Novartis Institutes for
Biomedical Research

# CAR-T therapies: insights in immunogenicity

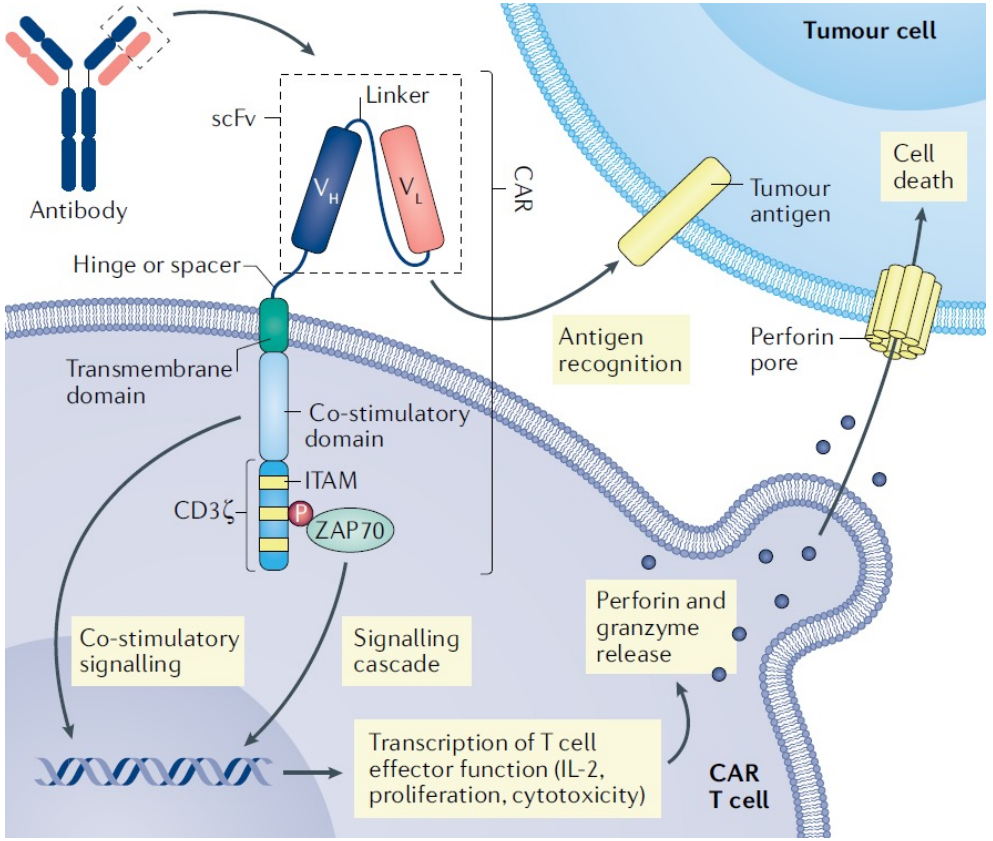
**Lydia Michaut**

**European Immunogenicity Platform**
**Scientific Symposium on Immunogenicity of Biopharmaceuticals**
**April 28th, 2023**

NOVARTIS | Reimagining Medicine

# Basic mechanism of a CAR-T cell

- Living drug
- Genetically-engineered T lymphocytes expressing an artificial T cell receptor, the **chimeric antigen receptor.**
  - Extracellular domain: binding to antigens expressed on target cells tumour) (*e.g. single-chain fragment variable (scFv) antibody construct)*
  - Co-stimulatory domain (CD28, 4-1BB..)
  - CD3ζ chain *(three immune receptor tyrosine-based activation motif (ITAM) domains that, upon phosphorylation, signal through ZAP70).*
- Establishment of an immunological synapse upon binding.

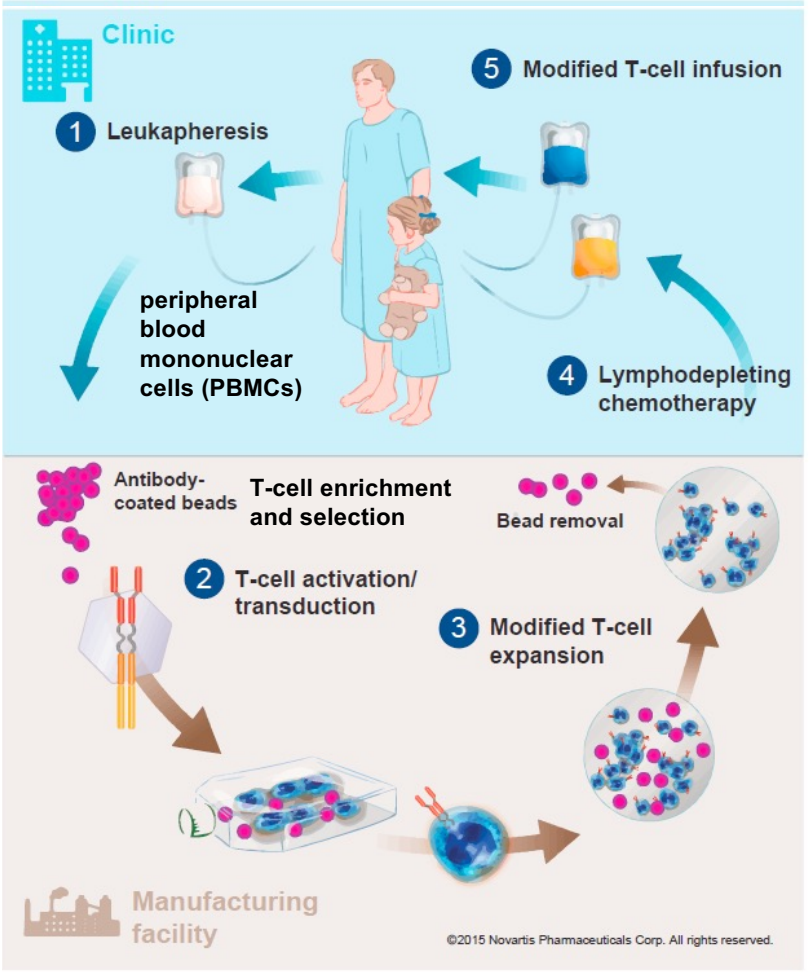



*Larson and Maus, Nat Rev Cancer. 2021 Mar;21(3):145-161.*

# CAR-T manufacturing

## Ex. of an autologous therapy

1) Collection of peripheral blood mononuclear cells (PBMCs) from a patient by leukapheresis after lymphodepleting
2) T cells are enriched, selected, activated and transduced with self-inactivating lentiviral vector containing anti-CD19 CAR transgene
3) Cell expansion, and isolation of transduced T cells
4) Once CAR/T cells are available, lymphodepleting chemotherapy is initiated
5) Infusion of modified T cells into the same patient who provided the cells




*Tyagarajan et al, Mol Ther Methods Clin Dev. 2019*

NOVARTIS | Reimagining Medicine

# Approved CAR-T therapies

| CAR T product | Brand name | Initial US approval | Company | Target | Antigen binding domain | Intracellular domain | Indication(s) |
|---|---|---|---|---|---|---|---|
| tisagenlecleucel[1] | KYMRIAH | 2017 | Novartis | CD19 | scFv | 4-1BB - CD3ζ | B cell acute lymphoblastic leukemia (ALL); diffuse large B cell lymphoma (DLBCL) |
| axicabtagene ciloleucel [2] | YESCARTA | 2017 | Kite / Gilead | CD19 | scFv | CD28 - CD3ζ | diffuse large B cell lymphoma (DLBCL); follicular lymphoma (FL) |
| brexucabtagene autoleucel [3] | TECARTUS | 2020 | Kite / Gilead | CD19 | scFv | CD28 - CD3ζ | mantle cell lymphoma (MCL) |
| idecabtagene vicleucel [5] | ABECMA | 2021 | Bluebird / Celgene / BMS | BCMA | scFv | 4-1BB - CD3ζ | multiple myeloma |
| lisocabtagene maraleucel [4] | BREYANZI | 2021 | Juno / BMS | CD19 | scFv | 4-1BB - CD3ζ | diffuse large B-cell lymphoma (DLBCL); high-grade B-cell lymphoma (HGBL); primary mediastinal large B-cell lymphoma (PMBCL); follicular lymphoma (FL) |
| ciltacabtagene autoleucel [6] | CARVYKTI | 2022 | Legend / J&J | BCMA | 2x$V_H$H | 4-1BB - CD3ζ | multiple myeloma |

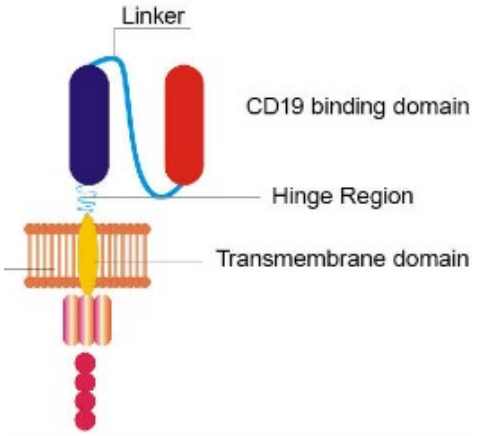


[1] *KYMRIAH prescribing information (US), 2020.*
[2] *YESCARTA prescribing information (US), 2021.*
[3] *TECARTUS prescribing information (US), 2021.*
[4] *BREYANZI prescribing information (US), 2021.*
[5] *ABECMA prescribing information (US), 2021.*
[6] *CARVYKTI SmPC (EMA), 2022.*

# Immunogenicity of CAR-Ts

- Both **humoral and cell-mediated responses** to CAR can occur and impact:
  - Patients' safety (anaphylaxis, cytokine release syndrome (CRS), infusion reactions, hypersensitivity, immune effector cell associated neurotoxicity syndrome (ICANS)...
  - Persistence and therefore efficacy of the treatment, mainly upon re-infusion.
- Concepts of protein-based therapeutics apply to CAR-T cell therapies ... PLUS ...

- Complex, "multidomain" structure
  - antigen binding domain: scFv (murine, humanized); camelid nanobody
  - hinge and linker expression of a protein encoded by several human genes in a single CAR construct creates fusion sequences at junctions that do not normally exist in humans.




- Intracellular domain: presentation on the cell surface can enhance CAR IG: CD8+ T-cells that recognize peptides from foreign transgene products presented by HLA class I molecules on transduced T-cells are a major mediator of immune mediated elimination
- Complex production process: residual impurities such as (lenti)viral proteins or other non-human proteins related to gene transfer process.

# Antibody response

## Potential implications

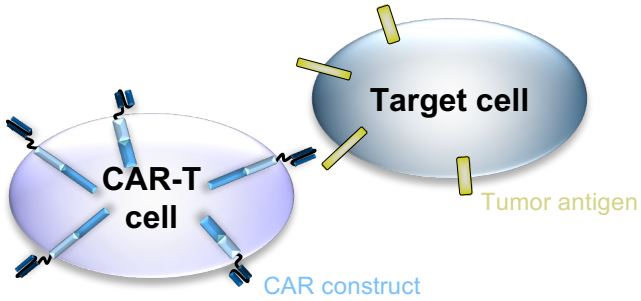
### Neutralization of binding

- Neutralizing ADAs
- Block CAR binding to the target antigen
- Impact on efficacy by preventing cell lysis

### ADCC and CDC

- Non-neutralizing ADAs (opsonins)
- Induction of:
  - **a**ntibody-**d**ependent **c**ellular **c**ytotoxicity (-> NK cells)
  - **c**omplement-**d**ependent **c**ytotoxicity (-> complement activation)

  => CART cell lysis or clearance; limit repeat dosing and therapeutic outcome




## Monitoring

The “to-be-or-not-to-be” of ADA assay format

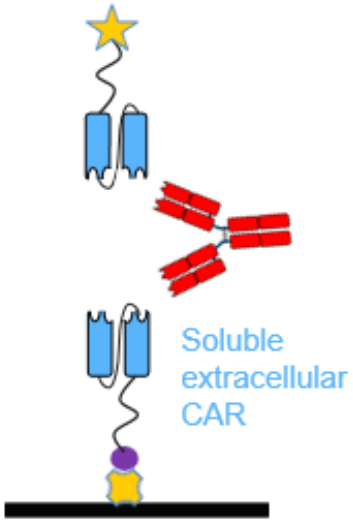
-> LBA (plate-based assay)?

-> CBA (cell-based assay)?

NOVARTIS | Reimagining Medicine

# ADA assay formats for approved CAR-T

## Bridging format LBA

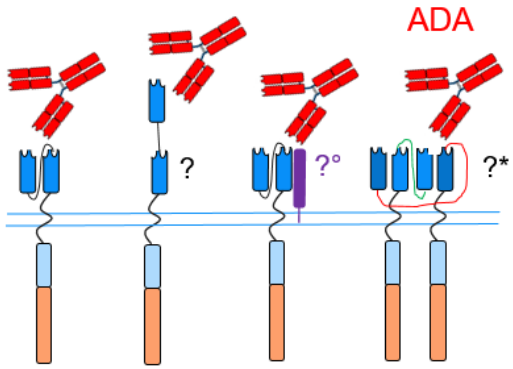



**Lack of solubility**
- CAR domains may be insoluble
- Soluble CAR does not represent the full extracellular domain

**Missed epitopes**
- Conformational
- Interactions with other membrane proteins

**Epitope masking**
- Labeling of CAR domain as reagent
- Endogenous glycosyation of CAR

## Cell-based assay




*ADA potentially more relevant ?*

**Label-free CAR**
No risk of masked epitopes

**Presentation in cell membrane**
Native environment & conformation, potential interaction partners

**CAR processed through T cell expression system**
Glycosylation

**Not limited by insoluble/hydrophobic domains**



°Salmerón et al. J Immunol. (1991); 147(9): 3047-3052
* Whitlow et al. Protein Engineering (1994); 7(8): 1017-1026

NOVARTIS | Reimagining Medicine

# ADA assay formats for approved CAR-T

| Product | Assay |
|---|---|
| KYMRIAH [1] | Cell-based flow cytometry assay for anti-CAR19 [7] |
| YESCARTA [2] | ELISA for anti-FMC63;<br>cell-based confirmatory assay |
| TECARTUS [3] | ELISA for anti-FMC63 |
| ABECMA [5] | Not specified |
| BREYANZI [4] | ECL for anti-extracellular CD19-binding domain |
| CARVYKTI [6] | Not specified |

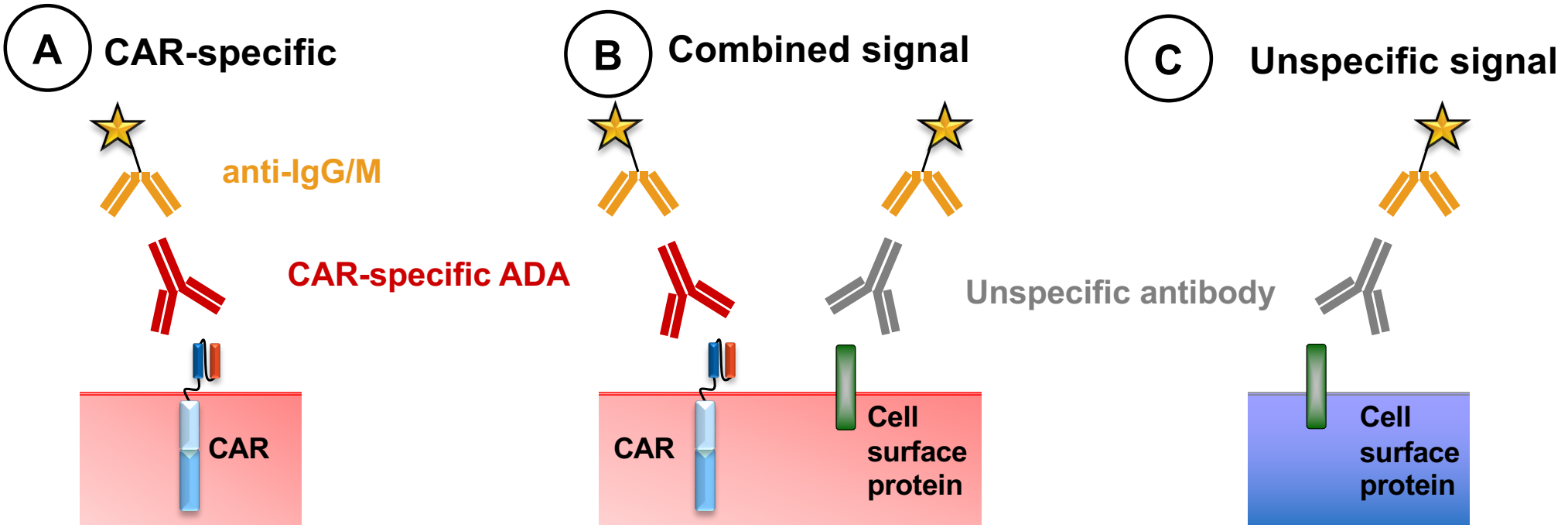
| Detected ADAs & Features | Bridging ELISA with soluble CAR | Bridging ELISA with source Antibody *(e.g. FMC63)* | Cell based assay |
|---|---|---|---|
| Anti-VAR | ✓ | ✓ | ✓ |
| Anti-scFv | ✓ | X | ✓ |
| Anti-Hinge, anti-Linker | ✓ | X | ✓ |
| Anti-membrane protein interaction epitopes | X | X | ✓ |
| Anti-insoluble extracellular domains | X | X | ✓ |
| Label-Free | X | X | ✓ |


References:

[1] *KYMRIAH prescribing information (US), 2020.*
[2] *YESCARTA prescribing information (US), 2021.*
[3] *TECARTUS prescribing information (US), 2021.*
[4] *BREYANZI prescribing information (US), 2021.*
[5] *ABECMA prescribing information (US), 2021.*
[6] *CARVYKTI SmPC (EMA), 2022.*
[7] Potthoff et al, (2020) J Immunol Methods.

# Cell-based ADA assay for anti-CAR antibodies

**CAR-specific signal (A) = B - C**




*CAR19-transduced cell line*

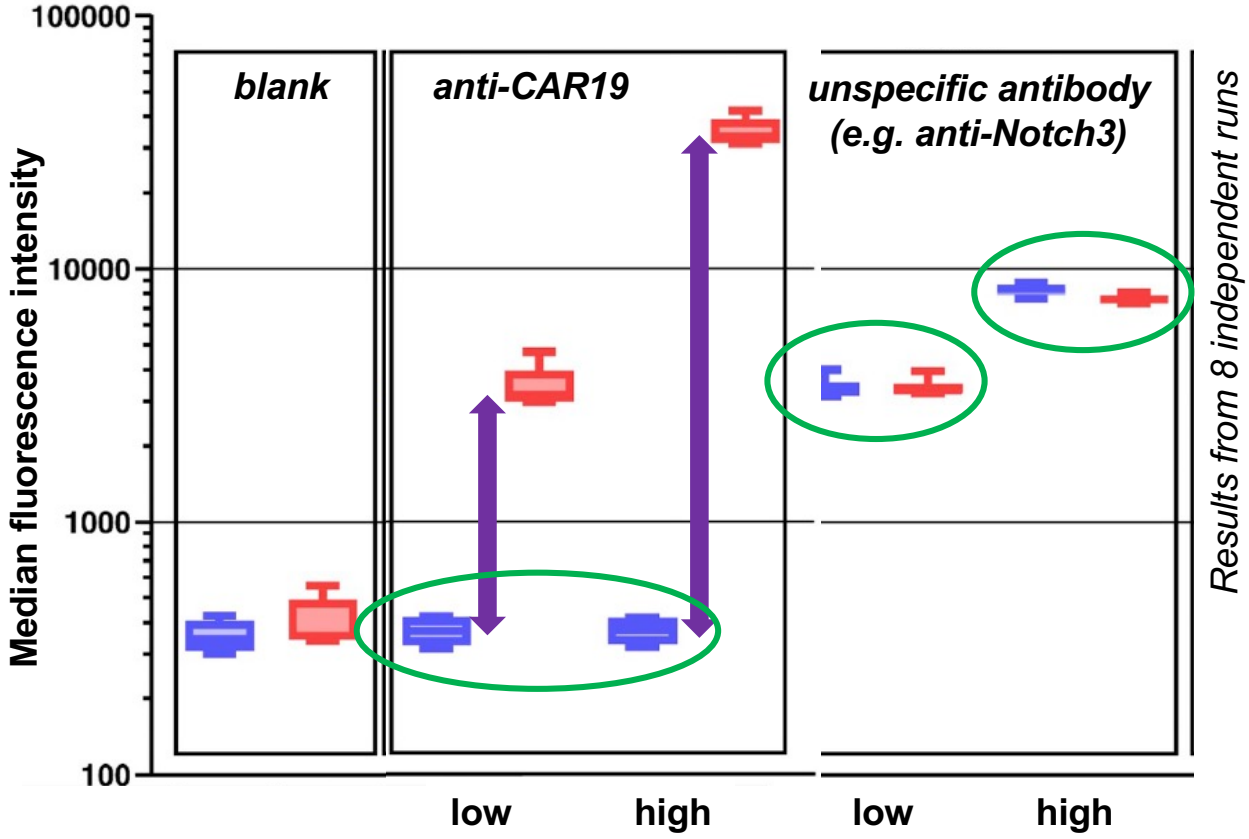
*Non-transduced cells*

*Adapted from Potthoff et al, J Immunol Methods. 2020 Jan;476:112692.*

# Integration of data from two cell lines

- Positive controls assessed with both **WT cells (blue)** and **CAR19 cells (red)**
  - Specific anti-CAR19 antibody does not bind to WT cells
  - Signal of unspecific antibody is comparable for both cell lines

**Signal subtraction between cell lines is meaningful = anti-CAR19 specific signal**




*Adapted from Potthoff et al, J Immunol Methods. 2020 Jan;476:112692.*

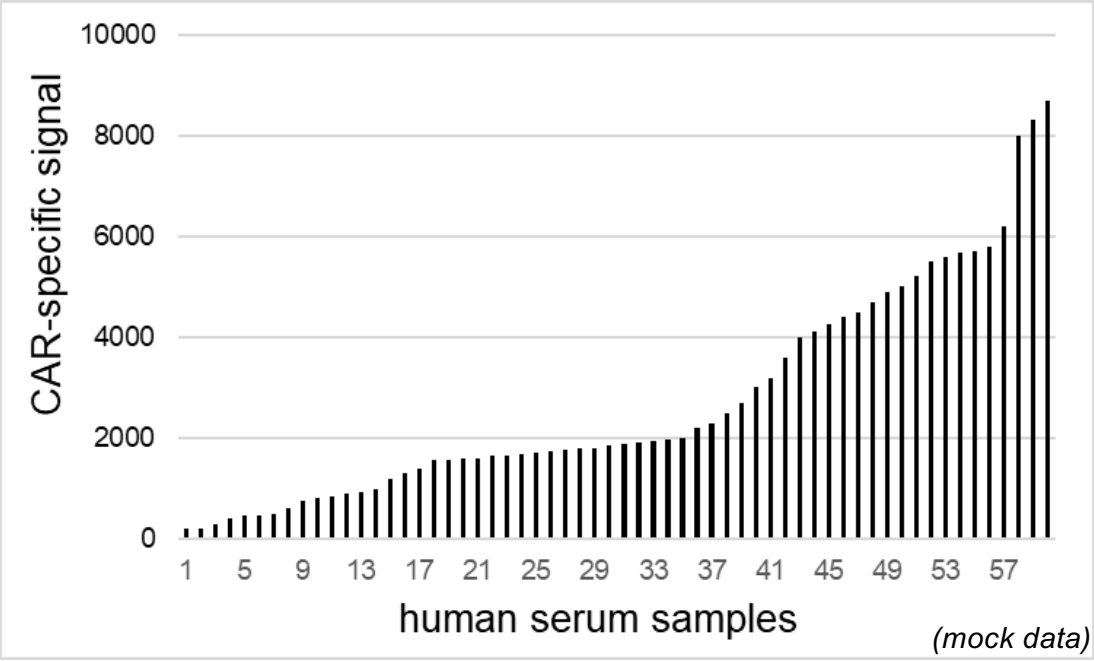


NOVARTIS | Reimagining Medicine

# Defining the assay cut point

**<u>Challenge</u>: pre-existing antibodies**

→ high signals in treatment-naive human serum samples (HAMAs)



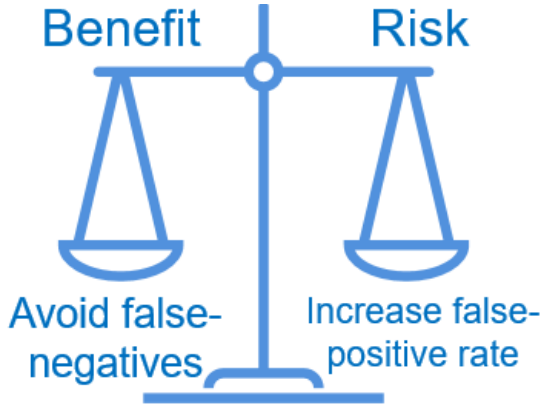

- No meaningful statistical outlier removal
- No calculation of representative cut point possible with naive human serum



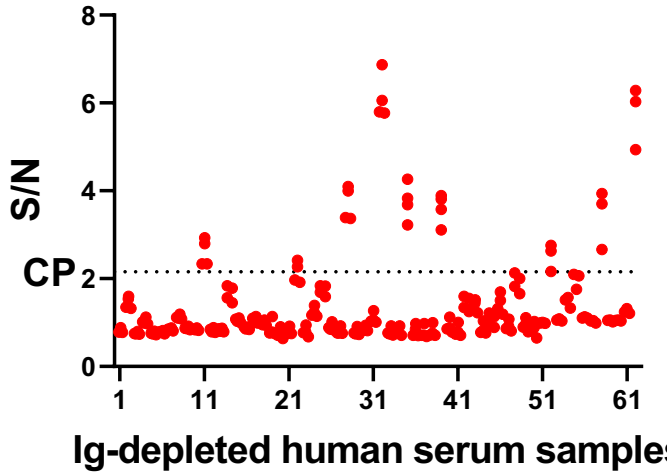
*Adapted from Potthoff et al, J Immunol Methods. 2020 Jan;476:112692.*

NOVARTIS | Reimagining Medicine

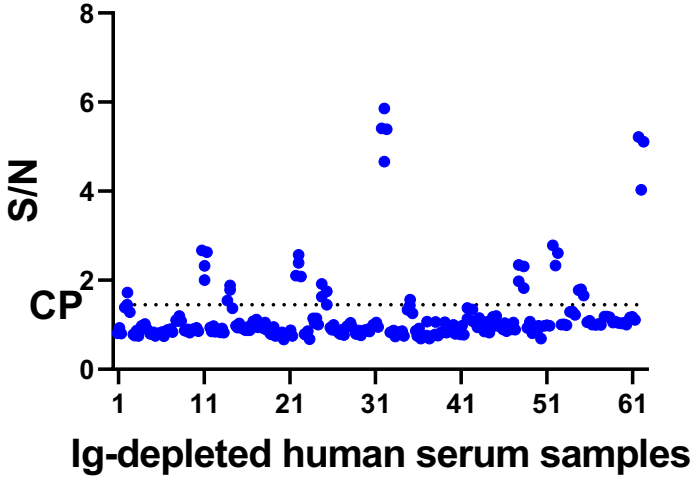
# Immunoglobulin-depleted sera




- Screened on both cell-lines
- Used for cut-point determination and negative control pool preparation




*CAR19-transduced cell line*




*Non-transduced cell line*



*Mock data*

*See also: Potthoff et al, J Immunol Methods. 2020 Jan;476:112692.*

NOVARTIS | Reimagining Medicine

# Reliable prediction of titer based on screening assay signal

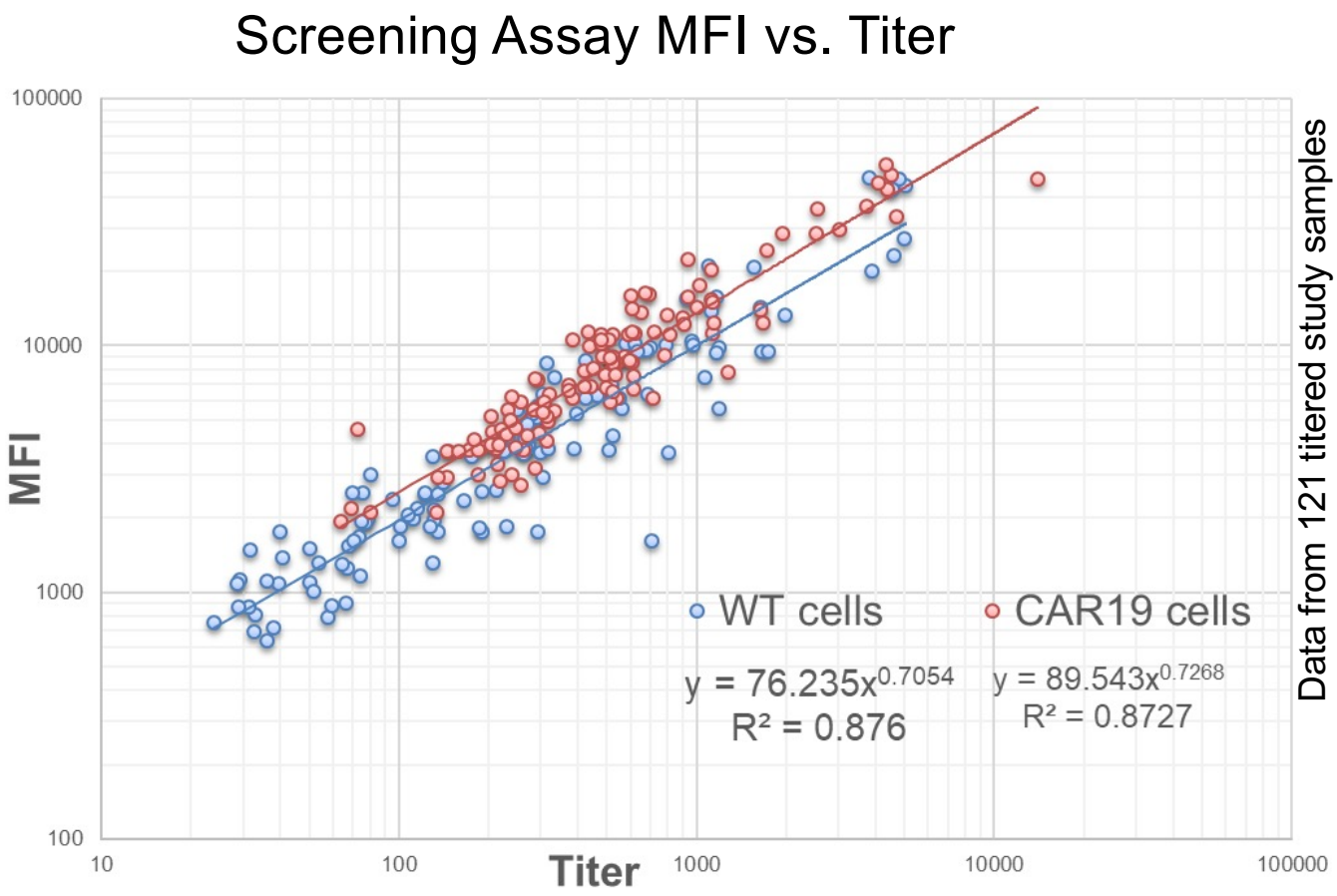
1. Classical approach

Dilution (1:X) of study samples until signal falls below titer cut point

2. Data analysis: screening assay signal reliably reflects titer

3. In further clinical trials: predict ADA response magnitude on the basis of the screening assay signal

*Titration only if screening signal is above threshold




NOVARTIS | Reimagining Medicine

# Antibody response to approved CAR/T

| Product | Assay | Prevalence | Incidence | Impa |
|---|---|---|---|---|
| KYMRIAH [1] | Cell-based flow cytometry assay for anti-CAR19 [7] | 86% - 91% | 5% (DLBCL) | - Per pos<br>- No |
| YESCARTA [2] | ELISA for anti-FMC63; cell-based confirmatory assay | 13% | Screening: 2%<br>Confirmatory: 0% | - No YE<br>- No |
| TECARTUS [3] | ELISA for anti-FMC63 | Not specified | Screening: 17pts<br>Confirmatory: 0 | - No TE<br>- No |
| ABECMA [5] | Not specified | 3% | 47% | - No anti AB |
| BREYANZI [4] | ECL for anti-extracellular CD19-binding domain | 11% | 11% | - Rel saf pat |
| CARVYKTI [6] | Not specified | Not specified | 19.6% (CARTITUDE-1) to 25% (pooled studies) | - Bas an initi |

## Humoral immunogenicity of approved CAR-T products

- No standardized approach for evaluation of ADA production
  Different detection methods result in different numerical values (%) for humoral immunogenicity
- No evidence for correlation of ADA with clinical outcomes (exposure, efficacy, safety)
- Do we need to build knowledge about non-neutralizing vs. neutralizing anti-CAR antibodies?

References:

[1] *KYMRIAH prescribing information (US), 2020.*
[2] *YESCARTA prescribing information (US), 2021.*
[3] *TECARTUS prescribing information (US), 2021.*
[4] *BREYANZI prescribing inform*
[5] *ABECMA prescribing inform*
[6] *CARVYKTI SmPC (EMA), 2*
[7] *Potthoff et al, (2020) J Immunc*

# Cellular immune response

Mediated by CAR-specific CD8+ cytolytic T cells (CTLs).

CAR-T cells display CAR-derived peptides via HLA class I molecules which can prime CD8+ cells.

CAR peptides from apoptotic or necrotic CAR T cells can be displayed via HLA I or HLA II by antigen-presenting cells and prime CD8+ and CD4+ T cell responses in secondary lymphoid organs

The presence of CAR-specific cytolytic T cells after infusion has been associated with treatment failure in some clinical trials

- Contrasts with anti-CAR antibodies

**May limit the success re-infusion:** clinical responses to second or subsequent infusions have generally been suboptimal, with complete remissions typically seen in <25% of patients

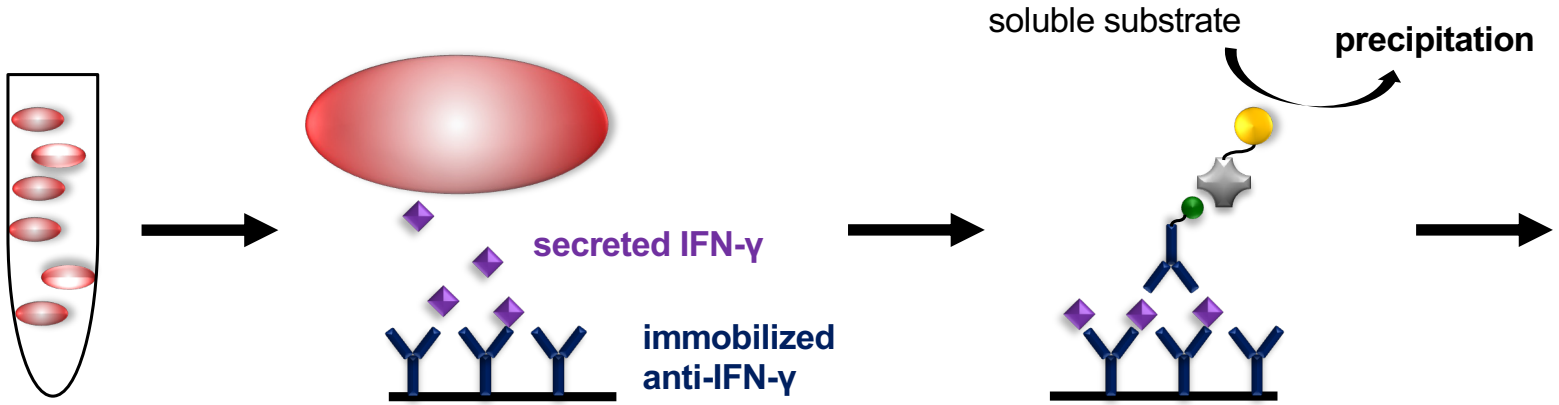
- Despite the high complete response rates to the first infusion of CD19-directed CAR-T cells after lymphodepletion in patients with several haematological malignancies, disease recurrence remains an issue, with approximately 30–50% of patients having disease relapse within 12 months.
- Antigen escape through loss of CD19 expression has been seen in 7–25% of patients, depending on the trial. For those with CD19-positive disease relapse, a repeat infusion could be considered

**MONITORING:**
Detection of CAR-specific CD8+ T cell response in post-infusion PBMCs

- ELISpot
- Chromium Release Assay
- Intracellular Cytokine Staining

# ELISpot Assay

- Enzyme-linked immune absorbent spot (ELISpot) assay to detect **cytokine secretion of single cells**
- Example: Interferon gamma (IFN-γ) detection as functional measure of antigen-specific cellular immune response to CAR



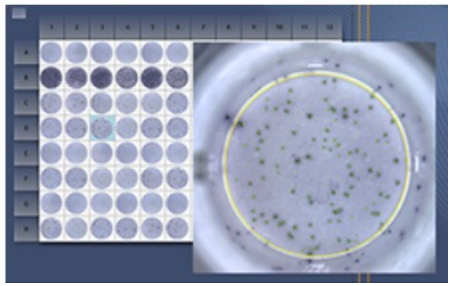

Blood collection and PBMC isolation

Incubation in presence or absence of antigen (peptide pools)

→ Secretion of IFN-γ

→ Binding of IFN-γ to immobilized capture antibody

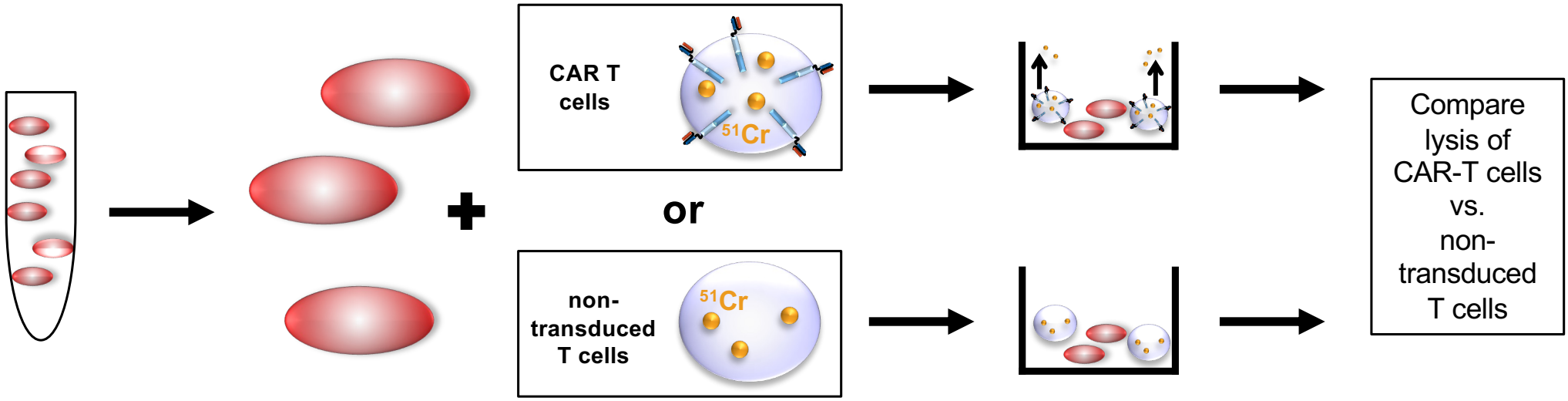
Addition of enzyme-conjugated detection system and precipitation of substrate



© AID Autoimmun Diagnostika GmbH

# Chromium Release Assay

- $^{51}$Chromium Release Assay to detect **lysis of target cells**
- Example: CAR-T cell lysis as functional measure of antigen-specific cellular immune response to CAR




Blood collection and PBMC isolation

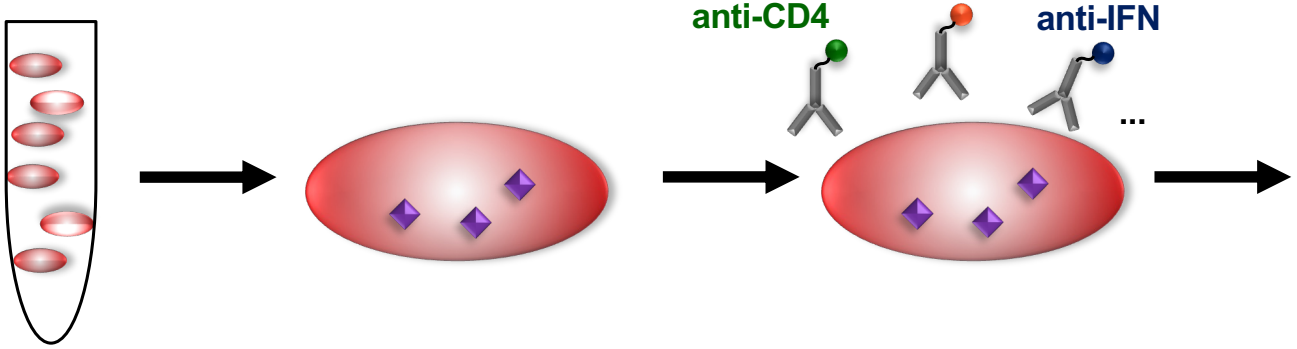
Stimulation of PBMCs and incubation with $^{51}$Cr-labeled autologous CAR-T cells **or** autologous non-transduced T cells

Cell lysis and release of $^{51}$Cr into the supernatant

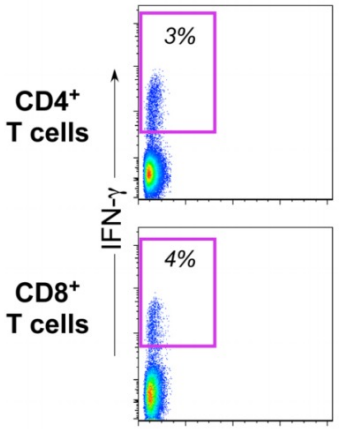
Analysis of supernatant in liquid scintillation counter

# Intracellular Cytokine Staining

- Intracellular Cytokine Staining (ICS) to detect **cytokine production in single cells**
- Example: Interferon gamma (IFN-γ) detection as functional measure of antigen-specific cellular immune response to CAR in T cell sub-populations




Gating hierarchy (example):
- PBMC
- Singlets
- Live cells
- CD3+, CD4+ or CD8+ T cells
- Cytokine producing CD4+ or CD8+ cells




*Horton et al., J Immunol Methods. 2007 May 31;323(1):39-54.*

Blood collection and PBMC isolation

Incubation in presence or absence of antigen (peptide pools) → Production of IFN-γ

Fixation, permeabilization, staining with different antibodies

Flow cytometry with sequential gating to identify specific T cell subsets



NOVARTIS | Reimagining Medicine

# Advantages and limitations of different assay formats

| | Advantages | Limitations |
|---|---|---|
| **ELISpot Assay** | ▪ High sensitivity<br>▪ Plates can be stored or shipped for spot counting<br>▪ Possibility of cytokine multiplex assay with fluorescent substrates (FluoroSpot) | ▪ No information about phenotype of cells<br>▪ No simultaneous analysis of cytokines in context of phenotypic markers<br>▪ No quantification of secreted cytokine per cell |
| **Intracellular Cytokine Staining** | ▪ Multiplex staining<br>→ simultaneous evaluation of multiple cytokines and/or phenotypic markers<br>→ parallel characterization of cell populations | ▪ Standardization of gating required<br>▪ Fixation increases hydrophobicity of cell proteins<br>→ increase of non-specific binding<br>→ potentially low signal-to-noise ratio |
| **Chromium Release Assay** | ▪ Relatively short duration<br>▪ Good well-to-well reproducibility<br>▪ Representative of CAR-T induced lysis *in vivo* | ▪ Requires radioactivity<br>▪ Relatively high background release<br>▪ Often low sensitivity<br>▪ Variability due to inconsistent loading with $^{51}Cr$ |

# Limitation of all assay formats

Patient T-cells

## Availabilty

- Prioritized use for release assays & treatment
- Limited availability for IG assays (development & controls)

## Quality and consistency

- Challenging logistics (PBMCs or stabilized blood)
- Impact on result quality and relevance

# FMC63 (mouse α-CD19)-derived CAR-T Therapies

| Drug | Humoral IG | Cellular IG |
|---|---|---|
| **Kymriah** (Tisagen-lecleucel) | **Screening CBA with full CAR** | PBMC stimulation with CAR peptide pool followed by **Intracellular Cytokine staining** (IFN-γ) |
| **Yescarta** (Axicabtagene ciloleucel) | 1st tier: **Screening LBA vs. source antibody** 2nd tier (only screening positive samples) **Confirmatory CBA vs. extracellular CAR (ScFv, hinge, linker)**[2] | PBMC stimulation with 4 peptide pools (15-mer peptides) followed by **ELISpot** (IFN-γ)[1] |
| **Breyanzi** (Lisocabtagene maraleucel) | **Screening LBA with ScFv** ("extracellular CD-19-binding domain")[3] | PBMC stimulation with irradiated autologous CAR-T cells + IL-2 followed by **Chromium release assay (CRA):** Lysis of T cells vs. CAR T cells; **ELISpot** to identify CAR peptides stimulating the identified anti-CAR T cell line[4] |


[1] Brudno et al, Nature Medicine (2020) 26(2): 270-280
[2] Package Insert - YESCARTA (fda.gov)
[3] Package Insert - BREYANZI (fda.gov)
[4] Turtle et al. Science Translational Medicine (2016) 8(355): 355ra116

# Cellular immunogenicity

Tisagenlecleucel:

Very low cellular immunogenicity observed in trials:

- Net responses < 1%
- Few cases of slightly increased responses were not correlated with clinical outcome.

Liso-cel

- Positive response definition in CRA:
  - Stimulated post-dose PBMCs lyse CAR-T cells but not non-transduced T cells.
  - Pre-dose PBMCs must lyse neither CAR-T nor non-transduced cells.
- 6 /11 patients show CAR-T cell lysis after first and/or second CAR-T cell infusion.
- **Second step:** T cells from one cell-based immunogenicity positive patient further assessed in IFN-γ ELISpot => Two scFv peptides found to stimulate IFN-γ expression more than T cells alone

Source: Turtle et al. Science Translational Medicine (2016) 8(355): 355ra116

# Mitigation of anti-CAR cellular immunity (1/2)

## Mouse scFv FMC63

- Used in several CD19-specific CARs
- *Turtle et al., (2016)*

T cell-mediated anti-CAR responses have been detected, to a lesser extent, with the use of fully human CAR constructs.

## Fully human constructs

## Intensified lymphodepletion (cyclophosphamide and fludarabine)

- identified as a factor that might reduce the extent of anti-CAR cellular immunity
- conditioning regimens containing both of these agents are currently considered the standard of care approach prior to initial administration of CD19-targeted CAR-T cells.



Wagner et al. (2021)

# Mitigation of anti-CAR cellular immunity (2/2)

## Other parts of the transgene

- *Brudno et al., (2020)*
- 3/19 patients
- signal peptide linker
- hinge domains

## Sequence analysis of the fusion sites between human components of the CAR

### Preliminary computational analysis (NetMHC)

-> CAR sequences not encoded by the human genome.

### Further screening based on MHC binding prediction

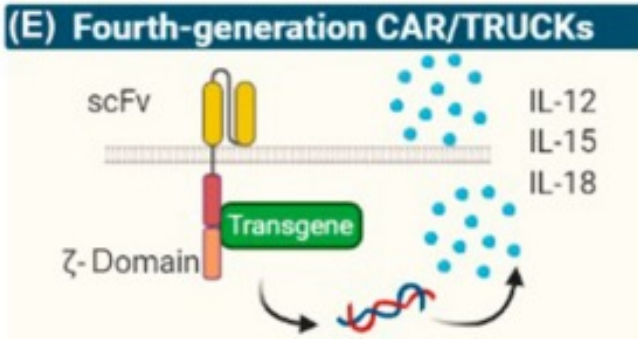
-> fusion site between CD28 and 4-1BB: **seven** 9-mer peptide sequences with <100nM affinity to several MHC class I molecules.

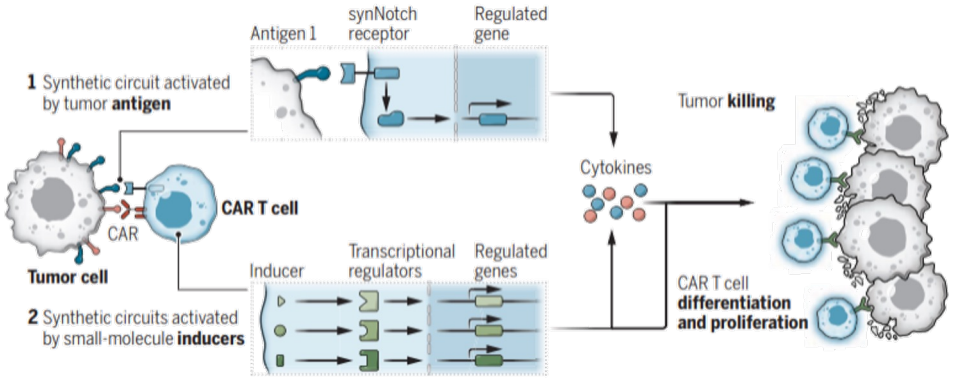
### Extension of CD28 sequence by two amino acids

=> only **one** predicted 9-mer peptide with affinity <100nmM

Sommermeyer et al (2017)

# Next generation




**Multiple antigen-targeting CARs**

Transgene integration at defined genomic loci

**Receptor – Ligand interaction**

**Inclusion of cytokines to improve efficacy**

-> TRUCKS (**T**-cell **r**edirected for **u**niversal **c**ytokine-mediated **k**illing)

**Conditional expression of CARs**

-> Temporal or / and spatial regulation of CAR activity

**Use in other immune cells**

e.g Natural killer cells

**Allogenic CAR-Ts => KO of endogenous TCR to reduce GVHD and immune rejection risk**

*e.g. host T cell mediated immune response leading to the clearance of allogenic double KO CAR-T (Benjamin et al., 2020)*

**Solid tumour antigen targets**

Targets not expressed on B cells => increased risk of inducing both cellular and humoral anti-CAR immunity



REVIEWED IN
Cortés-Selva et al (2020)

Wagner et al (2021)
Salazar-Cavazos and Altan-Bonnet (2022)

# Multiple layers of immunogenicity to be considered

- the foreignness of the cells (autologous vs allogeneic)
- whether they are genetically modified and/or are extensively passaged
- the nature of any transgene/gene editing
- the mechanism of action
- Co-administration of immunomodulatory molecules
- Trace amounts of vector used to deliver transgene

# Thank you

**PK Sciences**
Christian Joffroy
Fraser McBlane
Grzegorz Terszowski

**Preclinical Safety**
Franck Brennan
Andrea Kissling

**NIBR Biologics Center**
Annette Karle
Elisabetta Traggiai

**Former colleagues**
Bernd Potthoff
Britta Zehnpfennig
Denise Sickert
Sebastian Spindeldreher