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Monitoring CAR T cells in peripheral blood by flow cytometry following Tisagenlecleucel in Fundeni Clinical Institute, Bucharest

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ABSTRACT

Introduction: Over the past few years, the introduction of chimeric antigen receptor (CAR) T-cell therapy by the FDA has shown remarkable success in treating various hematologic malignancies. However, the limited response and resistance observed in some patients have hindered its broader application.

Methods: At Fundeni Clinical Institute, we implemented the use of Tisagenlecleucel, a second-generation CAR T cell therapy, in April 2022. This therapy targets CD19, an antigen expressed in all B lineage cells. To assess the cellular kinetics of CAR T cell-treated patients and conduct further research, we developed an 8-color/10-parameter flow cytometry tube. This tube utilizes a biotinylated CD19 CAR Detection Reagent with high sensitivity and specificity for CD19-targeted CARs, enabling us to effectively separate CAR T cells from normal T cells.

Results: Through immunophenotyping, we successfully identified circulating CAR T cells and distinguished various subtypes of immune cells in the peripheral blood of infused patients. Furthermore, we validated the accuracy of our flow cytometry panel for monitoring the progress of CAR T cell therapy.

Conclusions: This paper highlights the implementation of our flow cytometry monitoring panel for CAR T cells following Tisagenlecleucel therapy at Fundeni Clinical Institute. Our practical solution allows us to identify CAR T cells, assess B cell presence, and characterize different T cell subtypes in our patients. This standardized approach enhances our understanding and monitoring of CAR T cell therapy, leading to improved patient care and outcomes.

Keywords: CAR T, Tisagenlecleucel, cellular therapy, flow cytometry

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INTRODUCTION

Adoptive immunotherapy involves the infusion of immunocompetent cells to treat various cancers and infections [1]. Recently, this therapy has shown promising results in autoimmune diseases such as refractory systemic lupus erythematosus by targeting autoreactive B cells [2]. It also offers a potential solution to overcome challenges associated with experimental therapeutic vaccines, particularly for patients with compromised immune systems. However, one major drawback of immunotherapy is the destruction of immunity to "self" antigens, which presents a significant challenge for clinicians [1].

Chimeric antigen receptor (CAR) T-cell therapy aims to genetically engineer T lymphocytes obtained through leukapheresis to recognize CD19 and other B-cell surface proteins. This enables T cells to attack and destroy chemotherapy-resistant cancers. The first generation of CAR T cells involved the introduction of a tumor-specific CAR consisting of four regions: the extracellular binding domain(s), hinge, transmembrane (TM), and cytoplasmic signaling regions. Variations in the length and

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composition of the hinge region regulate the threshold of CAR signaling and antigen binding [3-6]. The absence of a costimulatory domain in the first generation CARs hampers their activation, growth, and survival [7].

To address these limitations, the second generation of CAR T cells incorporates an intracellular co-stimulatory molecule, such as CD28 or 4-1BB (CD137), which includes activation regions based on immunoreceptor tyrosine. These molecules play a crucial role in cellular metabolism, enhancing the activation, expansion, and long-term persistence of genetically modified T cells [8,9]. Both CD28 and 4-1BB domains have demonstrated high treatment response rates, with CD28 domain CAR T cells differentiating into effector memory T cells utilizing aerobic glycolysis, while 4-1BB domain CAR T cells differentiate into central memory T cells (TCM) [3, 10,11]. Notably, CAR T cells with 4-1BB costimulatory domains exhibit prolonged persistence compared to those with CD28 domains [12].

In 2017, the FDA approved Tisagenlecleucel (also known as Kymriah; Novartis), a second-generation CAR T cell therapy, for adult patients with refractory/relapsed non-Hodgkin lymphoma (NHL), as well as pediatric and young adult patients with refractory/relapsed B-cell acute lymphoblastic leukemia after multiple lines of chemotherapy or even after Autologous Stem Cell Transplant (ASCT) [12,13]. However, this innovative immunotherapeutic approach necessitates a highly standardized and robust method for detecting CAR T cells.

Multiparametric flow cytometry offers a rapid antibody-based analysis of cells in peripheral blood, bone marrow, and cerebrospinal fluid in leukemia patients. Although flow cytometry is a standardized method, it is subject to variability due to various factors, including operator handling, antibodies, and data analysis [14].

The aim of our paper is to describe, validate, and evaluate a new monitoring method using CD19 protein for the quantification of CAR T cells in patient blood following Tisagenlecleucel therapy using flow cytometry.

METHODS

Study population

Patients with relapsed/refractory non-Hodgkin lymphoma (NHL) and children or young patients with refractory/relapsed B-cell acute lymphoblastic leukemia are the target demographic eligible for Tisagenlecleucel. Thus, we selected 5 adult patients (40-60 years old) diagnosed with DLBCL, and 2 pediatric patients (13,14 years old) diagnosed with ALL B to receive Tisagenlecleucel in the Department of Hematology, Fundeni Clinical Institute. Prior to Tisagenlecleucel administration, the patients underwent the same lymphodepletion regimen. The panel implementation led to modifications in the frequency of blood sampling. Initially, peripheral blood analysis was conducted on day 1, day 3, day 7, day 10, day 14, day 21, day 28, followed by monthly sampling thereafter. However, upon observing the dynamics of CAR T cells, we adjusted the blood sampling frequency to once a week during the first month, and subsequently shifted to monthly sampling. We obtained informed consent from all patients and healthy volunteers as control samples and the ethical committee from Fundeni Clinical Institute approved the study design. Our study was accomplished in accordance with the Helsinki declaration.

Set up positive and negative threshold

Controls of 42 healthy patients were processed and compared to the patient specimens in order to check if there was any background staining. We measured fluorescence intensity of unstained cells in order to assess cellular autofluorescence (Figure 1A). To set the upper limits for background signal and to gate positive population, we used fluorescence minus one (FMO) for Anti Biotin Antibody (Figure 1B), and a modified FMO that excluded CD19 CAR detection reagent but kept Antibiotin Antibody in order to evaluate the nonspecific binding of Antibiotin Antibody (Figure 1C).

Staining protocol

We used a biotinylated CD19 CAR Detection Reagent (Miltenyi Biotec, Bergisch Gladbach, #130– 115-965, Germany) that binds sensibly and specifically CD19-targeted CAR. A fluorochrome-conjugated antibiotin antibody was added in a second incubation step. For the second tube, the peripheral blood was stained with BD Multitest™ 6-color TBNK reagent and BD Trucount™ tubes were used in order to evaluate the percentages and absolute counts of lymphocytes, as well as the subpopulations of T cells.

Processing of the samples followed a protocol recommended by the manufacturer. To optimize the quantity of blood required for the analysis, we adapted the protocol [15]. Instead of using the full 2 mL of whole blood, we determined that lysing 200 uL of blood would be sufficient to obtain approximately 10^6 nucleated cells per milliliter for the subsequent staining process.

Red blood cells were lysed with 4 mL of Red Blood Cell Lysing Solution 10x (1:10 dilution) (Miltenyi Biotec, Germany) in a 1:20 proportion (blood volume:lysing solution) and washed three times with Protein Extraction Buffer (PEB) (300 RCF for 10 minutes) before staining. The staining procedure consists of two steps: firstly, 100 uL of the resuspended washed leukocytes that contain a

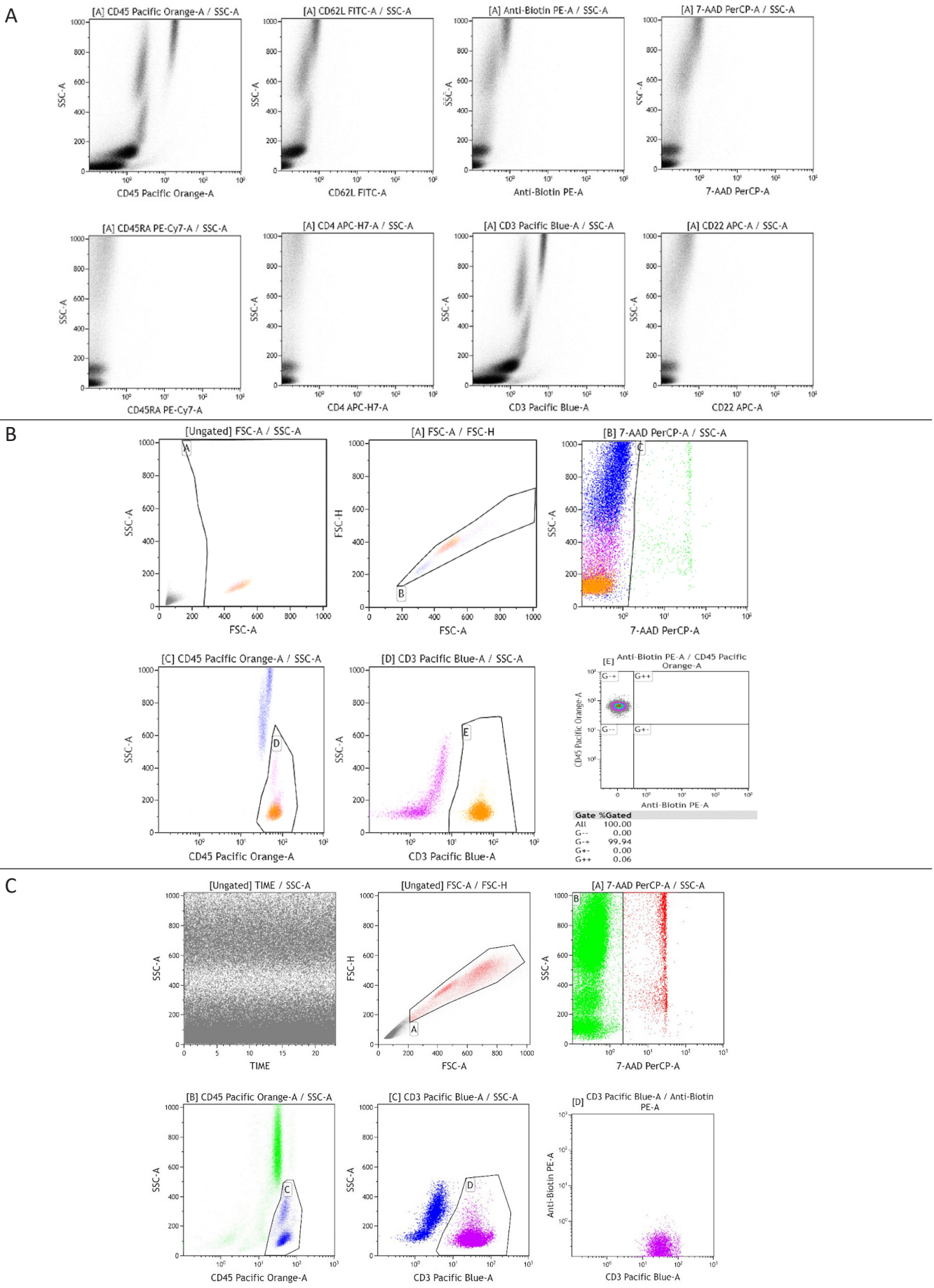

Fig. 1. A. Unstained control. Kaluza Software; B. FMO control for antibiotin antibody. Kaluza Software; C. Modified FMO control for CD19 CAR detection reagent. Kaluza Software.

maximum of 10^6 leukocytes are stained with 2 uL CD19 CAR Detection Reagent, human, Biotin (Miltenyi Biotec, Germany), and secondly (after washing), an antibody cocktail with a total volume of 100 uL is added. The antibody cocktail has the following constituents: CD62L FITC (16uL), Biotin Antibody PE ReAffinity™ (Miltenyi Biotec, Germany) (2uL), 7AAD (evaluated on PerCP Cy5.5 fluorescence channel) (5uL), CD45RA PECy7 (5uL), CD22 APC (5uL), CD4 APC H7 (5uL), CD3 PB (5uL), CD45 PO (5uL), PEB (52uL). PEB is made of 1:10 MACS BSA Stock Solution (Miltenyi Biotec, Germany): autoMACS Rinsing Solution (Miltenyi Biotec, Germany), and the total PEB volume needed should be estimated before the sample processing begins to fit the needs for the number of patients processed. CD22 APC, as a lineage B Lymphocyte marker, was introduced at a later date, following the need to immunophenotype the B Lymphocytes that could not be identified by using the CD19 antibody, secondary to the molecular neutralization between CD19 antibody and CD19 CAR Detection Reagent, and for the rare cases where CD19 disappears from the surface of B Lymphocytes following the CAR T cell infusion [15]. In order to calculate the absolute number of CAR T cells, we stained 50uL of whole blood with 20uL BD Multitest 6-color TBNK reagent (CD3 FITC / CD16 PE + CD56 PE / CD45 PerCP-Cy™5.5 / CD4 PE-Cy™7 / CD19 APC / CD8 APC-Cy™7) and we used BD Trucount tubes. CAR T cells/uL were calculated using the following formula (dual platform method) [16], where AntiBiotin(+) events and CD3(+) events are obtained from the CAR T tube, and CD3(+) events/uL are obtained from the TBNK kit:

$$\frac{AntiBiotin(+)\ events}{CD3(+)\ events} \times CD3(+)\ events/uL = CART\ cells/uL$$

Instrument Settings

After the preanalytical part, data acquisition was performed on a BD FACSLyric™ flow cytometer equipped with 3 lasers (blue, red, and violet), 12 fluorescence channels and 14 parameters using BD FACSuite™ Software and a standard filter configuration (BD Biosciences). Daily QC CS&T beads (BD Biosciences) were used to set up the instrument and for Daily Quality Control. For compensation, we used FC Beads to create the initial default compensation matrix, MACS® Comp Bead Kit, anti-REA for Anti Biotin Antibody PE, and single color-stained BD Compbeads (BD Biosciences) for the tandem antibodies in the panel. The compensation is an automatic process performed according to the manufacturer's instructions on the BD FACSLyric flow cytometer. A minimum threshold of 10.000 CD45+ lymphocytes was acquired for each analysis.

Gating strategy

The gating strategy for CAR T cell analysis was initiated by examining the TIME vs. SSC-A plot to ensure proper acquisition. Singlets were gated, and doublets and debris were excluded. To eliminate non-viable events, 7AAD vs. SSC-A gating was applied. A broad gate encompassing lymphocyte and monocyte populations was drawn on the CD45 vs. SSC-A dot plot to capture all CAR T cells, as these cells exhibit intermediate internal complexity between the aforementioned populations. Subsequently, the CD3 vs. Anti-Biotin dot plot isolated the CAR T cell population, characterized by CD3 positivity and Anti-Biotin positivity. A positive threshold was established based on controls, and CAR T cells comprising less than 0.5% of T lymphocytes were deemed negative. Following identification of the CAR T cell population, further characterization of subpopulations was performed by analyzing CD4+ and CD4- expression. To identify T cell differentiation, the CD62L vs. CD45RA plot was utilized (Figure 2).

Data Analysis

Dot plots were generated using FACSuite Software (BD Biosciences) or Kaluza Software version 1.8.

RESULTS

Through the implementation of this panel, we successfully captured the dynamics of CAR T cells in patients following infusion, allowing us to identify distinct in vivo evolution phases that align with existing literature. Additionally, we made modifications to the panel to gather more information by incorporating CD62L, CD45RA, and, at a later stage, CD22 (although data for CD22 is currently unavailable).

Evolution of CAR T in vivo

The dynamic evolution of CAR T cells in vivo can be categorized into three distinct phases. Firstly, an initial phase characterized by vigorous expansion takes place within 6 to 9 days after infusion. This is followed by a rapid contraction phase, during which the CAR T cell counts decline rapidly. Finally, a persistence phase ensues, marked by a gradual decrease in absolute CAR T cell counts.

Based on our observations, we found that the peak of CAR T cells typically occurred during the second week following infusion, with a median peak day of 10 and a range spanning from day 6 to day 14. Notably, the dynamics within the first week exhibited significant heterogeneity among patients, with generally lower absolute CAR T cell counts during this period (Figure 3, Table 1).

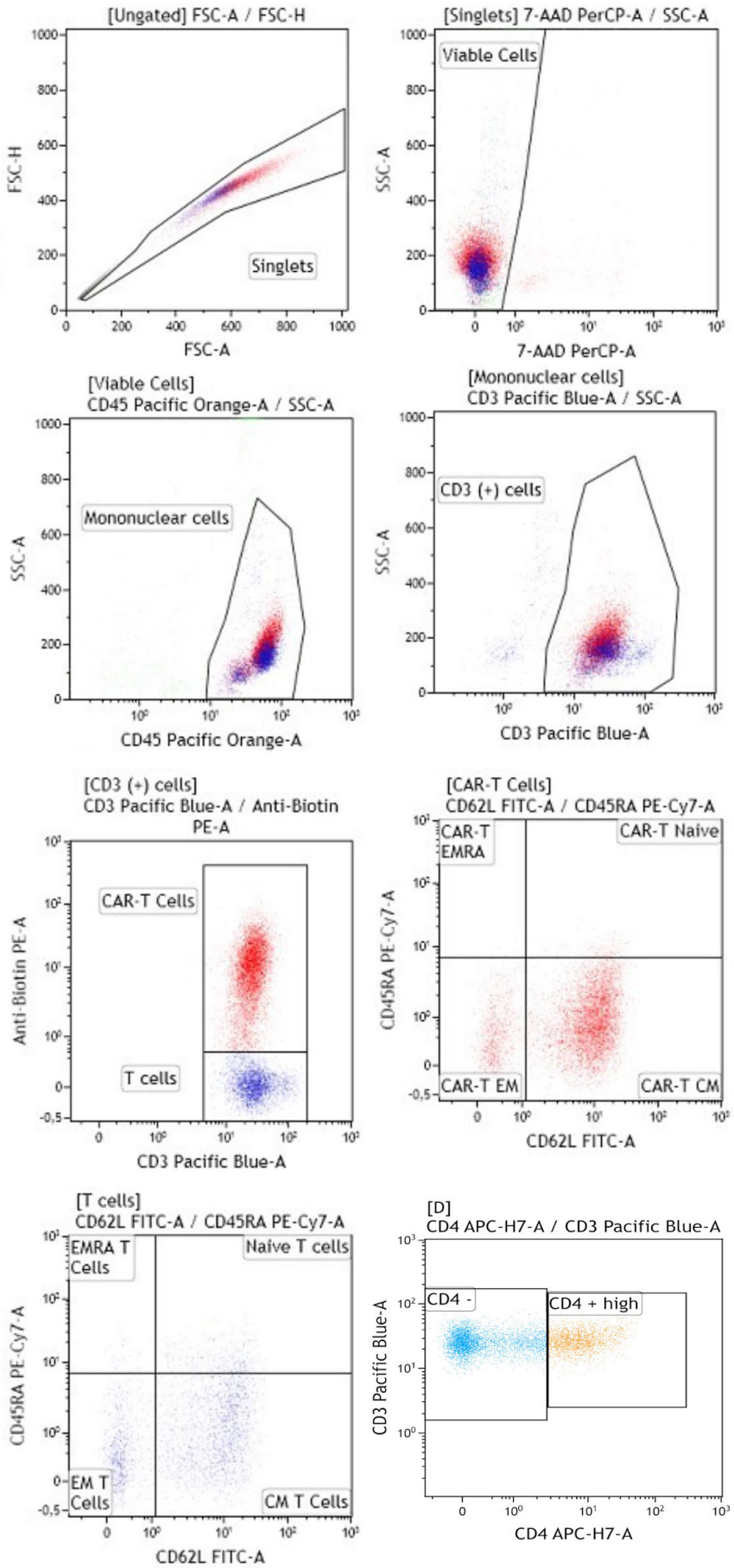

Fig. 2. Gating strategy used to identify CAR-T cells, subtypes of CAR-T cells, subtypes of T cells.

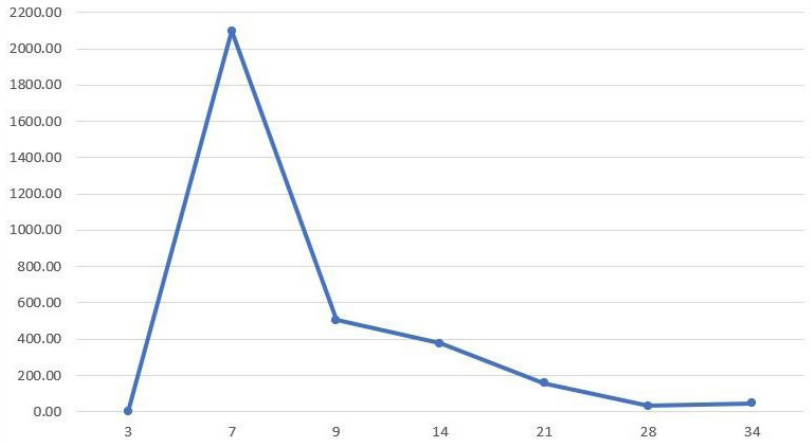

Fig. 3. CAR-T absolute counts long term monitorization. OX axis represents days after the CAR-T cell infusion. OY axis represents the absolute count of CAR-T cells in the peripheral blood.

Dynamics of CAR T cells and mature T cells

By incorporating CD62L and CD45RA markers, we examined the T cells subsets post-infusion in order to identify the T memory stem cells (TSCM, Naive T cells (TN), effector memory T cells (TEM), central memory T cells (TCM) and effector T cells (TEF). TEF is equivalent to Effector Memory T cells reexpressing CD45RA (EMRA). We observed a higher frequency of TEF and TCM during the expansion phase. Adult patients showed a predisposition towards TEM (with a median value of 340.51/µL; min=122.58; max=668.22), while pediatric patients displayed a higher proportion of TCM (with a median value of 389.32/µL; min=270.84; max=507.81). Subsequently, following the peak level, both subsets mentioned above decreased in favor of the TN subset. It is important to note that since specific markers for TSCM were not included, these cells are included within the TN category. Furthermore, we observed that the dynamics of the mature T cells and CAR T cells populations were similar (Table 1).

Residual B cells and B-cell aplasia (BCA)

After five days of lymphodepletion chemotherapy, all patients exhibited BCA. In the first 28 days following CAR T infusion, the median absolute count of B lymphocytes for all seven patients was 0. Relapses commonly occur when a decline in circulating CAR T cells coincides with the loss of BCA after CAR T cell therapy [17]. In our clinic, a pediatric patient experienced a relapse according to this pattern. On day 83, the patient had 2.98 CAR T cells/µL and only 1 B lymphocyte/µL, while on day 160 and day 198, the absolute counts of CAR T cells dropped to less than 0.5%, while B cells increased to 16/µL and 134/µL, respectively.

CD4+ and CD4- T cells and CAR T cells

We analyzed the distribution of CD4+ and CD4- cells to assess the heterogeneity of CAR T cells. During the first week following infusion, we noted a higher percentage of CD4- cells (with a median value of 25.5, min=1.09, max=3052.44) compared to CD4+ cells (with a median value of 1.25, min=0, max=240). However, the CD4+ cell subset exhibited gradual growth over time and eventually reached a balance with the CD4- subset. We further examined the cellular kinetics of CD4+ and CD4- cells in both T cells and CAR T cells, and, interestingly, we observed a resemblance between the two populations (Figure 4, Table 1).

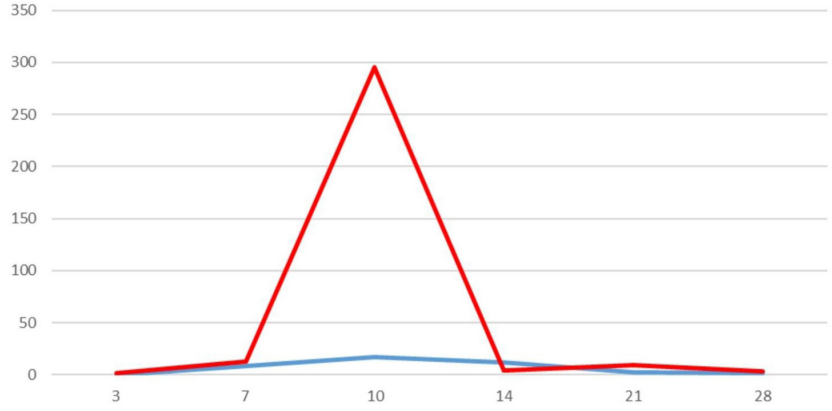

Fig. 4. CD4+ CAR (blue) and CD4- CAR (red) CAR-T absolute count dynamics in the first month

Table 1. Monitorization of absolute counts for CAR-T cell subtypes, T cell subtypes and B cells. The values presented for each subtype and for each day of monitorization are in this format: [Median (Min ; Max)].

		Day 3	Day 7	Day 10	Day 14	Day 21	Day 28
	CAR-T Cells	1.67 (0.05 ; 7.07)	17.53 (1.93 ; 2099.49)	313.28 (56.72 ; 505.71)	73.21 (10.98 ; 377.08)	11.81 (0.96 ; 158.12)	4.8 (0.96 ; 31.47)
	B Lymphocytes	1 (0 ; 8)	1 (0 ; 5)	2.66 (0 ; 8)	2 (0 ; 3)	1 (0 ; 2)	3 (0;19)
T CELLS	**T Lymphocytes**	192 (44 ; 607)	751 (258 ; 4744)	1013 (956 ; 1530)	1036 (738 ; 1332)	1385 (632 ; 2025)	1264 (352; 2198)
	CD4+	33 (11 ; 37)	218 (111 ; 584)	234 (35 ; 317)	328 (92 ; 739)	360 (85; 722)	391 (66 ; 647)
	CD8+	140 (16; 448)	347 (144 ; 4142)	957 (706 ;1097)	659 (368 ; 861)	976 (322 ; 1531)	811 (177 ; 1478)
	CD4/CD8	0.33 (0.25 ; 1.68)	0.77 (0.02 ; 1.68)	0.33 (0.03 ; 0.34)	0.68 (0.1 ; 1.8)	0.36 (0.11 ; 0.98)	0.3 (0.1 ; 0.97)
	Naïve T cells	13.55 (2.09 ; 98.75)	95.59 (13.82 ; 112.95)	87.07 (60.53 ; 123.82)	75.47 (22.98 ; 237.26)	54.95 (0 ; 289.74)	37.88 (0 ; 326.32)
	CM T cells	45.52 (9.71 ; 123.37)	222.73 (103.01 ; 638.64)	168.54 (137.63 ; 395.72)	310.69 (102.96 ; 425.03)	264.75 (0 ;486.63)	168.5 (0 ; 402.4)
	EM T cells	32.7 (12.39 ; 245.9)	262.8 (96.59 ; 770.33)	284.53 (35.5 ; 501.65)	371.37 (28.37; 668.22)	690.77 (77.92; 1998.34)	541.04 (65.83 ; 1844.44)
	EMRA T Cells	9.89 (6.23 ; 134.98)	95.52 (9.9 ; 183.35)	80.77 (2.84 ; 218.82)	128.33 (2.01 ; 335.29)	124.76 (0 ; 636.59)	321.81 (13.3 ; 602.47)
CAR-T Cells	**CD4+ CART**	0.08 (0 ; 2.86)	8.77 (0; 2099.49)	16.7 (14.98 ; 16.86)	11.71 (0 ; 47.72)	2.7 (0; 17.77)	1.91 (0 ; 8.89)
	CD8+ CART	1.09 (0.03 ; 4)	12.95 (1.94; 55.54)	295.53 (39.29 ; 482.14)	34.12 (10.78 ; 360.16)	9.05 (0.96 ; 137.49)	3.25 (0 ; 24.67)
	CD4/CD8 CART	0.09 (0 ; 1.21)	0.48 (0; 37.79)	16.7(14.98 ; 16.86)	11.71(0 ; 47.72)	0.19(0;0.5)	0.24(0;0.55)
	Naïve CART cells	0.25(0;2.03)	2.5 (0 ; 362.67)	41.83 (3.11; 104.31)	50.28(1.81 ; 176.89)	1.88 (0 ; 106.34)	2.3 (0 ; 27.06)
	CM CART cells	0.57 (0 ; 3.34)	7.59 (0.08 ; 1528.18)	155.13 (19.88 ; 250.04)	8.88 (3.44 ; 158.12)	2.34 (0.25 ; 26.65)	1.3 (0 ; 5.93)
	EM CART cells	0.03 (0 ; 1.61)	17.43 (7.05 ;399)	339 (3.52 ; 1391)	10.34 (0.27 ; 444)	13.59 (0 ; 165)	4.9 (0.52 ; 23)
	EMRA CART Cells	0.04 (0 ; 0.51)	0.47 (0: 22.4)	11.43 (1.79 ; 18.61)	3.33 (0.2 ; 19.64)	2.26 (0 ; 9.36)	0.39 (0 ; 14.09)

DISCUSSION

Immunotherapy has emerged as a promising approach to the treatment of cancer, offering hope to many patients. However, there is still a need to further understand the intricacies of CAR T cell immunotherapy to ensure its long-term success. Our study aimed to establish and optimize a monitoring panel for the identification of CAR T cells using flow cytometry in our center. By developing this panel, we aimed to enhance our understanding of CAR T cell immunotherapy and improve our ability to track and analyze these cells in patients.

We present a flow cytometry panel specifically designed for the detection of anti-CD19 CAR T cells, employing indirect staining with the CD19 CAR Detection Reagent from Miltenyi Biotec. Notably, this panel can be easily integrated into routine laboratory procedures, requiring only a small number of staining steps.

By establishing this flow cytometry panel, we aim to contribute to the advancement of CAR T cell immunotherapy by providing a reliable and efficient method for the identification of CAR T cells in our hospital. This will aid in monitoring the efficacy and persistence of these cells in patients, ultimately leading to improved treatment outcomes.

Demaret et al. conducted a study to evaluate different proteins for monitoring CAR T cells in peripheral blood. Three proteins were investigated: PE-CD19 protein from Acro Biosystems, FITC labeled CD19 protein from Acro Biosystems, and CD19 CAR Detection Reagent from Miltenyi Biotec. Based on their findings, the CD19 CAR Detection Reagent from Miltenyi Biotec (#130–115-965, Germany) demonstrated the best discrimination for CAR T cell subsets. Considering economic constraints, we made the decision to utilize the CD19 CAR Detection Reagent from Miltenyi Biotec for our monitoring purposes. This choice was driven by the ability of the reagent to provide optimal discrimination and accurate identification of CAR T cell subsets in peripheral blood samples [17].

We observed an expansion phase between 6- and 9-days post infusion, then a contraction phase and a constant decline in absolute count in the persistent phase. According to ongoing studies, even a low number of CAR T cells present in the persistence phase may be sufficient to maintain the effect of the therapy, but it is possible that the decrease below a threshold level contributes to the reduction of the efficiency of the anti-CD19 CAR T therapy [18, 19] The expansion and the persistence phase are crucial for the anti-tumoral effect after CAR T therapy [20].

In addition, we assessed CAR T cell heterogeneity in terms of CD4+ and CD4- T lymphocytes. The activation and differentiation of T cells depend on signals transduced by these categories of receptors: TCRs (CD4 and CD8 receptors in response to MHC-II and MHC-I displayed antigens), cytokine receptors and costimulatory receptors [21]. During the expansion phase, we identified among CAR T cells a high frequency of TEM and TCM. Also, we reported a similar dynamic between mature T cells and CAR T cell subsets. The subset of TCM recirculates in the blood stream to lymphoid organs while the subset of TEM migrates to non-lymphoid tissues [22]. It was observed that while TEF and TEM decrease constantly, the proportion of TSCM and TN may increase and could sustain the memory pool of long-lived CAR T cells [18,19].

Furthermore, we monitored the residual B cells in order to identify the potential CAR positive B cells and the regeneration of B cell line. The median absolute counts of B lymphocytes for all seven patients within the initial 28 days following CAR T infusion were 0. Ruella et al. mentioned that transduction of residual B lymphocytes with anti-CD19 CAR protein during the manufacturing process of the CAR T cell therapy is a rare but possible event. In consequence, it will bind and mask its own CD19 antigen resulting in the relapse of the therapy and a poor prognosis for the patient [23].

Assessing the subpopulations of CAR T cells in routine practice can serve as a predictive measure for the severity of cytokine release syndrome and neurotoxicity. We observed that during the first week after infusion, CD4- cells had a higher percentage than CD4+ cells, but the CD4+ cell subset gradually increased and eventually reached equilibrium with the CD4- subset. The kinetics of CD4+ and CD4- CAR T cell subsets provide valuable insights into the early complications of this therapy (cytokine release syndrome and neurotoxicity), offering a more comprehensive understanding of their underlying pathophysiology [17,19]. Due to the fact that generally CAR T cells could not persist for more than 2 months, it is necessary to recommend the use of a more sensitive assay such as real time PCR (qPCR) to identify the sequence of the integrated CD19 CAR transgene [24].

CONCLUSIONS

Our flow cytometry immunophenotyping panel demonstrated its effectiveness in detecting anti-CD19 CAR T cells, as evidenced by the results obtained in our study. These findings align with previous research conducted on Tisagenlecleucel, reinforcing the suitability and reliability of our panel for CAR T cell detection.

LIMITATIONS OF THIS STUDY

Other CD19 proteins and CAR antibodies are accessible from various suppliers, but an entire analogy has not been performed in our analysis. We consider it necessary to check bone marrow minimal residual disease at least once a month for the purpose of following the relapse or regeneration of B cell line. In very rare cases, loss of CD19 antigen after CAR T cells infusion may occur, so we only recently added CD22 for a better follow up of the relapse and regeneration of B cell line [23]. Due to the limited number of patients included for flow cytometry monitoring, no clinical correlations have been conducted.

ABREVIATIONS

AICD – activation-induced cell-death

ASCT – Autologous Stem Cell Transplant

BCA – B cell aplasia

CAR – chimeric antigen receptor

EMRA – Effector Memory reexpressing CD45RA

PEB – Protein Extraction Buffer

TCM – central memory T cells

TEF – effector T cells

TEM – memory effector T cells

TN – naïve T cell

TSCM – stem cell memory T cells

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AUTHORS' CONTRIBUTION

DCP –Conceptualization, Methodology, Validation, Formal Analysis, Writing original draft paper, Writing - Review and editing, Supervision

HMS –Conceptualization, Methodology, Validation, Writing original draft paper, Writing- Review and editing, Supervision

RS – Methodology, Validation, Writing original draft paper, Writing- Review and editing

VGŢ – Methodology, Validation, Writing original draft paper, Writing- Review and editing

AŞ – Methodology, Validation, Writing original draft paper, Writing- Review and editing

IŞ – Methodology, Validation, Writing original draft paper, Writing- Review and editing

CJ – Methodology, Validation, Writing original draft paper, Writing- Review and editing

DC – Conceptualization, Validation, Writing- Review and editing, Supervision

AT – Conceptualization, Validation, Writing- Review and editing, Supervision

AC – Conceptualization, Validation, Writing- Review and editing, Supervision

All authors have read and agreed to the published version of the manuscript

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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