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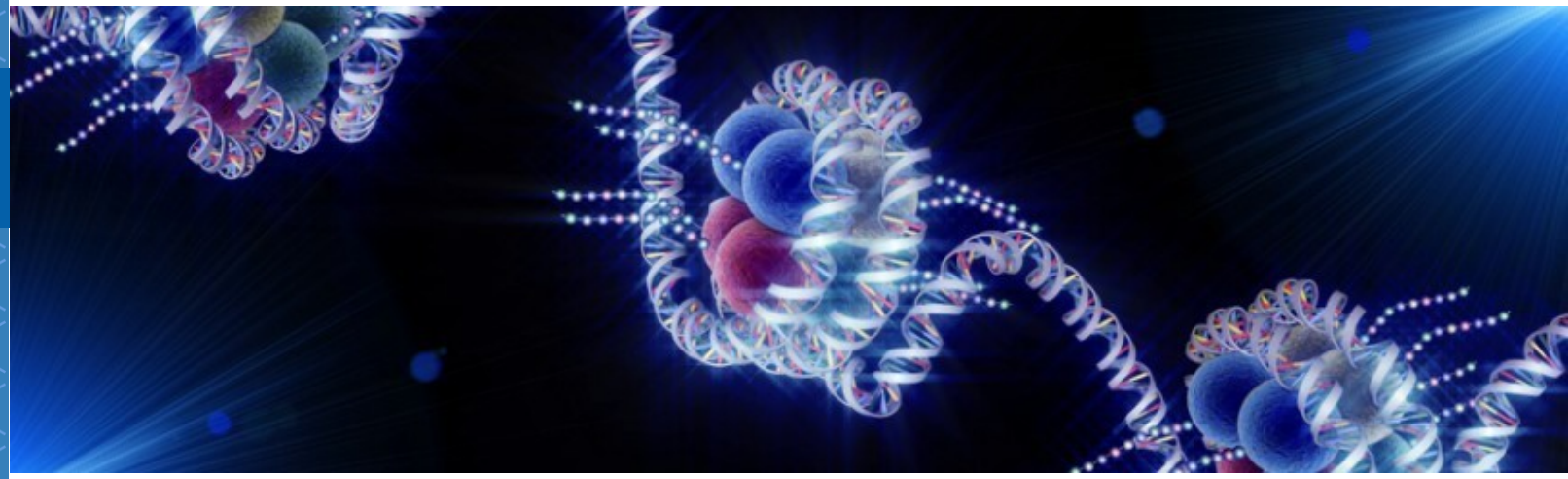

Flow cytometry based immunogenicity assays for CART

EBF Immunogenicity Training Day
23 - 24 March 2021

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Novartis Institutes for BioMedical Research
Translational Medicine
PK Sciences-Bioanalytics

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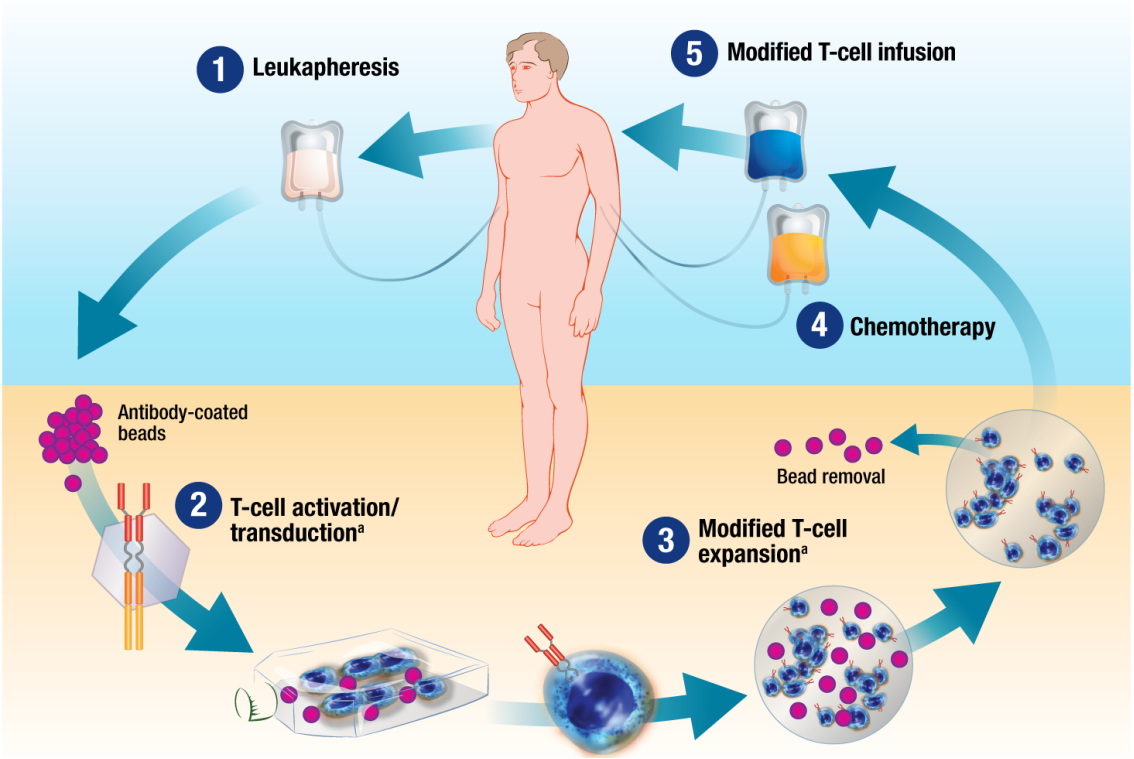
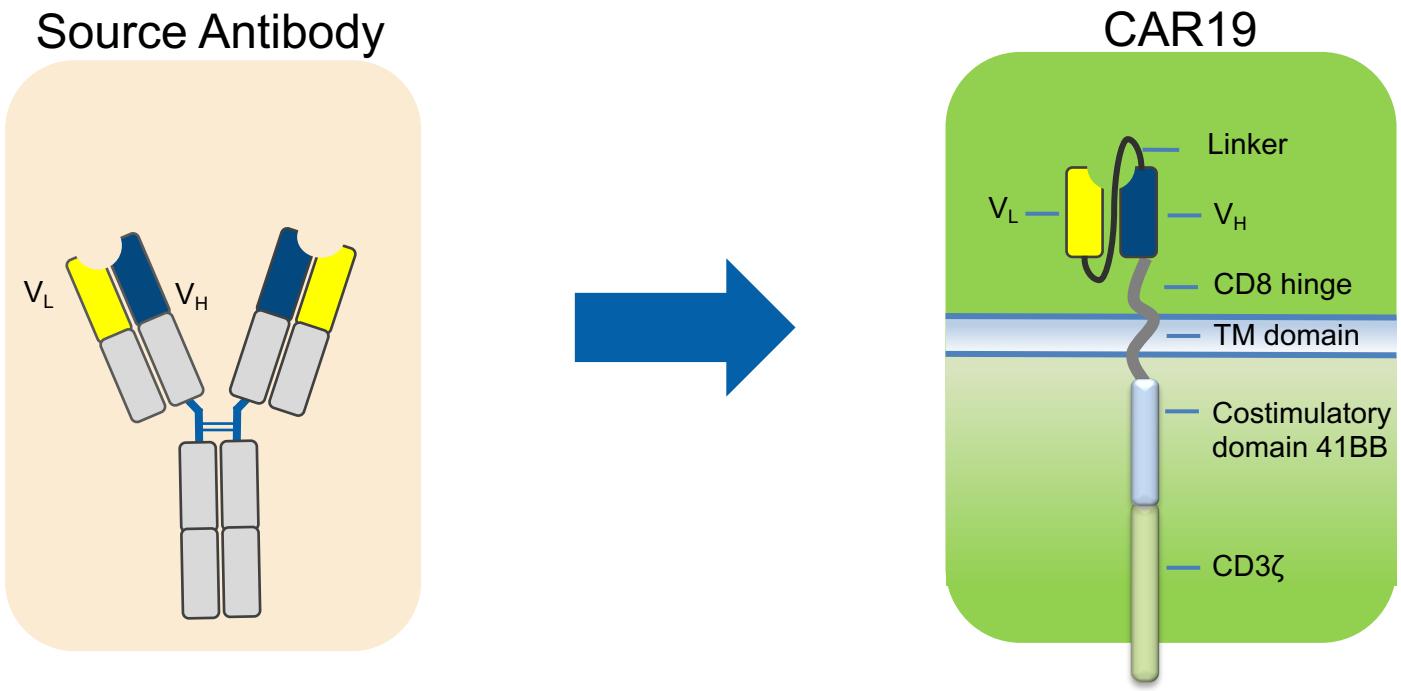
Chimeric antigen receptor T cells (CART)

1. **Leukapheresis**: patient's T cells are harvested
2. **T cells are activated** on antibody-coated beads and **genetically transduced ex vivo** with a construct encoding the anti-CD19 CAR
3. **CTL019 cells undergo ex vivo expansion** on antibody-coated beads
4. **Chemotherapy**: patient receives a preparative lymphodepleting regimen before T-cell infusion
5. **CTL019 cells are re-infused** into the patient, where they undergo in vivo expansion and target CD19+ cells for destruction



Composition of the CAR



The scFv (single chain variable fragment) targeting CD19 is based on a mouse hybridoma and has been characterized for its specificity to CD19 in several preclinical CAR T cell systems.

CAR-T immunogenicity

Humoral - ADA (anti-drug antibody)

Potential risks of anti-CAR antibodies:

- Neutralization, i.e. functional inhibition of CAR binding to its target
- Induction of CAR-T cell death by CDC, ADCC, uncontrolled receptor triggering

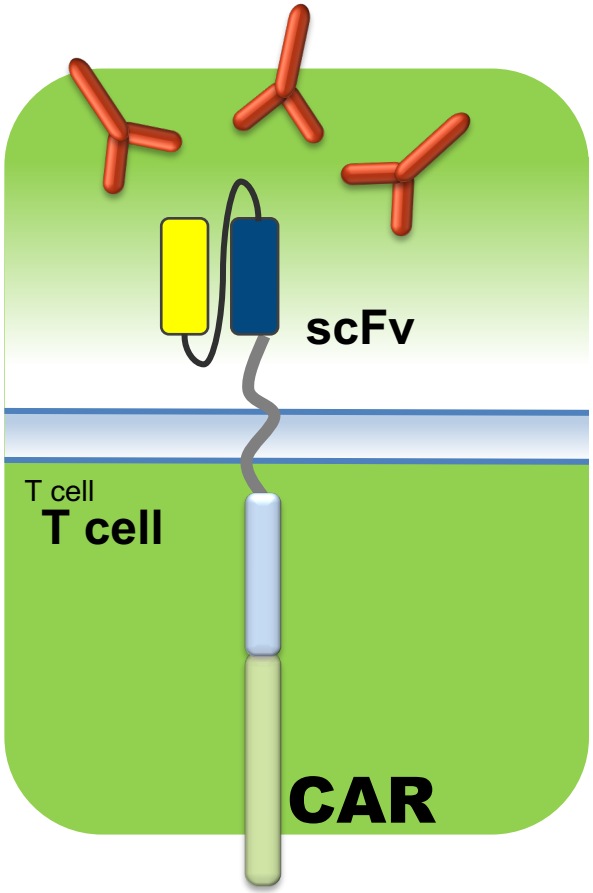
Cellular immunogenicity

Potential risks:

- Activation of cytotoxic CD8 T cells by CAR proteins
- Elimination of CAR T cells

Humoral immunogenicity assay (ADA)

Anti-CAR antibodies (ADA) in serum samples from patients



anti-CAR ADA assay formats

- Cell-based
  - Capture ADA with CAR-expressing T-cell line
  - Mimics membrane CAR on CAR-T cell
  - Flow cytometry output
- LBA (Ligand Binding Assay)
  - Soluble CAR or CAR fragments (can be difficult)
  - Parent mAb used to derive CAR-scFv
  - No membrane context
  - ELISA type assay

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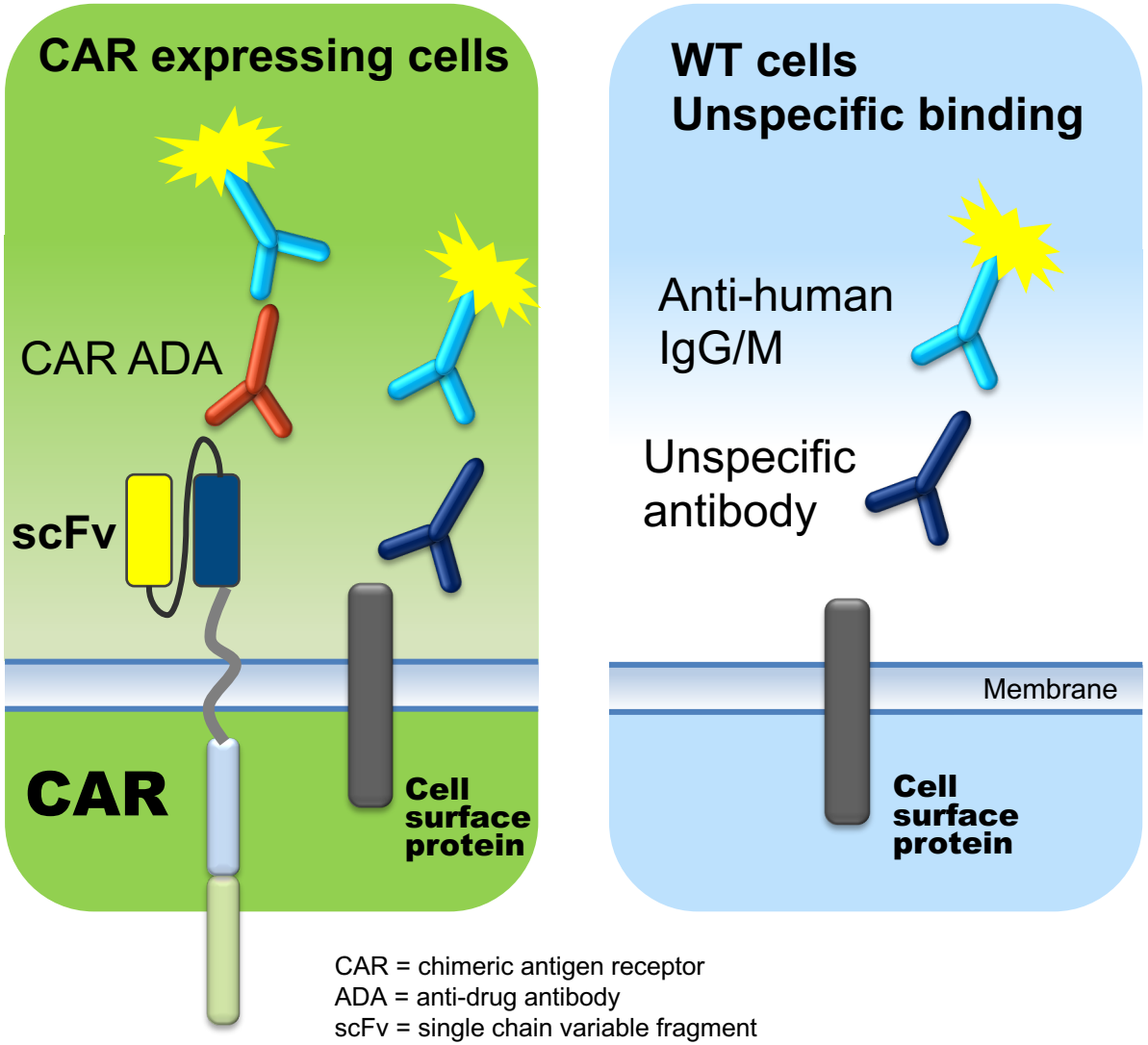
Cell based humoral IG assay

Jurkat cell lines (CAR+ vs. wild type) are used to detect ADAs in clinical samples

Calculation of CART ADA signal: Signal on CAR+ cells minus signal on WT cells (background)

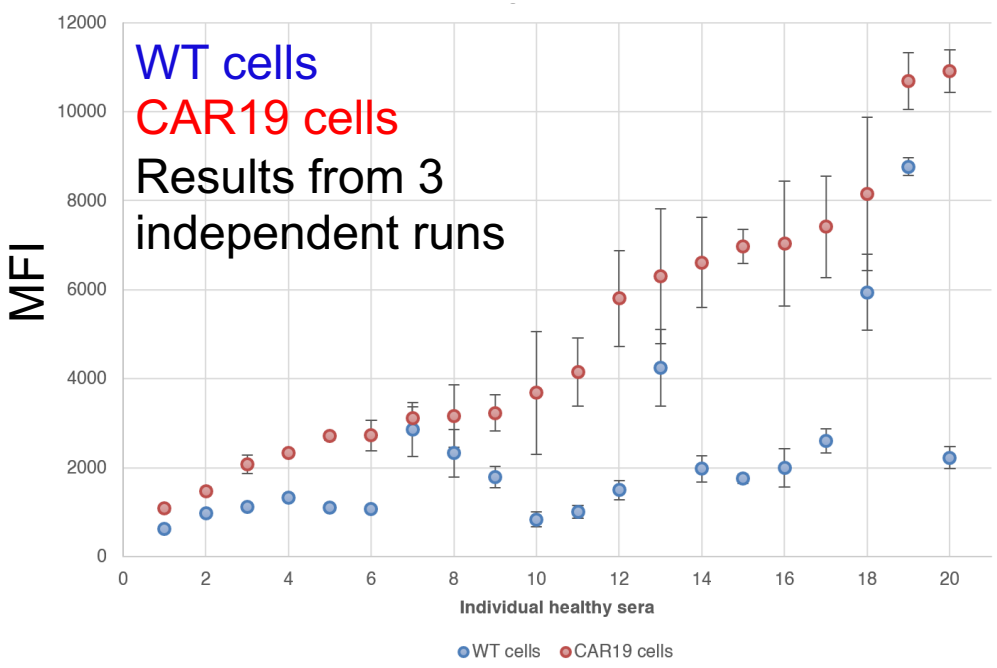
Comparable cell numbers?
Staining of Notch3 receptor



CAR = chimeric antigen receptor
ADA = anti-drug antibody
scFv = single chain variable fragment
WT = wild type cells

Pre-existing ADA & Cut point

Pre-existing antibodies in >80% of individual sera (validation) and patients (clinical studies)



Potthoff B et al- (2020) "A cell-based immunogenicity assay to detect antibodies against chimeric antigen receptor expressed by tisagenlecleucel, JIM, Vol. 476,112692, https://doi.org/10.1016/j.jim.2019.112692

- high signals of healthy donor sera didn`t allow for meaningful cut-point evaluation and outlier removal
- CAR19 specific signals (inhibition with CAR19 protein)

=> Immunoglobulin depleted sera were used for CP evaluation

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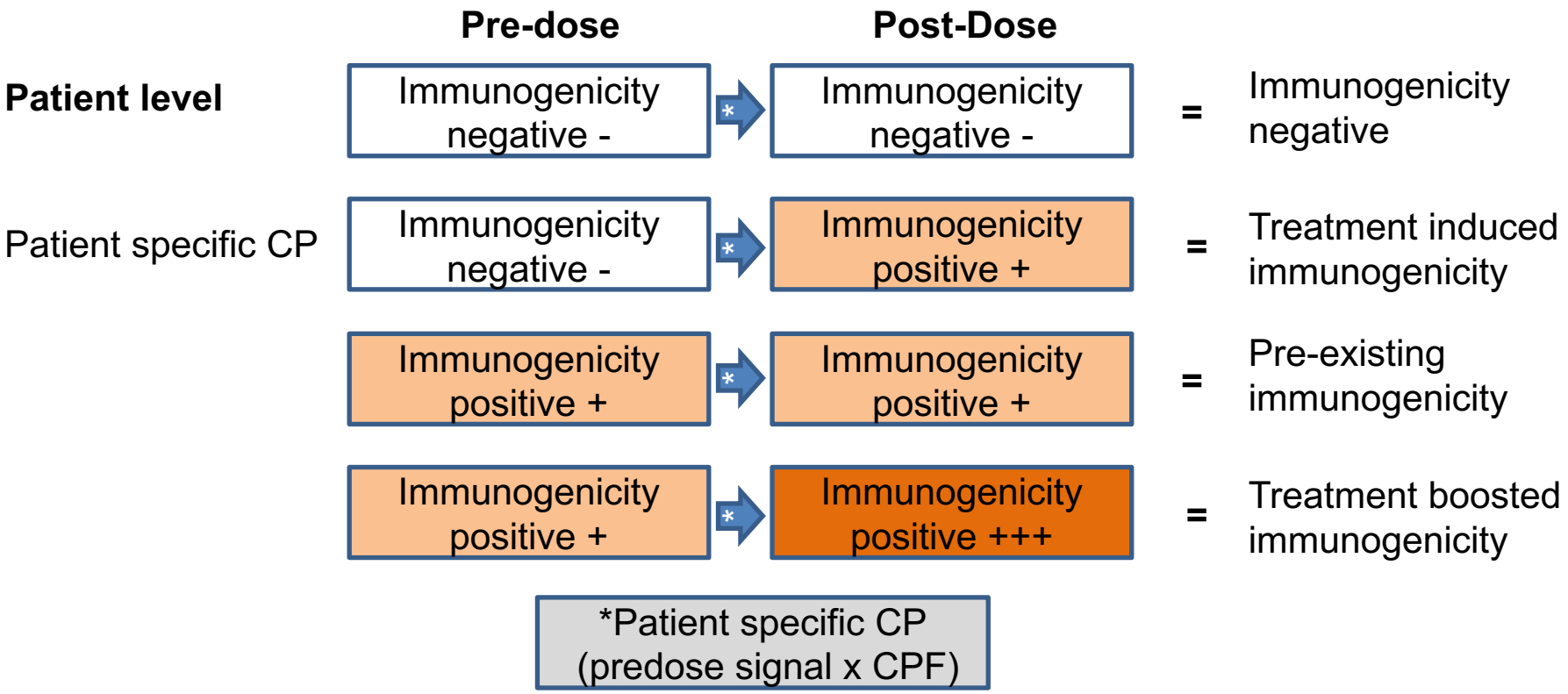
Data interpretation - Patient



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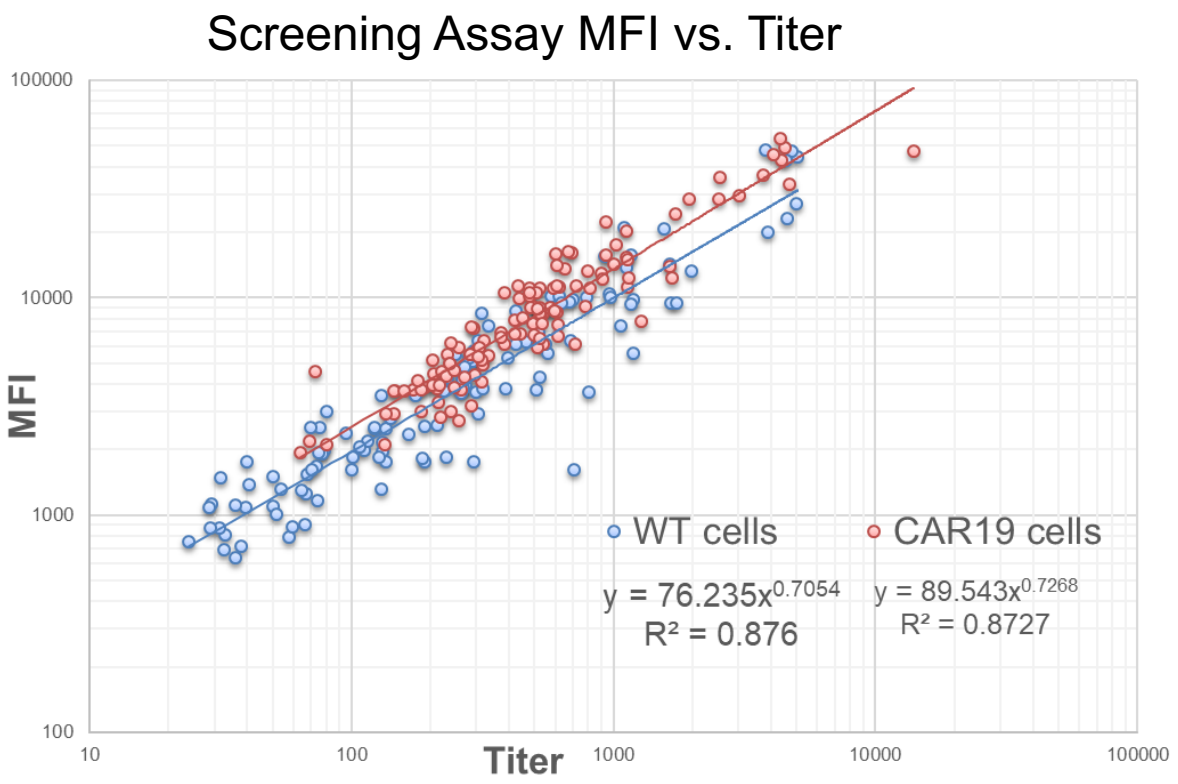
Correlation of screening and titration results

- Good correlation between screening (MFI) and titer results shown during validation and clinical sample analysis
- Possible since screening signal is not “capped” but correlates well with the analyte even at higher titers
- Screening step is reported since it does not only indicate whether samples are positive or negative but already reflect the different amounts of ADA



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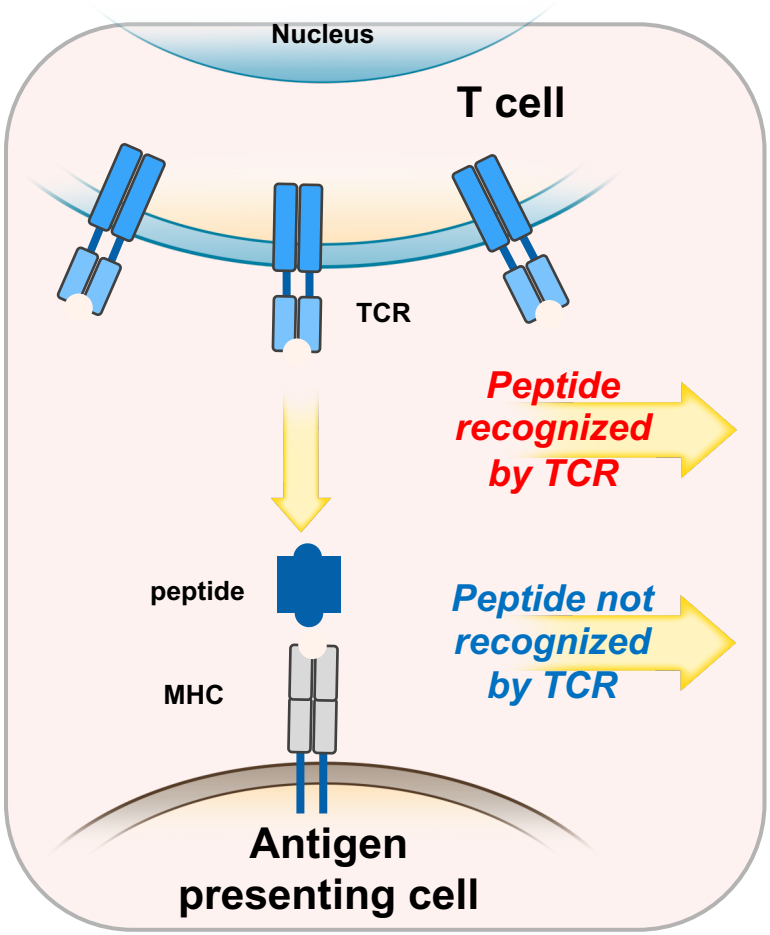
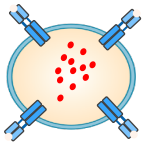
Cellular immunogenicity - T cell activation assay

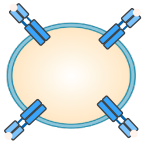


- Patient`s PBMCs are collected and incubated with CAR peptides
- T cell activation is measured by Flow cytometry (intracellular IFNg staining)



T cells are activated and express interferon gamma

T cell



No activation

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Cellular immunogenicity assay

Controls:

- **SEB** (staphylococcal enterotoxin B)
- **CEF**: pool of 27 peptides corresponding to viral and vaccine sequences frequently recognized by CD4+ and CD8+ T lymphocytes
- **FMO:** stimulation with SEB and incubating with all fluorescently labeled antibodies except IFNγ
- **DMSO** (Negative control, used to dissolve peptides)

Actual stimulation:

- **2 CAR peptide pools** (15-mer overlapping peptides)

Read out: percentage of IFNγ+CD4+ T cells and IFNγ+CD8+ T cells with respect to their parent populations. No threshold/cut point is applied. Percentages of IFNg positive cells are reported.

Cellular immunogenicity assay

Ranges observed in exemplary clinical study

Population	Treatment	Values
IFNγ+ CD4 T cells	SEB treated	1.610-27.300%
	CEF treated	<LOD-0.093%
	Pool 1 treated	<LOD-0.091%
	Pool 2 treated	LOD-0.081%
IFNγ+ CD8 T cells	SEB treated	2.710-25.600%
	CEF treated	0.076-2.450%
	Pool 1 treated	<LOD
	Pool 2 treated	<LOD-0.051%

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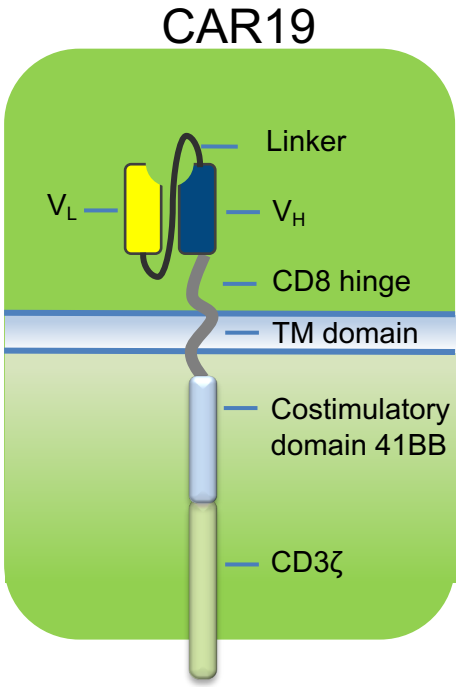
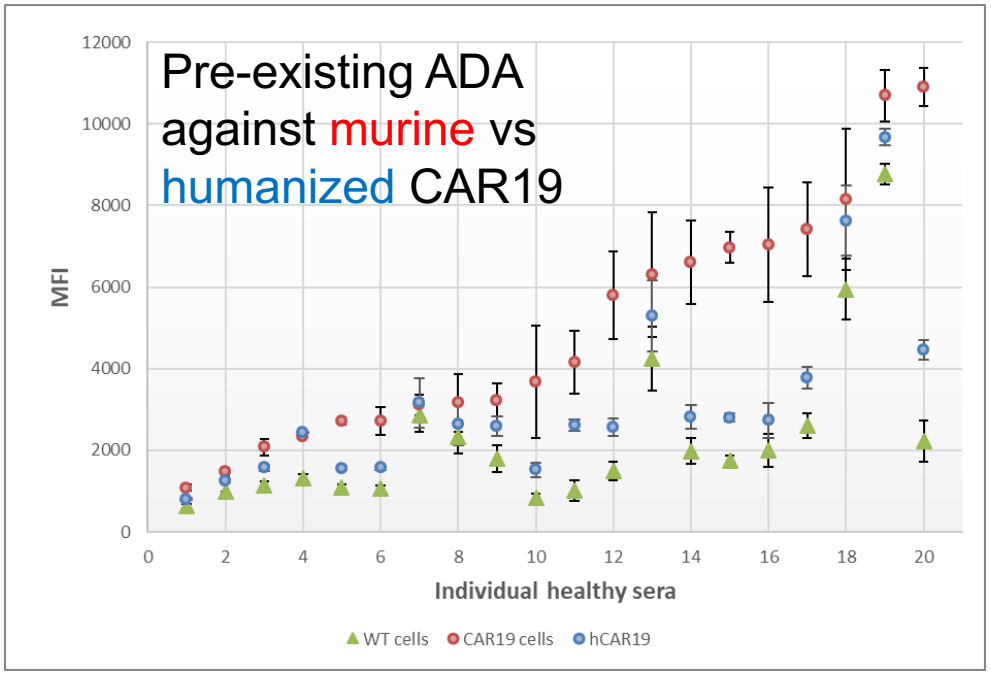
Outlook – further characterization of humoral immunogenicity against CAR19

- No impact of detected ADAs on efficacy, safety or exposure
- Identification of the predominant CAR19 ADA binding sites
- Spiking of extracellular domains (murine and humanized version of CAR19) and peptides





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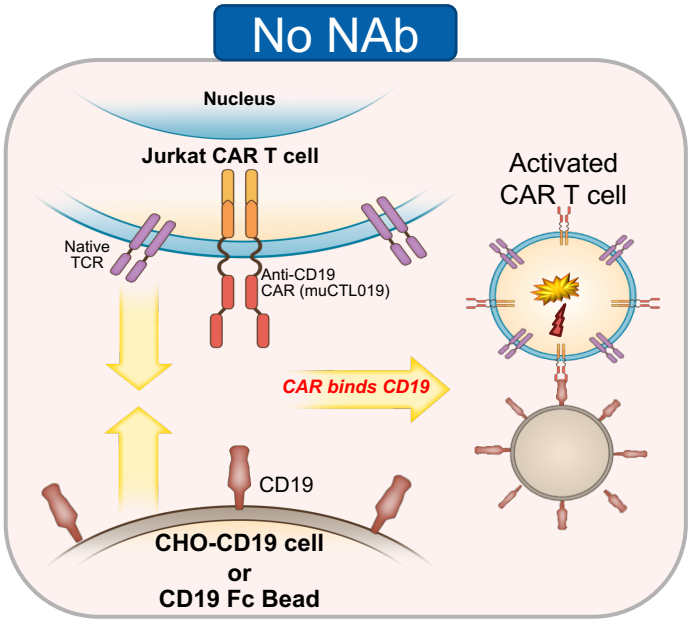
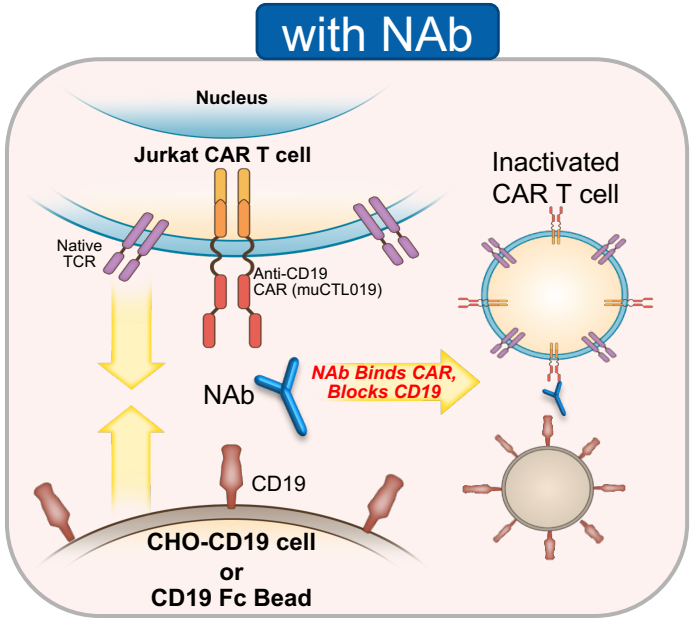
Outlook – potential cell-based CAR T NAb assay





- Jurkat-muCTL019 binds CD19 (CHO or Beads)
- Jurkat activated → produces measurable response (Luciferase or IFNγ)

- NAbs present in sample, bind CAR and block CAR-CD19 interaction
- Jurkat remains inactivated → limits measurable response (Luciferase or IFNγ)

Thank you

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