Multiparameter characterization of CAR T cells

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Introduction

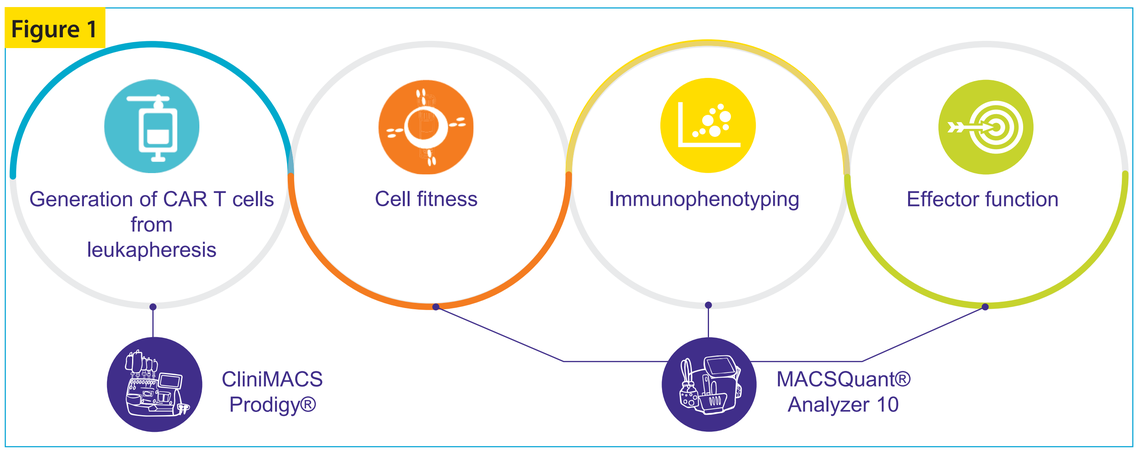
Adoptive cell transfer of chimeric antigen receptor (CAR) modified T cells has demonstrated great therapeutic success against certain malignancies. However, treatment efficacy varies according to mechanisms not fully understood or controlled to date. Multiple avenues for new CAR T cell-based therapies continue to be developed. During all phases of pre-clinical and clinical development, it is fundamental to characterize CAR T cells using reliable tools and methods. We established a set of various *in vitro* assays for multiparameter characterization of CAR T cell products, which can be applied throughout all phases of drug development.

Methods and Results

1 General workflow

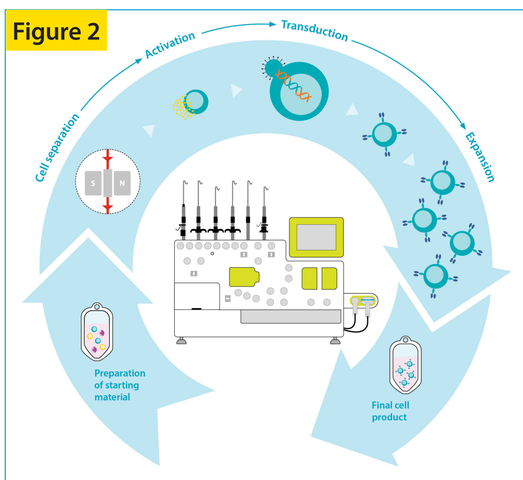
We have established a workflow (Figure 1) for *in vitro* multiparameter characterization of engineered CAR T cells, which includes comprehensive analysis of CAR T cell fitness, immunophenotypes, and effector functions. Automated CAR T cell manufacturing was performed using CliniMACS Prodigy®. Cell fitness, phenotypic and functional characterization was analyzed using MACSQuant® Analyzer.

Figure 1



2 Generation of CAR T cells using CliniMACS Prodigy®

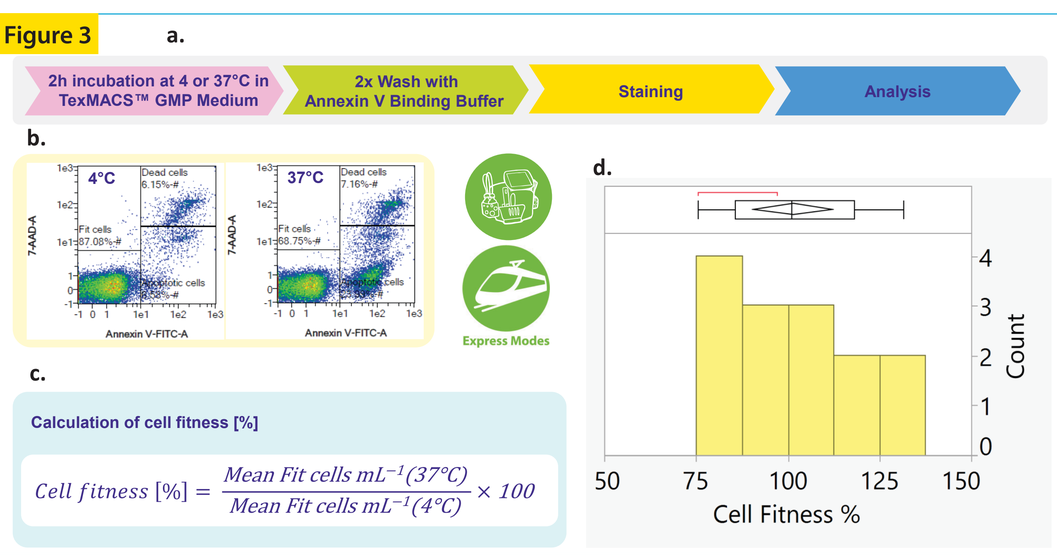
Figure 2



Leukapheresis from healthy donors were collected to generate CAR T cells using the GMP-compliant CliniMACS Prodigy® platform, which enables an automated and closed engineering of T cells in a highly reproducible manner. During the manufacturing process, CD4+ and CD8+ T cells were isolated magnetically with MACS® Cell Separation system and activated with MACS® GMP T Cell TransAct™ following by the lentiviral transduction with a second generation CAR. For expansion, CAR T cells were cultured in TexMACS™ GMP Medium supplemented with inactivated human AB serum, IL-7 and IL-15, which was replaced with serum-free TexMACS™ GMP Medium on day 5. The CAR T cells were harvested on day 12 and used for the multiparameter characterization.

3 Characterization of cell fitness

Figure 3



Cell fitness was assessed by analyzing the proportion of cells that remain fit when cultured at 37°C, as compared to at 4°C (Figure 3.a). Identification and enumeration of fit (live and non-apoptotic) cells was performed using the Annexin V-FITC Kit and 7-AAD Staining Solution. CAR T cells were incubated at either 4°C or 37°C for 2 hours. After incubation, cells were washed twice with Ca^{2+}-containing Annexin V Binding Buffer and consequently stained for flow cytometric analysis (Table 1).
Fully automated measurement and analysis was done by using Express Mode (Figure 3.b). Cell fitness was calculated using the indicated formula (Figure 3.c). The cell fitness of 14 donor-derived CAR T cells (Figure 3.d) varied between 75.4 and 131.5 with the mean value of 101.4 and SD of 18.7.

4 Characterization of immunophenotypes

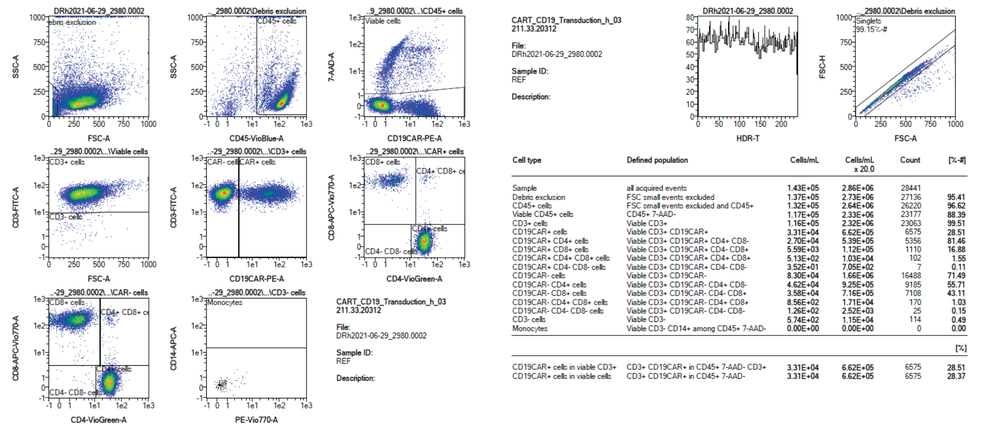
To assess immunophenotypes of generated CAR T cells, several panels for flow cytometric analysis have been designed (Table 1). Labeling with CAR detection reagent (CAR DR) was used to determine transduction efficiency and immunophenotypes of CAR+ T cells. REAfinity™ Recombinant Antibodies were used for standardized, background-free flow analysis. Automated acquisition was performed using MACSQuant® Analyzer 10.

Table 1

Panel		Cellular composition	Transduction efficiency	Differentiation	Proliferative ability	Exhaustion	Activation	Cell fitness
Channel on MQ10	Fluorochrome	Antibodies/ Labeling reagents						
V1	Vioblue®	CD45 (REA747)	CD45 (REA747)	CD27 (M-T271)	CD27 (M-T271)	CD223 (11C3C65)	CD154 (REA238)	
V2	VioGreen™	CD4 (REA623)	CD4 (REA623)	CD8 (REA734)	CD8 (REA734)	CD8 (REA734)	CD8 (REA734)	CD4 (REA623)
B1	FITC	CD3 (REA613)	CD3 (REA613)	CD3 (REA613)	CD3 (REA613)	CD3 (REA613)	CD3 (REA613)	Annexin V
B2	PE	CD16/CD56 (REA423 /REA196)	CAR DR	CAR DR	CAR DR	CAR DR	CAR DR	
B3		7-AAD	7-AAD	7-AAD	7-AAD	7-AAD	7-AAD	7-AAD
B4	PE-Vio® 770	CD19 (REA675)		CD62L (145/15)	CD279 (REA1165)	CD279 (REA1165)	CD25 (REA570)	
R1	APC	CD14 (REA599)	CD14 (REA599)	CD45RO (REA611)	CD127 (REA614)	CD366 (REA635)	CD137 (REA765)	CD3 (REA613)
R2	APC-Vio® 770	CD8 (REA734)	CD8 (REA734)	CD4 (REA623)	CD4 (REA623)	CD4 (REA623)	CD4 (REA623)	CD8 (REA734)

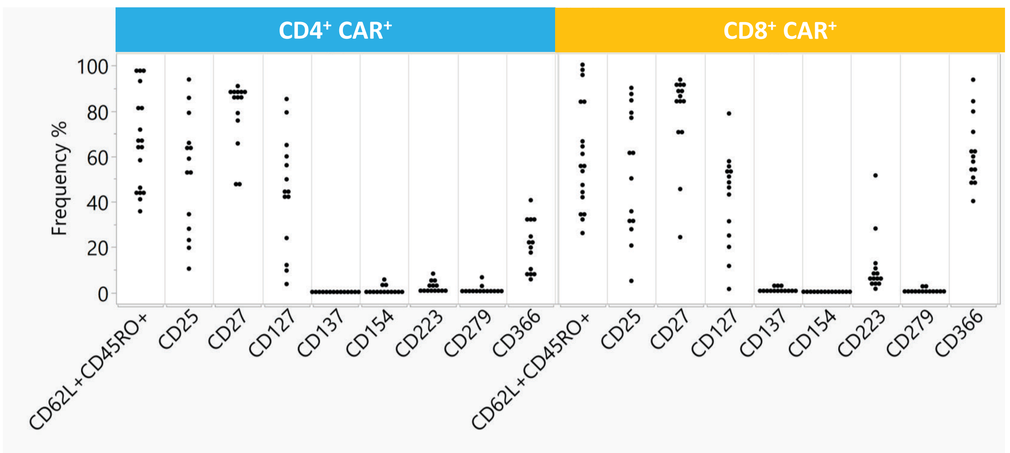
Figure 4

a.



Cell type	Defined population	Cells/mL	Cells/mL x 20.0	Count	[%-#]
Sample	all acquired events	1.43E+05	2.86E+06	28441	
Debris exclusion	FSC small events excluded	1.37E+05	2.73E+06	27136	95.41
CD45+ cells	FSC small events excluded and CD45+	1.32E+05	2.64E+06	26220	96.62
Viable CD45+ cells	CD45+ 7-AAD-	1.17E+05	2.33E+06	23177	88.39
CD3+ cells	Viable CD3+	1.16E+05	2.32E+06	23063	99.51
CD19CAR+ cells	Viable CD3+ CD19CAR+	3.31E+04	6.62E+05	6575	28.51
CD19CAR+ CD4+ cells	Viable CD3+ CD19CAR+ CD4+ CD8-	2.70E+04	5.39E+05	5356	81.46
CD19CAR+ CD8+ cells	Viable CD3+ CD19CAR+ CD4- CD8+	5.59E+03	1.12E+05	1110	16.88
CD19CAR+ CD4+ CD8+ cells	Viable CD3+ CD19CAR+ CD4+ CD8+	5.13E+02	1.03E+04	102	1.55
CD19CAR+ CD4- CD8- cells	Viable CD3+ CD19CAR+ CD4- CD8-	3.52E+01	7.05E+02	7	0.11
CD19CAR- cells	Viable CD3+ CD19CAR-	8.30E+04	1.66E+06	16488	71.49
CD19CAR- CD4+ cells	Viable CD3+ CD19CAR- CD4+ CD8-	4.62E+04	9.25E+05	9185	55.71
CD19CAR- CD8+ cells	Viable CD3+ CD19CAR- CD4- CD8+	3.58E+04	7.16E+05	7108	43.11
CD19CAR- CD4+ CD8+ cells	Viable CD3+ CD19CAR- CD4+ CD8+	8.56E+02	1.71E+04	170	1.03
CD19CAR- CD4- CD8- cells	Viable CD3+ CD19CAR- CD4- CD8-	1.26E+02	2.52E+03	25	0.15
CD3- cells	Viable CD3-	5.74E+02	1.15E+04	114	0.49
Monocytes	Viable CD3- CD14+ among CD45+ 7-AAD-	0.00E+00	0.00E+00	0	0.00
					[%]
CD19CAR+ cells in viable CD3+	CD3+ CD19CAR+ in CD45+ 7-AAD- CD3+	3.31E+04	6.62E+05	6575	28.51
CD19CAR+ cells in viable cells	CD3+ CD19CAR+ in CD45+ 7-AAD-	3.31E+04	6.62E+05	6575	28.37

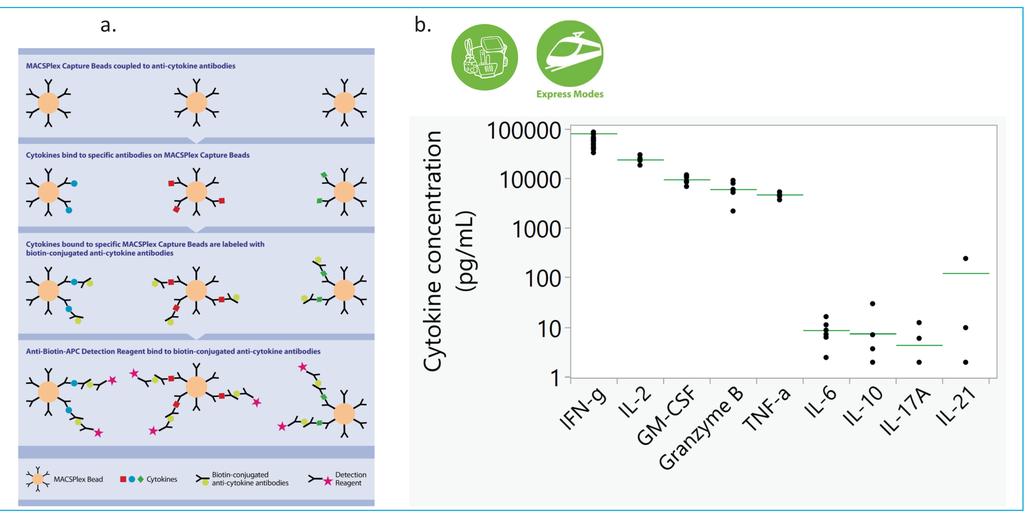
b.



Analysis of panels was predominately performed with Express Modes as an add-on for the MACSQuantify™ software. The fully automated gating procedure of the Express Mode adapts to each data file via pre-defined algorithms, which yields a high level of standardization across samples, avoids operator caused variability, and increases reproducibility (example in Figure 4.a). Frequencies of marker expression of generated CAR T cells (n = 14) within CD4+/CAR+ and CD8+/CAR+ subpopulations were analyzed (Figure 4.b), where donor dependent variability was assessed.

5 Characterization of effector function

Figure 5



Functionality of the generated CAR T cells was assessed by measuring cytokine concentration in the supernatants after an overnight co-culture with target-expressing cells. Secretion of the indicated cytokines was simultaneously measured and the concentrations were determined using the MACSPlex Cytotoxic T/NK Cell Kit or the MACSPlex IFN-g Kit (Figure 5.a). Automated acquisition and analysis were done with Express Modes. After overnight co-culture, secretion of IFN-g, IL-2, TNF-a, GM-CSF and Granzyme B was greatly induced (Figure 5.b), whereas other cytokines were hardly detectable over background when stimulated with non-antigenic target cells (data not shown).

Conclusion and outlook

Here we show a workflow for multiparameter characterization of CAR T cells, using automated and reproducible analysis with a high degree of standardization. This workflow provides comprehensive phenotypic and functional characterization of engineered CAR T cells, and can be widely applied during all phases of CAR T cell research and development, such as CAR engineering process and drug formulation development, stability assessments, as well as identification of biomarkers that are associated with clinical efficacy of CAR T cell therapy.

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