Received: 22 January 2024 | Accepted: 5 February 2024

DOI: 10.1002/jha2.871

eJHaem

British Society for Haematology

RESEARCH ARTICLE

Early CAR$^-$ CD4$^+$ T-lymphocytes recovery following CAR-T cell infusion: A worse outcome in diffuse large B cell lymphoma

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Abstract

CAR$^-$ CD4$^+$ T cell lymphopenia is an emerging issue following CAR-T cell therapy. We analyzed the determinants of CD4$^+$ T cell recovery and a possible association with survival in 31 consecutive patients treated with commercial CAR-T for diffuse large B-cell (DLBCL) or mantle cell lymphoma. Circulating immune subpopulations were characterized through multiparametric-flow cytometry. Six-month cumulative incidence of CAR$^-$ CD4$^+$ T cell recovery (≥200 cells/μL) was 0.43 (95% confidence interval [CI]: 0.28–0.65). Among possible determinants of CD4$^+$ T cell recovery, we recognized infusion of a 4-1BB product (tisagenlecleucel, TSA) in comparison with a CD28 (axicabtagene/brexucabtagene, AXI/BRX) (hazard ratio [HR] [95% CI]: 5.79 [1.16–24.12] $p = 0.016$). Higher CD4$^+$ T cell counts resulted with TSA at month-1, -2 and -3. Moderate-to-severe infections were registered with prolonged CD4$^+$ T cell lymphopenia. Early, month-1 CD4$^+$ T cell recovery was associated with a worse outcome in the DLBCL cohort, upheld in a multivariate regression model for overall survival (HR: 4.46 [95% CI: 1.12–17.71], $p = 0.03$). We conclude that a faster CAR$^-$ CD4$^+$ T cell recovery is associated with TSA as compared to AXI/BRX. Month-1 CAR$^-$ CD4$^+$ T cell subset recovery could represent a "red flag" for CAR-T cell therapy failure in DLBCL patients.

KEYWORDS
cell therapy, immunophenotype, non-Hodgkin lymphoma

1 | INTRODUCTION

Several studies investigated peripheral blood immune cell reconstitution following CAR-T cell therapy for hematological malignancies. Overall, a delayed CAR$^-$ CD4$^+$ T cell recovery emerged in comparison to CD8$^+$ T and NK cells [1, 2, 3, 4], which can translate in a profound CD4$^+$ lymphopenia (below 200 cells/μL) [4] and CD4$^+$/CD8$^+$ ratio inversion [1], sometimes exceeding the year in duration. In this context, determinants of CAR$^-$ CD4$^+$ T cell recovery have not been fully elucidated. Disease burden, preexisting cytopenias and inflammation, differences among CAR-T products and the severity of inflammatory complications may have a role.

Beyond determinants of immune-recovery, it has to be elucidated whether dynamics of non-CAR expressing T cells can potentially impact efficacy. Recently, CAR-T cell exhaustion [5, 6] and identification of regulatory CAR-T cells [7, 8] were associated to poor treatment responses. On the contrary, efforts to identify connections between peripheral, non-CAR expressing circulating lymphocytes and therapy efficacy were not conclusive: no associations were found between day 0 absolute lymphoid counts (ALC) and kinetics of relapse for ALL [1], neither between baseline ALC and the remission rate for B-cell lymphoma, in a post-hoc analysis of patients treated with axi-cel on ZUMA-1 and ZUMA-9 study [2].

We therefore conducted a timepoint-oriented, flow-cytometry based analysis, aimed to: (1) identify determinants of CAR^- $CD4^+$ T cell recovery; (2) identify possible associations between CAR^- $CD4^+$ T cell counts and CAR-T therapy efficacy, focusing on early timepoints.

2 | METHODS

2.1 | Patient population

This is an observational, single institution study including 31 consecutive adult patients, treated with commercial CAR-T cells for aggressive B-cell lymphomas at the IRCCS Ospedale Policlinico San Martino, Genova, Transplant Program, between October 30, 2020, and January 31, 2023. Patients had a documented diagnosis of diffuse large B-cell lymphoma (DLBCL; $n = 28$) or mantle cell lymphoma (MCL; $n = 3$). Fludarabine/cyclophosphamide (Flu/CY) lymphodepleting (LD) chemotherapy and CAR-T cells were administered according to standard clinical indications (tisagenlecleucel [TSA]: Flu 25 mg/m^2/day and CY 250 mg/m^2/day, days -5, -4, -3; axicabtagene [AXI]/ brexucabtagene [BRX]: Flu 30 mg/m^2/day and CY 500 mg/m^2/day, days -5, -4, -3).

As CD28-based products AXI and BRX share LD chemotherapy dosage and construct characteristics, for the purposes of immune-recovery analysis, data from AXI and BRX were pooled together (AXI/BRX) and compared to 4-1BB based TSA.

Cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) were classified according to ASTCT consensus grading [9] and treated in accordance with European Hematology Association/European Society for Bone and Marrow Transplantation (EHA/EBMT) guidelines [10].

Adverse events, including pre/post-infusion cytopenias, were graded according to CTCAE v5.0 criteria.

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All biological samples were collected after obtaining informed consent from the patients. The study was approved by the Regione Liguria Ethical Committee (574/2022).

2.2 | Flow cytometry analysis

Etylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood samples underwent multiparametric flow cytometry analysis at pre-specified timepoints (i.e., pre-lymphodepletion and at 0–3–6–10–14 days, 1–2–3–6 months following infusion) to assess absolute counts and relative proportions of CAR^+ $CD4^+$, CAR^+ $CD8^+$, CAR^- $CD4^+$, CAR^- $CD8^+$ T, NK and NKT cells. Cells counts were reported as cells/μL.

2.3 | Response assessment

Patients received a pre-infusion Positron Emission Tomography with Computed Tomography (PET/CT) scan to assess the baseline disease burden. Response to treatment was assessed with PET/CT at standardized time-points (i.e., 1–2–3–6–12 months following infusion) or as per clinical-need, according to Lugano criteria [11].

2.4 | Statistical analysis

The primary objective of this study was to determine the recovery dynamics of CAR^- lymphocyte ($CD4^+$ T, $CD8^+$ T, NK, NKT) populations following CAR-T cells therapy for aggressive B-cell lymphomas, at our center. Patients were grouped to assess the impact of possibly relevant variables on immune-recovery.

The secondary objective was to assess the prognostic impact of early CAR^- $CD4^+$ T cell recovery on overall survival (OS) and progression-free survival (PFS).

Population descriptive data are composed of continuous and categorical variables. The Mann–Whitney test was used to compare continuous variables and the Fisher exact test to compare categorical data.

At each time-point, immune subpopulations were represented as a mean and the respective standard deviation and compared through the Student's *t*-test. Within each group, outlier values ($\leq$3 interquartile range; IQR) were included and extreme values (>3 IQR) excluded.

The probability of CAR^- $CD4^+$ T cell recovery was generated using cumulative incidence estimates, with a new-anti-lymphoma therapy considered as a competing event. Univariate analysis for CAR^- $CD4^+$ T cell recovery was performed using the Gray's test, considering the product type (4-1BB, CD28-based), baseline inflammation (C-reactive protein; ferritin), tumor load (lactic dehydrogenase [LDH]), cytopenias (pre-lymphodepletion $CD4^+$ T cell and platelets counts; CAR-HEMATOTOX score) inflammatory complications (moderate to severe CRS/ICANS; high-dose dexamethasone) and a previous autologous stem cell transplant (ASCT) as relevant variables.

OS and PFS analysis were calculated from the date of CAR-T infusion to death for OS, death or relapse for PFS. For the purpose of OS calculation, patients who relapsed and subsequently achieved an allogeneic transplant were censored at transplant date. The probabilities of OS and PFS were calculated using the Kaplan–Meier estimator and groups were compared through the log-rank test. Univariate analysis was performed using Cox regression for OS and PFS. A significance level of $p = 0.1$ was chosen for subsequent entry into multivariate model. Results are reported as hazard ratio (HR) with corresponding 95% confidence interval (CI).

TABLE 1 Clinical description of the CAR-T treated patients at baseline.

Characteristic		All patients (31)	TSA treated patients (18)	AXI/BRX treated patients (13)	*p*-Value
Age, median (range)		61 (28–75)	60.5 (28–70)	61 (42–75)	0.78
Sex, *n* (%)	Male Female	16 (51.6) 15 (48.4)	8 (44.4) 10 (55.6)	8 (61.5) 5 (38.5)	0.47
Disease, *n* (%)	DLBCL MCL	28 (90.3) 3 (9.7)	18 (100.0) 0 (0.0)	10 (76.9) 3 (23.1)	0.06
Previous therapies, median (range)		2 (2–4)	2 (2–4)	2 (2–4)	0.98
Previous ASCT, *n* (%)	No Yes	20 (64.5) 11 (35.5)	12 (66.7) 6 (33.3)	8 (61.5) 5 (38.5)	1.0
Bridging therapy, *n* (%)	Chemo-based RxT-based Combined	15 (48.4) 6 (19.4) 10 (32.2)	10 (55.6) 1 (5.6) 7 (38.8)	5 (38.45) 5 (38.45) 3 (23.1)	0.07
Cytopenia (CTCAE ≥2),[a] *n* (%)	Neutropenia Anemia Thrombocytopenia	7 (22.6) 15 (48.4) 4 (12.9)	5 (27.8) 9 (50) 2 (11.1)	2 (15.3) 6 (46.1) 2 (15.3)	0.69 1.0 1.0
Serum CRP (mg/dL),[a] median (range)		8 (3–134)	7 (3–134)	22 (3–89)	0.37
Ferritin (ng/mL),[a] median (range)		283 (11–1507)	296.5 (65–1507)	206 (11–1434)	0.87
LDH > ULN,[a] *n* (%)	No Yes	10 (32.2) 21 (67.8)	7 (38.8) 11 (61.2)	3 (23.1) 10 (76.9)	0.45
HEMATOTOX,[a] *n* (%)	Low High	13 (41.9) 18 (58.1)	5 (27.8) 13 (72.2)	8 (61.5) 5 (38.5)	0.07

Abbreviations: ASCT, autologous stem cell transplantation; AXI, axicabtagene; BRX, brexucabtagene; CRP, C-reactive protein; CTCAE, common terminology criteria for adverse events; DLBCL, diffuse large B-cell lymphoma; LDH, lactic dehydrogenase; MCL, mantle cell lymphoma; TSA, tisagenlecleucel; ULN, upper limit of the normal range.

[a]As assessed before lymphodepletion. ¶ The CAR-HEMATOTOX is a five-item score used to assess the risk of prolonged cytopenia following CAR-T cell therapy. A high risk score (≥2 points) identifies patients with pronounced myelosuppression after CAR-T cell therapy. The Mann–Whitney test for continuous variables and the Fisher exact test for categorical data were used to assess significance.

Two-sided *p*-values are reported, with a $p < 0.05$ considered as statistically significant. Statistical analysis was performed with SPSS 28.0.1.0 (SPSS Inc.) for the whole analysis, with the except of cumulative incidence functions and Gray test, which was performed with NCSS N.23.0.2 (NCSS LLC).

3 | RESULTS

3.1 | Cell product, disease and patient characteristics

We identified 31 consecutive patients treated with commercial CAR-T cells for aggressive B-cell lymphoma. Among 28 DLBCL affected patients, 18 received TSA and 10 AXI; three patients affected by MCL received BRX. Table 1 summarizes patient demographics and baseline characteristics, with data from AXI and BRX gathered together as stated above. The median age at CAR-T was 61 years, the median number of lines before CAR-T was two. A total of 11 patients (35%) had received an ASCT, 25/31 (80%) needed chemo or combined chemo/radiation bridging therapy and 21/31 (68%) had higher-than-normal LDH levels at lymphodepletion. Seven patients (23%) were neutropenic at lymphodepletion, 18/31 (58%) had a high-risk CAR-HEMATOTOX score. No baseline differences among TSA and AXI/BRX emerged (Table 1). The median duration of the follow-up was 16 months.

3.2 | Kinetics of the peripheral blood immune reconstitution after CAR-T cell therapy

Following CAR-T infusion, we observed a rise in peripheral blood CAR^- $CD8^+$ and CAR^- $CD4^+$ T cell counts, which may represent the impact of LD chemotherapy in promoting T-cell proliferation (Figure 1A). $CD8^+$ T cells peaked early at month-1 as compared to $CD4^+$ T cells at month-2, and both the populations declined thereafter, achieving 6-months mean values superimposable to pre-lymphodepletion (pre-LD) levels ($CD8^+$: 251 cells/μL [95% CI: 157–344] vs. 308 [95% CI: 169–441], $p = 0.40$; $CD4^+$: 139 cells/μL [95% CI: 103–176] vs. 176 [95% CI: 92–260], $p = 0.27$). For NK cells we observed a different

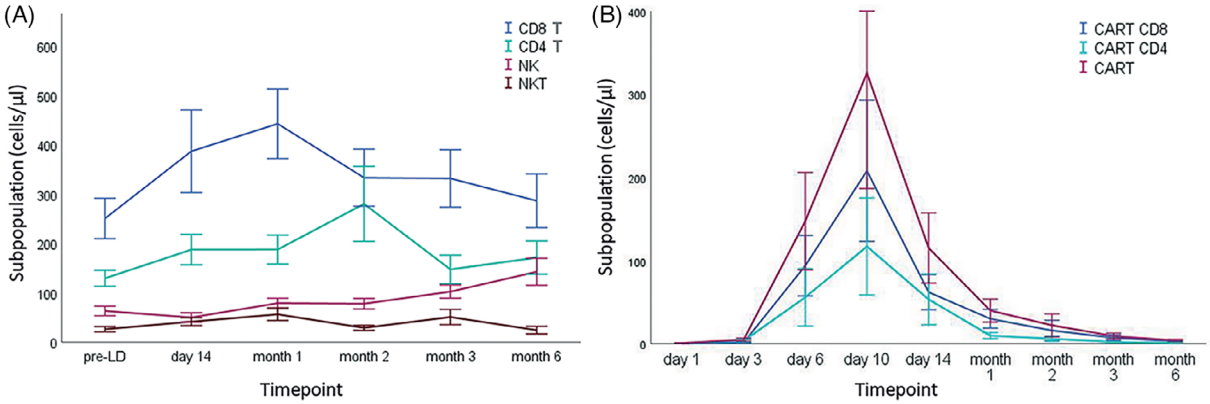

FIGURE 1 Kinetics of CAR$^-$ (CD4$^+$ T, CD8$^+$ T, NK, and NKT, panel A) and CAR$^+$ (CD4$^+$ T and CD8$^+$ T, panel B) cell populations over the time in the entire cohort of CAR-T treated patients ($n = 31$; CAR$^-$ CD8$^+$ T cells in blue, CAR$^-$ CD4$^+$ T cells in light blue, NK cells in red, and NKT cells in brown, panel A; CAR$^+$ CD8$^+$ T cells in blue, CAR$^+$CD4$^+$ T cells in light blue, and total CAR$^+$ T cells in red, panel B). Results are reported as mean ± SD. SD, standard deviation.

behavior, with a progressive increase overtime and month-6 mean levels significantly higher than baseline (NK: 67 cells/μL [95% CI: 45–89] vs. 135 [95% CI: 83–186], $p = 0.01$) (Figure 1A). Mean CAR-T cells peaked on day 10 at 207 and 116 cells/μL for CD8$^+$ and CD4$^+$, respectively, and declined thereafter (Figure 1B).

3.3 | The CAR product impacts peripheral blood CAR$^-$ CD4$^+$ T cell recovery

Six-month cumulative incidence of peripheral blood CAR$^-$ CD4$^+$ T cell recovery (i.e., ≥200 cells/μL) was 0.43 (95% CI: 0.28–0.65). In univariate analysis, the product type was significantly associated with CD4$^+$ T cell recovery: patients treated with TSA had a higher probability to achieve the 200 cells/μL cut-off (HR = 5.30 [95% CI: 1.16–24.12], $p = 0.03$) (Table 2 and Figure 3A). No other significant interactions were observed, including pre-LD CD4$^+$ T cell levels, the occurrence of moderate to severe CRS/ICANS and the need to administer steroids (Table 2).

We next compared CAR$^-$ CD4$^+$ T cell mean values between TSA and AXI/BSA at regular timepoints (Figure 2B). Month-1, -2, and -3 CAR$^-$ CD4$^+$ T cell counts were significantly higher for TSA, with a tendency to peak and subsequently decrease. The AXI/BRX counterpart, on the contrary, had a more stable trend overtime. No differences emerged between TSA and AXI/BRX for CAR$^-$ CD8$^+$ T, NK and NKT cells (Figure 2A,C,D). Pre-LD cell counts between TSA and AXI/BSA were superimposable across subpopulations, excluding significant baseline differences.

3.4 | CAR$^-$ CD4$^+$ T cell lymphopenia and infectious complications

Prolonged CD4$^+$ T cell lymphopenia might impact the infectious risk. We therefore assessed the infection rate occurring from day 28 in patients alive and free from new lymphoma therapies at the month-6 timepoint: 6/9 (66%) patients with persistent CD4$^+$ T cell lymphopenia developed an infection, as compared to 2/11 (18%) in those who experienced CD4$^+$ T cell recovery within the observation period. In the first group, infections required hospitalization in 3/6 (50%) of cases (one cytomegalovirus pneumonia; one *Aspergillus niger* pneumonia; one SARS-CoV2 pneumonia), two patients developed mild-grade symptoms from SARS-CoV2 and influenza virus, respectively, and one experienced a localized herpes simplex virus type-1 reactivation. Two mild-grade upper respiratory infections, one from SARS-CoV2 and one from respiratory syncytial virus-B, were documented in patients with CD4$^+$ T cell recovery.

3.5 | Early peripheral blood CAR$^-$ CD4$^+$ T cell recovery is associated with a worse clinical outcome in DLBCL

We addressed whether achieving an early peripheral blood CAR$^-$ CD4$^+$ T cell recovery (i.e., CD4$^+$ T cells ≥200/μl at month-1 timepoint) correlated with survival. For this purpose, we focused on the DLBCL cohort, considering that response and persistence rates might be different from MCL, thus representing a bias.

For the 28 DLBCL affected patients, the median follow-up duration was 16 months (range, 1–30). Twelve-month estimated OS and PFS were 0.65 (95% CI: 0.47–0.83) and 0.41 (95%CI: 0.23–0.59), respectively.

Within the early CAR$^-$ CD4$^+$ T cell recovery group, 9/11 (82%) progressed, versus 8/17 (47%) within those who did not experience early CAR$^-$ CD4$^+$ T cell recovery. Median PFS was 1 and 9 months, respectively ($p = 0.01$).

The results of univariate and multivariate for survival outcomes are reported in Table 3. Both PFS and OS were significantly worse in the early CAR$^-$ CD4$^+$ T cell recovery group versus the no-recovery group (HR for PFS = 3.17 [95% CI: 1.15–8.70], $p = 0.02$), (HR for OS = 5.24 [95% CI: 1.47–18.73], $p = 0.01$) (Figure 3B,C). In univariate analysis,

TABLE 2 Univariate analysis for CAR$^-$ CD4$^+$ T cell recovery.

CD4 recovery (DLBCL + MCL)			
Variables		**HR (95% CI)**	***p*-Value**
CAR-T product	4-1BB vs. CD28	5.30 (1.16–24.12)	**0.03**
Previous ASCT	Yes vs. no	1.37 (0.46–4.11)	0.56
LDH[a]	>ULN vs. <ULN	0.83 (0.29–2.35)	0.73
Ferritin[a] (ng/mL)	>650 vs. <650	1.31 (0.41–4.25)	0.64
Serum CRP[a] (mg/dL)	>3 vs. <3	0.62 (0.21–1.86)	0.39
HEMATOTOX[a]¶	High vs. low	1.17 (0.40–3.35)	0.78
CD4$^+$ T cells[a] (cells/μL)	<200 vs. > 200	0.40 (0.13–1.24)	0.11
Platelets[a] ($\times 10^9$/L)	<100 vs. >100	1.11 (0.35–3.57)	0.85
CRS-ICANS	Gr.3–4 vs. Gr.0–2	0.21 (0.02–1.63)	0.13
Dexamethasone (mg)	>40 vs. ≤40	0.29 (0.03–2.28)	0.24

Abbreviations: ASCT, autologous stem cell transplantation; CI, confidence interval; CRP, C-reactive protein; CRS, cytokines release syndrome; DLBCL, diffuse large B-cell lymphoma; HR, hazard ratio; ICANS, immune effector cell-associated neurotoxicity syndrome; LDH, lactic dehydrogenase; MCL, mantle cell lymphoma; ULN, upper limit of the normal range.

[a]As assessed before lymphodepletion. ¶ The CAR-HEMATOTOX is a five-item score used to assess the risk of prolonged cytopenia following CAR-T cell therapy. A high risk score (≥2 points) identifies patients with pronounced myelosuppression after CAR-T cell therapy. The Gray's test was used to compare the subdistribution hazard between groups. The bold characters indicate a statistically significant *p*-value ($p < 0.05$).

OS also differed for patients with high pre-LD ferritin levels (HR: 4.39 [95% CI: 1.33–14.43], $p = 0.01$) and for those who received high-dose dexamethasone (HR: 3.71 [95% CI: 1.04–13.29], $p = 0.04$).

In a COX multivariate regression model for OS including early CAR$^-$ CD4$^+$ T cell recovery, ferritin levels and high-dose dexamethasone as categorial covariates, early CAR$^-$ CD4$^+$ T cell recovery retained its impact (HR: 4.47 [95% CI: 1.12–17.71], $p = 0.03$).

4 | DISCUSSION

Peripheral blood immune recovery following CAR-T cell therapy for hematological malignancies follows different rules as compared to conventional chemo-immunotherapy. Cytokine storm induced by LD chemotherapy, medications used to modulate inflammatory complications, and the CAR-T cell activity itself may individually fine-tune its dynamics. Prolonged neutropenia emerged as a determinant of mortality [12]. B-cell aplasia is unavoidable following anti-CD19 constructs and an indirect marker of residual CAR-T activity [13]. Peripheral blood CD4$^+$ T cell lymphopenia is less described, less predictable but retains significant implications for infectious complications [2, 3]. In this context, a cut-off of 200 cells/μL has been employed for CD4$^+$ T cell recovery estimation [14]. Our study provides a longitudinal description of peripheral blood CD4$^+$ T cells recovery following anti-CD19 CAR-T therapy for aggressive B cell lymphomas. In 31 patients treated with commercial CAR-T cells for DLBCL or MCL at our center, less than half achieved CD4$^+$ T cell recovery within 6 months. Thus, our study is in line with previous observations, which reported a prolonged delay in CD4$^+$ T cell recovery following CAR-T cells [2, 3, 4]. Importantly, in our experience none of the potential determinants (i.e., disease burden, preexisting cytopenias and inflammation, inflammatory complications following CAR-T infusion) singularly impacted CD4$^+$ T cell recovery but one: the product type (Table 2). Patients who received TSA had a 5.3 HR to achieve recovery, as compared to AXI/BRX. Differences in LD chemotherapy might explain our findings: patients undergoing AXI/BRX receive a CY dose which doubles that of TSA (1500 mg/m^2 vs. 750 mg/m^2, total dose). Experiences with high-dose Post Transplant Cyclophosphamide (PTCY) to prevent Graft Versus Host Disease (GVHD) in the allogeneic transplant setting show that CD4$^+$ T cells are particularly sensitive to CY, and this sensitivity is more pronounced in comparison to CD8$^+$ T cells [15, 16]. Moreover, in a mouse model testing different strategies of GVHD prophylaxis, PTCY decreased the CD4$^+$/CD8$^+$ ratio by reducing CD4$^+$ T cell percentages [17]. Despite clear distinctions with allogeneic transplant, these observations could represent a rationale for our data, as differences between products, and their relative LD strategies, emerged only for CD4$^+$ T cells and not for CD8$^+$ T, NK and NKT cells (Figure 2).

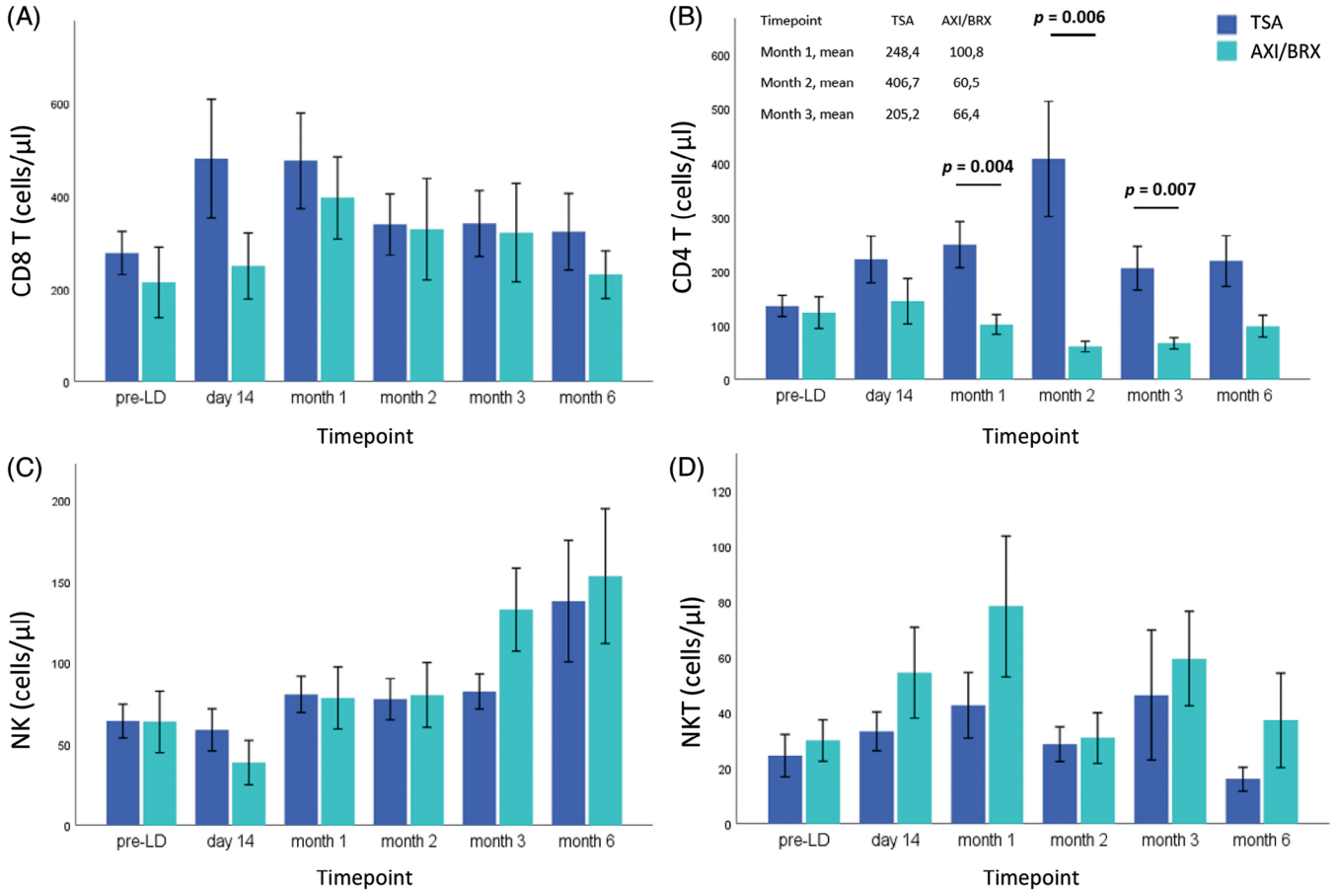

FIGURE 2 Kinetics of CAR$^-$ (CD8$^+$ T, CD4$^+$ T, NK, and NKT) immune cell populations over the time, comparing TSA ($n = 18$, in blue) and AXI/BRX CAR-T treated patients ($n = 13$, in light blue). Results are reported as mean ± SD. Student's *t*-test was used to compare groups at each time-point and a two-sided *p*-value was considered for significance. Statistically significant *p*-values are labeled above the corresponding comparisons. AXI/BRX, axicabtagene/brexucabtagene; SD, standard deviation; TSA, tisagenlecleucel.

From a clinical perspective, infections represent the most common cause of non-relapse-mortality (NRM) following CAR-T therapy [12, 18, 19]. Prolonged (i.e., > 100 days) neutropenia is common following AXI [20] and a major determinant of late-NRM, in the majority of cases from bacterial infections [3, 12]. Beyond neutropenia, prolonged, severe CD4$^+$ T cell lymphopenia emerged as a risk factor for herpetic (Herpes simplex virus/Varicella-zoster virus, HSV/VZV) reactivation and *Pneumocystis jirovecii* pneumonia (PJP), frequently following primary prophylaxis discontinuation [2, 3]. We observed severe, non-HSV/VZV or -PJP infectious complications (two viral, one fungal pneumonia) exclusively in patients with persistent CD4$^+$ lymphopenia. All the three patients were relatively young (respectively 41, 45, and 50 years of age), affected by a refractory, symptomatic disease at the moment of CAR-T infusion, and developed Gr. 3–4 ICANS in 2/3. Our experience suggests that prolonged peripheral blood CD4$^+$ T cell lymphopenia may identify a cohort of high-risk features patients who might experience severe infectious complications, in line with severe neutropenia and Gr. 3–4 ICANS as risk factors for late-NRM [12].

Peripheral blood CD4$^+$ T cell recovery is per se desirable, but its early achievement might represent an alert. We describe an impact of early CAR$^-$ CD4$^+$ T cell recovery on survival, following CAR-T cell therapy. Early, month-1 recovery emerged as the sole factor associated with PFS and the most impacting for OS, the latter in a multivariate model which included high-dose steroids and high pre-LD ferritin levels. The need to administer high-dose dexamethasone might identify those patients at higher risk of NRM, due to severe infections or inflammatory complications. On the contrary, early CAR$^-$ CD4$^+$ T cell recovery candidates as a marker of CAR-T therapy refractoriness, as nearly 80% relapsed, most of them within 2 months since CAR-T infusion. Early CD4$^+$ T cell recovery was more common among patients treated with TSA (10/18; 55%) as compared to AXI (1/10; 10%). Nevertheless, the sole patient who experienced the event following AXI relapsed shortly after. In our experience, baseline characteristics were superimposable between TSA and AXI (Table 1) and no differences emerged in terms of response rate and survival, in line with much larger registry reports [21]. Thus, we hypothesize that early CD4$^+$ T cell recovery represents a marker of CAR-T disfunction independently of the infused product, which, however, is more prone to emerge following TSA. One may assume a role for the less intense LD chemotherapy in advance of TSA: lower-dose CY might lead to a reduced toxicity among lymphocyte subpopulations, including CD4$^+$ T cells, and a faster recovery thereafter. In this case, early CD4$^+$ T cell recovery would be

TABLE 3 Univariate and multivariate analysis for progression-free survival and overall survival.

Survival outcomes, diffuse large B cell lymphoma							
		Progression-free survival		Overall survival			
Variables		Univariate HR (95% CI)	*p*-Value	Univariate HR (95% CI)	*p*-Value	Multivariate HR (95% CI)	*p*-Value
CAR-T product	4-1BB vs. CD28	0.72 (0.27–1.91)	0.51	0.79 (0.20–3.05)	0.73		
Previous ASCT	Yes vs. no	1.18 (0.43–3.22)	0.74	0.90 (0.23–3.42)	0.88		
LDH[a]	>UNL vs. <UNL	1.22 (0.45–3.32)	0.69	1.77 (0.46–6.73)	0.40		
Ferritin[a] (ng/mL)	>650 vs. <650	1.94 (0.72–5.27)	0.19	4.39 (1.33–14.43)	**0.01**	2.31 (0.60–8.81)	0.22
CRP[a] (mg/dL)	>3 vs. <3	1.36 (0.50–3.68)	0.46	1.77 (0.47–6.69)	0.39		
HEMATOTOX[a]¶	High vs. Low	1.01 (0.38–2.66)	0.98	3.30 (0.71–15.32)	0.12		
$CD4^+$ T cells[a] (cells/μL)	<200 vs. >200	0.78 (0.24–2.48)	0.96	1.31 (0.27–6.22)	0.73		
Platelets[a] ($\times 10^9$/L)	<100 vs. >100	1.04 (0.34–3.20)	0.94	2.04 (0.59–7.01)	0.25		
CRS-ICANS	Gr.3–4 vs. Gr.0–2	1.09 (0.35–3.36)	0.87	2.37 (0.69–8.16)	0.87		
Dexamethasone (mg)	>40 vs. ≤40	1.57 (0.50–4.86)	0.43	3.71 (1.04–13.29)	**0.04**	3.64 (0.90–14.69)	0.06
$CD4^+$ T cells, m1 (cells/μL)	≥200 vs. <200	3.17 (1.15–8.70)	**0.02**	5.24 (1.47–18.73)	**0.01**	4.47 (1.12–17.71)	**0.03**

Abbreviations: ASCT, autologous stem cell transplantation; CI, confidence interval; CRP, C-reactive protein; CRS, cytokines release syndrome; HR, hazard ratio; ICANS, immune effector cell-associated neurotoxicity syndrome; LDH, lactic dehydrogenase; m1, at month-1 evaluation; ULN, upper limit of the normal range.

[a]As assessed before lymphodepletion. ¶ The CAR-HEMATOTOX is a five-item score used to assess the risk of prolonged cytopenia following CAR-T cell therapy. A high risk score (≥2 points) identifies patients with pronounced myelosuppression after CAR-T cell therapy. The Cox proportional hazards regression model was used to investigate the role of prognostic factors and to obtain hazard ratios. The bold characters indicate a statistically significant *p*-value ($p < 0.05$).

a marker of insufficient LD effect, which is needed to abate immune suppressive populations and induce a sustained cytokine peak. On the other side, higher-dose CY might be needed to eradicate $CD4^+$ regulatory T (Treg) subpopulations, which otherwise may expand following LD chemotherapy and hamper CAR-T cell activity, due to their intrinsic immune-suppressive properties. The hypothesis might be complementary, deserving therefore a deeper analysis. Noteworthy, experimental models with PTCY in the haploidentical allogeneic transplant setting showed a preferential recovery of $CD4^+CD25^+Foxp3^+$ Treg cells within the first month following transplant [17, 22]. As aldehyde dehydrogenase confers CY resistance to Treg cells following a strong (allo)antigen stimulation [23], it is possible, in an autologous setting such as that of therapy with commercial CAR-T cell products, that CY simply acts as a non-cycle-specific agent, whose efficacy in eradicating $CD4^+$ T cells, included Treg cells, occurs in a dose-dependent way: higher CY doses, higher Treg depletion. In this view, a recent work in the setting of chronic lymphocytic leukemia identified high $CD4^+$ T cell counts following a CY-based regimen (FCR; fludarabine, cyclophosphamide, rituximab) as associated with a reduced PFS. Strikingly, phenotype analysis revealed that the majority were $CD4^+$ $CD25^+$ $CD127^-$ $Foxp3^+$, likely belonging to the Treg cell subset [24].

Our work has limitations. First, the small cohort of patients does not allow to generalize our observations and could mask determinants of resistance and survival. Data were recorded in a retrospective fashion, nevertheless timepoints for phenotype analysis were accurately distributed throughout the follow-up period. Cytokines and immune cell subpopulations analysis would be necessary to elucidate our hypothesis. In this sense, we propose to further analyze our data.

5 | CONCLUSION

In conclusion, CAR-T cell therapy represents a curative option for aggressive B-cell lymphomas, but inefficacy is a common issue. Mechanisms of resistance are heterogeneous and might involve multiple factors, other than the duo cancer—CAR-T cell. Our study provides a relatively simple marker of early CAR-T cell failure in DLBCL, which

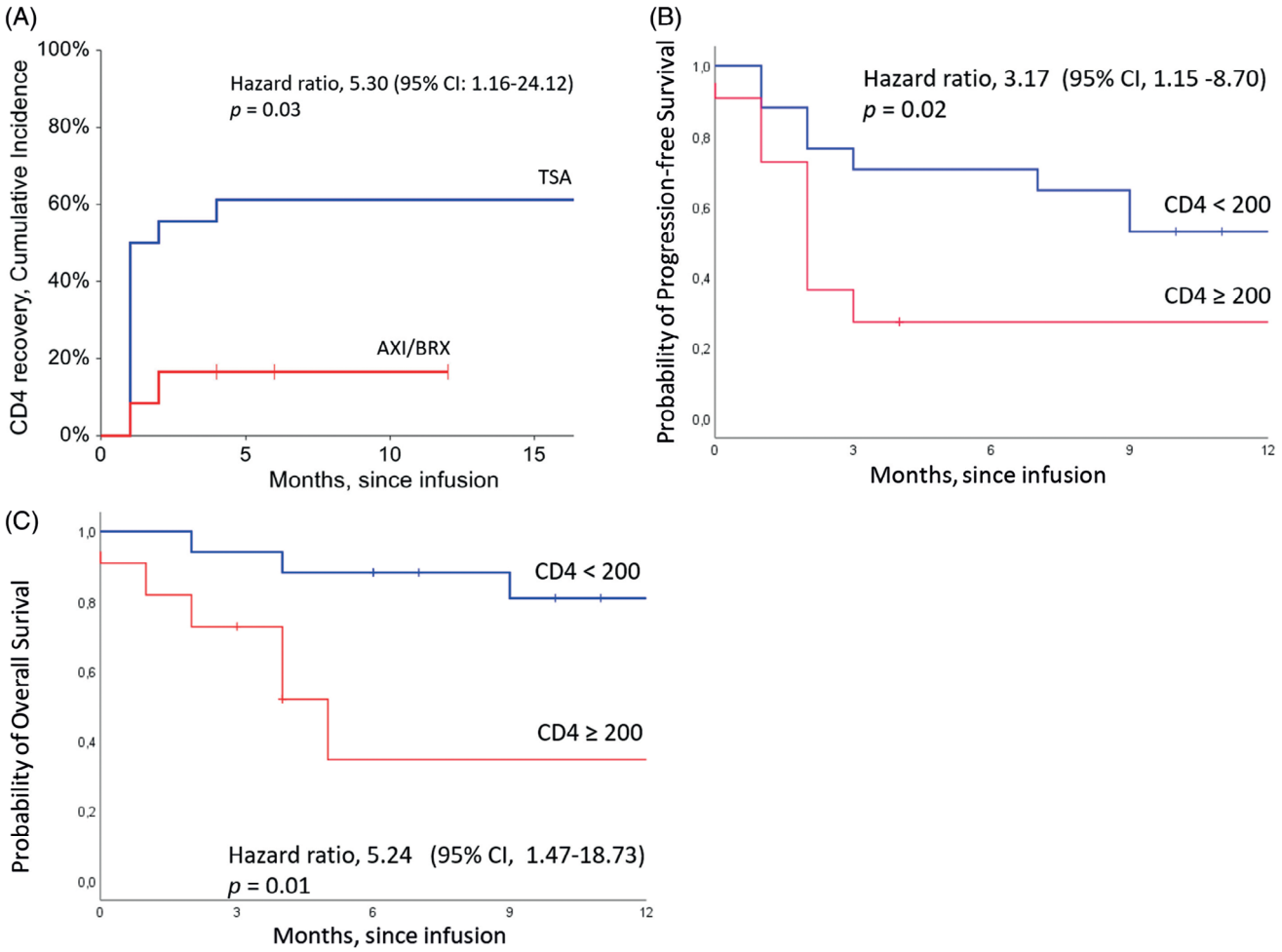

FIGURE 3 Cumulative incidence of peripheral blood CAR^{-} $CD4^{+}$ T cell recovery stratified by product, tisagenlecleucel (TSA) versus axicabtagene/brexucabtagene (AXI/BRX) (panel A). Kaplan–Meier analysis of progression-free survival (panel B) and overall survival (panel C) stratified by peripheral blood CAR^{-} $CD4^{+}$ T cell recovery, lower versus upper than 200 cells/μL, at month-1 evaluation. Gray's test for cumulative incidence, and Cox regression for survival analysis were used to calculate hazards and the corresponding *p*-values.

might origin from LD chemotherapy. Understanding of the underlying reasons may help to optimize the treatment.

AUTHOR CONTRIBUTIONS

Massimiliano Gambella and Anna Maria Raiola conceived the article; Elisabetta Tedone, Rosa Mangerini, Nicoletta Colombo, and Alessia Parodi performed the flow cytometric analysis and acquired data; Massimiliano Gambella, Simona Carlomagno, Chiara Setti, Simona Sivori, and Anna Maria Raiola interpreted data; Chiara Ghiggi, Livia Giannoni, Elisa Coviello, Silvia Luchetti, Alberto Serio, Antonella Laudisi, Monica Passannante, and Alessandra Bo critically revised the article; Simona Sivori and Emanuele Angelucci supervised the process. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors would like to thank all members of the study team, the patients, and their family. They would also like to thank the Scientific Direction of the IRCCS Ospedale Policlinico San Martino for the support.

Open access funding provided by BIBLIOSAN.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interests.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article. Further inquiries about the data access can be directed to the corresponding author.

ETHICS STATEMENT

The study was approved by the ethical committee of the Regione Liguria (574/2022) and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

CLINICAL TRIAL REGISTRATION

The authors have confirmed clinical trial registration is not needed for this submission.

PATIENT CONSENT STATEMENT

All patients included in the study provided informed consent.

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How to cite this article: Gambella M, Carlomagno S, Mangerini R, Colombo N, Parodi A, Ghiggi C, et al. Early CAR^{-} CD4^{+} T-lymphocytes recovery following CAR-T cell infusion: A worse outcome in diffuse large B cell lymphoma. eJHaem. 2024;5:360–68. https://doi.org/10.1002/jha2.871