

# Monitoring of CAR-T cell therapy by flow cytometry

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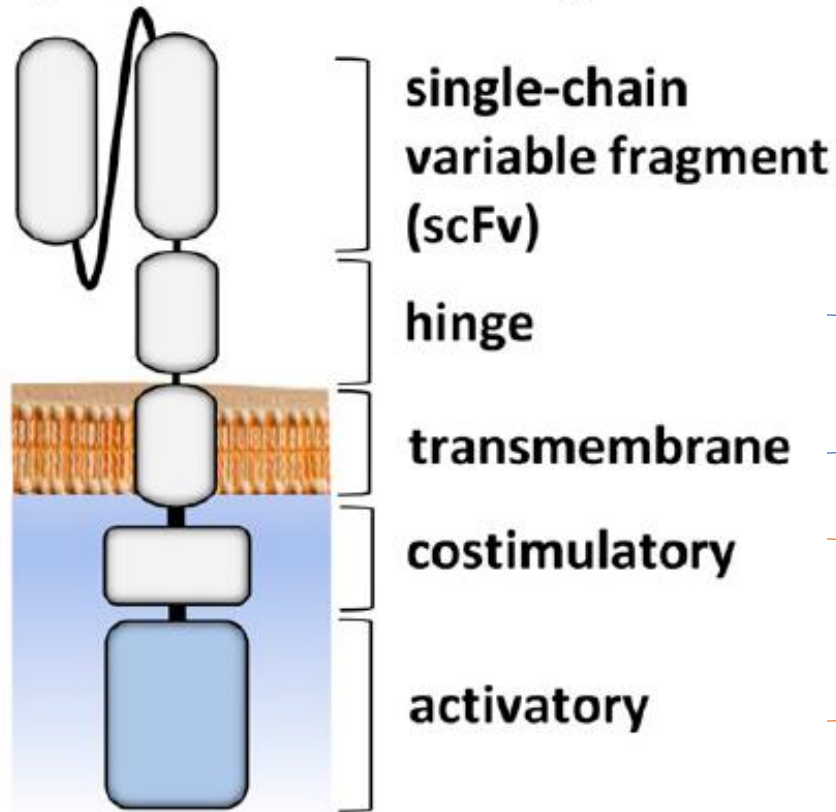
# Outline

- Structure of CAR and indications of CAR T cells
- Why monitor CAR T cells?
- Analytical validation of CAR T cell detection/quantification by flow cytometry
- Recommendations for immune monitoring and real life EBMT survey

# The chimeric antigen receptor

variable region  
light chain ( $V_L$ )

variable region  
heavy chain ( $V_H$ )



single-chain  
variable fragment  
(scFv)

hinge

transmembrane

costimulatory

activatory

CD8 $\alpha$ -, CD28-  
or IgG-derived

4-1BB/CD28-CD3 $\zeta$

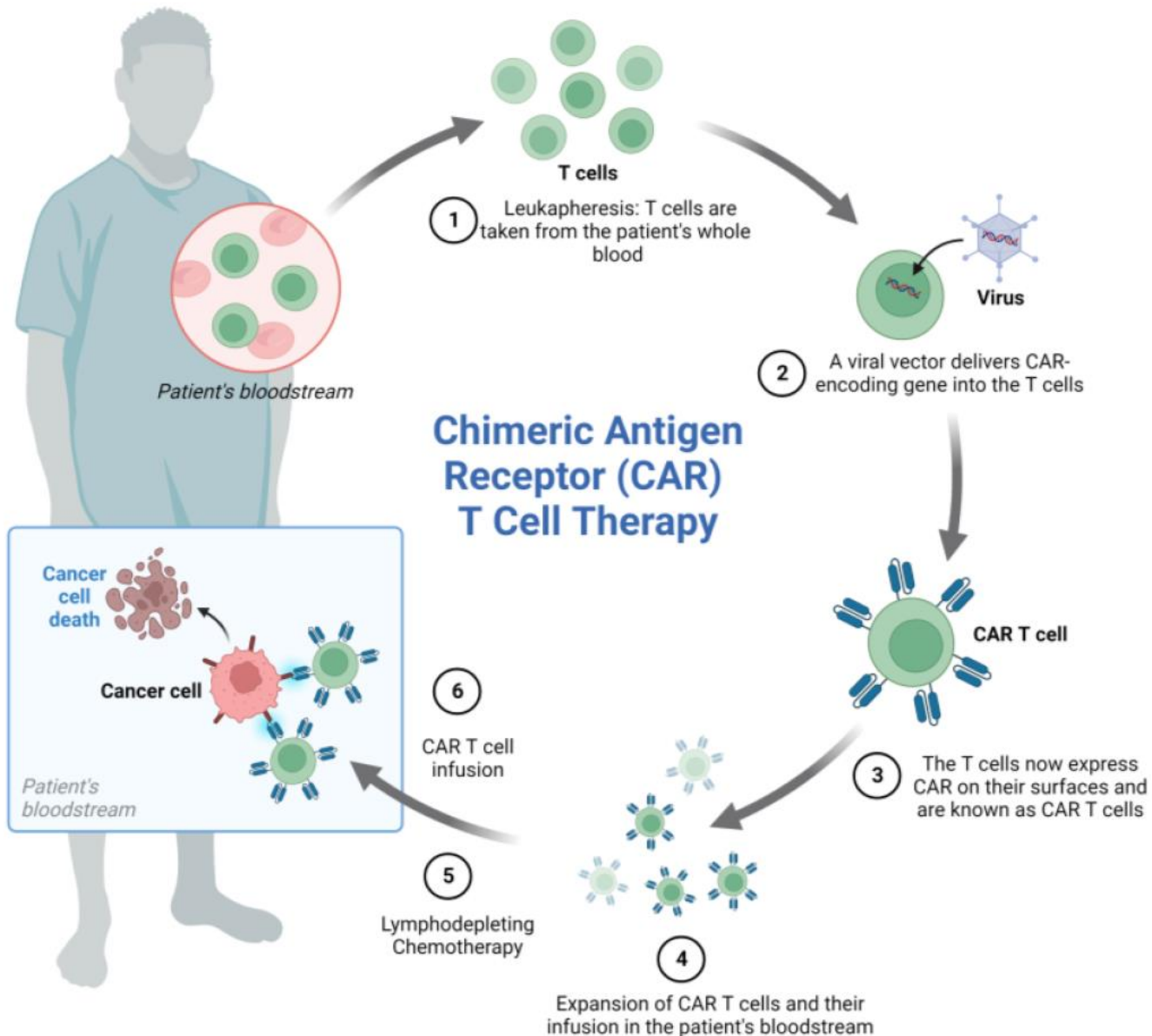
specificity CD19, BCMA

adapt to antigen density and length

signal transduction  
persistence/exhaustion

**Chimeric Antigen Receptor**

# Process and indications of CAR T cells



- B-ALL
  - DLBCL
  - CLL
  - Multiple myeloma
- CD19
- BCMA

in resistance to conventional treatment

# Why monitor CAR T cells?

1. Anti-tumor efficacy ~ proliferation/persistence
  - Peak expansion higher in responding patients
  - Persistence correlate with EFS
2. Adverse effects:
  - Cytokine release syndrome (CRS) w or w/o immune effector cell-associated neurotoxicity syndrome (ICANS)
    - Peak CART expansion correlated with cytokine levels
  - On target/off tumor effects: B cell depletion

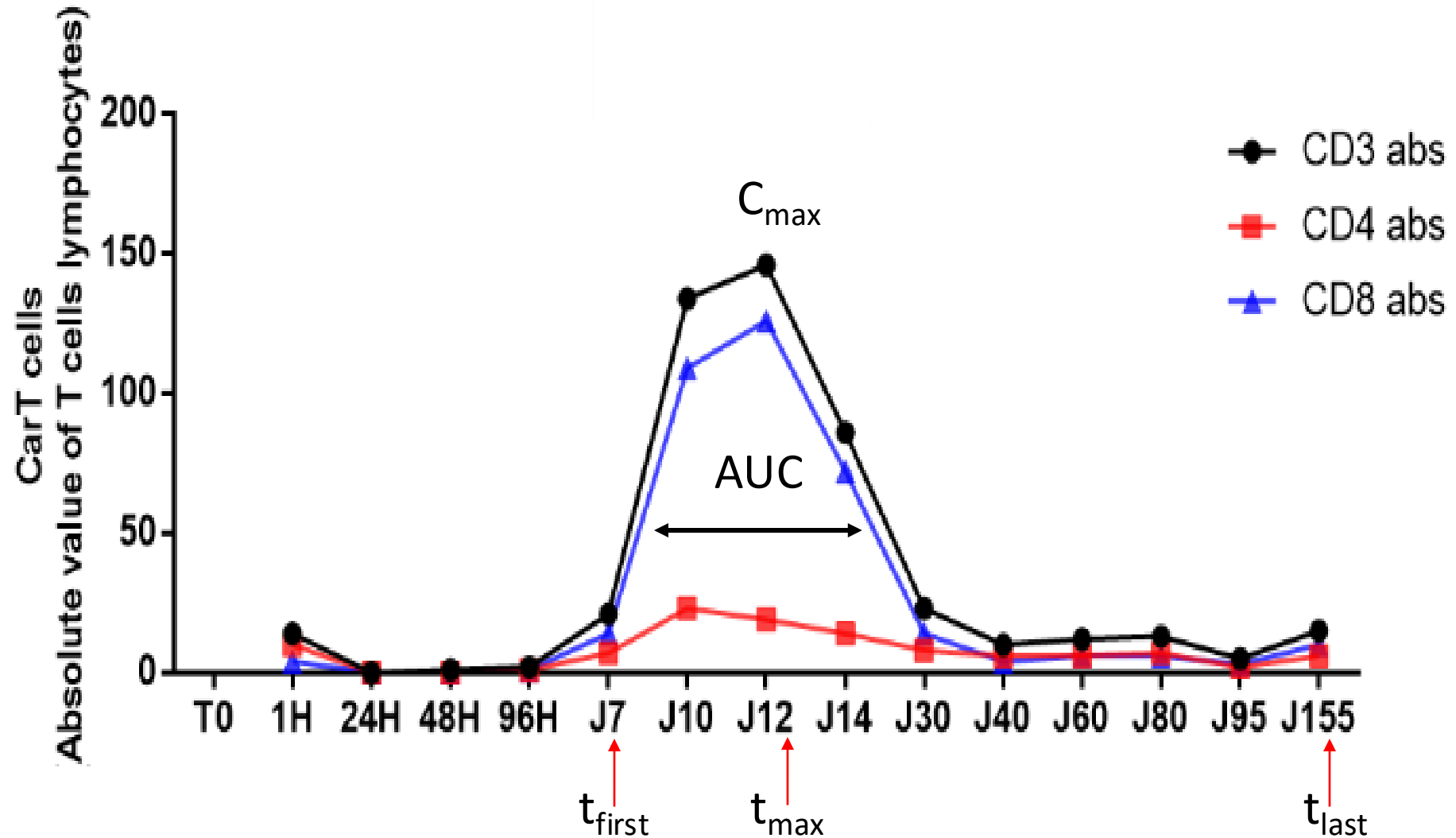
## Methodologies:

- Phenotype by flow cytometry: cells expressing chimeric receptor
- Genotype QPCR: transgene incorporation, # copies

Mueller KT, Blood 2017, 130-2317

Mueller KT, Clin Cancer Res 2018, 24-6175

# CART expansion kinetics



# Monitoring CAR T cells by flow cytometry

## Advantages

- Direct visualisation of CAR-T cells
- Speed: ~2h
- Multiplexing: minimal residual disease
- Additional phenotypic characterization (*viability, activation status, deep T cell phenotyping*)

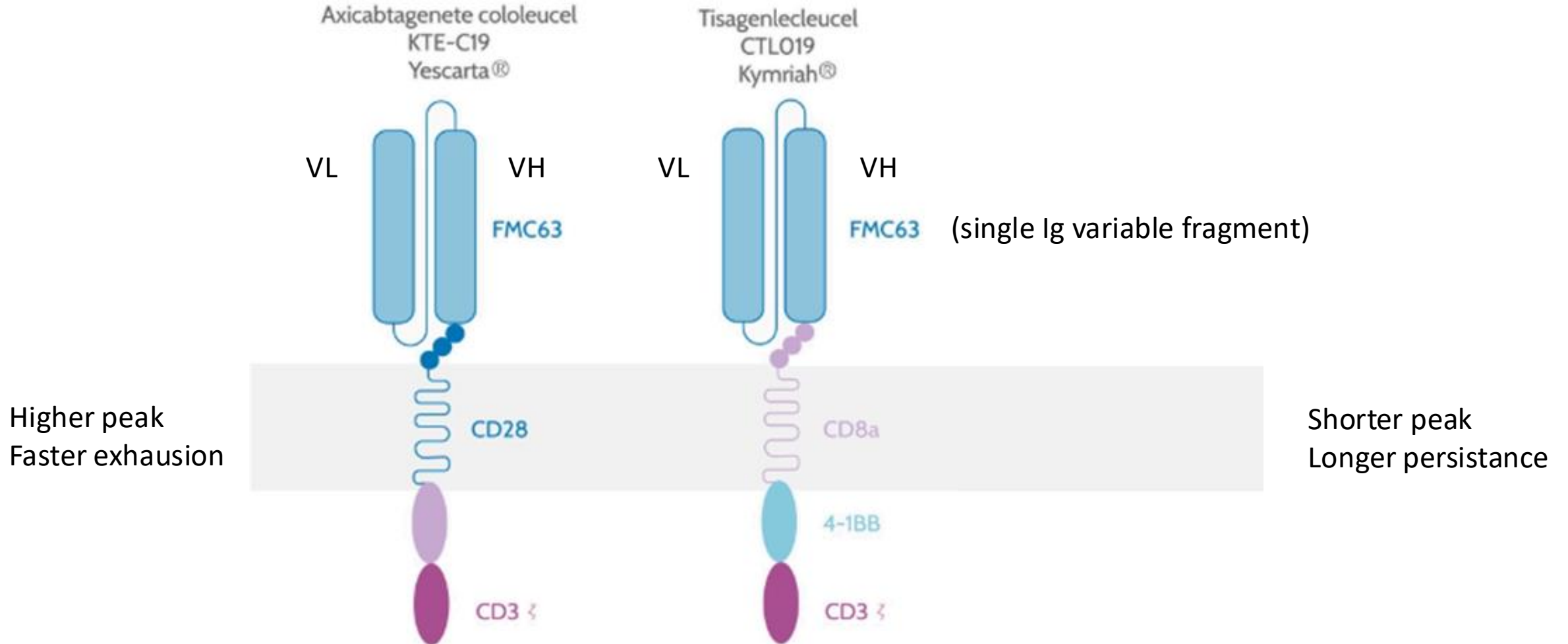
## Shortcomings

- limited sensitivity for long term monitoring
- non-specific interference and background signals
- expertise in analysis of complex data

# Analytical validation - outline

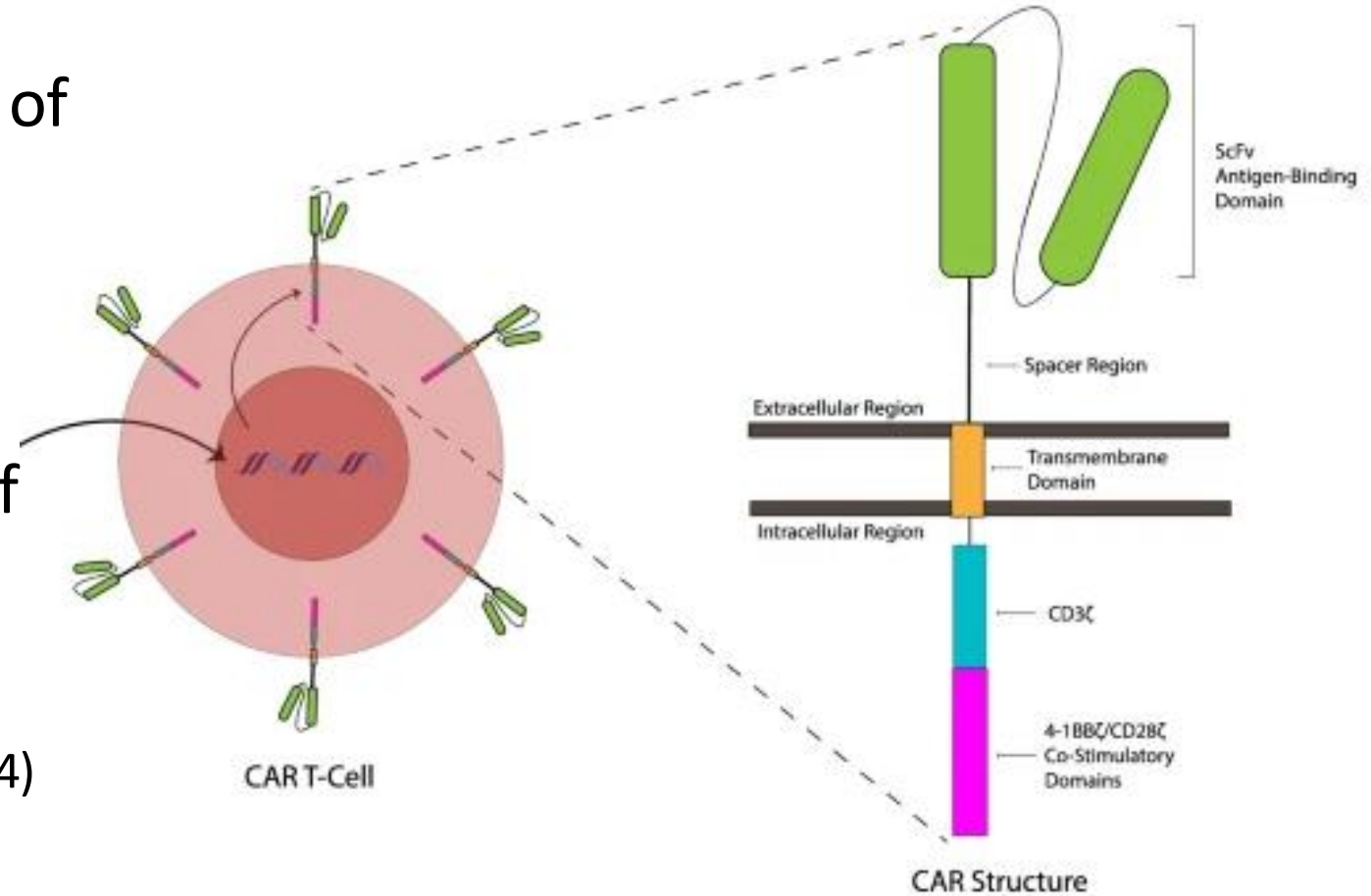
- Available reagents for CART detection by flow cytometry
- Resolution and specificity
- Lower limit of quantification
- Viability in unstained samples
- Engraftment kinetics, plausibility values
  
- Literature survey and own data

# CAR-T19 in B-ALL and DLBCL



# Reagents available to target CAR-T cells

- Universal detection reagents of Ig light chain
  - Protein L (1)
  - goat anti-mouse Ig (2)
- Specific detection reagents of FMC63 scFv
  - rCD19 protein
    - His-Tag (3)
    - Fused to IgG1 Fc ~ « CAR DR » (4)
  - Anti-FMC63 (5)

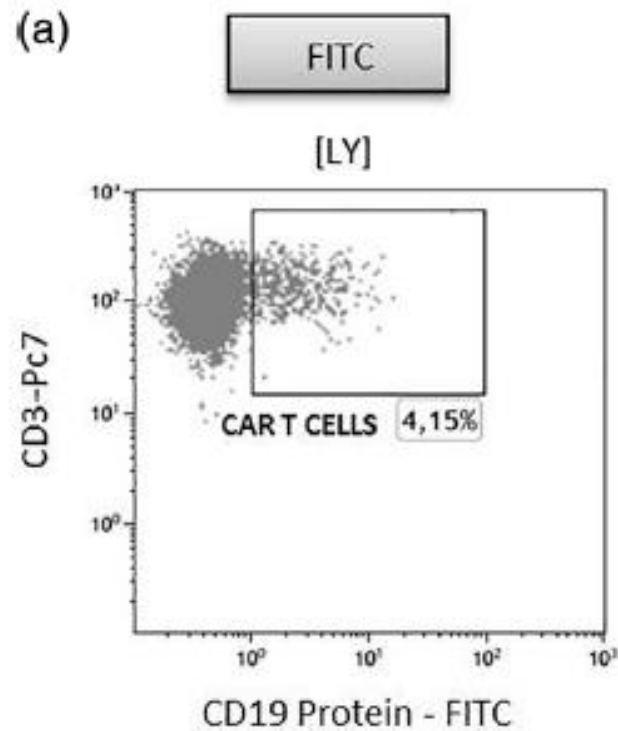


# Technical issues of CAR reagents

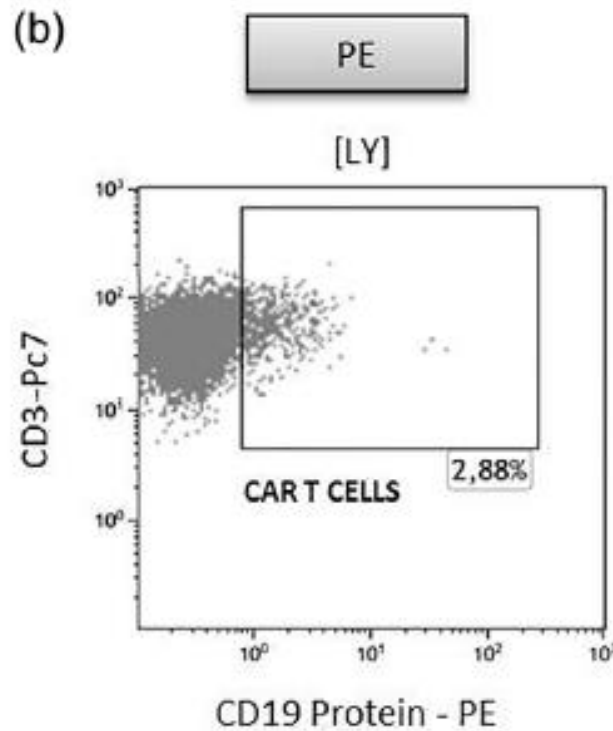
		Target	Compatibility w/ Ab Panels	One-step	Two-step (Anti-biotin/Streptavidin)	Stability	Off-targets
(1)	Protein L	IgL	No	X	X	High	Mon, B LY
(2)	GAM Ig *	IgL	No	X	X	High	
(3)	CD19-Fc (CAR DR)	scFv FMC63	Yes	X	X	High	
(4)	CD19-His	scFv FMC63	Yes	X	X	Low	
(5)	Anti-idiotypic *	scFv FMC63	Yes	X	X	High	

\* Use Fab'<sub>2</sub> or mutated Fc-engineered Ab

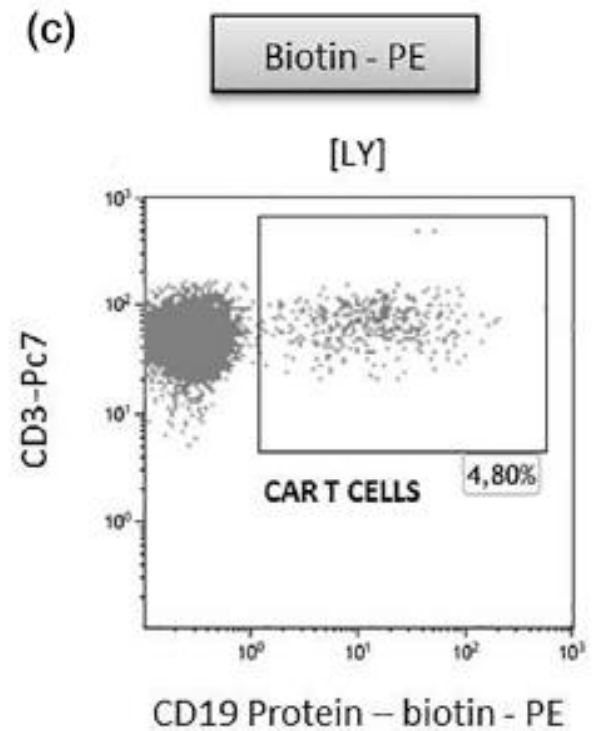
# Comparison direct (1-step) vs indirect (2-steps)



His-Tag



Fc-Tag

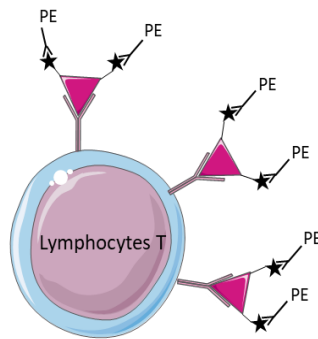
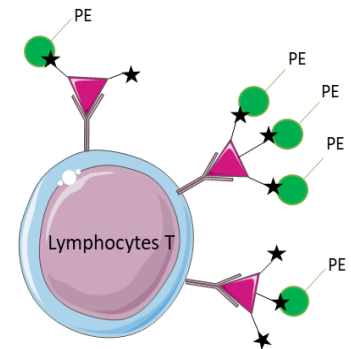


Anti-Biotin-PE

# Comparison of 2 Ag-based assays Anti-biotin vs streptavidin

Acro Biosystems

Miltenyi



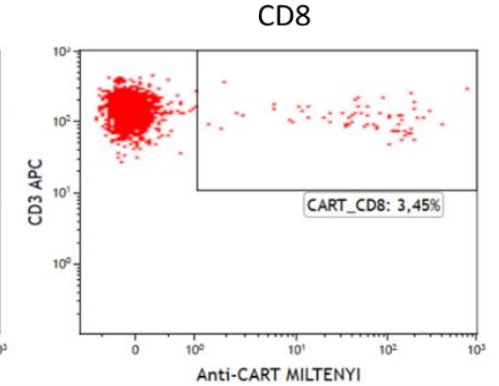
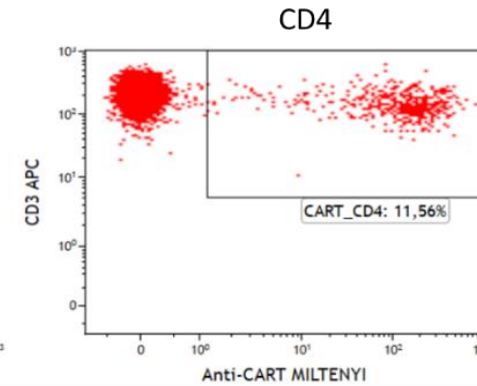
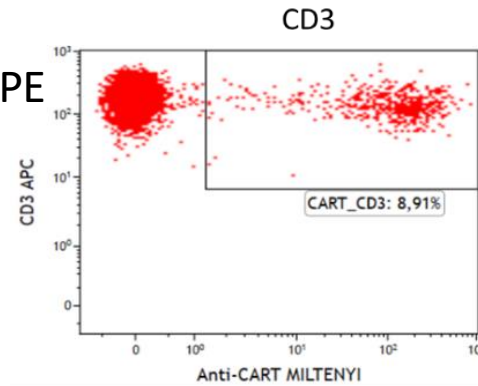
Y Récepteur CAR  
(Chimérique  
Antigène Receptor)

● PE Streptavidine  
couplée au  
fluorochrome PE

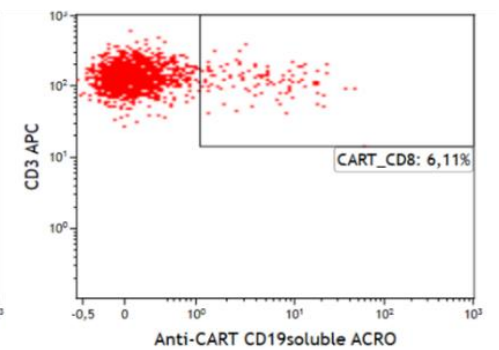
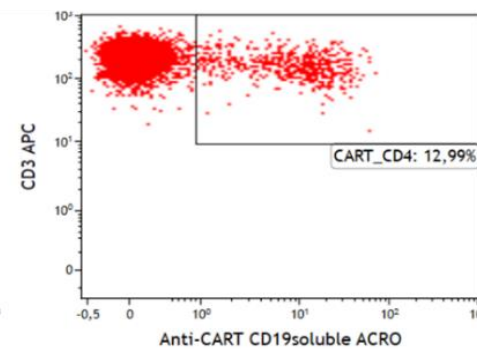
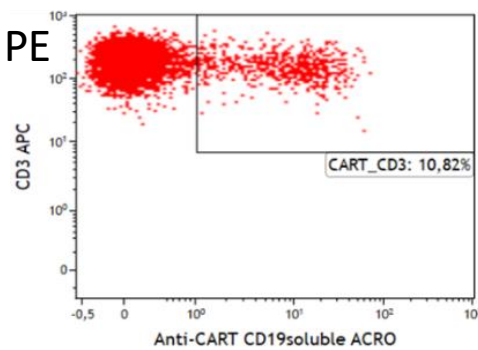
▲ CD19 recombinant  
Couplé à la biotine

▲ PE Anti-Biotine couplé  
au fluorochrome PE

Anti-Biotin PE



Streptavidin PE



# Background staining of CAR T reagents

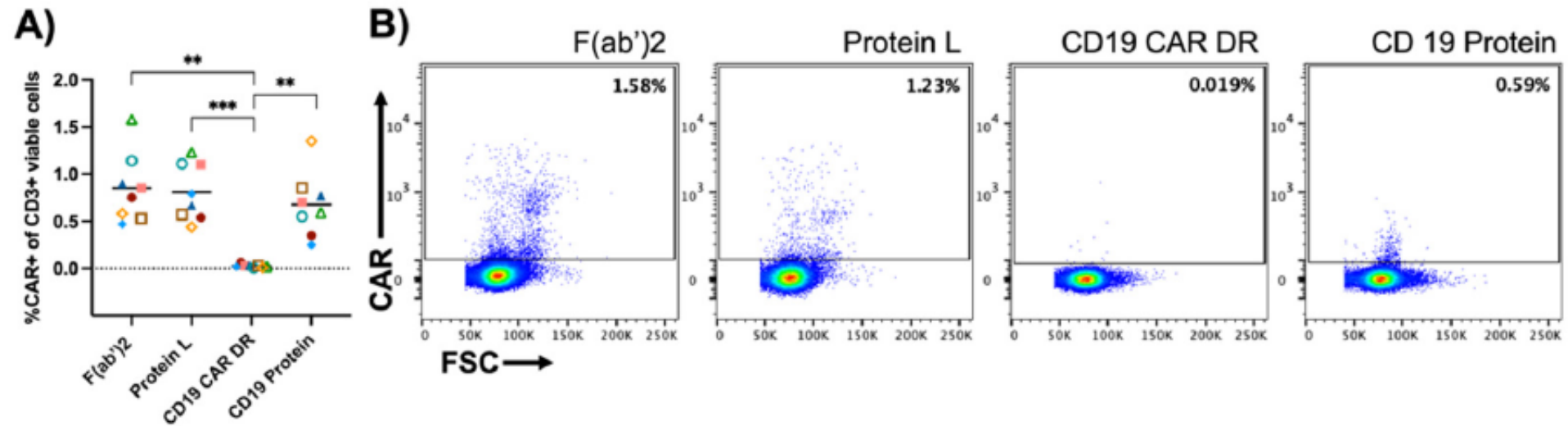
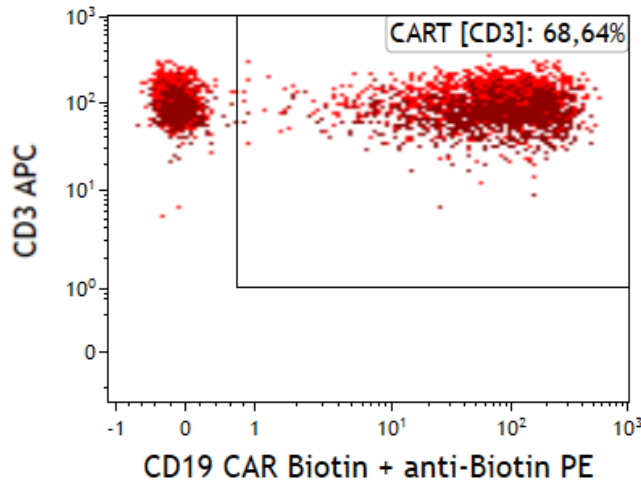


Figure 4. Specificity of the different detection reagents. PBMCs were stained with the respective CAR-detecting reagents to assess background staining. (A) shows the percentage of CD19.CAR-T cells in PBMCs only, for eight different donors. (B) displays results from one donor stained with different detection reagents. Data are representative of eight different HDs acquired in one experiment. (\*\*)  $p < 0.01$ ; (\*\*\*)  $p < 0.001$  by one-way ANOVA.

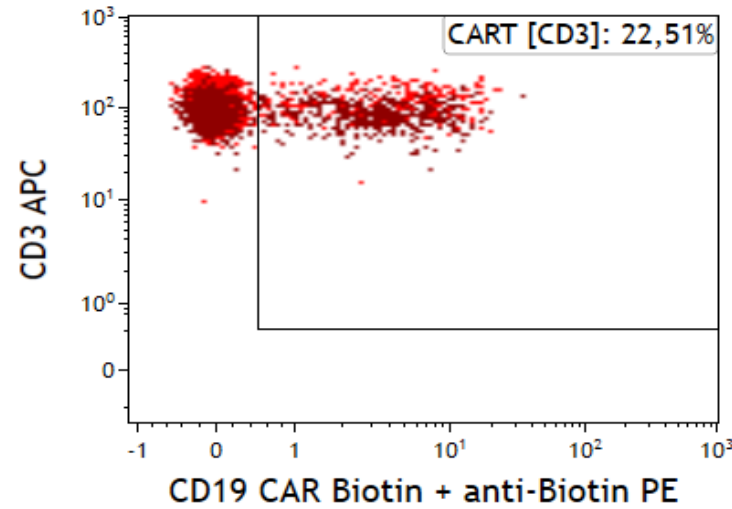
# Comparison CAR DR vs anti-FMC63



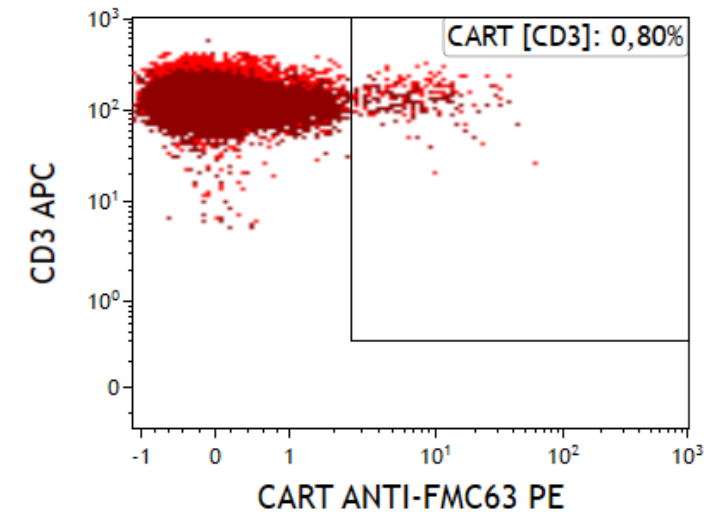
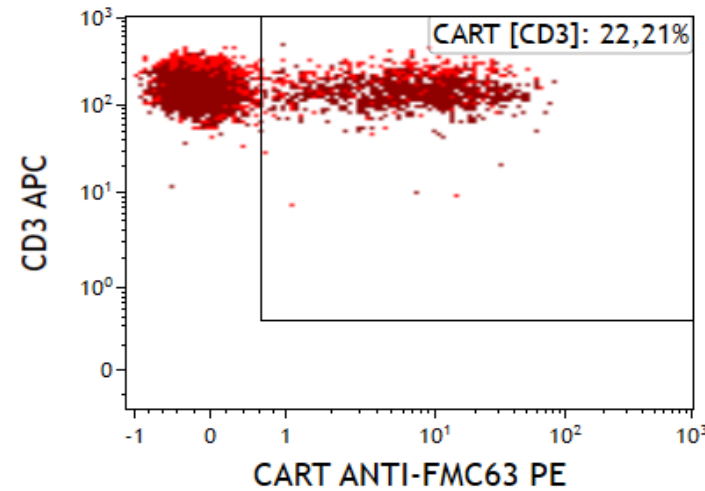
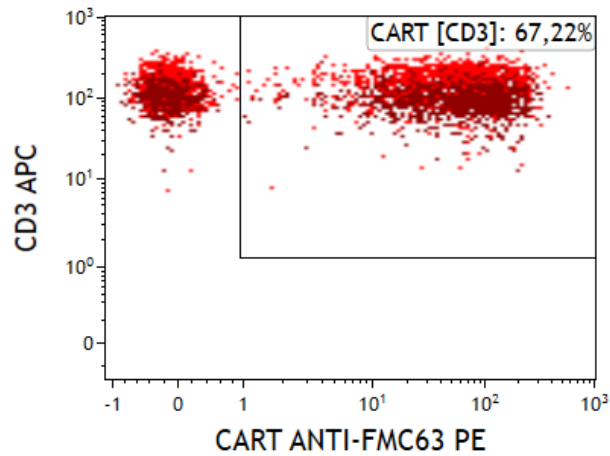
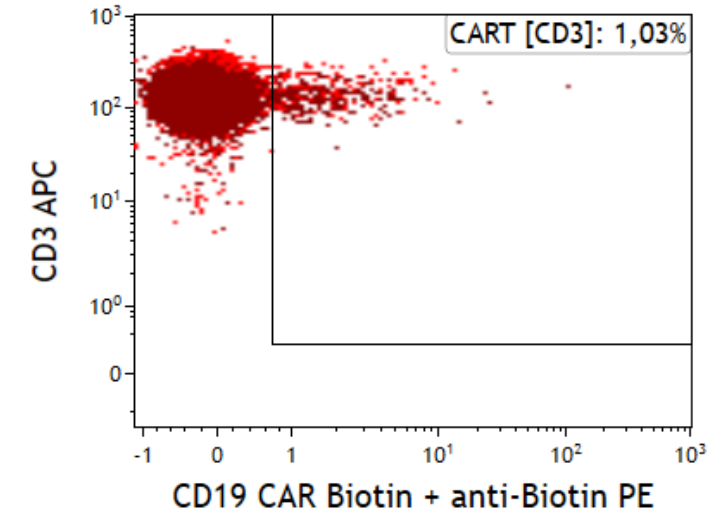
Day 7



Day 18



Day 29



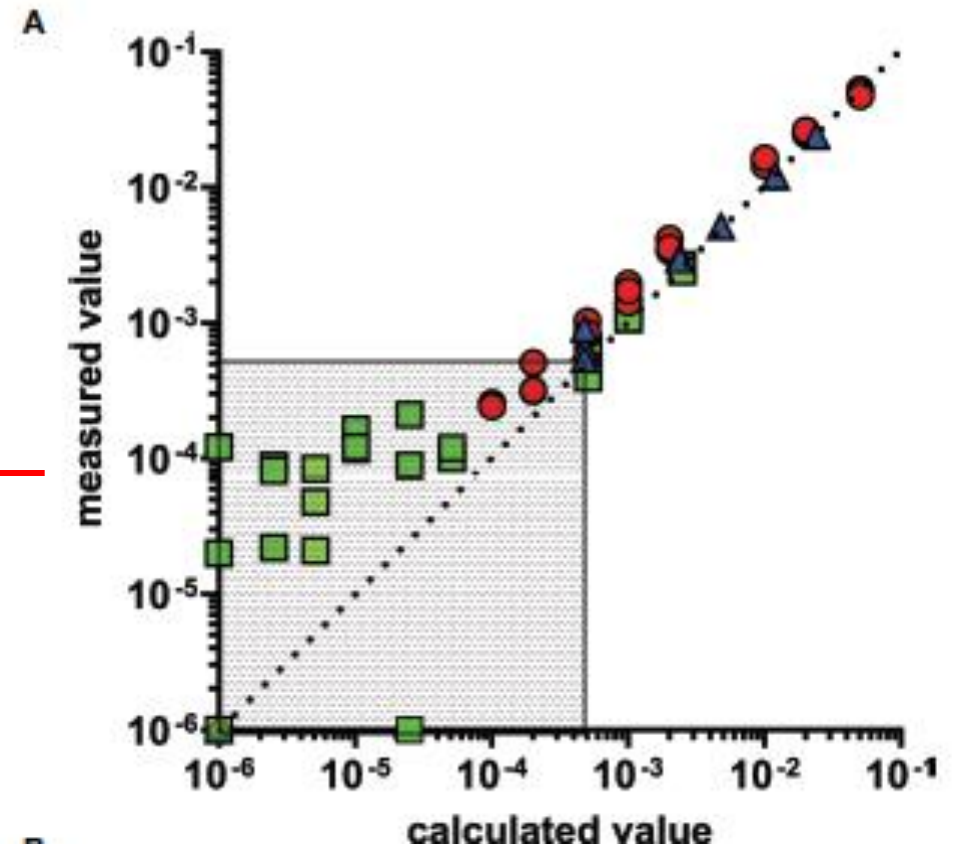
# Lower limit of linearity - experimental

Background ~ 0,05%

CAR DR-biot + anti-Biot-PE



Dilution curve



# Background staining – comparison CAR DR vs anti-FMC63

	CAR DR biot + anti-biot PE	Anti-FMC63 PE
Limit of blank = mean FMO + 1,645 SD	0,057	0,066
Limit of detection = mean FMO + 3 SD	0,078	0,102

- 8 patient samples, FMO method
- Panel: CD45v500, CD3APC, CD4FITC, CD8APC-H7
  - Anti Biotine PE
  - ∅

# Lower limit of quantification – statistical

$$R = (100/CV)^2$$

TABLE 1: Determination of database/sample size that will provide a given precision in rare event analysis.<sup>(a)</sup>

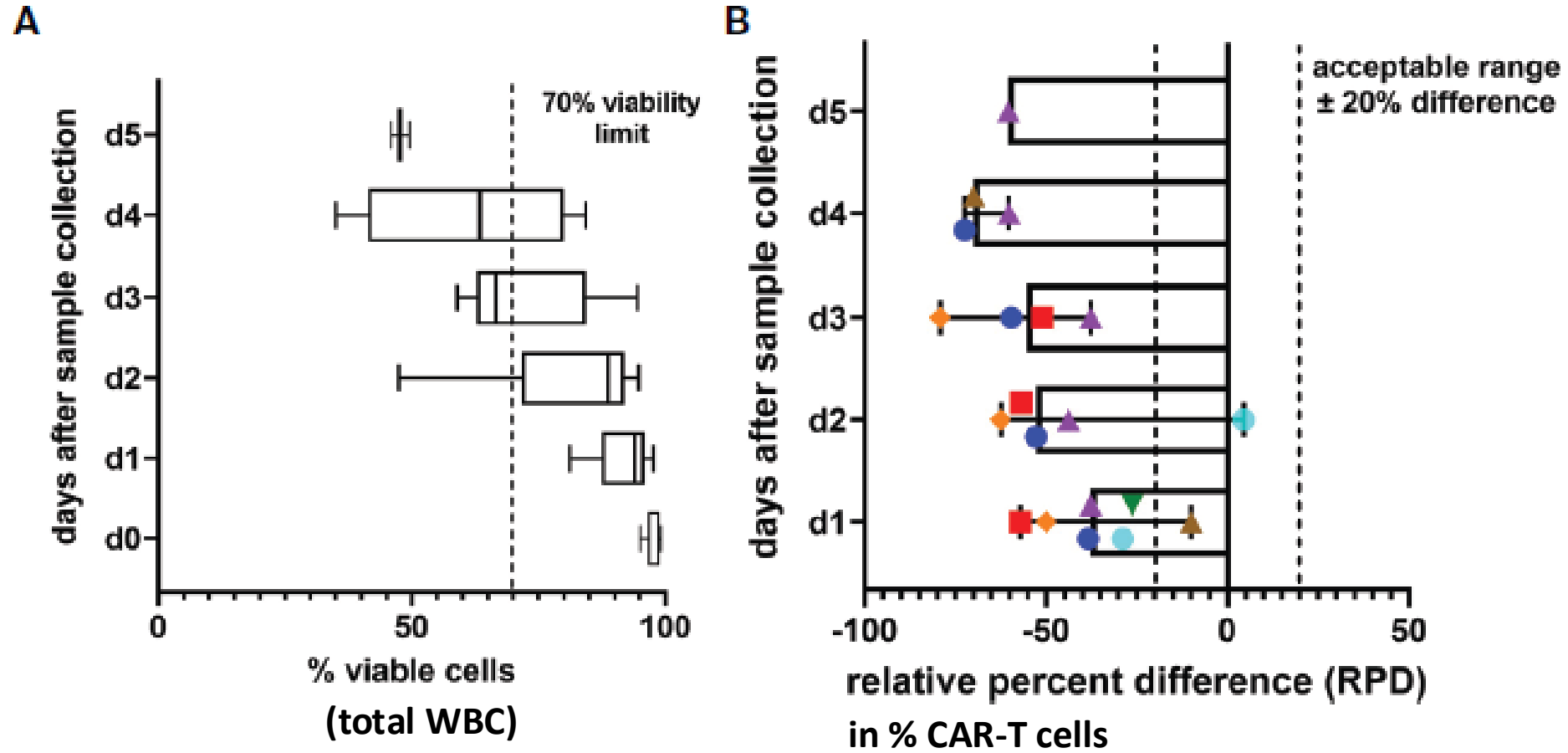
Desired CV (%) →		1	5	10	20	40
$r = \text{no. of events of interest} \rightarrow$		10000	400	100	25	6
When occurring at a frequency of (%)	1 : $n$	Total no. of events which must be collected <sup>(b)</sup>				
10	10	$10^5$	$4 \times 10^3$	$10^3$	$2.5 \times 10^2$	$6.3 \times 10^1$
1	100	$10^6$	$4 \times 10^4$	$10^4$	$2.5 \times 10^3$	$6.3 \times 10^2$
0.1	1000	$10^7$	$4 \times 10^5$	$10^5$	$2.5 \times 10^4$	$6.3 \times 10^3$
0.01	10,000	$10^8$	$4 \times 10^6$	$10^6$	$2.5 \times 10^5$	$6.3 \times 10^4$
0.001	100,000	$10^9$	$4 \times 10^7$	$10^7$	$2.5 \times 10^6$	$6.3 \times 10^5$
0.00001 <sup>(c)</sup>	10,000,000	$10^{11}$	$4 \times 10^9$	$10^9$	$2.5 \times 10^8$	$6.3 \times 10^7$

Sample volume for 100,000 lymphocytes (P25, n=39):

- J7: 1379  $\mu\text{l}$
- J14: 1052  $\mu\text{l}$



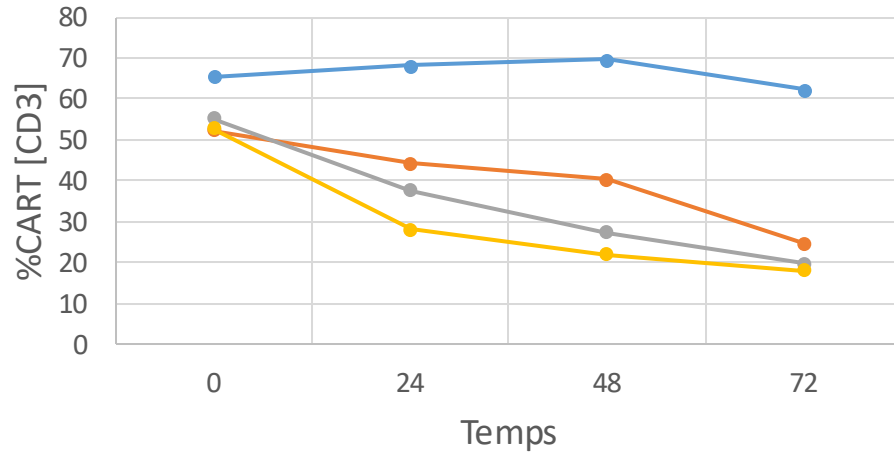
# Viability of CAR-T cells in unprocessed samples



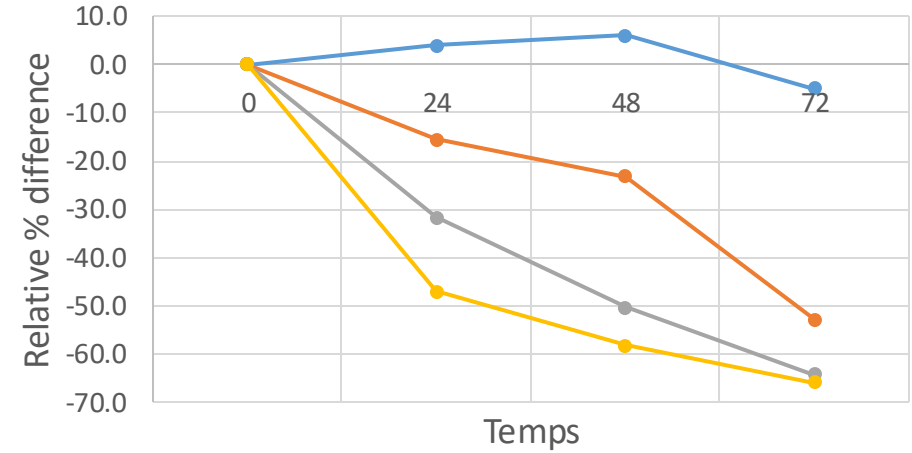
# CAR T cell viability

1 colour/sample

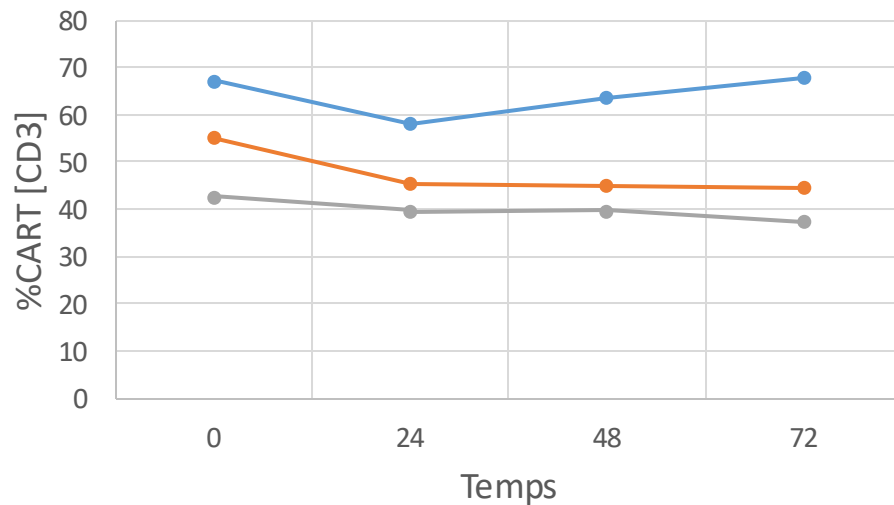
### RT stability



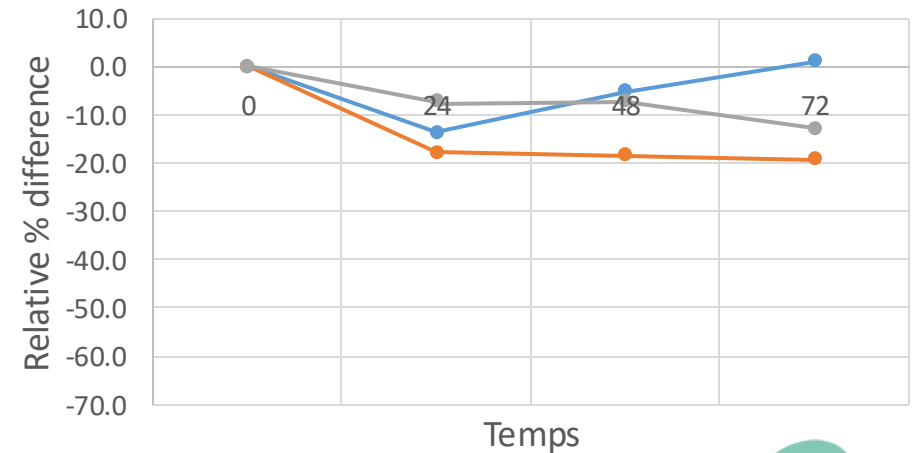
### RT stability

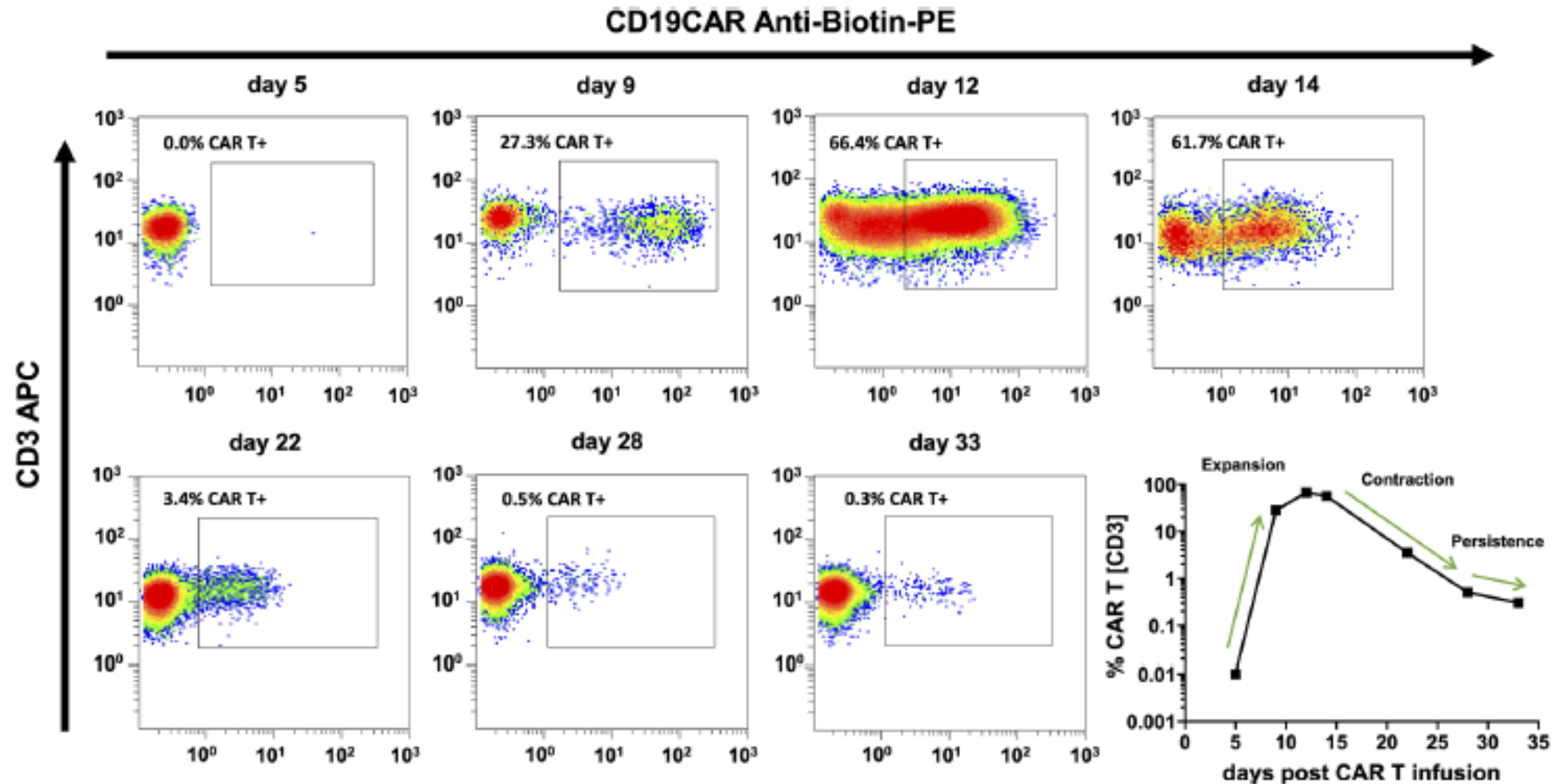


### 4°C stability



### 4°C stability



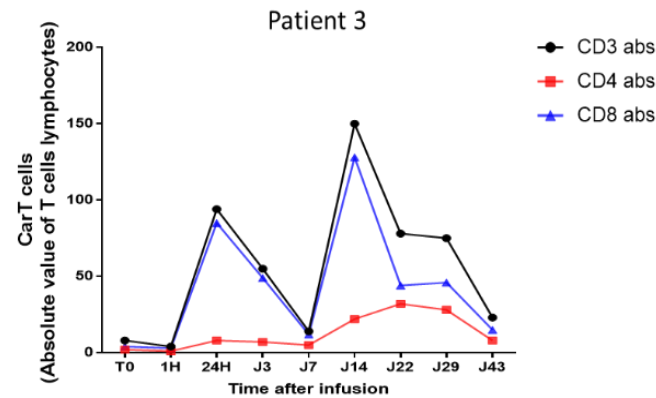
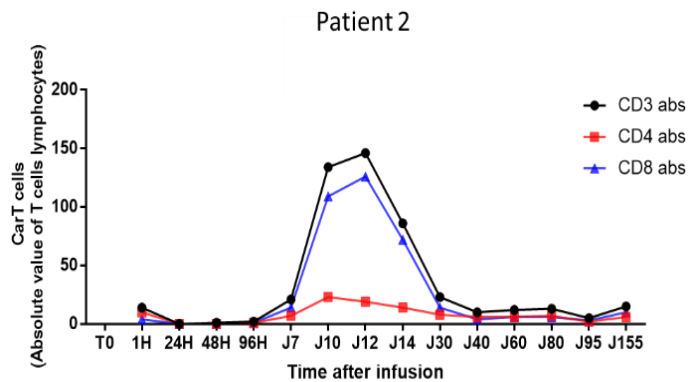
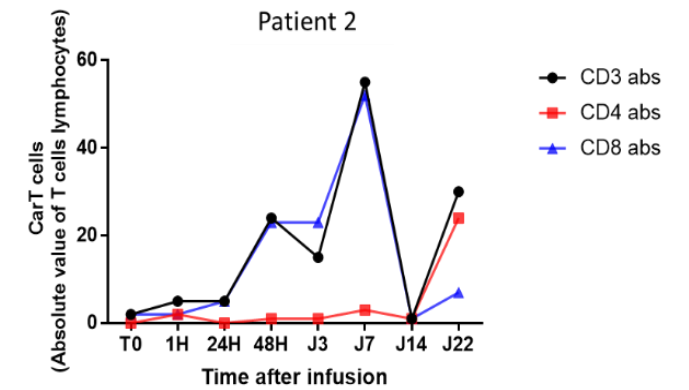
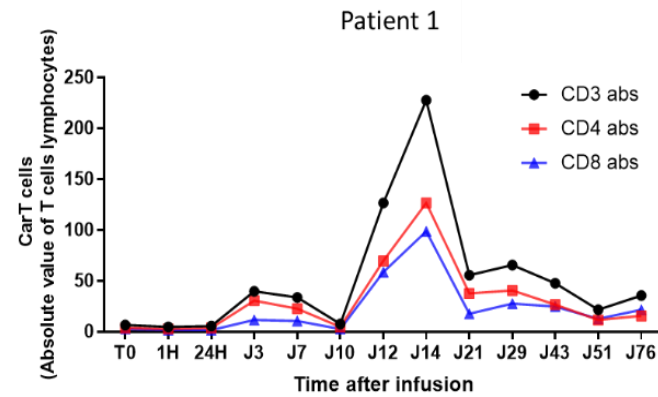
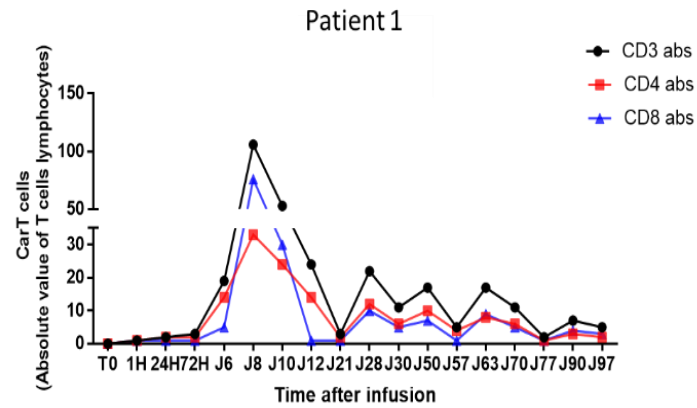


**FIGURE 4 |** CAR T cell monitoring curves match well with common models of CAR T cell cellular kinetics. CAR T cell cellular kinetics can be broken up into an initial period of exponential growth (expansion), a period of rapidly falling CAR T cell numbers (contraction), and a gradual decline over months or years (persistence). Flow cytometry plots and the bottom right curve depict the CAR T cell monitoring course of the same individual patient. Cellular kinetic phases can be easily identified and are indicated in the bottom right curve.

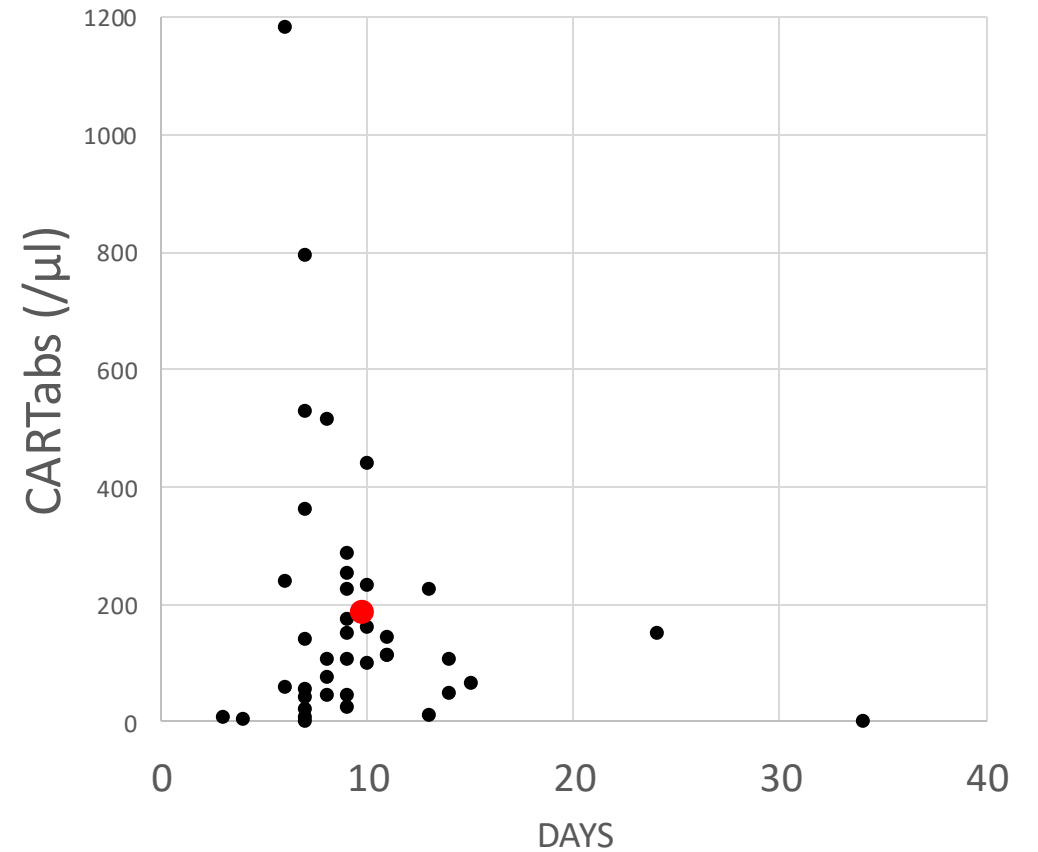
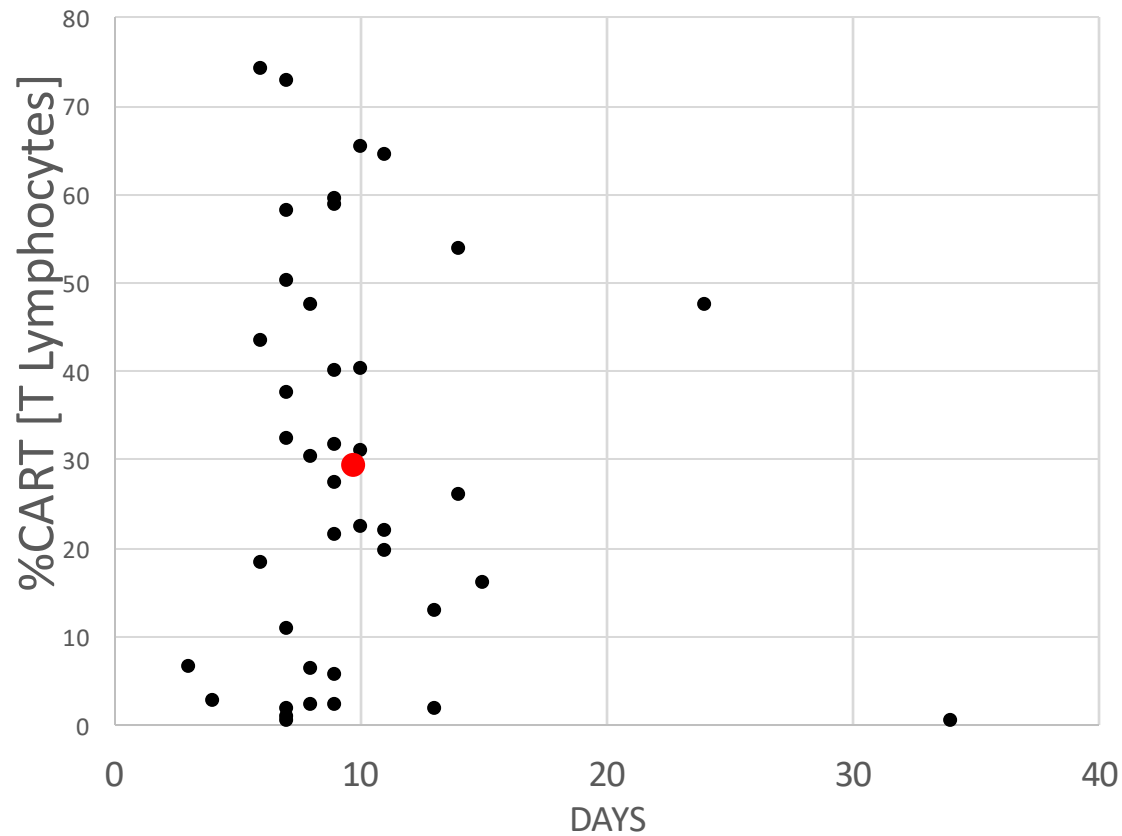
# Kinetics of CAR T cell engraftment

B-ALL

DLBCL



# Kinetics of CAR T cell engraftment



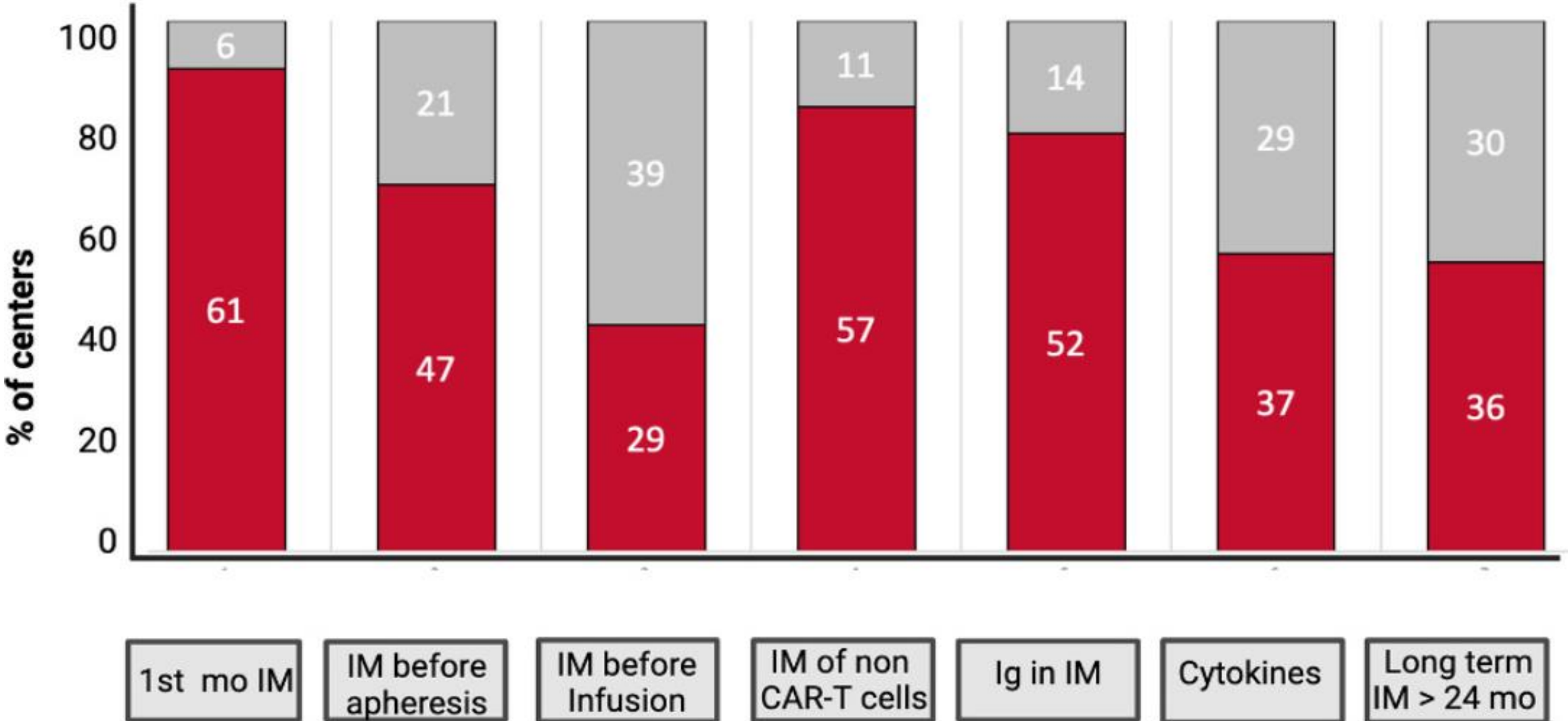
# Predictive factors of clinical response

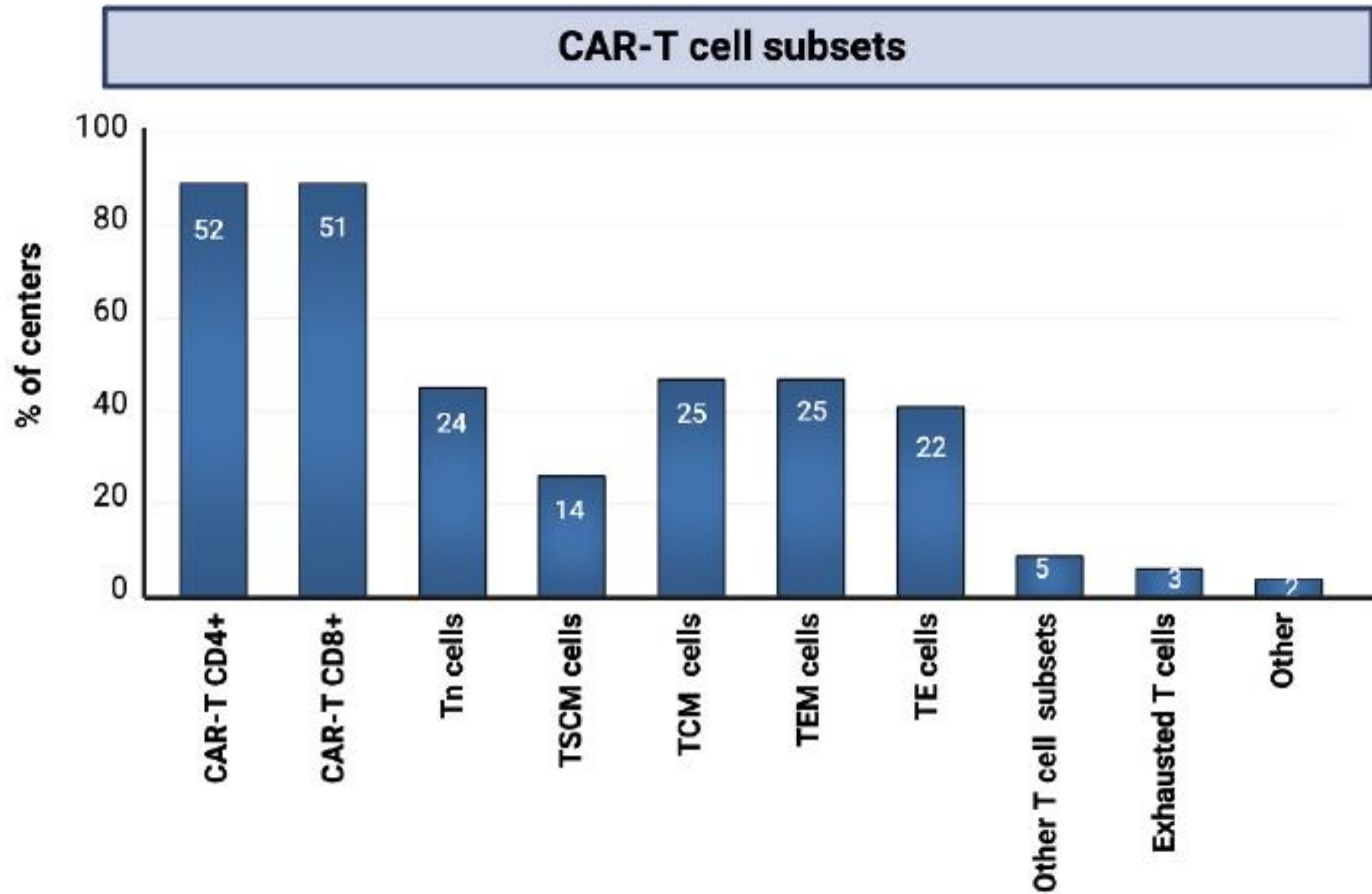
- Expansion kinetics
  - Peak/AUC ~ response
  - Persistence ~ long term remission
- Prior treatments
  - Depletion of naive and early memory T cells (CCR7+CD27+) → ↓ expansion
  - Exhaustion markers (PD1, LAG3, TIM-3) → ↓ efficacy
- Lymphodepletion
  - IL-7/IL-15 availability → ↑ expansion
- Product characteristics
  - Naive/early memory vs exhaustion ~ response

# Immune monitoring recommendations

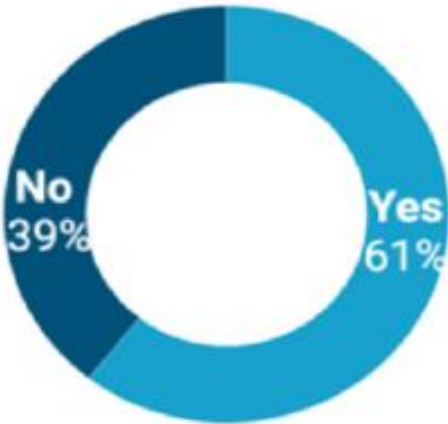
- **Mandatory**
  - CART quantification by FCM 2x/week for 3 weeks, M1, M2, M3, M6, 2x/year until undetectable
  - Quantification of lymphocytes CD3, CD4, CD8, **B**, NK
  - sIgG M1, M2, M3, M6
- **Recommended**
  - T cell subpopulations at apheresis
  - CART quantification by PCR when undetectable by FCM
- **Optional**
  - Deep T cell phenotype 1x/week
    - Naive/memory
    - Exhaustion
    - Activation

# EBMT survey

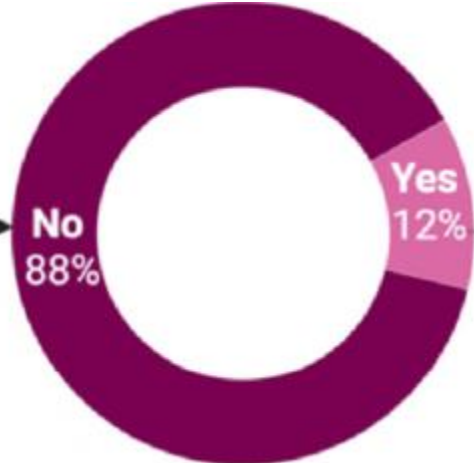




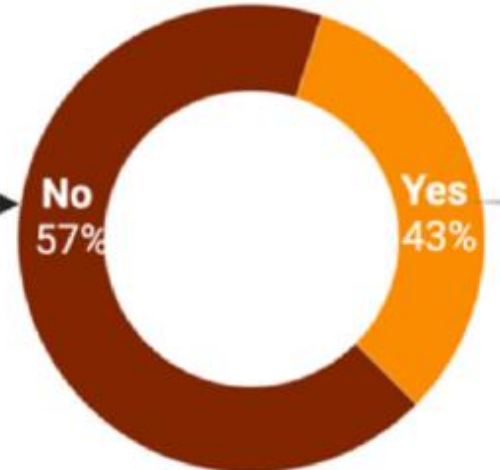
Do the results of immune monitoring have an impact on the decision-making process in your center?



Do the results of immune monitoring prompt you/your colleagues to adapt/modify the ongoing treatment for an established CRS and ICANS?



Do the results of immune monitoring prompt you/your colleagues to start a disease consolidation therapy or any other anti-tumor treatment?



« disconnection between availability of immune monitoring data and its clinical application »

# Conclusions

Flow cytometry is a specific, fast and quantitative method to monitor CAR T cell therapy.

Technical tips:

- Higher background with non-specific detection reagents
- Two-step CD19-Biotin/anti-Biotin-PE or single-step anti-FMC63-PE
  - comparison of LLOQ in progress
- LLOQ ~ 0,05%, acquire 100,000 lymphocytes (1-2 ml PB, bulk lysis)
- Process sample immediately or store at 4°C (CART viability low!)
- Peak expansion reached in 10-14 days, ~30% T lymphocytes or 200 CART/ $\mu$ l

Merci

Thank you

Dank u

