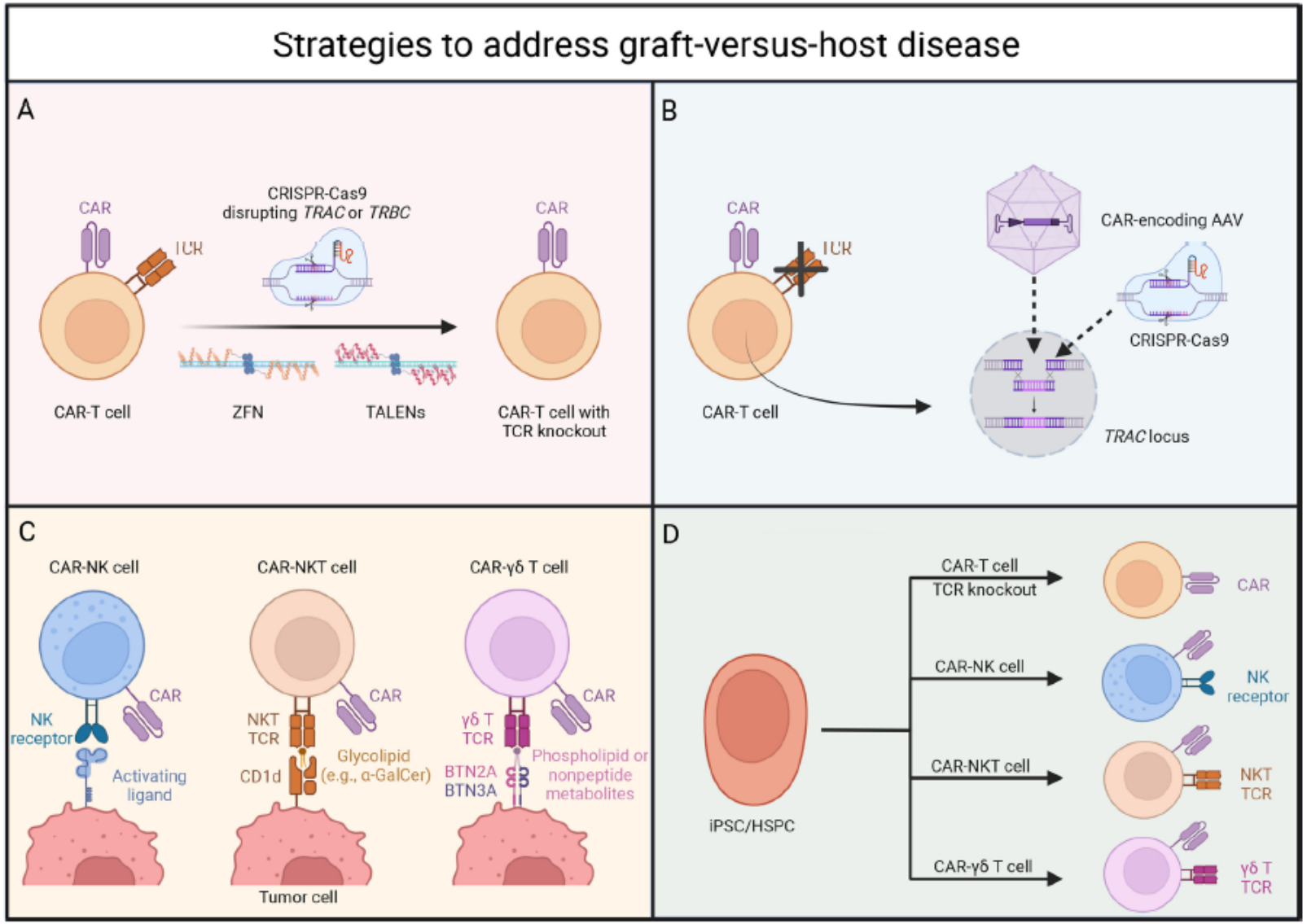

Reference: Lyu et. al. Exp Hematol Oncol. 2025; 14(1):66

Allogeneic CAR-T cell: Strategies to Overcome Host Rejection

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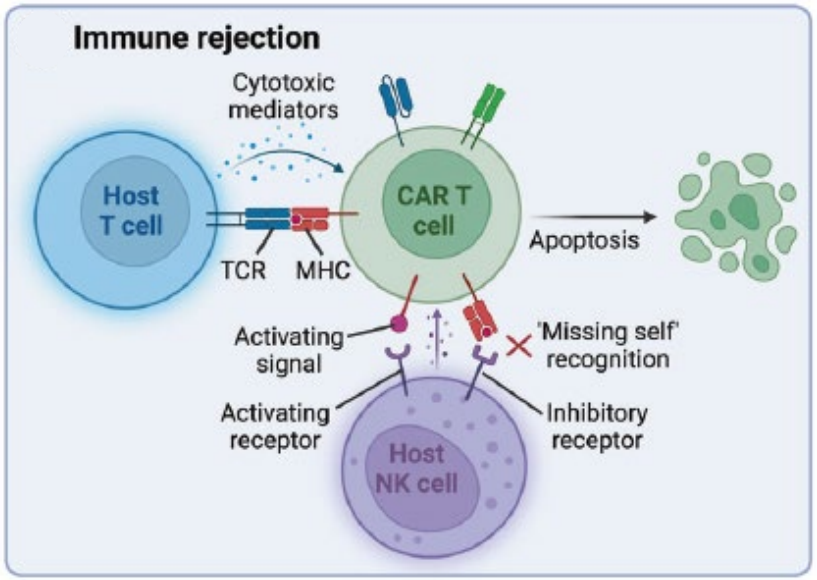

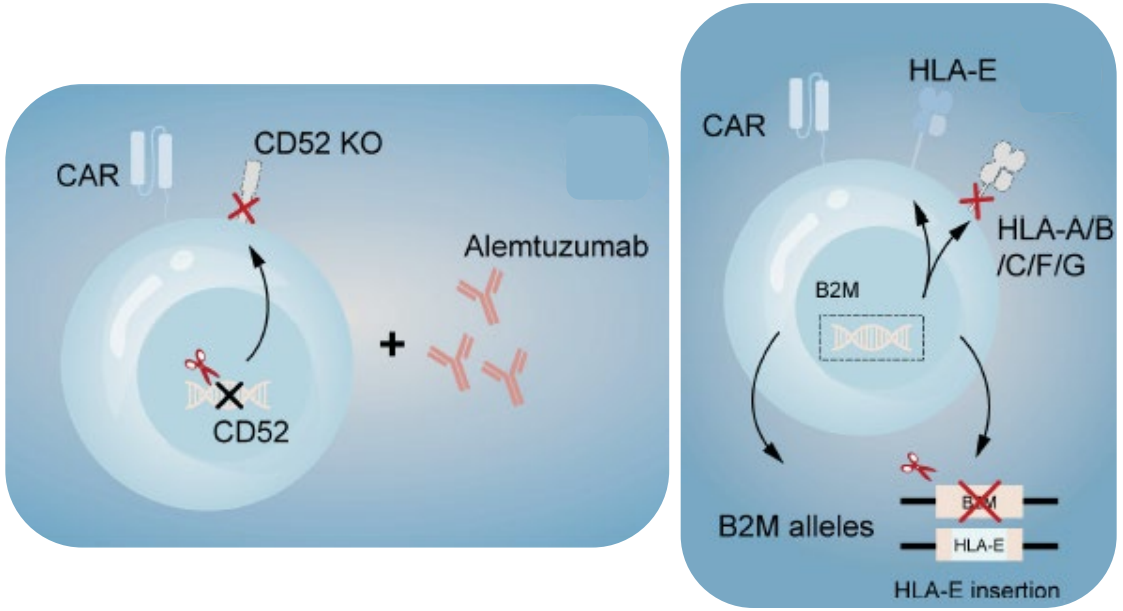

Ongoing Clinical Trials for Allogenic CAR-T cells : Some Highlights

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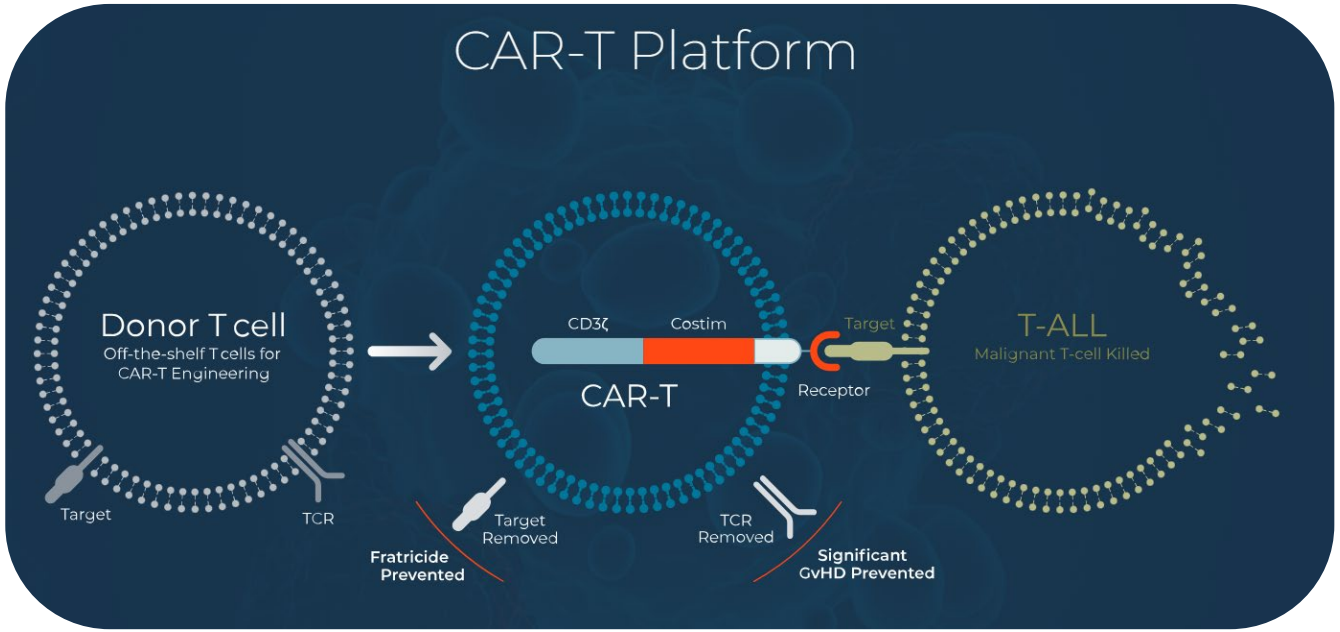
Sofi-cel (WU-CART-007) from Wugen

- Has received breakthrough therapy designation from the FDA in heavily pretreated R/R T-ALL and T-LBL
- Targeting CD7
- CRISPR-Cas9 for deletion of CD7 and T-cell receptor alpha constant (TRAC)
- Phase 1/2 trial data showed a 90.9% overall response rate and 72.7% composite complete remission rate in heavily pretreated patients



Reference: https://wugen.com/car-t-platform/

Ongoing Clinical Trials for Allogenic CAR-T cells : Some Highlights

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Sponsor	Study NCT number	Phases	Product	Condition	Target	Gene editing technology
Allogene Therapeutics	NCT07085104	PHASE1	ALLO-329	multiple autoimmune diseases	CD19 and CD70	Dagger® Technology
	NCT04696731	PHASE1	ALLO-316	clear cell renal cell carcinoma	CD70	
	NCT04093596	PHASE1	ALLO-715	multiple myeloma	BCMA	Cellectis TALEN® technologies.
	NCT03939026	PHASE1	ALLO-501	large B-cell lymphoma or follicular lymphoma	CD19	
	NCT04416984	PHASE1|PHASE2	ALLO-501A	large B-cell lymphoma, CLL/SLL	CD19	
	NCT06500273	PHASE2	cema-cel	large B-cell lymphoma (LBCL)	CD19	
AvenCell Europe GmbH	NCT05949125	PHASE1	Allo-RevCAR01-T-CD123	AML	CD123	CRISPR-Cas9
	NCT07284433	PHASE1|PHASE2	Allo-QuadCAR01-T (AVC-203-01)	B-cell cancers	CD19 and CD20	
Beam Therapeutics Inc.	NCT05885464	PHASE1|PHASE2	BEAM-201	T-ALL or T-LL	CD7	Quadruplex base-editing
Cellectis S.A.	NCT03190278	PHASE1	UCART123v1.2	AML	CD123	Cellectis TALEN® technologies.
	NCT05607420	PHASE1|PHASE2	UCART20x22	B-Cell Non-Hodgkin Lymphoma (B-NHL)	CD20 and CD22	
	NCT04150497	PHASE1|PHASE2	UCART22	B-cell acute Lymphoblastic Leukemia (B-ALL)	CD22	

Reference: https://clinicaltrials.gov/

Ongoing Clinical Trials for Allogenic CAR-T cells : Some Highlights

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Sponsor	Study NCT number	Phases	Product	Condition	Target	Gene editing technology
CRISPR Therapeutics	NCT05643742	PHASE1|PHASE2	Zugocel (CTX112)	B-cell malignancies	CD19	CRISPR-Cas9
	NCT06925542	PHASE1	Zugocel (CTX112)	multiple autoimmune diseases	CD19	
	NCT05795595	PHASE1|PHASE2	CTX131	Relapsed/Refractory Solid tumors	CD70	
	NCT06492304	PHASE1|PHASE2	CTX131	Relapsed/Refractory Hematologic Malignancies	CD70	
Fate Therapeutics	NCT07570862	PHASE2	FT819	lupus nephritis	CD19	iPSC derived, CRISPR-Cas9
	NCT06308978	PHASE1	FT819	systemic lupus erythematosus	CD19	
Fundamenta Therapeutics, Ltd.	NCT05576181	PHASE1	ThisCART19	B-ALL	CD19	non-gene-editing platform named ThisCART (TCRαβ/CD3 and/or HLA-I intracellular sequestered)
	NCT05106907	PHASE1	ThisCART19	B-NHL	CD19	
	NCT04384393	PHASE1	ThisCART19	CD19 positive B cell malignancies	CD19	
	NCT04601181	PHASE1	ThisCART22	B Cell Malignancies	CD22	
	NCT05106946	PHASE1	ThisCART22	B-NHL	CD22	
	NCT05127135	PHASE1	ThisCART7	CD7 positive T cell malignancies	CD7	

Reference: https://clinicaltrials.gov/

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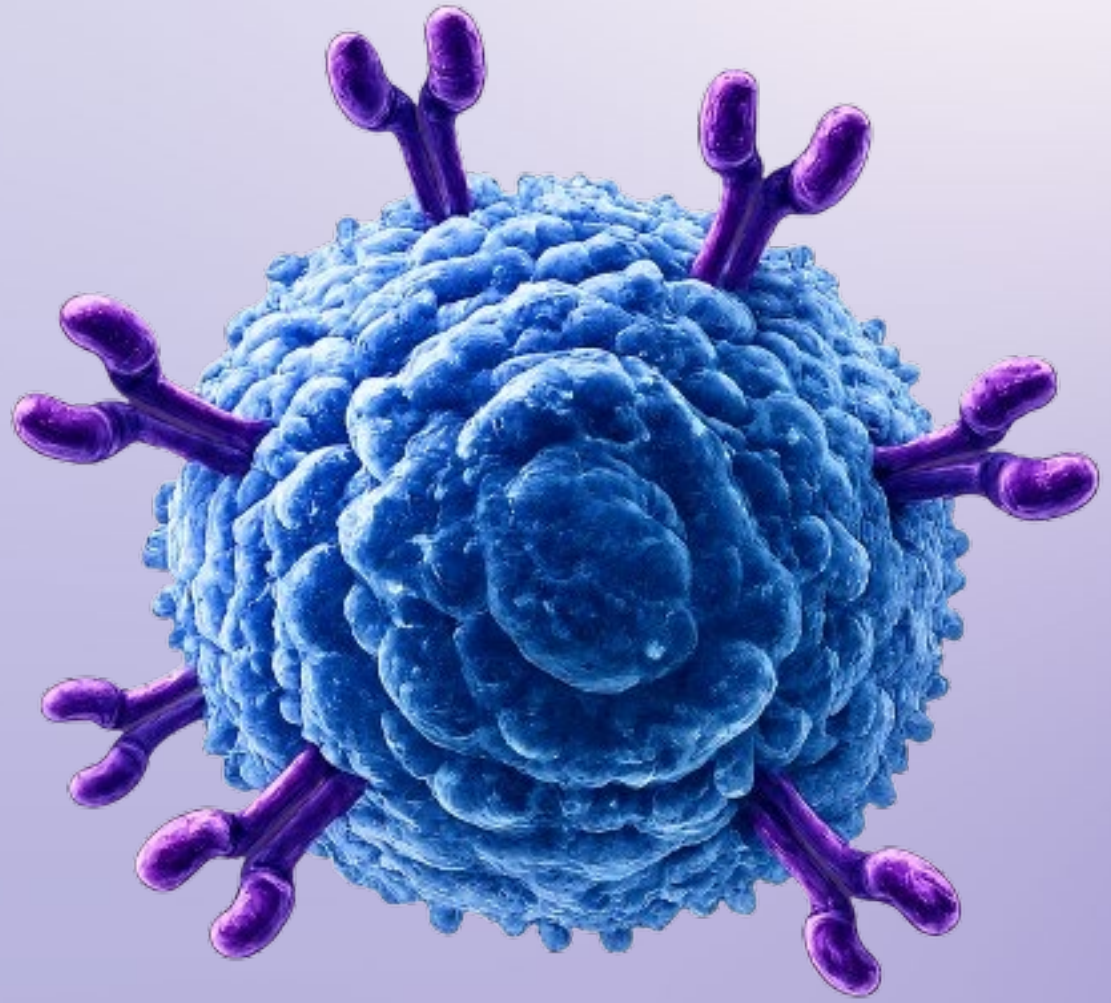

Role for Flow Cytometry in CAR-T cell Clinical Trials

Wide range of applications

Role for Flow Cytometry in CAR-T cell Trials

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Flow cytometric evaluation supporting CAR-T cell therapy:
Flow cytometry is used throughout the CAR-T cell therapy life cycle, from the manufacturing phase to post-infusion.

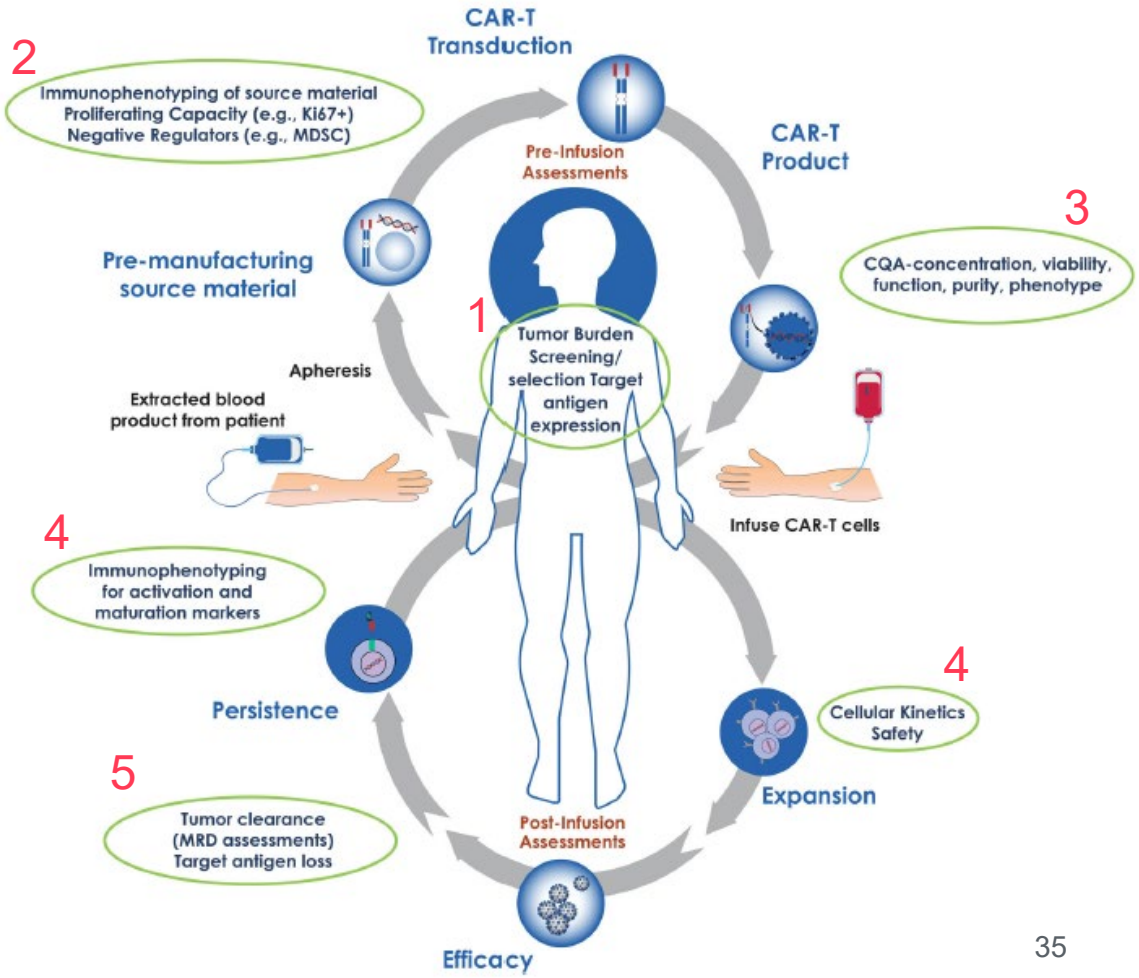
Received: 11 September 2020 | Revised: 10 December 2020 | Accepted: 15 December 2020
DOI: 10.1002/cyto.b.21985

ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Best practices for the development, analytical validation and clinical implementation of flow cytometric methods for chimeric antigen receptor T cell analyses

Ghanashyam Sarikonda[1] | Mélissa Mathieu[2] | Mahwish Natalia[3] | Anil Pahuja[1] | Qiong Xue[3] | Piotr L. Pierog[3] | Paul C. Trampont[4] | Vilma Decman[5] | Susan Reynolds[6] | Laïla-Aïcha Hanafi[2] | Yongliang S. Sun[7] | Steven Eck[8] | Michael N. Hedrick[7] | Jennifer J. Stewart[9] | Shabnam Tangri[1] | Virginia Litwin[2] | Naveen Dakappagari[1]



Reference: Sarikonda et. al. Cytometry B Clin Cytom. 2021; 100(1):79-91

Role for Flow Cytometry in CAR-T cell Trials - Pre-infusion

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1. Disease state of the patient:

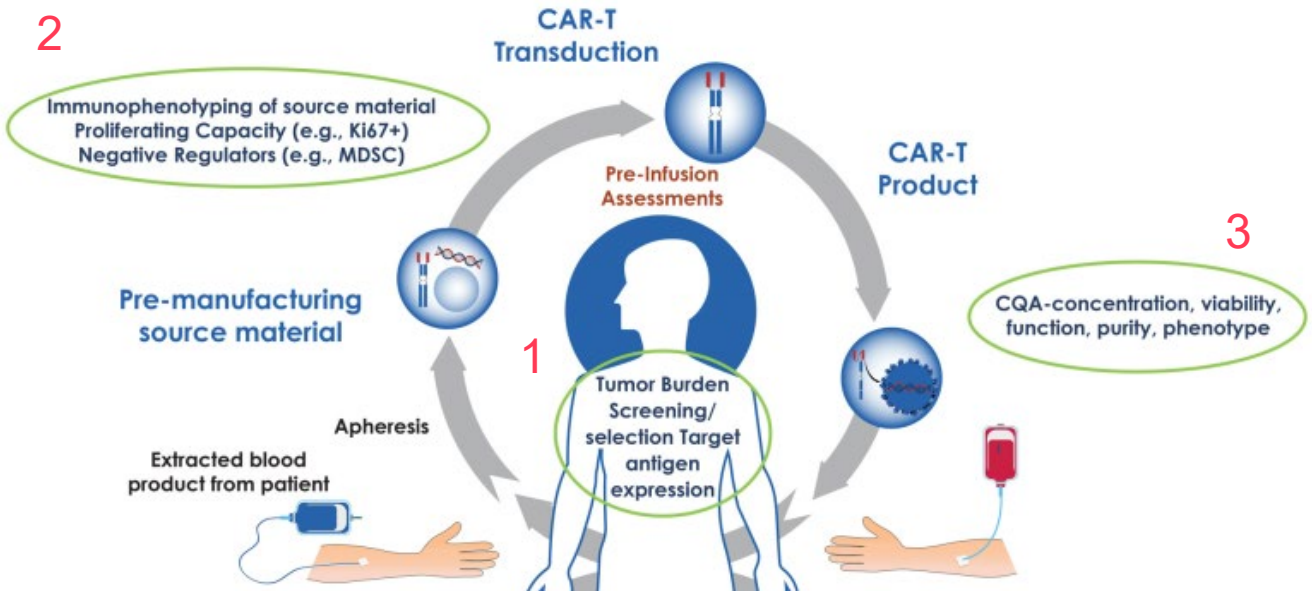
- **Screening:** What is the tumor burden?
- **Target antigen:** Do the cancer cells express the target of the CAR-T cell therapy?

2. Characterization of apheresis product:

- **Phenotypic characterization of source material:** presence of negative regulators, activation state of T cells, CD4:CD8 ratio

3. Product release:

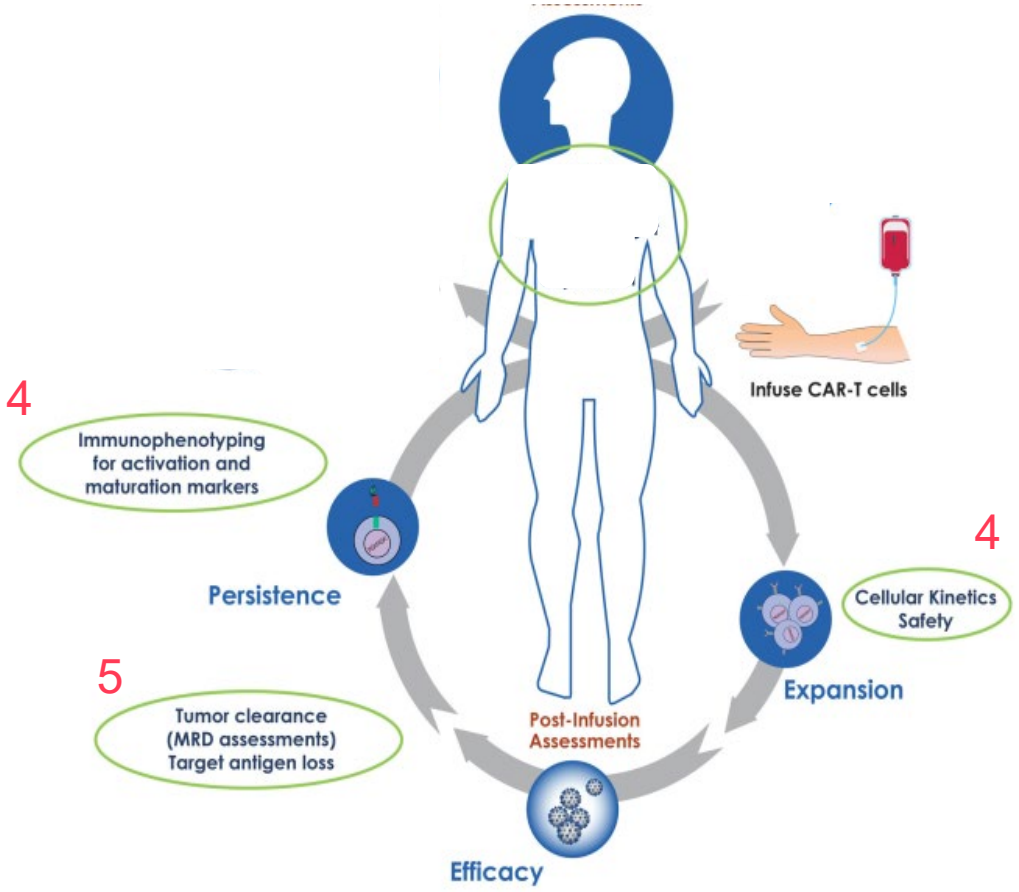
- **Transduction efficiency:** % CAR positivity
- **Purity and Viability:** any unwanted cells?
- **CAR-T cell function:** assess killing of target cells



Reference: Sarikonda et. al. Cytometry B Clin Cytom. 2021; 100(1):79-91

Role for Flow Cytometry in CAR-T cell Trials - Post-infusion

Cerba Research



4. Monitoring CAR-T cells post-infusion:

- **CAR-T cell pharmacokinetics (PK):** enumeration of CAR+ T-cells at various time points.
- **CAR-T cell persistence:** long term or short term?
- **CAR-T cell phenotypic characterization:** activation, exhaustion and memory phenotypes

5. Disease state of the patient

- **Efficacy - Monitoring tumor clearance:** including minimal residual disease (MRD) assessment.
- **Endogenous immune phenotyping**

Reference: Sarikonda et. al. Cytometry B Clin Cytom. 2021; 100(1):79-91

Flow Cytometry for PK Testing

Cerba Research

While qPCR was initially the preferred technique, flow cytometry can now be used for PK testing in global clinical trials.

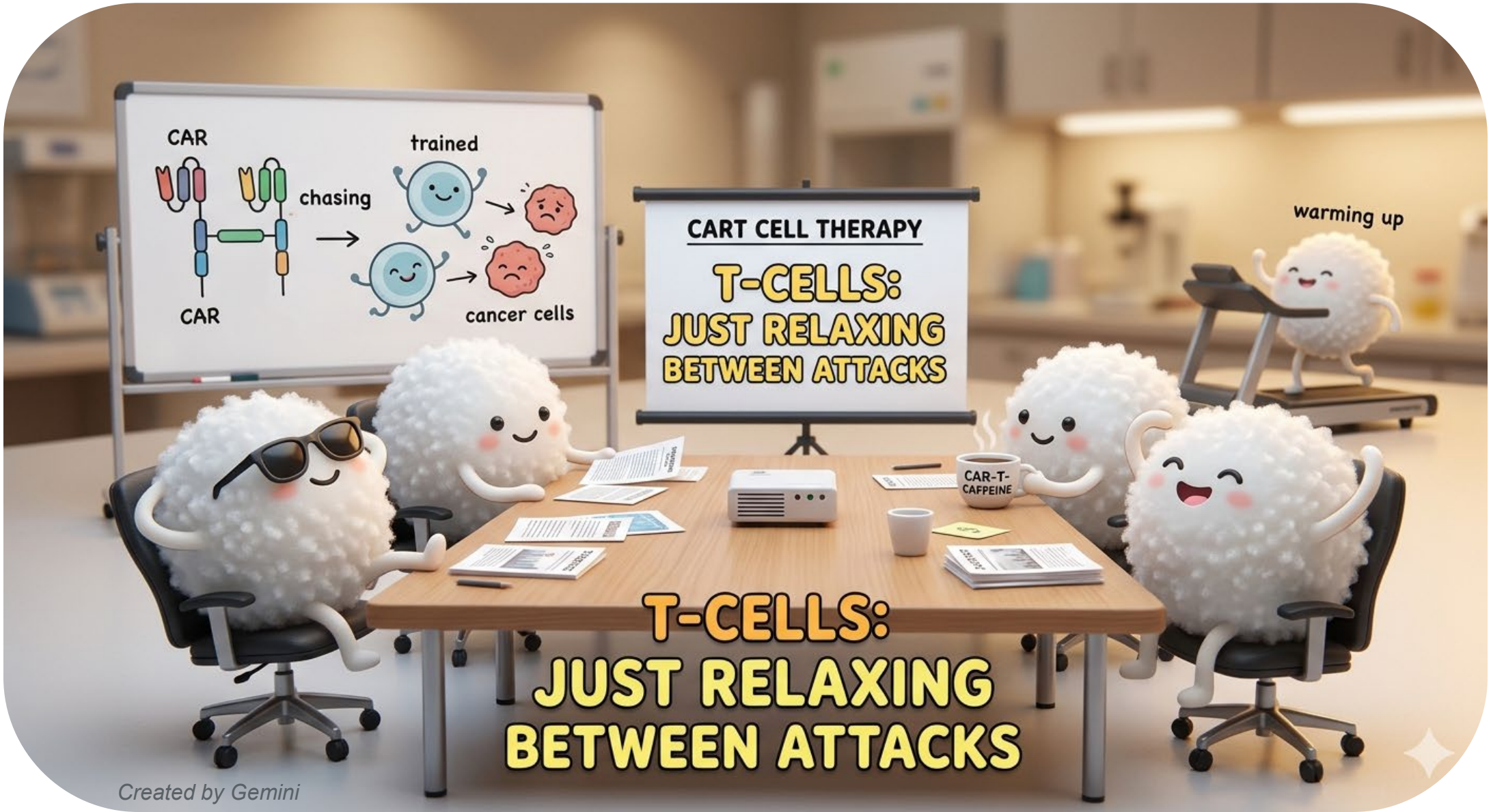
- Current cytometers allow standardized testing on a global scale
- High sensitivity using high-affinity anti-CAR reagents
- Absolute counts with quantification beads

	Flow cytometry	**qPCR/ddPCR**
Measures	Surface CAR **protein on living cells**	CAR Transgene copy (DNA) in dead/live cells
Speed	**Same-day** result	Longer turnaround
Phenotyping	**CD4/CD8, memory, activation, exhaustion**	Not possible
Sample stability	Limited (fresh blood)	Longer (frozen samples)

Reference: Ling et. al. Cytometry B Clin Cytom. 2026; 1-12

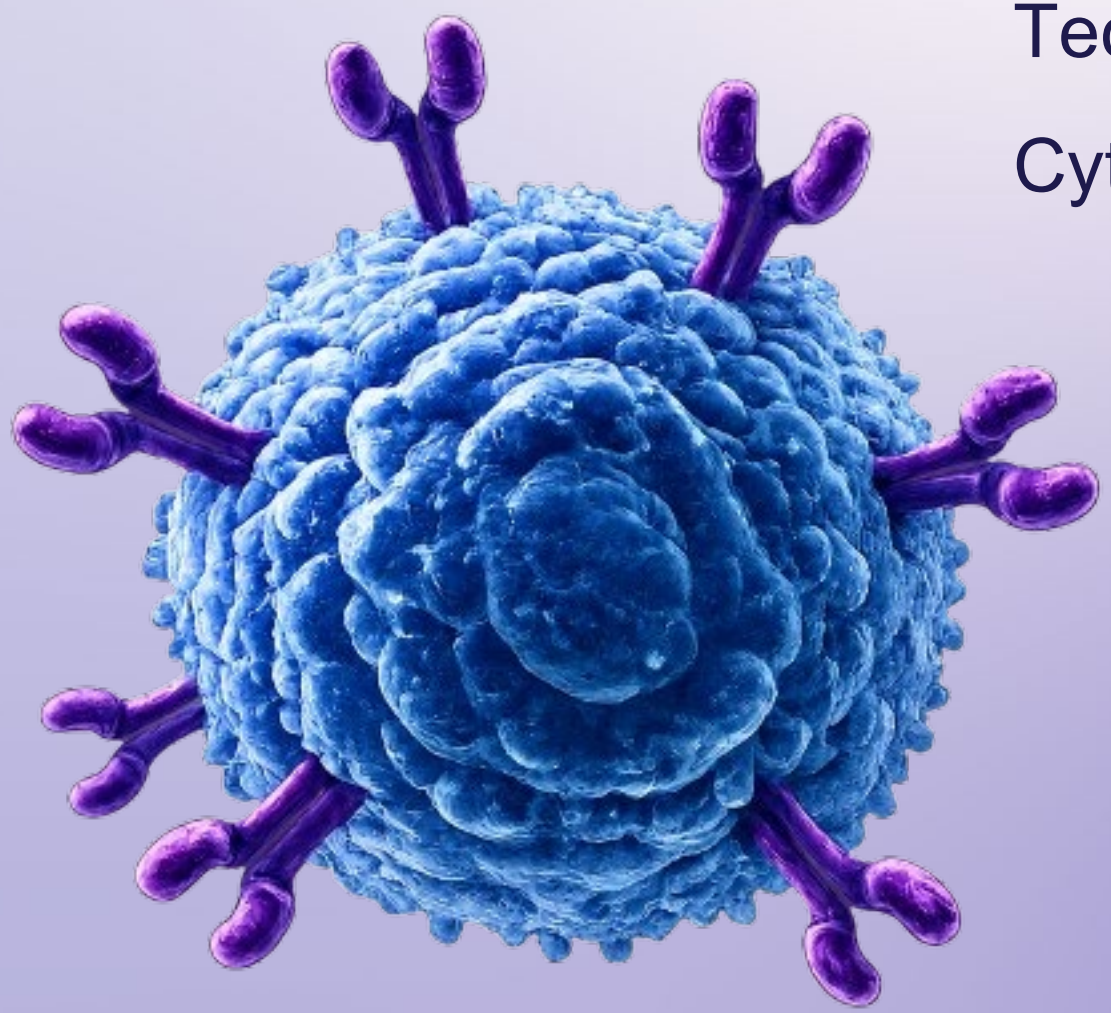
Take a Deep Breath!

Cerba Research



Created by Gemini

Cerba Research



Technical Challenges for Flow Cytometry in CAR-T Cell Clinical Trials

Detection of CAR-T cells

Detection of CAR-T cells

Cerba Research

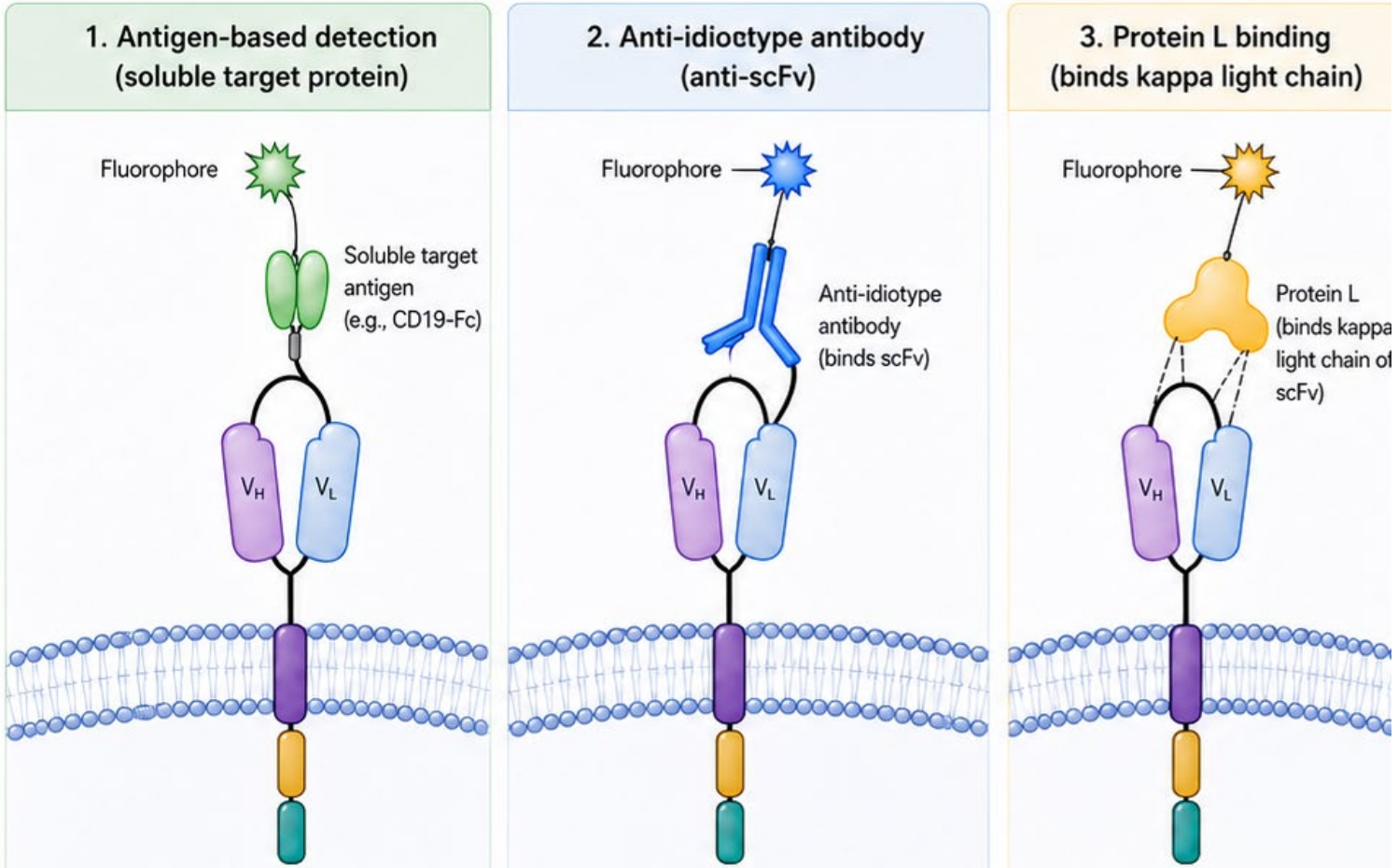
CAR construct (on CAR T cell)

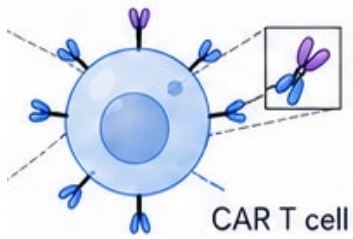
scFv (antigen-binding domain)

Transmembrane domain (embedded in cell membrane)

Co-stimulatory domain (e.g., CD28 or 4-1BB)

CD3ζ signaling domain





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Detection of CAR-T cells

Cerba Research

CAR construct (on CAR T cell)

scFv (antigen-binding domain)

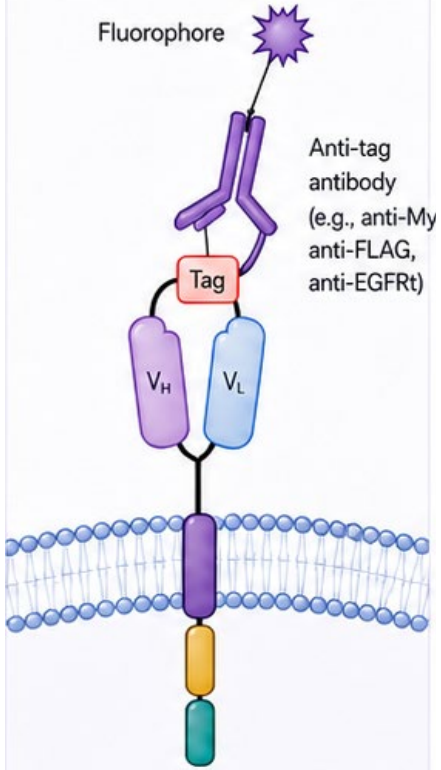
Transmembrane domain (embedded in cell membrane)

Co-stimulatory domain (e.g., CD28 or 4-1BB)

CD3ζ signaling domain

Tag (epitope tag: Myc, FLAG, EGFRt, etc.)

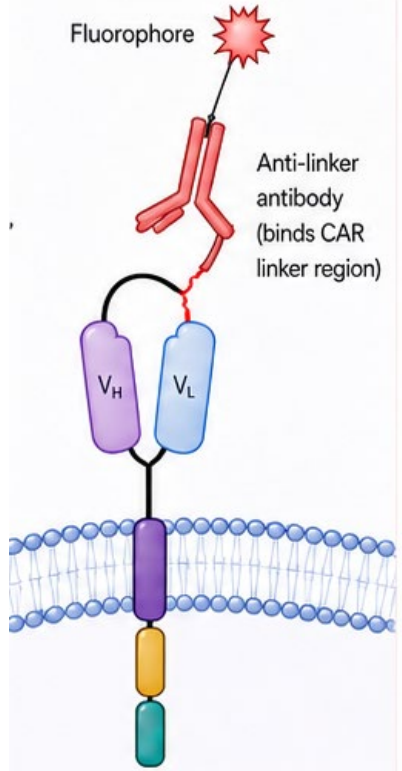
4. Tag-based detection (epitope tag)



How it works:

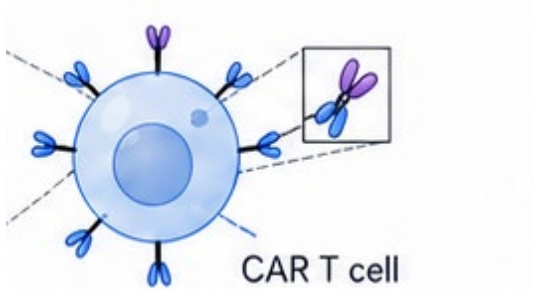
An antibody against an engineered epitope tag on the CAR binds to the tag.

5. Anti-linker antibody (binds CAR linker sequence)



How it works:

An antibody specific for the CAR linker sequence binds to the linker region between scFv and hinge.



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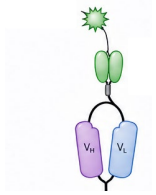
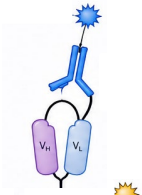
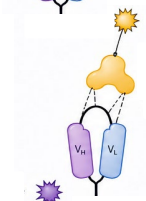
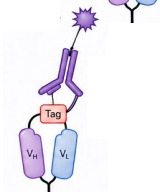
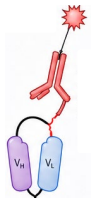
Detection of CAR-T cells

Cerba Research

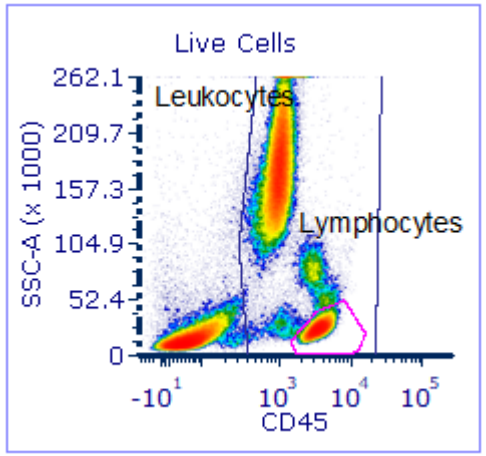
	Advantages	Disadvantages
 **Antigen-based detection**	Applicable across constructs targeting the same antigen, commercially available options (CD19, BCMA, CD22, CD33, …) reduce assay development time, high specificity	Lower affinity compared to anti-idiotype, risk of receptor activation/internalization
 **Anti-idiotype antibody**	High specificity and affinity, no risk of receptor activation/internalization	Must be custom-developed for each CAR (not universal). Only few commercially available options (CD19 FMC63 scFv, GD2 14G2a scFv)
 **Protein L binding**	Simple and inexpensive	Lower specificity, can bind endogenous immunoglobulins (background in patient samples)
 **Tag-based detection**	Highly consistent and reproducible across batches, can double as a safety switch marker (EGFRt for depletion)	Requires unique tag build in CAR construct, tag expression may not perfectly correlate with CAR surface expression, potential immunogenicity
 **Anti-linker antibody**	Applicable across constructs having same linker, commercially available options (Whitlow/218, G4S) reduce assay development time	Depends on linker accessibility

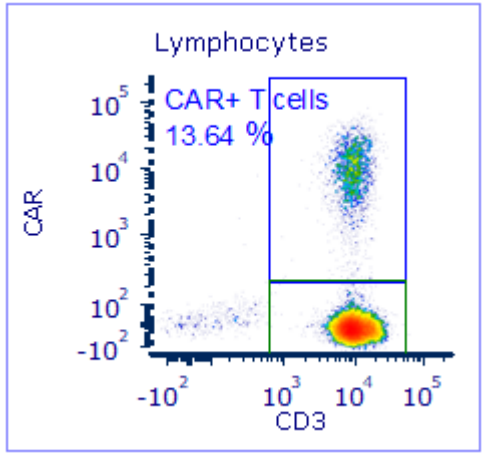
Reference: Ling et. al. Cytometry B Clin Cytom. 2026; 1-12

Detection of CAR-T cells - Example

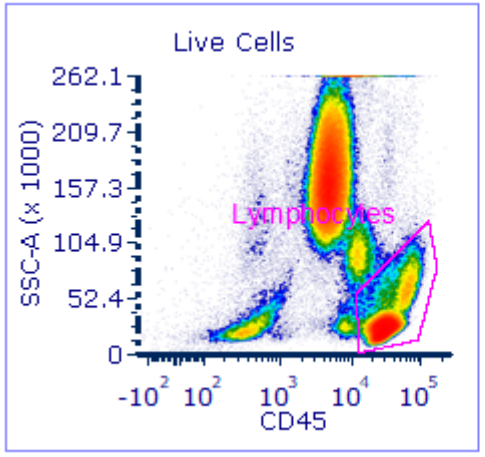
Cerba Research

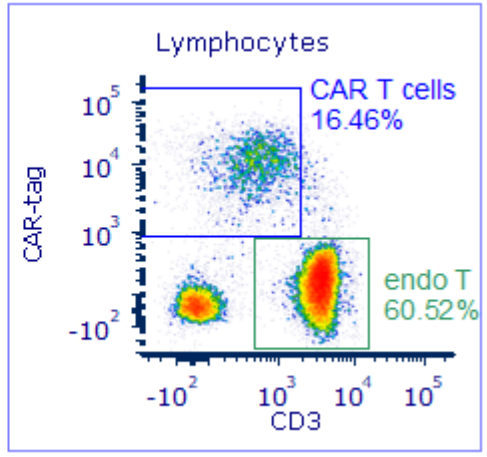
Autologous CAR-T cell using anti-idiotype antibody



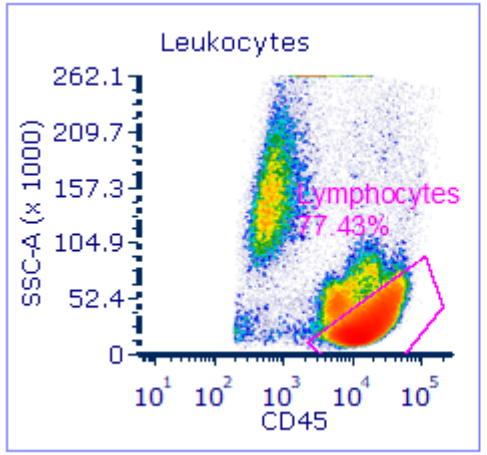


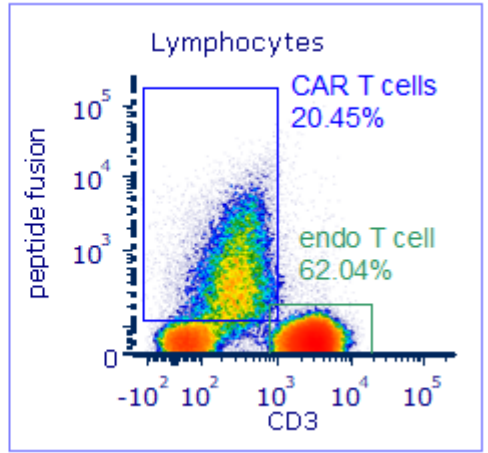
Allogenic CAR-T cell using anti-tag antibody





Allogenic CAR-T cell using peptide fusion

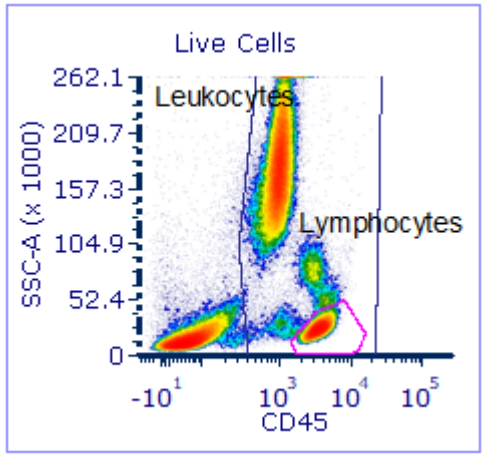


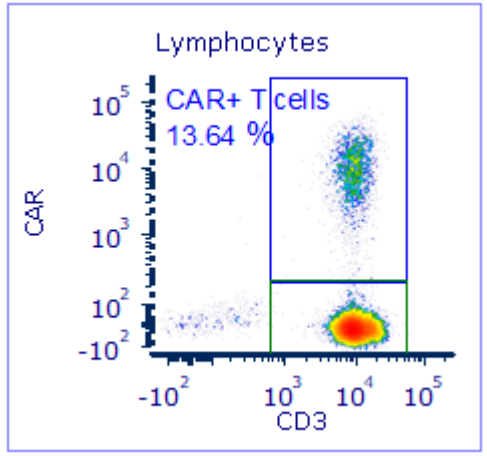


Detection of CAR-T cells – Gating Strategy for Autologous vs Allogenic

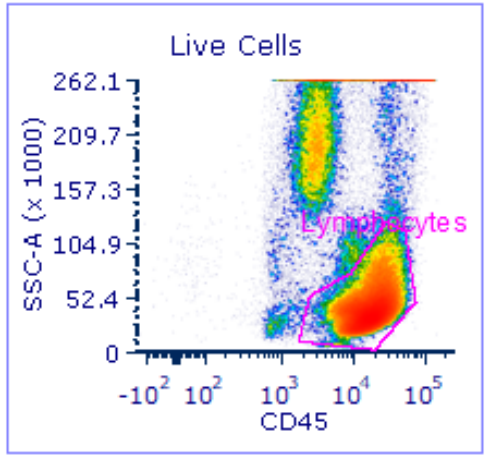
Cerba Research

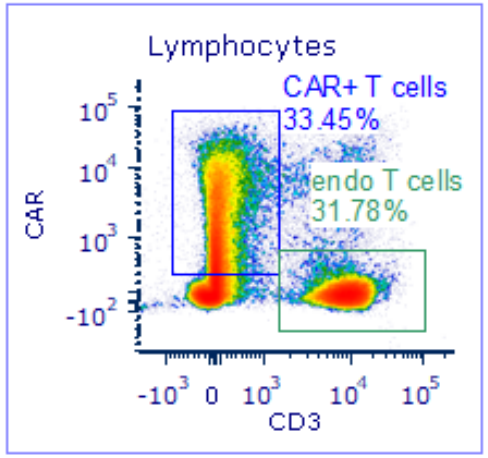
Autologous → CAR-T cells are within CD3 positive lymphocyte compartment



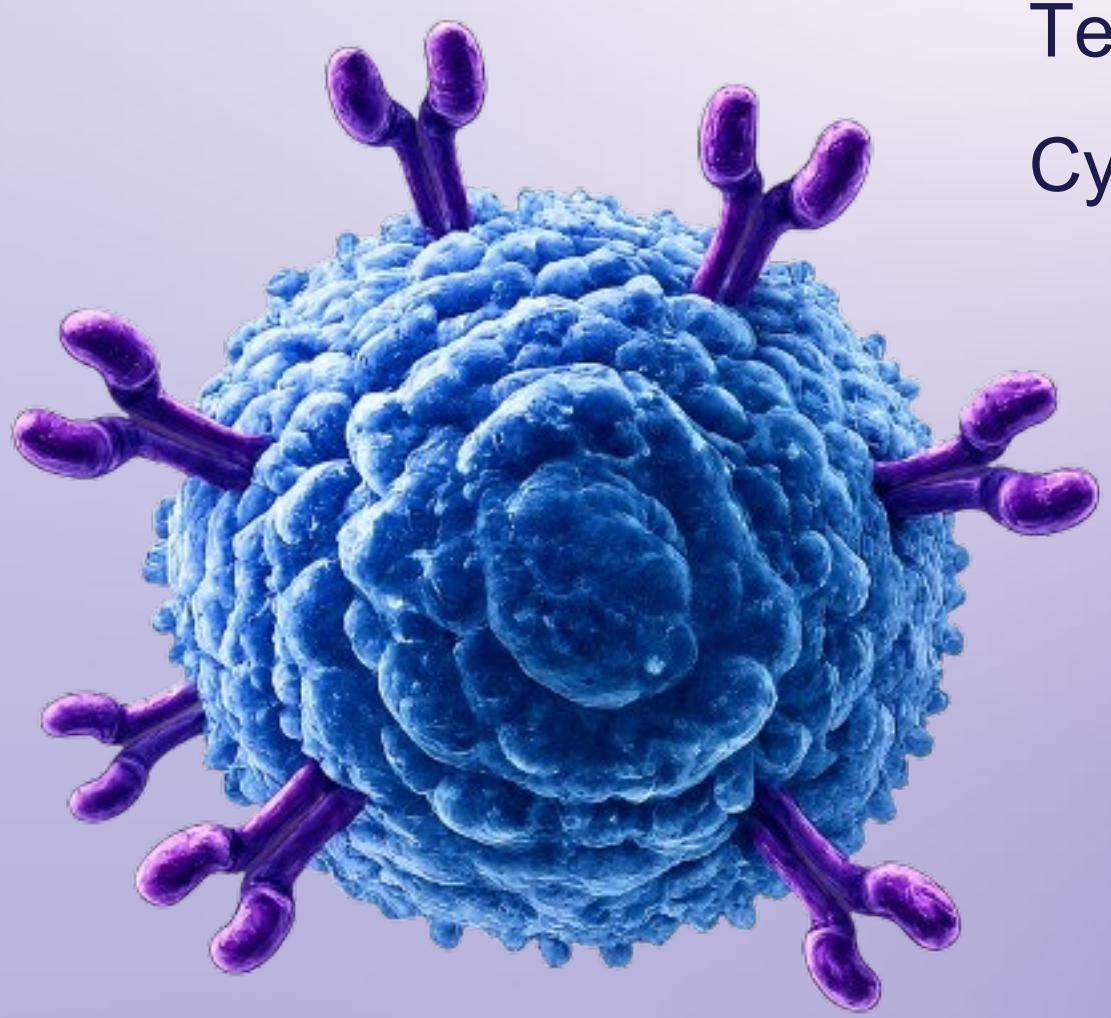


Allogeneic → CAR-T cells are within CD3 negative lymphocyte compartment





Cerba Research



Technical Challenges for Flow Cytometry in CAR-T Cell Clinical Trials

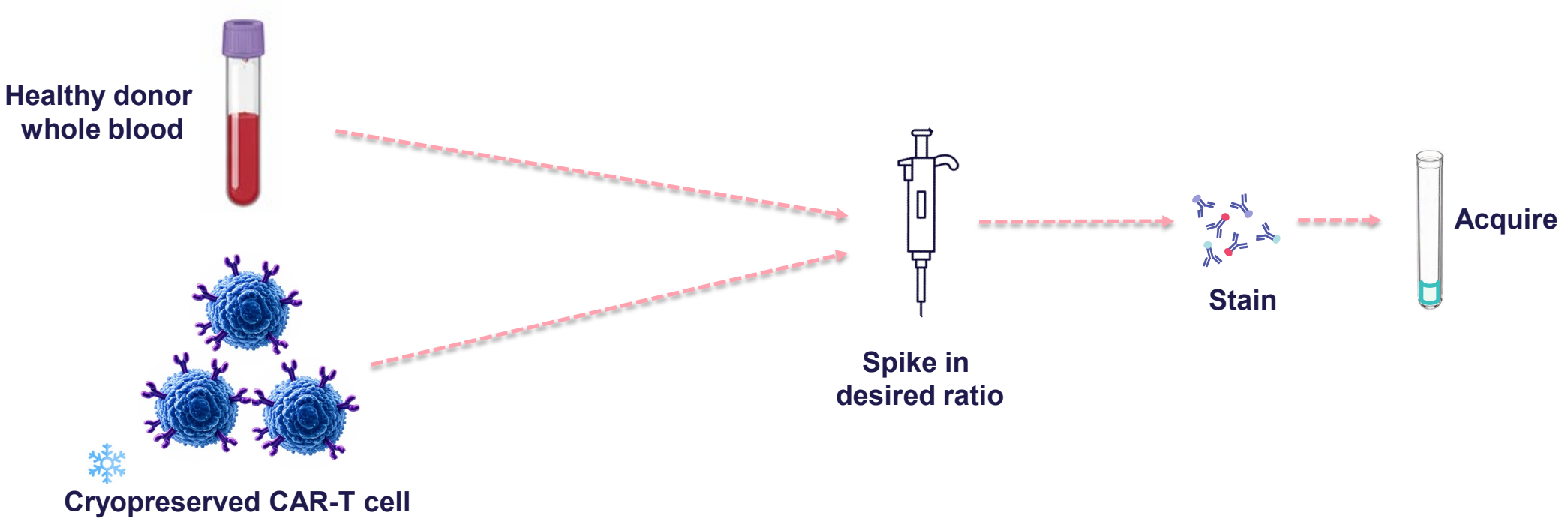
Samples for assay development and validation

Generation of CAR+ Validation Samples

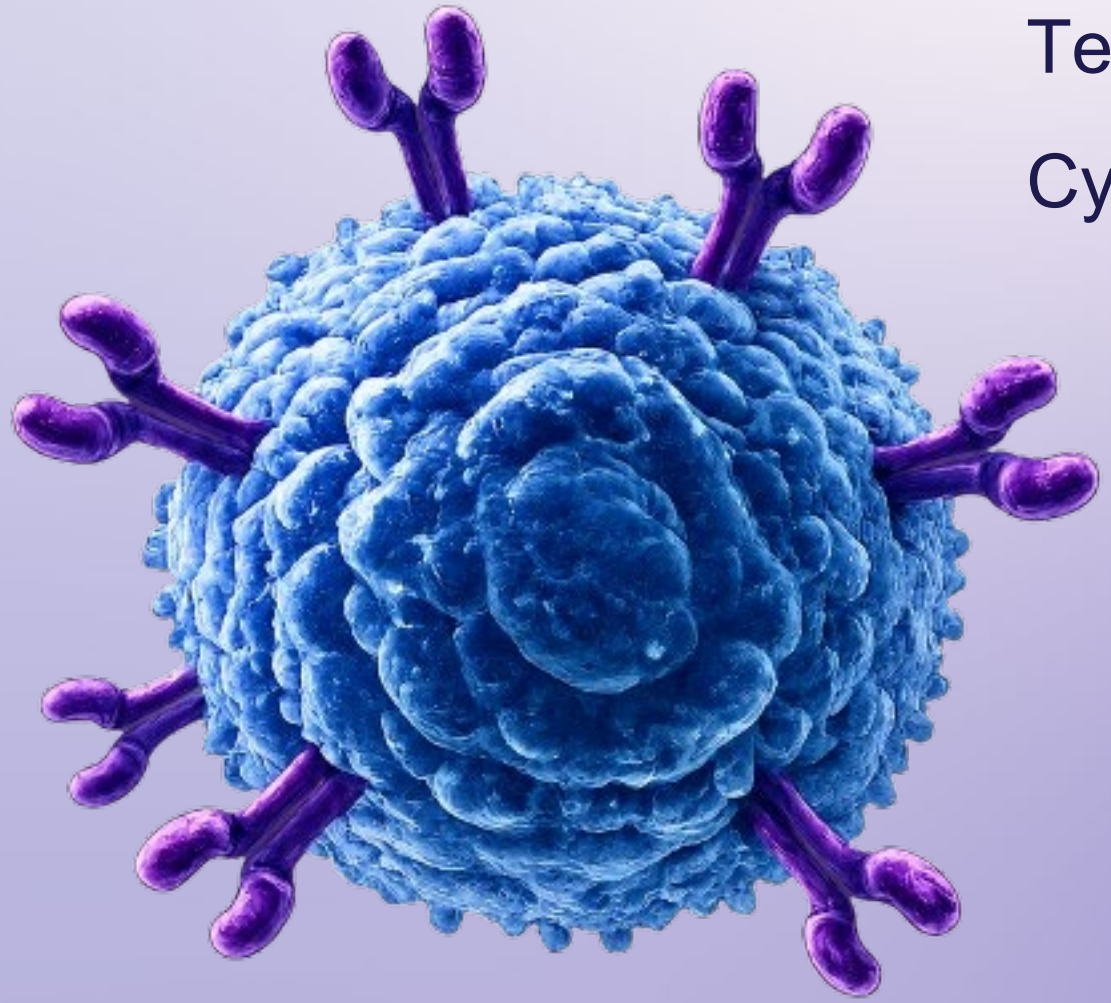
Cerba Research

During validation of a flow cytometry assays for detect CAR-T cells, you typically do not have access to samples from CAR-T cell treated patients.

So…… you will need to be a bit creative!



Cerba Research

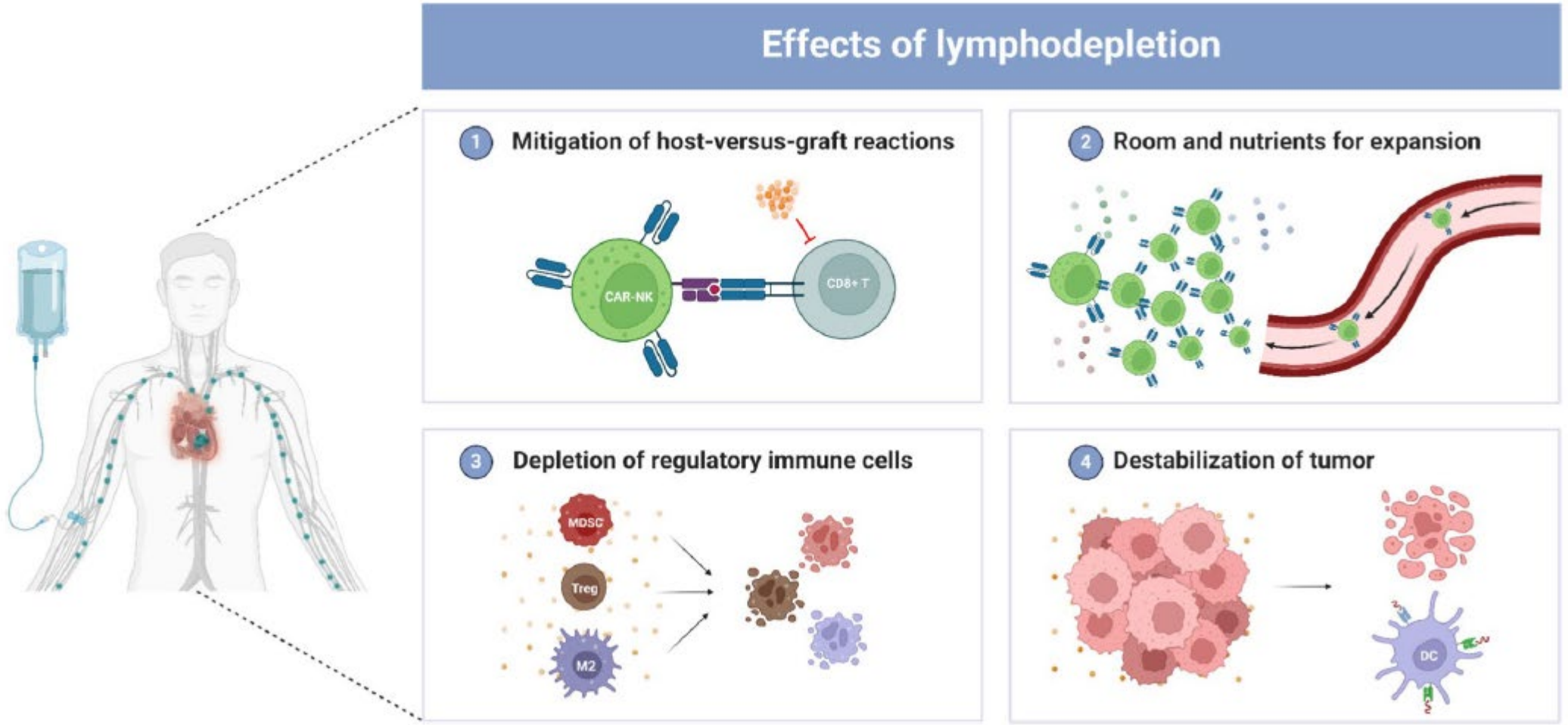


Technical Challenges for Flow Cytometry in CAR-T Cell Clinical Trials

Dealing with lymphodepleted samples

Lymphodepletion:
A Crucial Preparatory Step in CAR-T cell Therapy

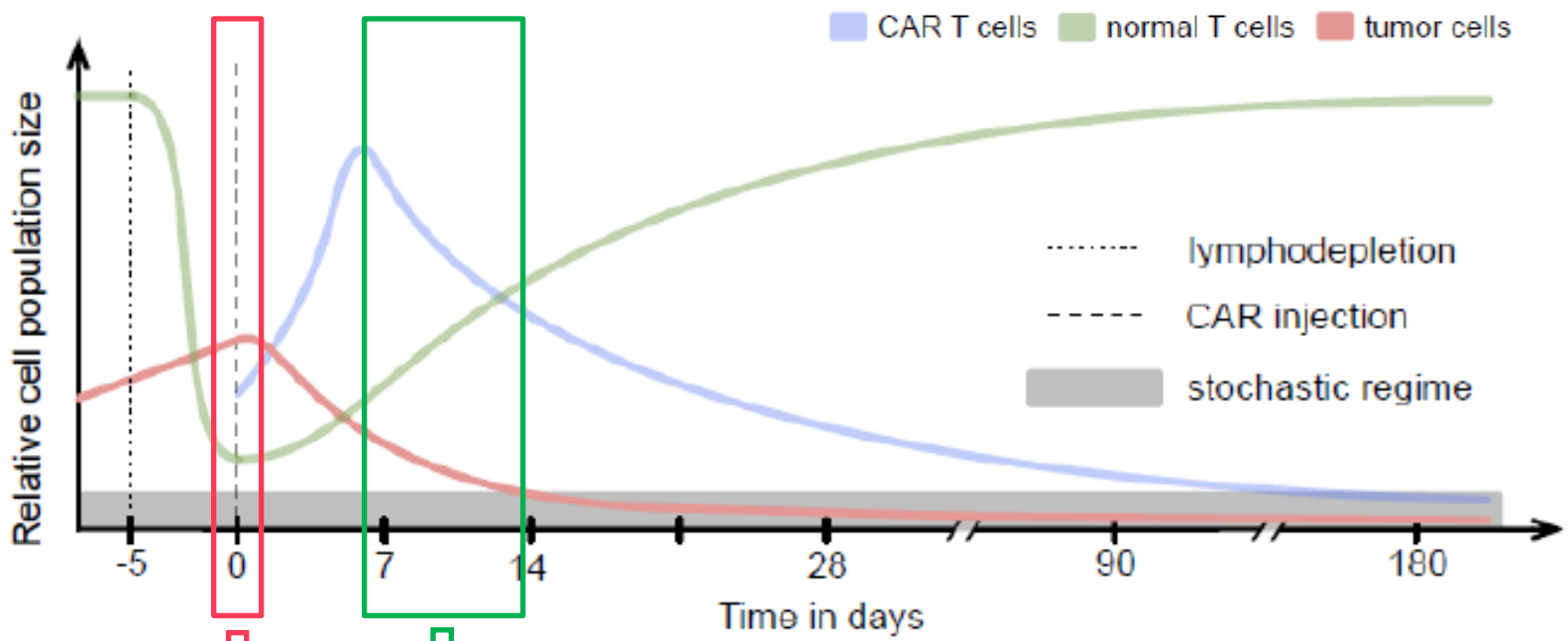
Cerba Research



Lymphodepletion and CAR-T cell Dynamics

Cerba Research

Lymphodepletion leads to variable cell numbers in your flow cytometry sample



low cell number
in day 0 visit

higher cell number due to CART expansion
and recovery of host cells after lymphodepletion

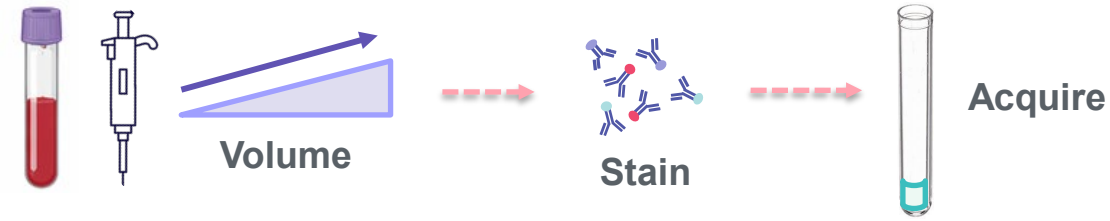
Reference: Kimmel et. al. Proc Biol Sci. 2021; 288:20210229

Lymphodepleted Samples - Options to Acquire More Cells

Cerba Research

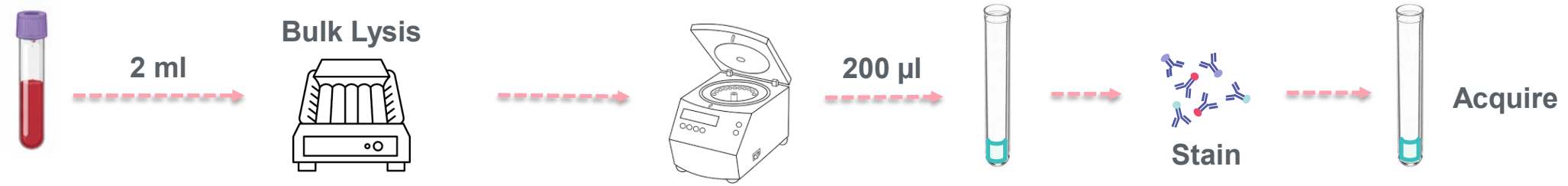
1. Increase sample volume

- Start with a higher sample volume before staining: 100 µl WB → 200 µl WB

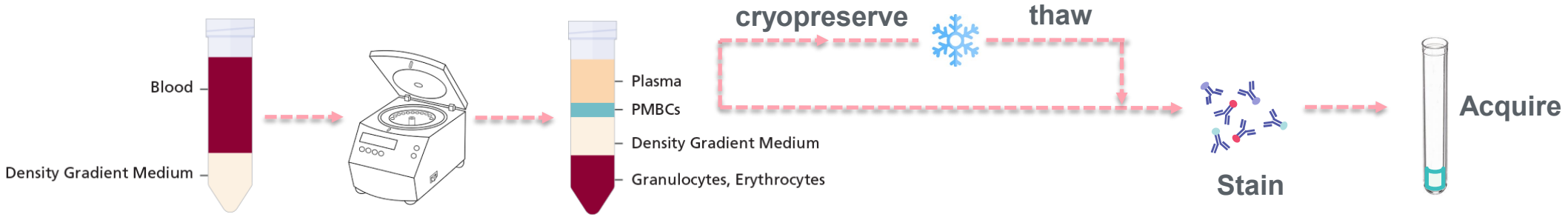


2. Concentrate sample before staining

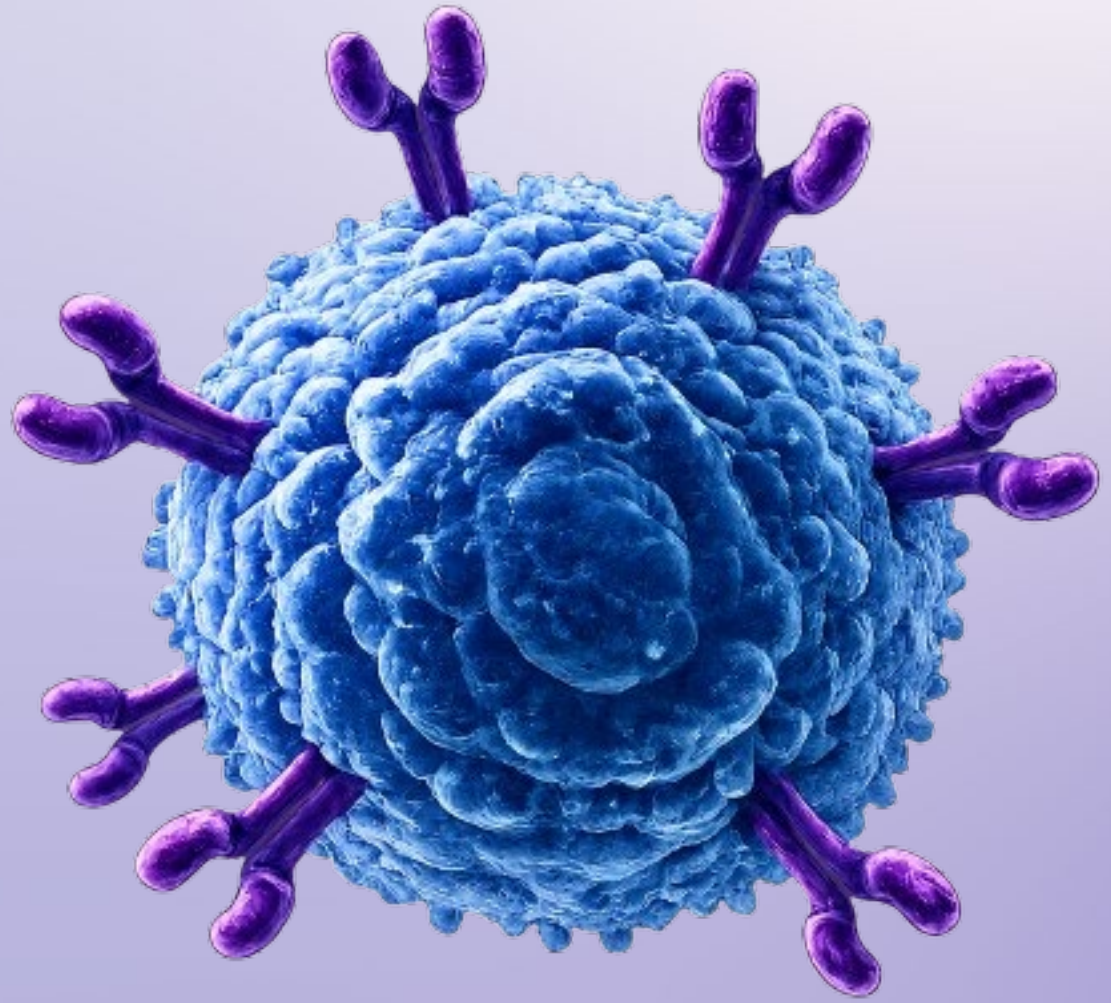
- **Bulk lysis:** bulk lyse a high volume of blood before staining, concentrate cells in lower volume (2 ml → 200 µl)



- **PBMC isolation:** perform PBMC isolation before staining to concentrate cells of interest, stain for instance 1 x 10^6 PBMCs per tube



Cerba Research



Case studies

Case study 1:

Enumeration and Characterization of CAR-T cells (autologous CAR-T cells)

CAR-T cell Panel

Cerba Research

Panel: T cell markers and CAR

Fluorochrome	V500	PE	PerCP-Cy5.5	PE-Cy7	AF700	APC-H7
(Filter)	(528/45)	(586/42)	(700/54)	(783/56)	(660/10)	(720/30)
Tube 1 (FMO)*	CD45		CD3	CD4	Viability	CD8
Tube 2	CD45	CAR	CD3	CD4	Viability	CD8
Tube 3**	CD45					

*FMO: gating control

** Trucount™ tube for absolute count calculation

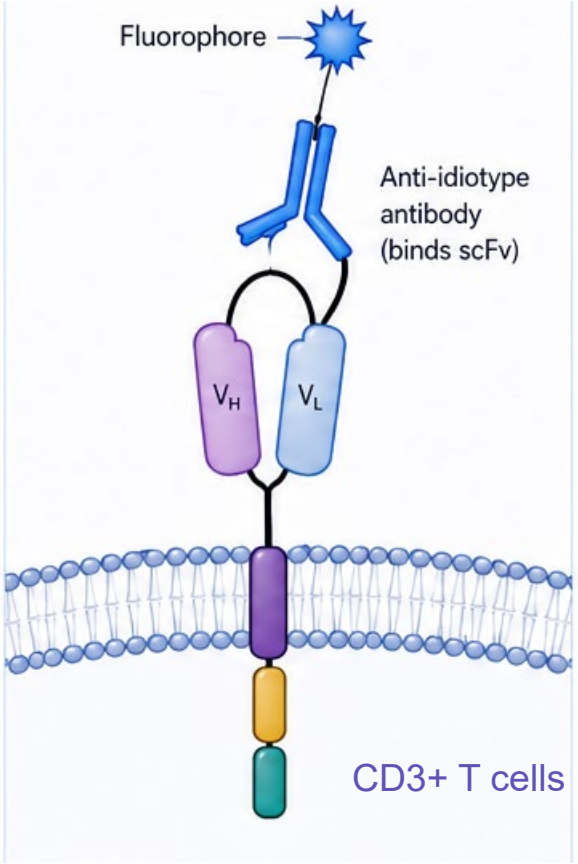
Instrument: BD FACSLyric (3-laser)

Lab: CAP/CLIA lab

Context of use of the data: Secondary end point (supports additional labeling claims)

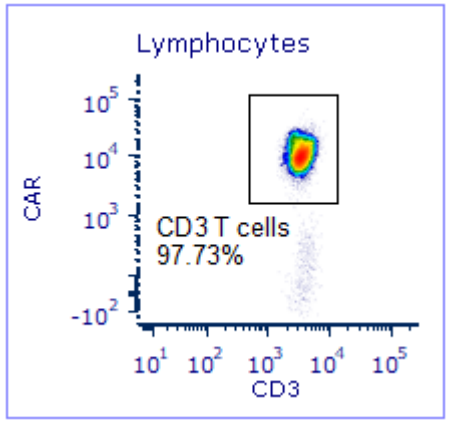
CAR-T cell Detection

Cerba Research



How it works:

An antibody specific for the unique idiotype of the CAR scFv binds to the antigen-binding domain.



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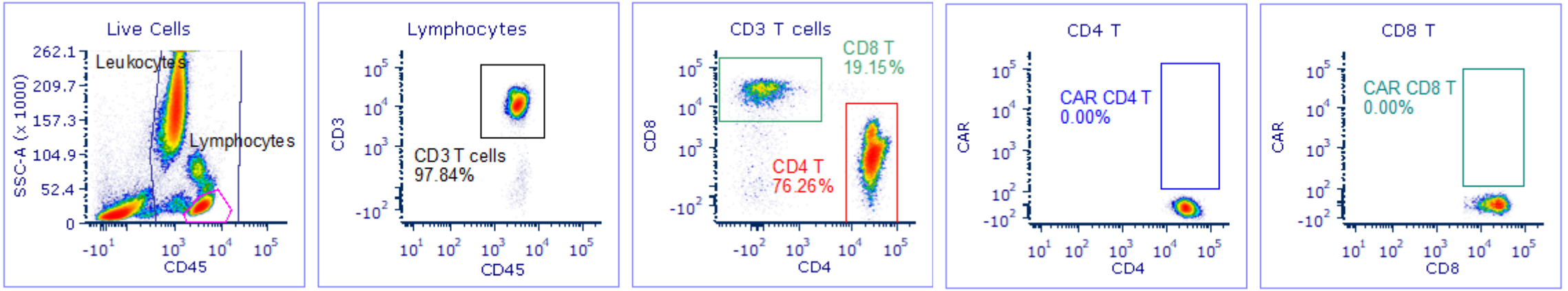
Enumeration and Characterization of CAR-T cells

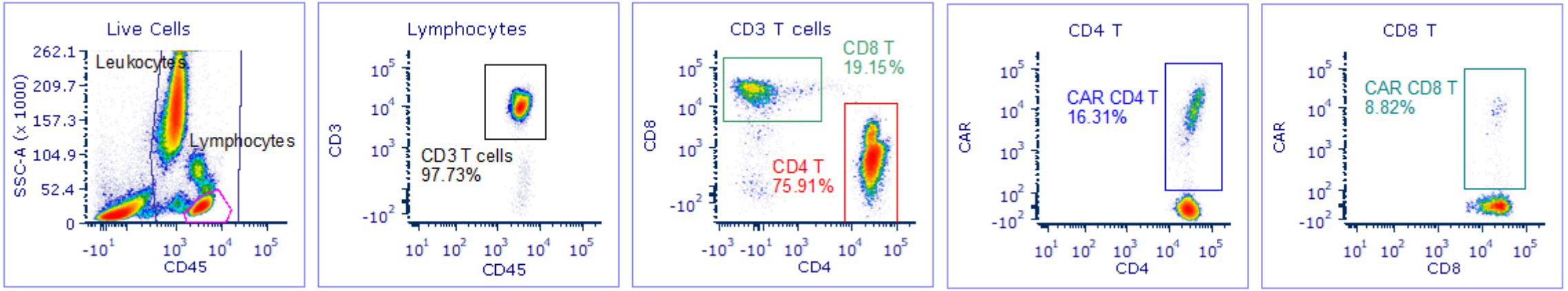
Cerba Research

CAR-T cell Panel

FMO for CAR



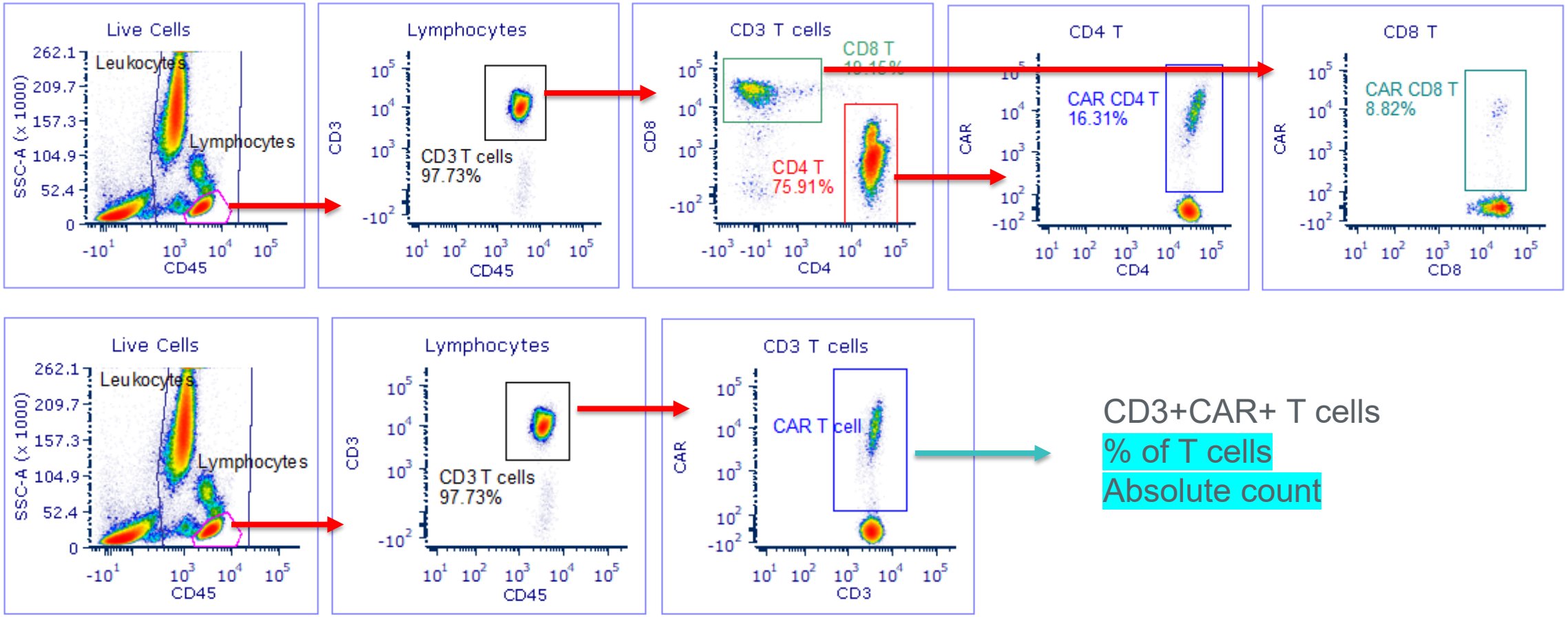
Fullstain



Enumeration and Characterization of CAR-T cells

Cerba Research

Gating Strategy



CD3+CAR+ T cells

% of T cells

Absolute count

Absolute Count Calculation

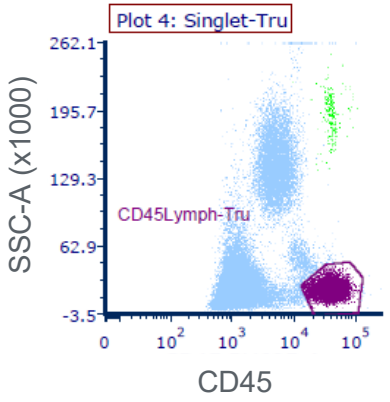
Cerba Research

Fluorochrome	V500	PE	PerCP-Cy5.5	PE-Cy7	AF700	APC-H7
(Filter)	(528/45)	(586/42)	(700/54)	(783/56)	(660/10)	(720/30)
Tube 1 (FMO)*	CD45		CD3	CD4	Viability	CD8
Tube 2	CD45	CAR	CD3	CD4	Viability	CD8
Tube 3**	CD45					

*FMO: gating control

** Trucount™ tube for absolute count calculation

Example:



Test Description	Events
CD45Lymph	38155
Bead Events	7961
CD45Lymph Absolute count (cells/uL)	2334

CAR-T cell (% of lymphocytes) = 5.0

CAR-T cell (cells/µL) = (5/100)*2334
= **117**

Lymphocyte absolute count from Trucount™ tube based on CD45+ lymphocytes is calculated as follows:

$$\text{Lymphocyte absolute count}\left(\frac{cells}{\mu L}\right) = \frac{\text{Lymphocytes Events} \times \text{lot specific bead concentration}}{Bead\ events \times sample\ volume}$$

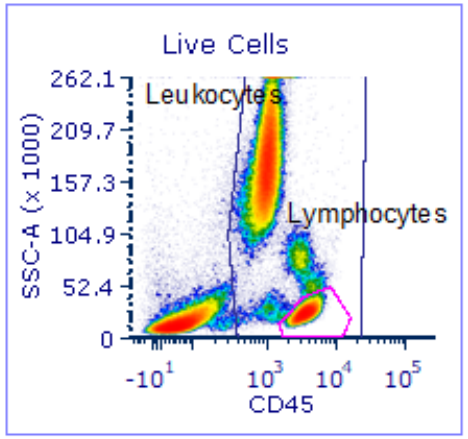
$$\textbf{CAR} - \textbf{T cell absolute count}\left(\frac{\text{cells}}{\mu\text{L}}\right) = \frac{\text{CAR} - \text{T cells (\% of Lymphocytes)}}{100} \times \text{Lymphocyte absolute count}$$

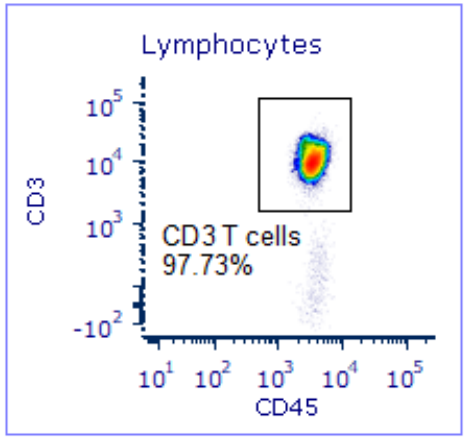
Quantitation of CAR Expression

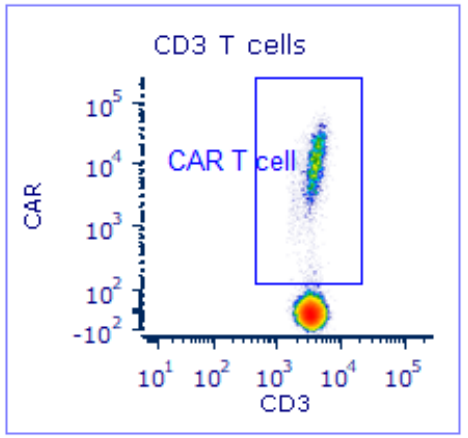
Cerba Research

Added extra tube to acquire Quantibrite beads

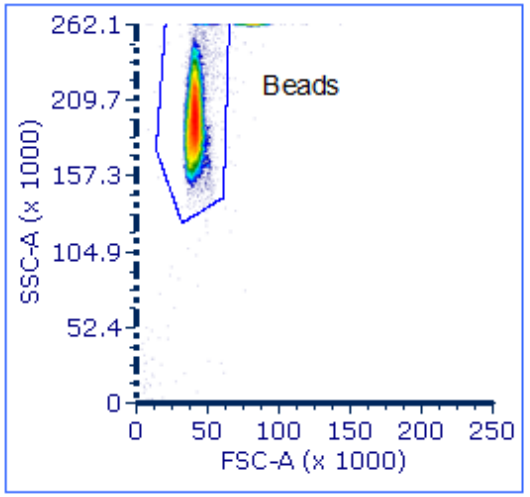
Fullstain

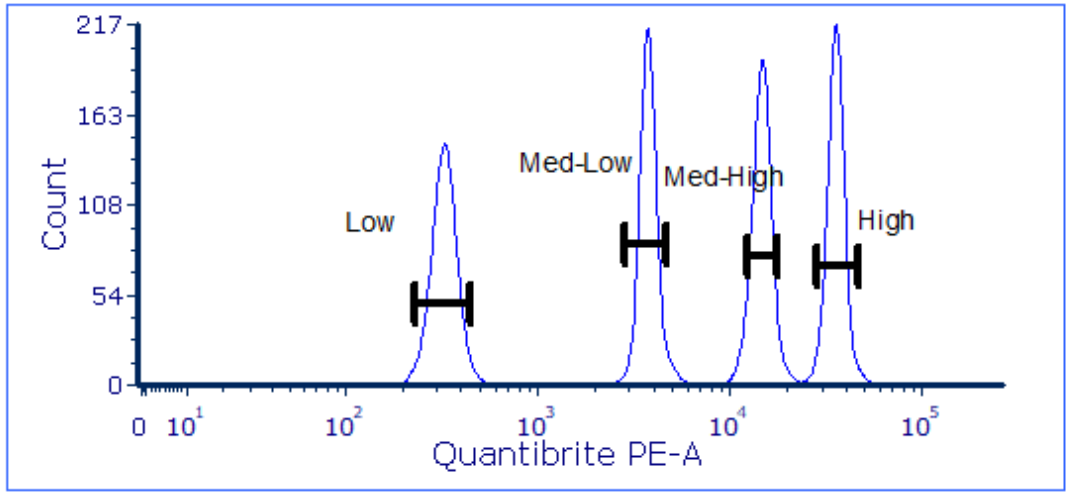






Quantification beads





CAR+ T cells

CAR MFI 8170

CAR+ T cells

MESF calculation

25878

MESF calibration enables cross-day normalization of fluorescence signals, supporting reproducible estimation of CAR expression levels by flow cytometry.

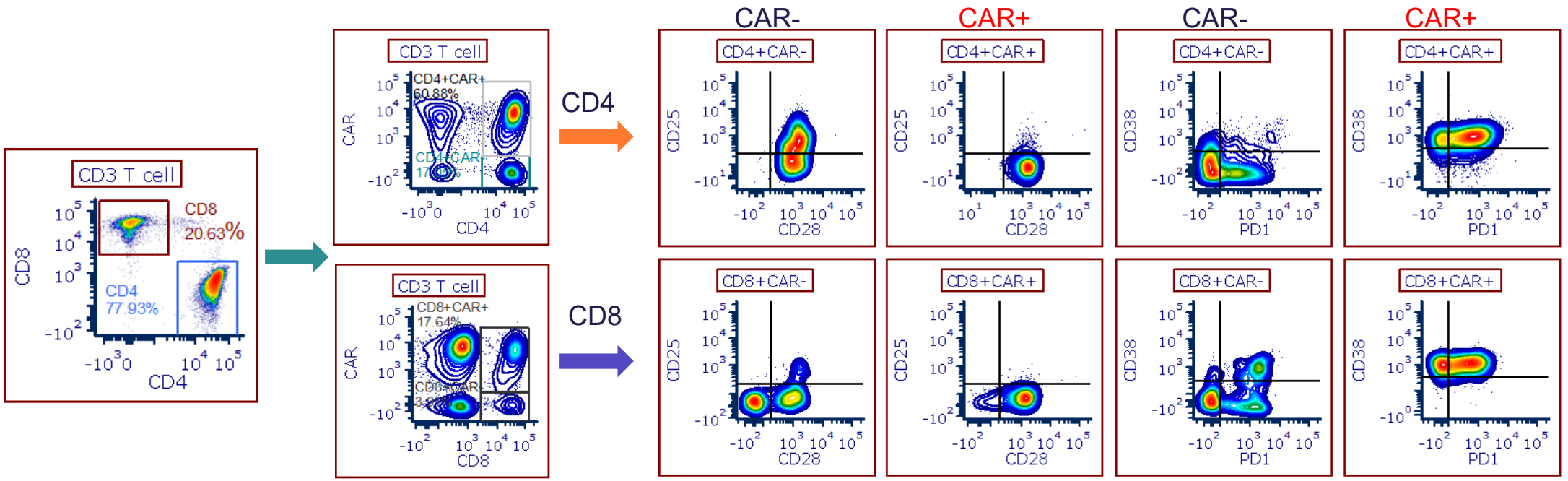
MESF: Molecules of Equivalent Soluble Fluorochrome

Characterization of CAR-T cells

Cerba Research

New panel for characterization of CAR-T cells with activation, exhaustion and Treg markers

Activation/exhaution markers: CD25, CD28, CD38, PD1

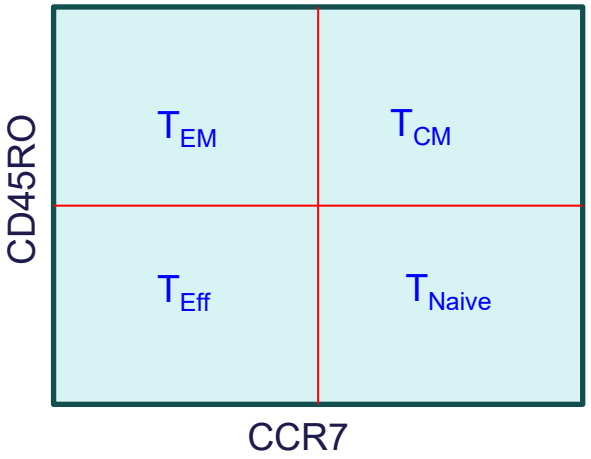


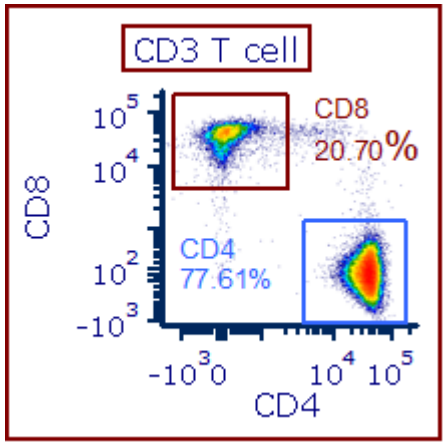
- ✓ Assessment of activation/exhaustion markers may provide insights into the phenotypic evolution and functional states of CAR+ T-cell populations in individual patients.
- ✓ In addition, assessment of endogenous (CAR-) CD4+ and CD8+ T cells could help elucidate overall status of the immune system in individual patients

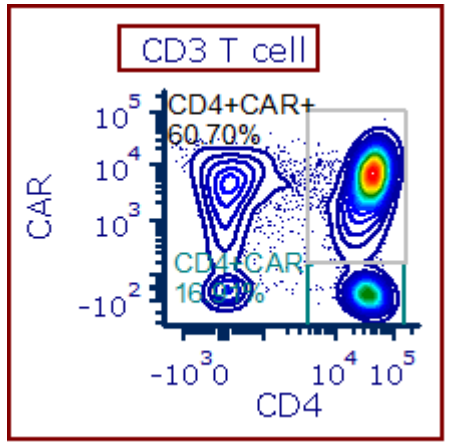
Characterization of CAR-T cells

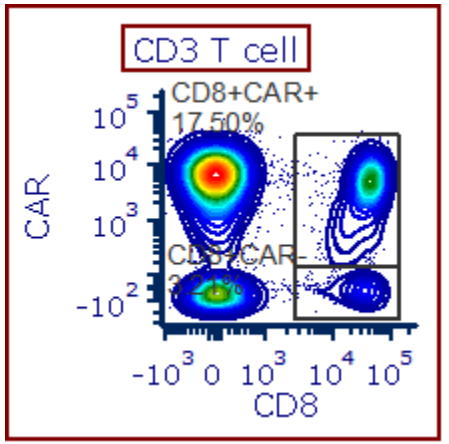
Cerba Research

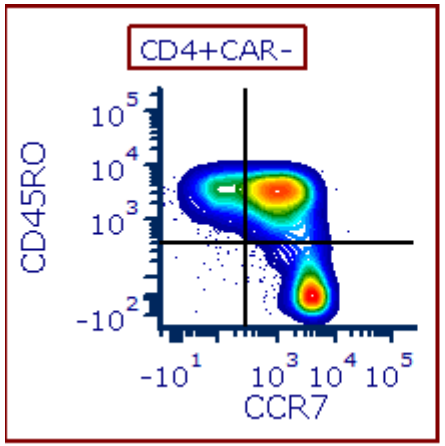
Memory phenotypes

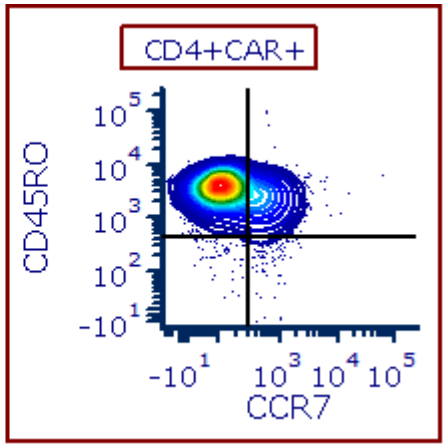


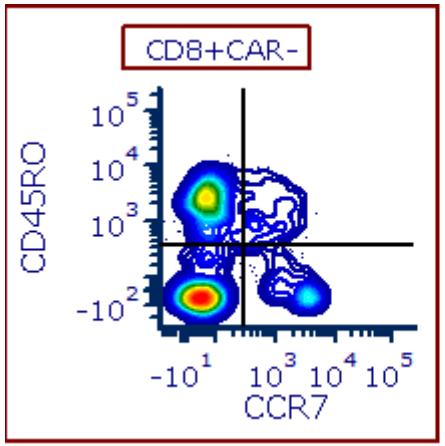


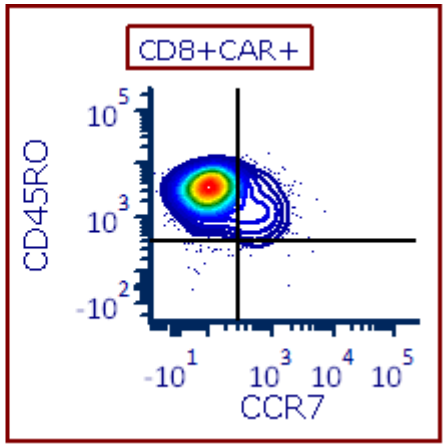












✓ It is critical to understand the differentiation of CAR+ T cells into distinct memory subsets, as variation in these subsets influences the effectiveness of CAR+ T-cell therapy across individual patients.

Characterization of CAR-T cells

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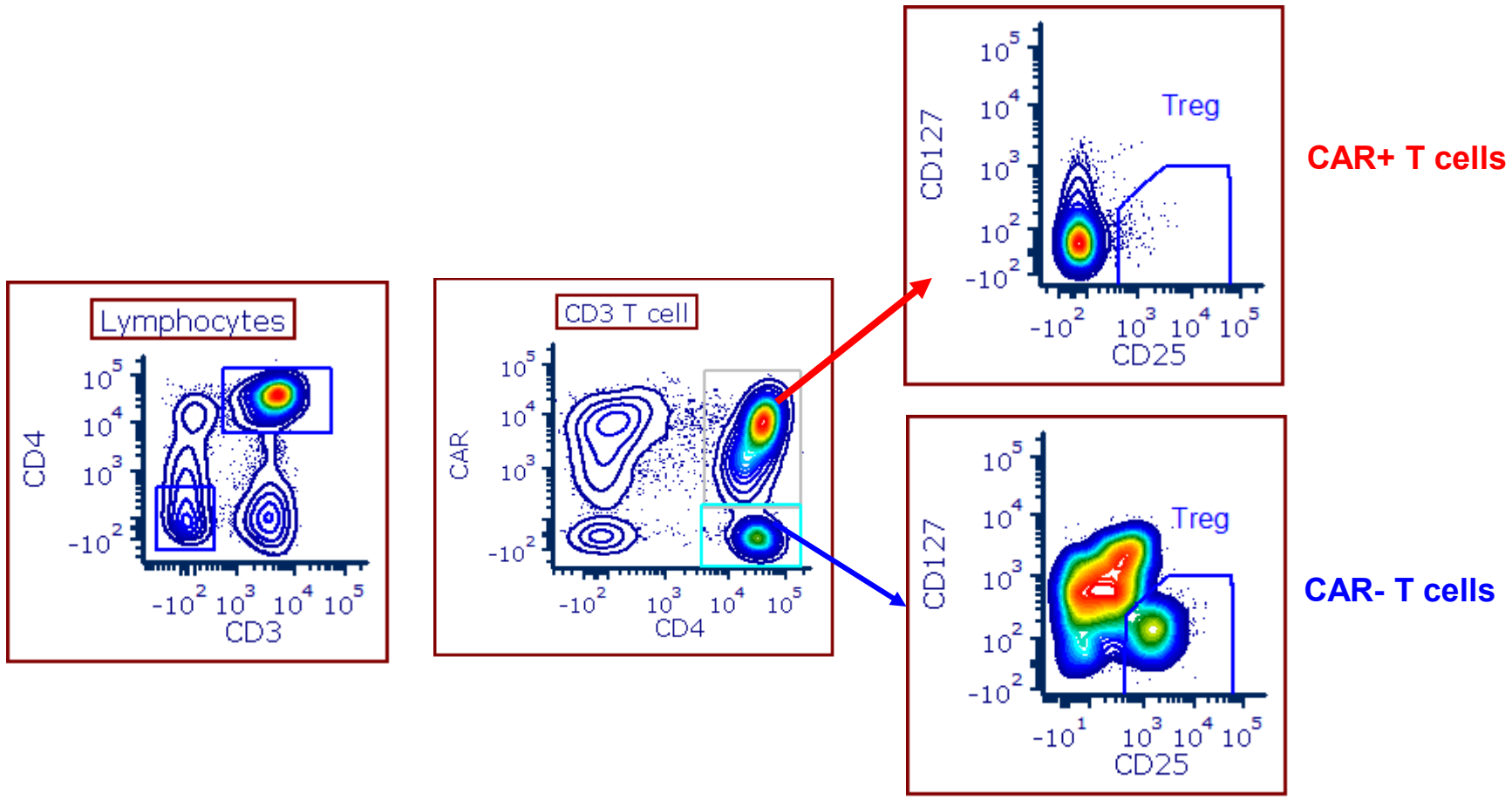
Regulatory T cells (Treg)



CAR+ T cells

CAR- T cells

- ✓ Assessment of CAR⁺ Treg cells may provide insights into the phenotypic evolution and functional states of CAR⁺ T-cell populations in individual patients.
- ✓ Assessment of CAR⁺ Treg cells could help elucidate regulatory mechanisms influencing CAR⁺ T-cell function in individual patients
- ✓ Assessment of endogenous (CAR-) Treg cells could help elucidate regulatory mechanisms influencing CAR⁺ T-cell function in individual patients

Memory Subset of CAR-T cells

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- ✓ CAR+ T cells differentiate into memory subsets (e.g., TSCM, TCM, TEM).
- ✓ These subsets strongly influence persistence, expansion, and clinical efficacy.
- ✓ This is well-supported in literature.

Cell

CellPress
OPEN ACCESS

Article

Distinct *in vivo* dynamics of donor-derived stem cell memory CAR T cells post-allogeneic HSCT relapse

Luca Gattinoni,[1,2,3,4,15,16,*] Gabriele Inchingolo,[1,15] Dennis C. Harrer,[5] Alberto Susana,[6] Simone Puccio,[6,7] Dragana Slavkovic-Lukic,[1] Danielle A. Natrakul,[8] Nicholas Strieder,[9] Christoph Heuser-Loy,[1] Jeremy G. Baldwin,[1]

Highlights

- CAR T_{SCM} cells expand and persist longer, achieving complete responses at low doses
- CAR T_{SCM} cells induce mild cytokine release syndrome, primarily driven by IFN-γ
- CAR T_{SCM} cells exhibit distinct *in vivo* dynamics, repopulating the T_{SCM} compartment
- CAR T_{SCM} maintenance is sustained by clonal succession waves

Cell *189*, 1–15, June 11, 2026

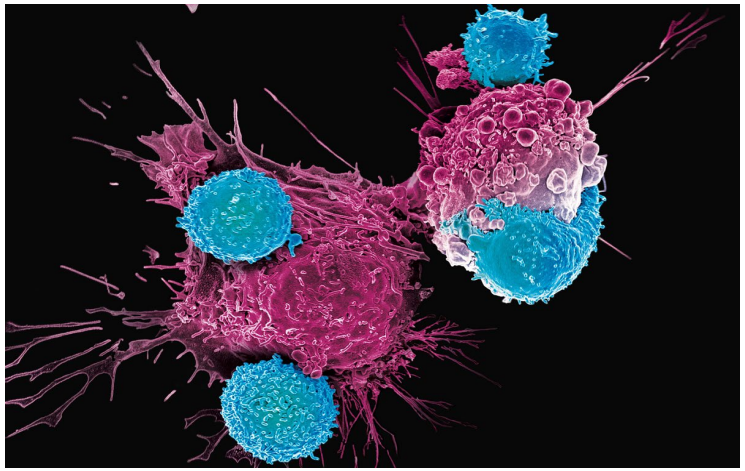
T_{SCM}:CCR7+CD45RA+CD45RO-CD95+

Flow cytometry will play a critical role in characterizing CAR-T cells during pre-infusion and post-infusion!

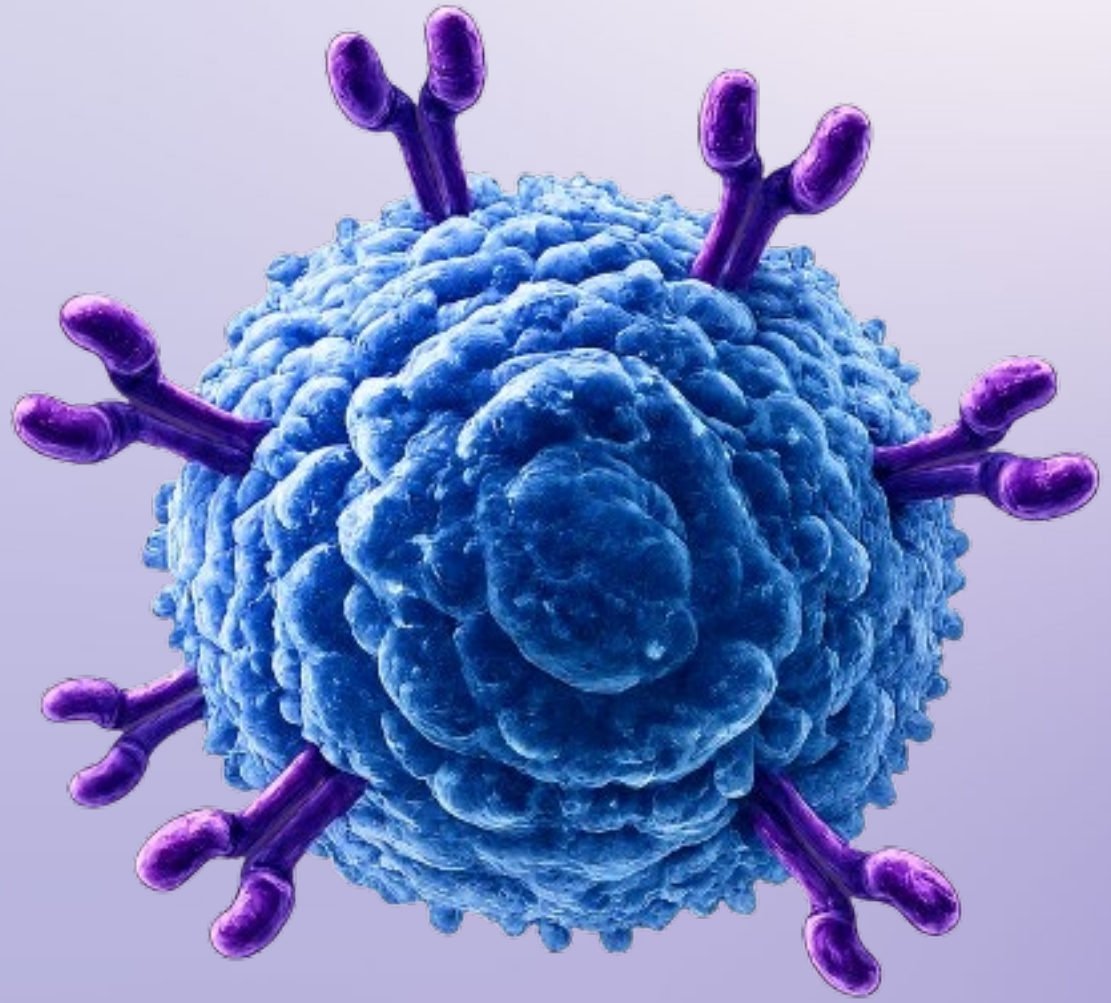
Conclusion

Cerba Research

- Reliable CAR-T cell enumeration and phenotypic characterization are critical for linking product attributes to **clinical response and durability.**
- Quantification of CAR+ cells, along with assessment of key subsets (e.g., memory, exhausted, activated), provides insight into **in vivo expansion, persistence, and efficacy**
- Standardized flow cytometry workflows enable **consistent monitoring across manufacturing and post-infusion timepoints**
- Ultimately, integrating high-quality analytical data with clinical outcomes will drive **improved patient stratification and next-generation CAR-T optimization**



This electron microscopic image shows CAR-T cells (blue) attacking cancer cells (pink). Reference: Dr. Prasad Adusumilli, www.mskcc.org/news



Case studies

Case study 2:

TBNK assay in allogeneic CAR-T cell

Case Study 2

Cerba Research

TBNK for assessing T cells, B cells and NK cells

What is the challenge with the standard 6-color TBNK assay for patients receiving Allogeneic CAR-T cells ?

Detector (laser)	FL1 (405)	FL2 (405)	FL3 (405)	FL4 (405)	FL5 (405)	FL6 (488)	FL7 (488)	FL8 (488)	FL9 (488)	FL10 (640)	FL11 (640)	FL12 (640)
Fluorochrome (Filter)	BV421 (448/45)	BV510 (528/45)	BV605 (606/36)	BV711 (715/50)	BV785 (755LP)	FITC (527/32)	PE (586/42)	PerCP-Cy5.5 (700/54)	PE-Cy7 (783/56)	APC (660/10)	APC-R700 (720/30)	APC-Cy7 (783/56)
6-color TBNK						CD3	CD16/56	CD45	CD4	CD19		CD8

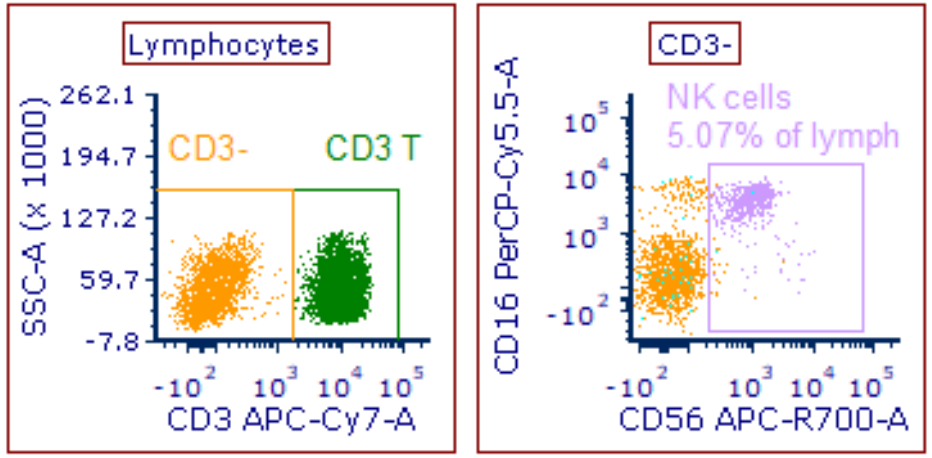
Case Study 2

Cerba Research

Traditional gating from standard 6-color TBNK

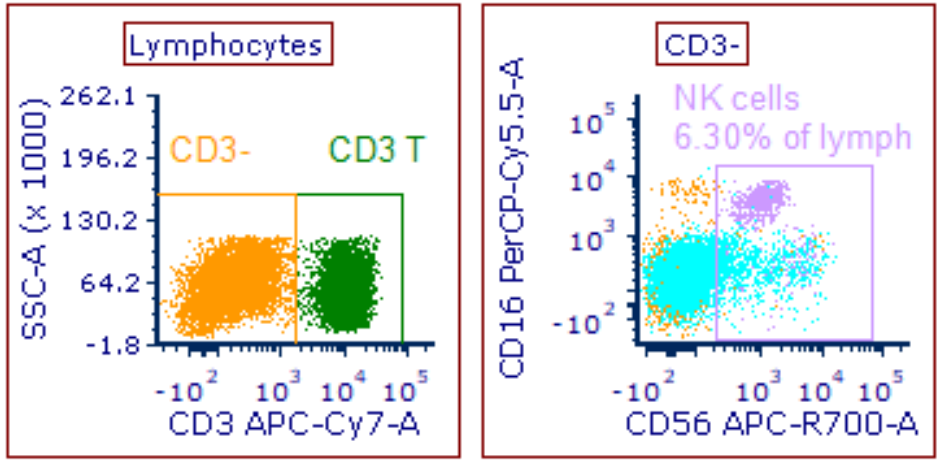
leads to inclusion of $CD56^{+}CD3^{-}$ allogeneic CAR-T cells in the NK-cell population

Healthy volunteer whole blood



CD3- lymphocytes
CD3+ lymphocytes
NK cells
Allogeneic CAR-T cells

Healthy volunteer whole blood spiked with allogeneic CAR-T cells



Case Study 2

Cerba Research

TBNK redesign

Detector (laser)	FL1 (405)	FL2 (405)	FL3 (405)	FL4 (405)	FL5 (405)	FL6 (488)	FL7 (488)	FL8 (488)	FL9 (488)	FL10 (640)	FL11 (640)	FL12 (640)
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6-color TBNK						CD3	CD16/56	CD45	CD4	CD19		CD8

same Lyse/No Wash assay in Trucount tube
100 µl whole blood instead of 50 µl

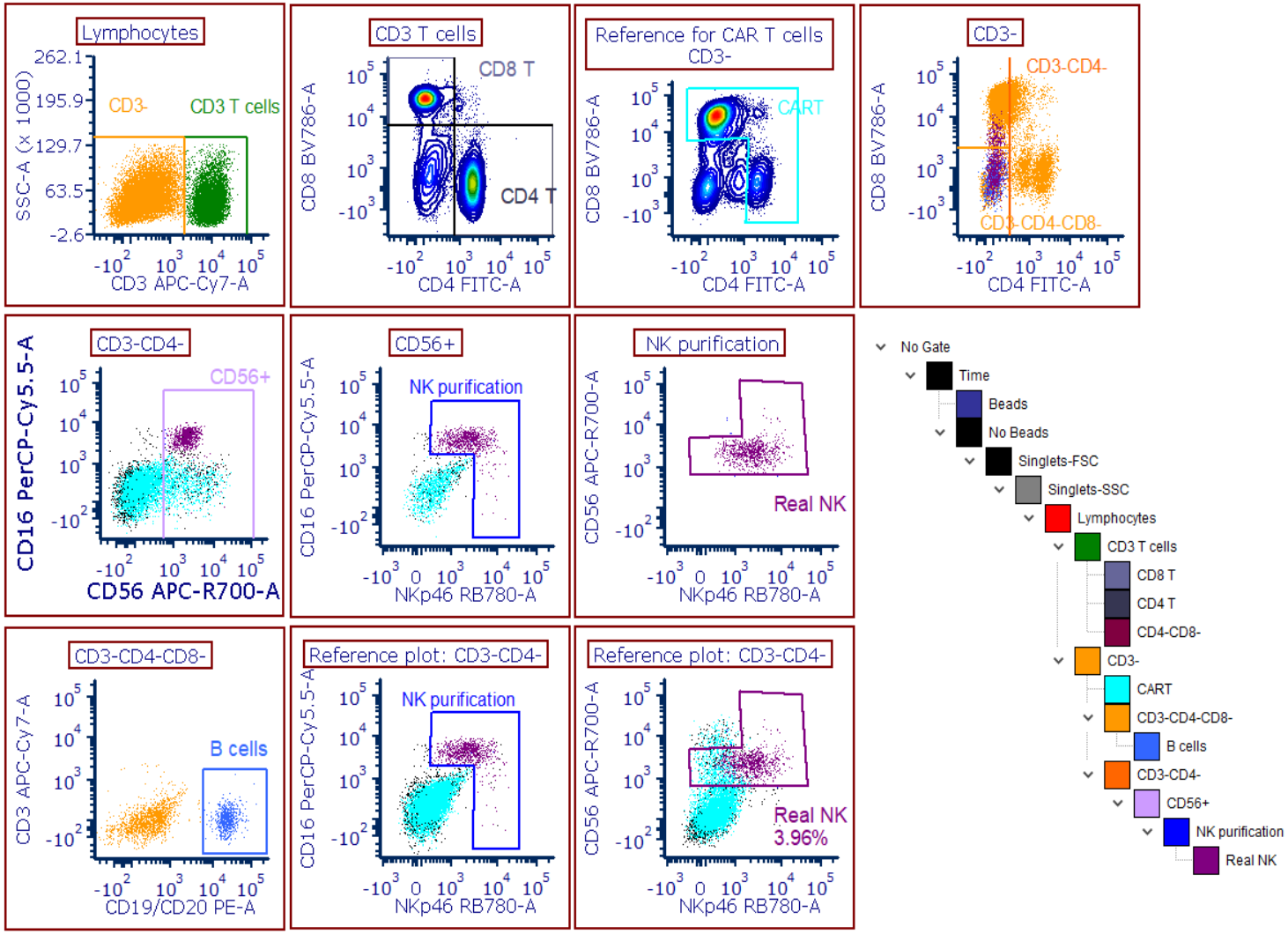
addition of NKp46 → extra NK cell marker
addition of CD20 → extra B cell marker in patients with anti-CD19 therapy
CD16 and CD56 split over two fluorochromes

Detector (laser)	FL1 (405)	FL2 (405)	FL3 (405)	FL4 (405)	FL5 (405)	FL6 (488)	FL7 (488)		FL8 (488)	FL9 (488)	FL10 (640)	FL11 (640)	FL12 (640)
Fluorochrome (Filter)	BV421 (448/45)	BV510 (528/45)	BV605 (606/36)	BV711 (715/50)	BV785 (755LP)	FITC (527/32)	PE (586/42)		PerCP-Cy5.5 (700/54)	RB780 (783/56)	APC (660/10)	APC-R700 (720/30)	APC-Cy7 (783/56)
Optimized TBNK			CD45		CD8	CD4	CD19	CD20	CD16	NKp46		CD56	CD3

Case Study 2

Cerba Research

Addition of NK cell marker NKp46 allows for proper distinction of host NK cells from CD56+CD3- CAR-T cells



Case Study 2

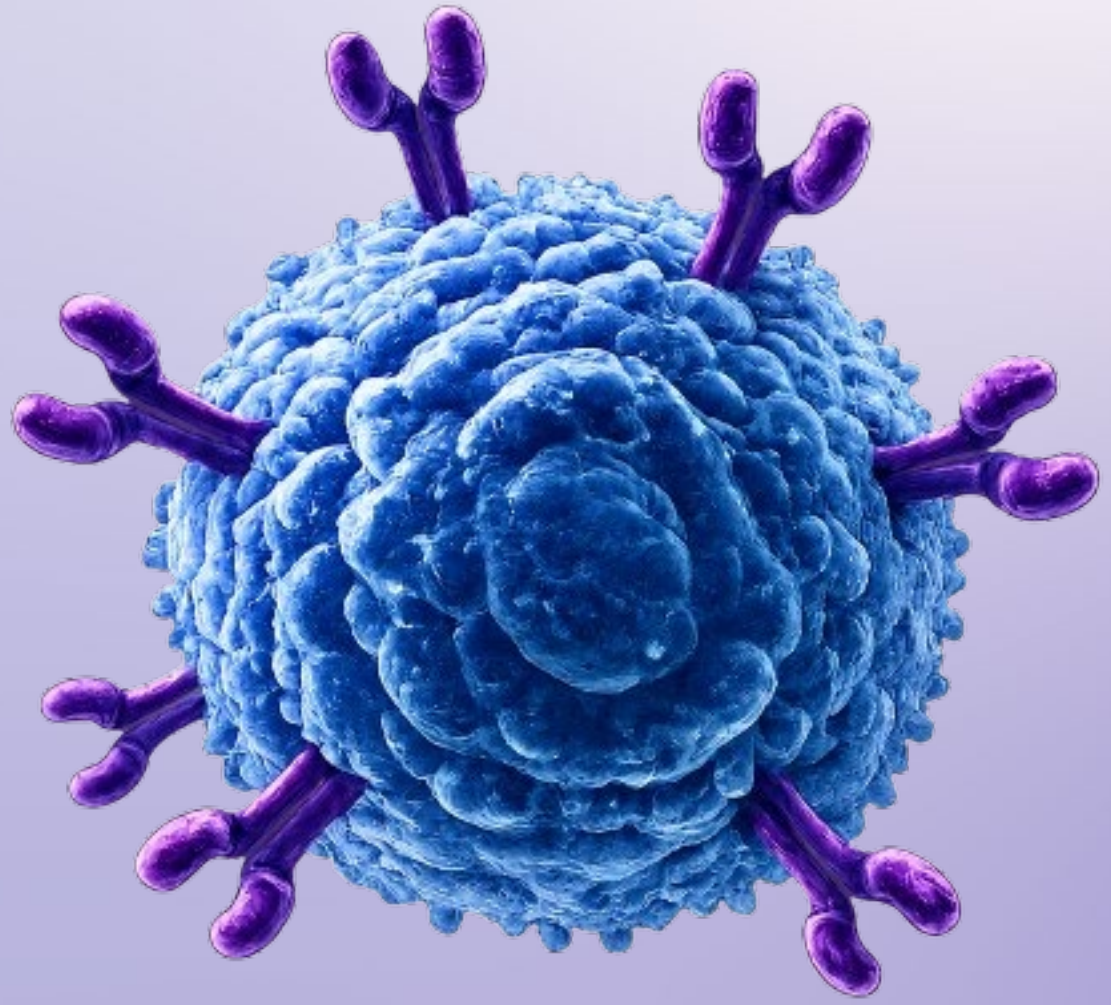
The optimized TBNK was validated for secondary endpoint according to the CLSI H62 guidelines.

Visit our poster for more information on assay validation

Poster #P034: Analytical constraints of the standard 6-color TBNK panel in allogenic CAR-T cell trials and strategies for resolution

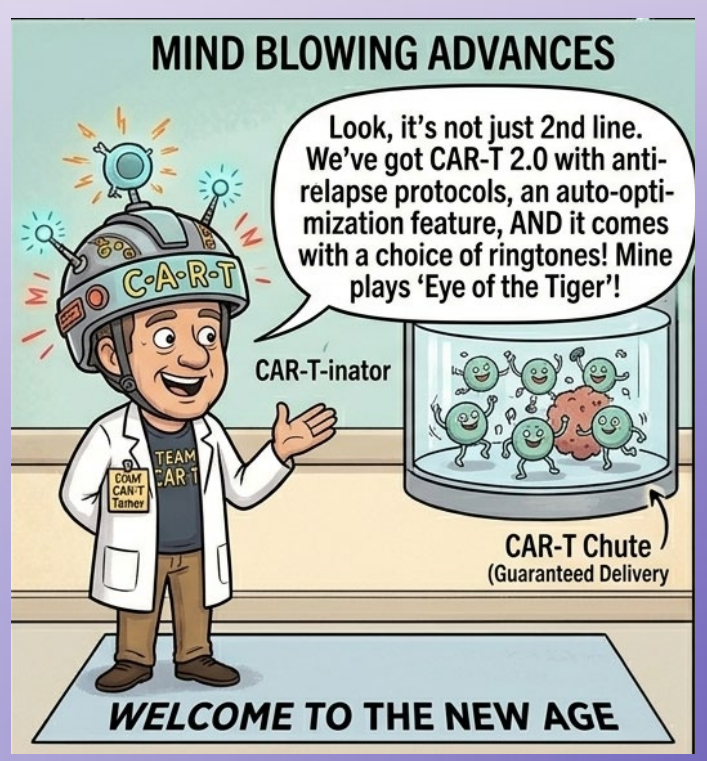
Tuesday, **June 9th, 2026**
5:00pm – 7:00pm, Hall AB

Cerba Research



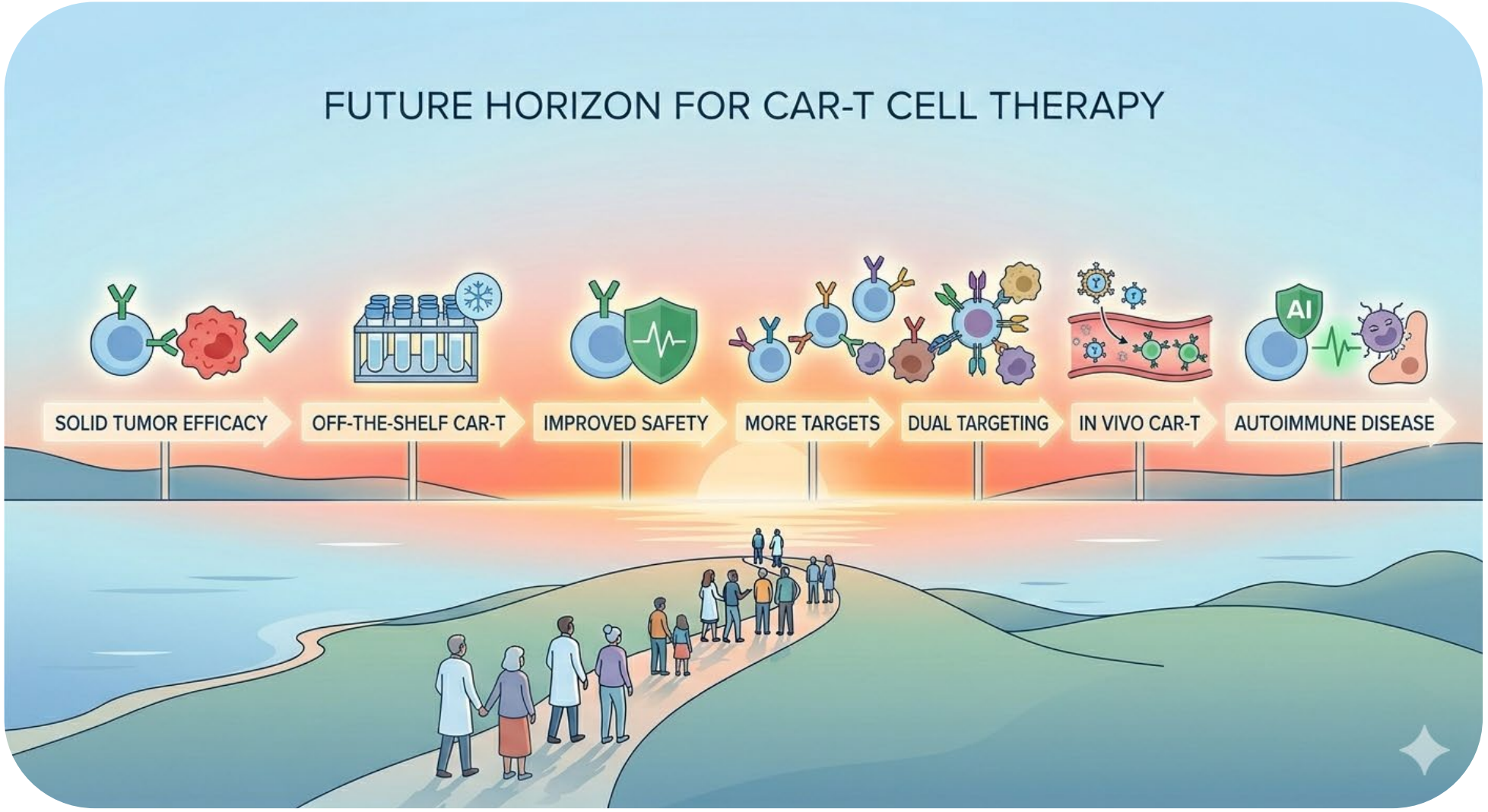
Future Frontiers

What's next for CAR!



Future is Hopeful to Treat Multiple Diseases!

Cerba Research



- ✓ CAR-γδ T cells,
- ✓ CAR-NK,
- ✓ CAR-iNKT,
- ✓ CAR-Macrophage

ClinicalTrials.gov
ID NCT06839976
Sponsor: Children's Hospital of Philadelphia

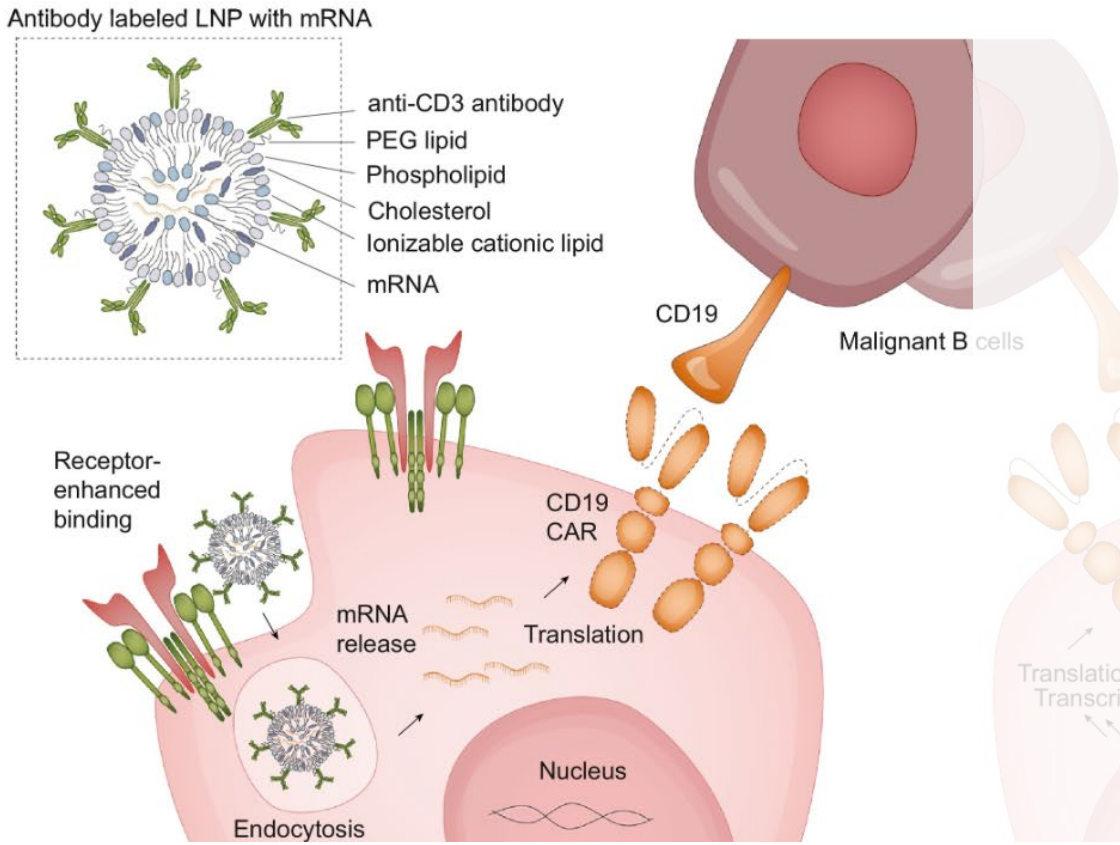
This is a single-center, single-arm, open-label phase 1/2 study of CART19 in children and young adults with refractory Systemic lupus erythematosus (SLE).

INCIPIENT Trial: A recent phase 1 clinical trial conducted by the Mass General Cancer Center evaluated a novel CAR-T therapy (**CARv3-TEAM-E**) for patients with recurrent glioblastoma.

What is In-vivo CAR-T cell Therapy?

Cerba Research

Method 1



- Transient CAR expression provides a safety advantage but may require multiple doses to provide a strong therapeutic effect.
- Serial dosing may:
  - Elicit an adaptive immune response against the CAR.
  - Enable real-time dose adjustment to minimize toxicities.
  - Prevent exhaustion in T cells.

Method 2

- A single dose can permanently modify T cells and provide long-term protection.
- Transduction of off-target cells (malignant cells, germ cells, or stem cells) carries the risk of epitope masking, germline modification, or novel malignancies, respectively.
- Low transduction efficiency may be overcome by proliferation.

Reference: Short et. al. Trends Pharmacol Sci. 2024; 45(5):406-418

What is In-vivo CAR-T cell Therapy?

Cerba Research

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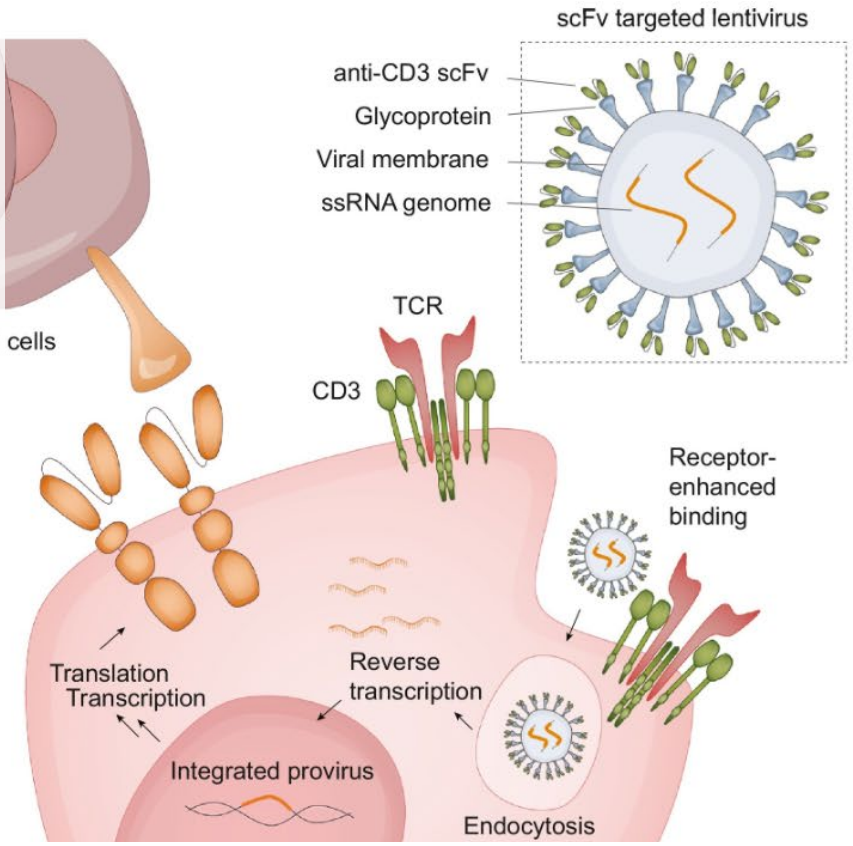

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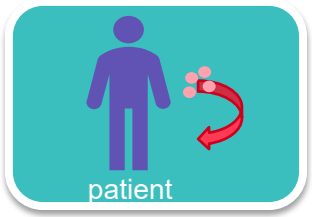
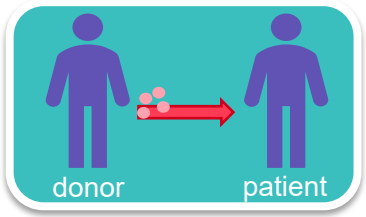
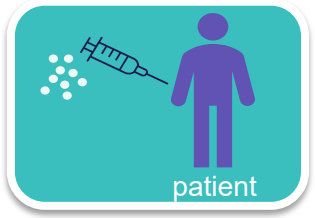
Autologous vs Allogenic vs In-vivo CAR-T cell Therapy

Cerba Research







Feature	Autologous CAR-T	Allogeneic CAR-T	In vivo CAR-T
Source	The patient's own blood. T-cells might be affected by previous treatment.	A healthy third-party donor.	CAR-T generated inside the patient.
Manufacturing	Custom-made for each individual. Complex logistics.	Mass-produced in large batches. Industrialized and standardized.	No external manufacturing
Availability / Timelines	3–4 week wait (Vein-to-vein).	Immediate ("Off-the-shelf").	Immediate ("Off-the-shelf").
Persistence	Intermediate to long (months to years).	Short to intermediate (weeks to months).	Transient expression in case of non-integrating delivery.
Redosing	Limited by number of cells in patient.	Possible, not limited by cell number.	Possible.
Challenges / Risks	Dealy in treatment could lead to exacerbation of the disease.	Risk of GvHD and rejection.	Targeting CAR construct to sufficent T-cells.
Cost	Extremely high (labor intensive).	Potential for lower cost per dose.	Potential for lower cost per dose and broader access.

In-vivo CAR-T cell

Cerba Research

Some of the pharmaceutical investment in in-vivo CAR-T cell therapy

- **AbbVie:** Acquired **Capstan Therapeutics** for $2.1 billion. Capstan is currently running a Phase 1 first-in-human trial for **CPTX2309**. This is an anti-CD19, targeted lipid nanoparticle (tLNP) delivering mRNA to preferentially reprogram CD8+ T cells *in situ* for autoimmune diseases. Beacon Intelligence
- **Bristol Myers Squibb (BMS):** Acquired **Orbital Therapeutics** for $1.5 billion to capture their next-generation *in vivo* RNA delivery platforms. www.drugdiscoverytrends...
- **AstraZeneca:** Acquired **EsoBiotec** for $1 billion. They are integrating EsoBiotec's *ENaBL* lentiviral vector platform to engineer *in vivo* cell therapies directly inside the patient. www.drugdiscoverytrends... + 1
- **Eli Lilly:** Acquired **Orna Therapeutics** (along with its clinical-ready lead program **ORN-252**, an *in vivo* circular RNA CAR targeting CD19 for B-cell driven autoimmune diseases), alongside acquiring **Engage Biologics** for its non-viral DNA delivery mechanics. www.dcatvci.org

Gemini search

CAR-T cell Therapy for Autoimmune Disease

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- Indications clustered around:
  - **Lupus (most advanced)**
  - **Myositis, systemic sclerosis**
  - **Emerging**: Myasthenia Gravis, Multiple sclerosis, and Rheumatoid arthritis
- Dominant strategy: **B-cell depletion (CD19 ± BCMA; CD70)**
- Expanding into:
  - **Dual-target CAR-T**
  - **Allogeneic/off-the-shelf CAR-T**
  - **mRNA CAR-T (transient expression)**
  - Majority of trials are **early-phase (Phase 1–2)**

- AbbVie acquired Capstan, including its lead asset CPTX2309, a potential first-in-class *in vivo* targeted lipid nanoparticle (tLNP) anti-CD19 CAR-T therapy candidate for the treatment of **B cell-mediated autoimmune diseases.**
- Orna Therapeutics has anti-CD19 in-vivo CAR for **B cell driven autoimmune disease** (panCAR™ Immune cells)

CAR-T cell Therapy for Solid Tumors

Cerba Research

- Historically, solid tumors have resisted CAR-T therapy due to physical barriers and immunosuppressive environments.
- 2026 has seen the first major clinical successes
- **Targeting Claudin18.2:** The therapy **Satri-cel (CT041)** has shown a ~41% objective response rate in advanced gastric and pancreatic cancers, marking a milestone as the first CAR-T treatment specifically for gastrointestinal solid tumors.

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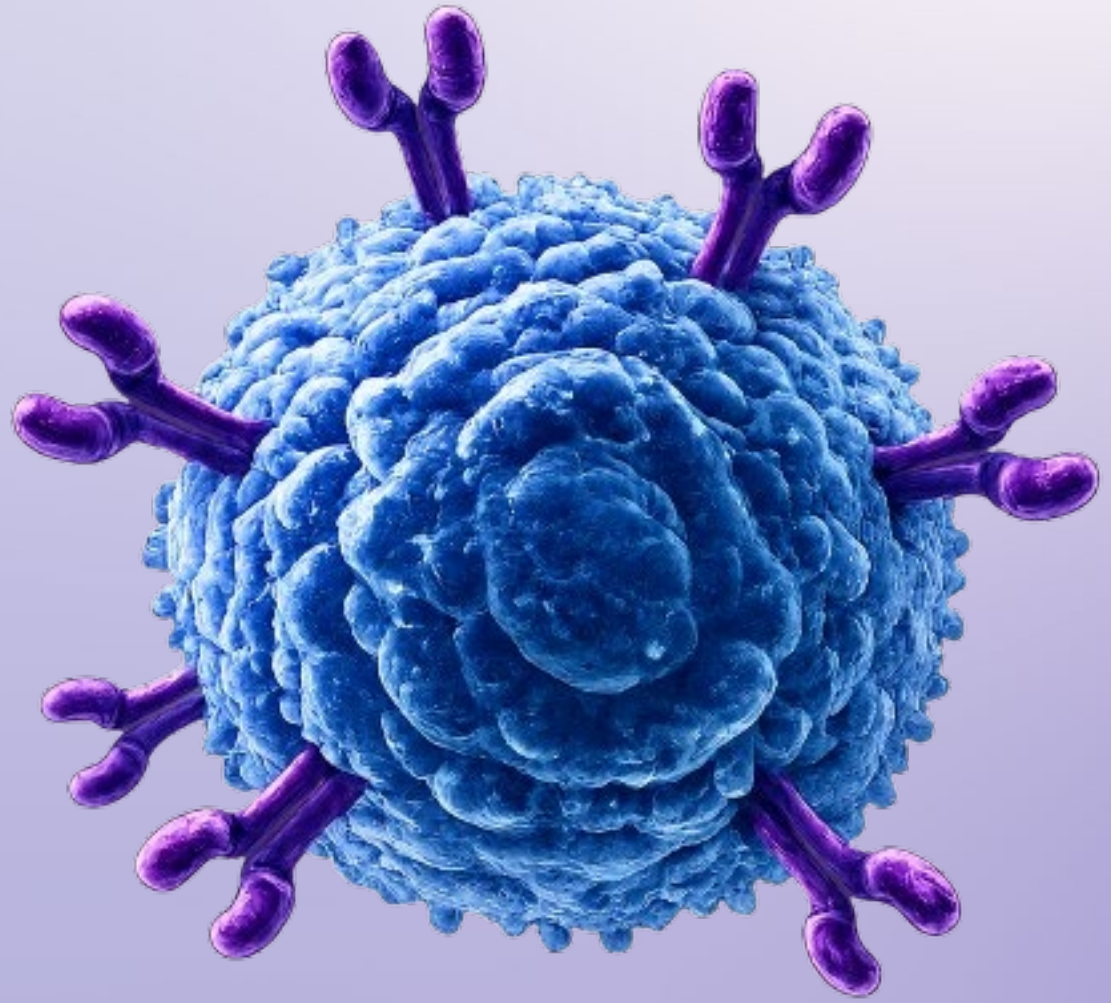

Key Take Aways

Flow Cytometry in CAR-T cell Therapy

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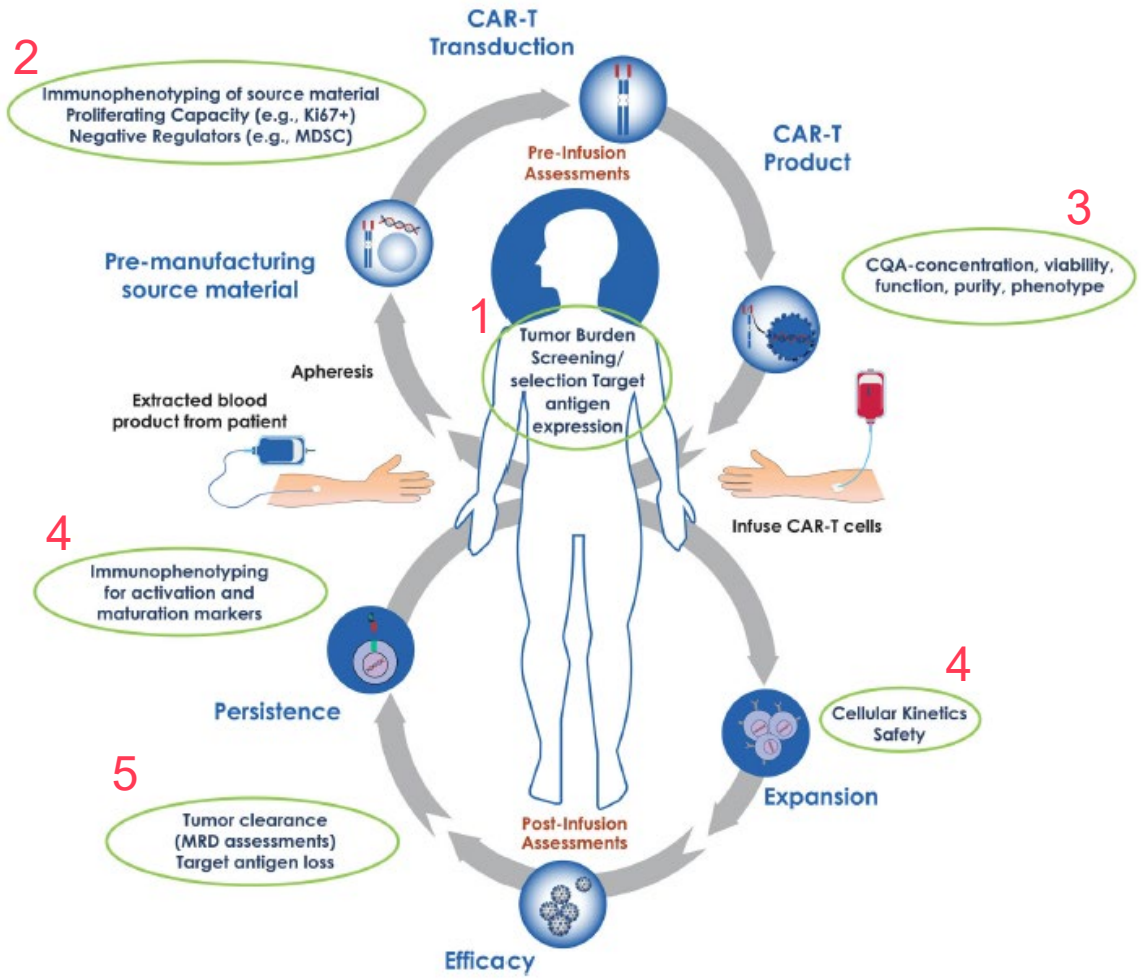
Wide range of applications for flow cytometry in CAR-T trials



Critical questions addressed by Flow cytometry:

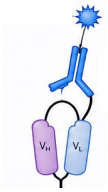
- ✓ What is the quality of the CAR-T product pre-infusion (viability, transduction efficiency, subset composition)?
- ✓ What is the expansion and persistence kinetics post-infusion?
- ✓ What is the absolute count, frequency and phenotype of CAR-T cells in patient samples over time?
- ✓ What is the distribution of memory subsets (naïve, central memory, effector)?
- ✓ Do CAR-T cells show markers of activation or exhaustion?
- ✓ How does the CAR-T cell therapy impact endogenous immune populations (T, B, NK, myeloid cells)?
- ✓ …….

Reference: Sarikonda et. al. Cytometry B Clin Cytom. 2021; 100(1):79-91

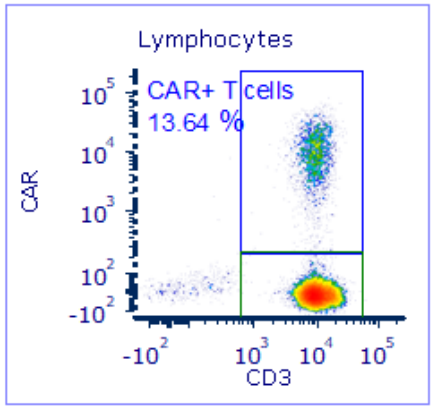
Detection Reagent

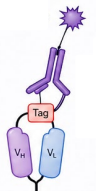
Cerba Research

Choose the correct detection method for your CAR

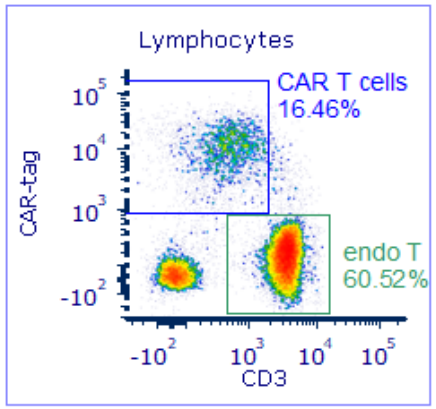


anti-idiotype antibody

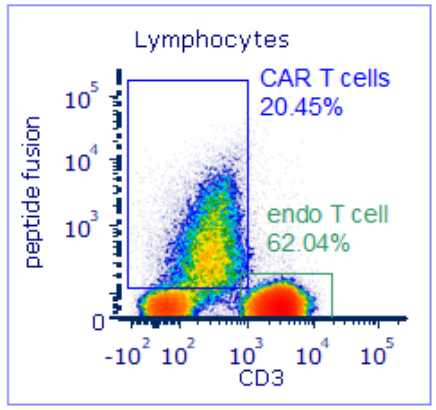




anti-tag antibody



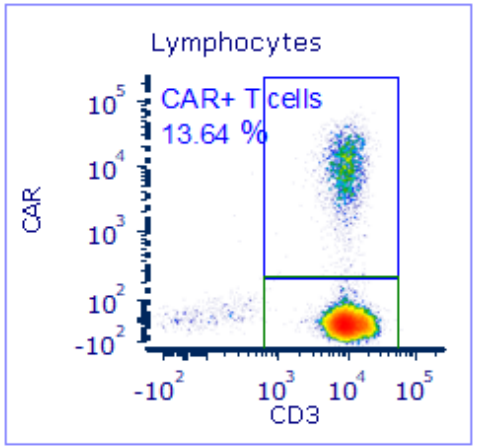
Peptide-fluorochrome



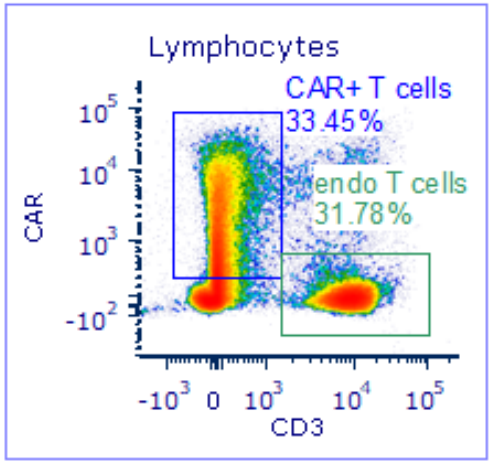
Gating Strategy for Autologous vs Allogenic CAR-T cell

Cerba Research

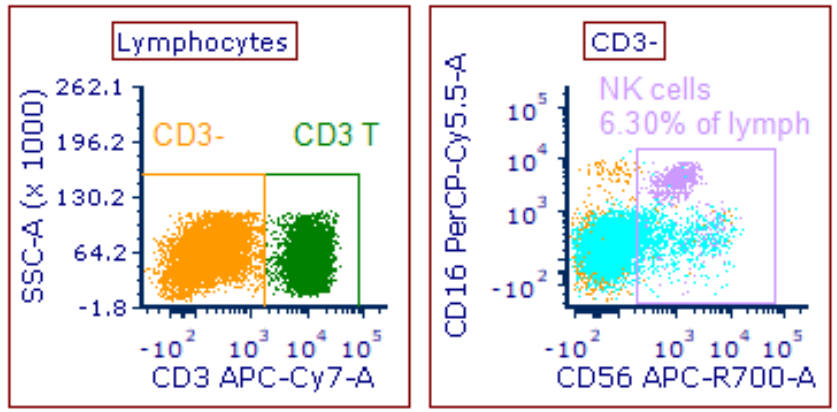
Autologous:
CD3 positive lymphocyte compartment



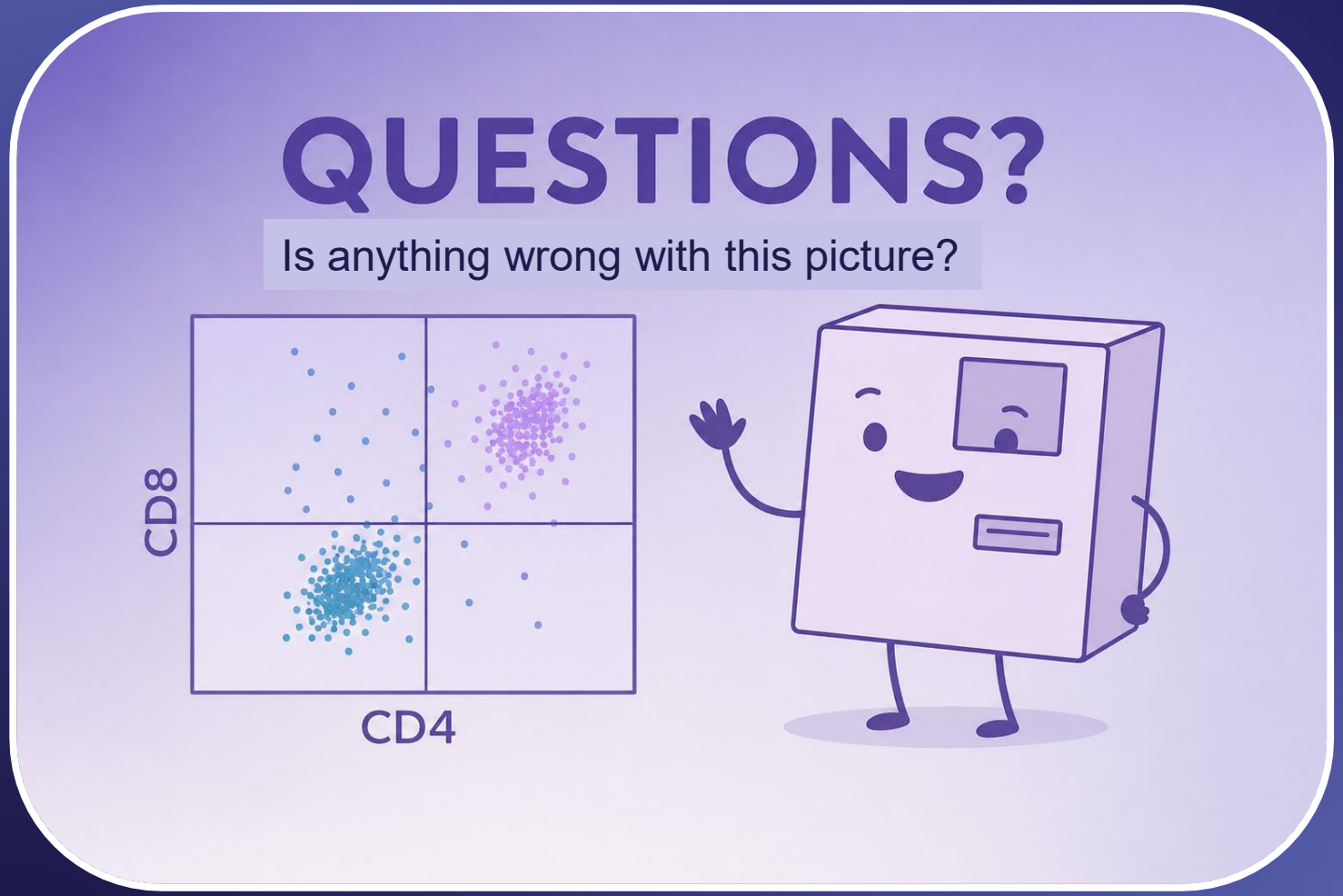
Allogenic:
CD3 negative lymphocyte compartment



Be aware of CAR-T cells contaminating other populations in your gating strategy
Specifically, NK cells in TBNK assay



Cerba Research



Visit our **Poster #P034**
Analytical constraints of the standard 6-color TBNK panel in allogenic CAR-T cell trials and strategies for resolution

Tuesday, **June 9th, 2026**
5:00pm – 7:00pm, Hall AB