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ORIGINAL PAPER

The CD4/CD8 ratio of infused CD19-CAR-T is a prognostic factor for efficacy and toxicity

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Summary
$CD4^+$ and $CD8^+$ chimeric antigen receptor T cells (CAR-T) play different roles in the in vivo anti-tumour response, but the role of the $CD4^+/CD8^+$ ratio among infused CAR-T has not been clearly defined yet. We analysed leftovers from infused anti-CD19 CAR-T bags of 31 patients with aggressive B-cell lymphomas. The median ratio was 1.44, lower for brexu-cel compared to tisa-cel and axi-cel. The CAR+$CD4^+/CD8^+$ ratio was influenced by lactate dehydrogenase levels at apheresis, not by age, previous treatments or the $CD4^+/CD8^+$ ratio in peripheral blood. Patients with a response at 3 months after CAR-T (M3) had a lower CAR+$CD4^+/CD8^+$ ratio in the infused products compared to non-responders (ratio 0.74 vs. 2.47, $p = 0.011$). A CAR+$CD4^+/CD8^+$ ratio higher than the cut point of 1.12 was associated with an increased risk of treatment failure at M3 (OR 23.3, $p = 0.012$) and M6 (OR 10, $p = 0.028$). The median 6-month PFS was 76% for patients with a ratio lower than 1.12% vs. 31% for the others. The prognostic role of the CAR+$CD4^+/CD8^+$ ratio was independent of the costimulatory domain (CD28 vs. 4-1BB) of the product (OR 16.41, $p = 0.041$). Our data indicate a crucial role for $CD8^+$ CAR-T and the CAR+$CD4^+/CD8^+$ ratio in predicting CAR-T efficacy.

KEYWORDS
apheresis, CAR-T cells, CD4/CD8 ratio, phenotype, prognostic factors

INTRODUCTION

The advent of chimeric antigen receptor T cells (CAR-T) has revolutionized the therapy for several relapsed/ refractory (R/R) B-cell malignancies. Currently, three autologous anti-CD19 CAR-T products have gained commercial availability for aggressive B-cell lymphomas: tisagenlecleucel (tisa-cel), axicabtagene ciloleucel (axi-cel) and brexucabtagene autoleucel (brexu-cel). These CAR-T therapies use different vectors, namely lentivirus and retrovirus, and exhibit distinct costimulatory domains, such as 4-1BB and CD28. The choice of costimulatory domains has been implicated in influencing expansion kinetics, long-term persistence and intracellular activating pathways. However, the clinical significance of these differences is not clearly defined.[1–3]

While several variables related to the characteristics of patients and their disease and CAR-T-cell products have been identified as predictors of efficacy and outcome, a precise prognostic tool is still lacking. Notably, lymphocyte fitness, patient-specific factors and tumour burden appear to be the key prognostic factors.[4–6]

The success of CAR-T therapies heavily relies on the composition and the quality of the final CAR-T-cell product. Recent studies have suggested that the composition of T-cell subsets in the apheresis product significantly influences the fitness and effectiveness of the resulting CAR-T cells.

Eugenio Galli and Silvia Bellesi have equally contributed.

Specifically, the presence of naïve and stem central memory (SCM) T-cells has been observed to contribute to robust expansion and proliferation during the in vitro generation of CAR-T cells. However, it has been reported by Das et al. in paediatric cancer patients that the cumulative cycles of chemotherapy can lead to the depletion of these early lineage T-cell subsets, resulting in a decline in successful ex vivo stimulation and potentially impacting the overall efficacy of CAR-T-cell therapy.[7]

Furthermore, patients with B-cell malignancies exhibit significant variations in the frequency of $CD8^+$ and $CD4^+$ T-cell subsets in their peripheral blood (PB). This heterogeneity can arise from factors such as age, prior pathogen exposure and the lymphocytotoxic effects of chemotherapy. Most patients with B-cell malignancies tend to have a higher proportion of $CD8^+$ T cells and a lower proportion of $CD4^+$ T cells.[8] Consequently, these differences in T-cell subset frequencies manifest as a considerable variation in the composition of CAR-T-cell products. The distinctive frequencies of $CD4^+$ and $CD8^+$ T cells within CAR-T-cell products have notable implications for their functionality. $CD4^+$ CAR-T cells have been shown to produce an increased repertoire of Th1 cytokines, including interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2). Moreover, they demonstrate robust proliferative capacity upon the recognition of tumour cells. Conversely, $CD8^+$ CAR-T cells exhibit a higher degree of specific lytic activity. The strategic combination of $CD4^+$ and $CD8^+$ CAR-expressing subsets in a precisely defined 1:1 ratio has demonstrated synergistic anti-tumour effects in vivo.[8–10] The TRANSCEND NHL study 001's findings suggest that liso-cel, with its specific composition of CAR+ $CD4^+$ and $CD8^+$ T cells, can effectively target and treat R/R large B-cell lymphoma while minimizing severe CRS and neurological events.[10] Currently, this CAR-T product is not commercially available in Italy.

Aim

Our study aimed to assess the phenotypic composition of CAR-T cells infused from three commercially available products and investigate potential implications for prognosis. The phenotype of in vivo CAR-T cells was also analysed.

METHODS

We have included all patients with aggressive B-cell lymphoma who were treated at our institution with commercially available CAR-T therapy. The patients were eligible to receive CAR-T if presenting with a R/R disease with two or more previous lines of therapy and if they fulfilled criteria outlined by the Italian Medicines Agency (AIFA) (https://farmaci.agenziafarmaco.gov.it/bancadatifarmaci/home). We included only patients for whom leftover samples from CAR-T bags were available for analysis.

To collect leftovers for analysis, we performed a procedure, where the empty CAR-T bags were washed with 5 mL of sterile 0.9% NaCl. This saline solution was then agitated within the bag to harvest any micro-residue adhering to the inner walls, which was subsequently collected in a vial.

We also obtained PB samples from the patients at specific time points after CAR-T infusion at Days 7, 14, 21, and 1–3 months (M).

The percentage and absolute counts of $CD3^+$ $CD4^+$ $CD8^+$ lymphocytes were obtained with a single-platform method using a standard antibody cocktail TETRA-1 by AQUIOS cytometer (Beckman Coulter). The CAR-T-cell identification was performed through an indirect method with a human CD19 His Tag protein in conjunction with an anti-His APC-labelled secondary antibody.

We performed two extra-cellular staining, with the following monoclonal antibodies:

- Tube1-negative control with CD45 (V-500); CD3 (FITC); CD8 (PC7); CD4 (APC-H7).
- Tube2 CAR-T detection tube with CD45 (V-500); CD3 (FITC); CD8 (PC7); CAR-T (APC); CD4 (APC-H7).

Flow cytometry was performed with a 'wash/stain/lyse' method (20 min for staining and 10 min for lyses).

Data were acquired with BD FACSCanto II cytometer and analysed with FACSDiva Software (BD Biosciences). A minimum of 200 000 $CD45^+$ events per tube were recorded.

In order to visualize the population of interest properly and consistently, appropriate gating strategies were adopted. The first tube was used for setting the positivity of CAR-T cells with a fluorescence minus one (FMO)-negative control. The second tube was performed to define the percentage of $CD3^+$ CAR-T on total $CD3^+$ lymphocyte population and the percentage of CAR-T $CD4^+$ and CAR-T $CD8^+$ with ratio CAR-T $CD4^+$/CAR-T $CD8^+$ (Figure 1A–E).

The results obtained from phenotypic characterization were correlated with patient characteristics and outcomes. We took into account variables such as the characteristics of patients including previous treatments and current tumour burden. Additionally, we collected relevant biological data on haematological and biochemical parameters.

Responses to CAR-T-cell therapy were evaluated at 1 month (M1), 3 months (M3) and 6 months (M6) following CAR-T infusion, in accordance with national guidelines. The response was determined with ^{18}FDG PET–CT scans and assessed based on the Deauville/Lugano criteria. Patients who achieved a complete response (CR) or partial response (PR) were classified as 'responders', while all other patients were considered 'non-responders'.

Statistical analysis

Categorical and continuous variables were analysed using appropriate descriptive statistical tests, such as chi-square

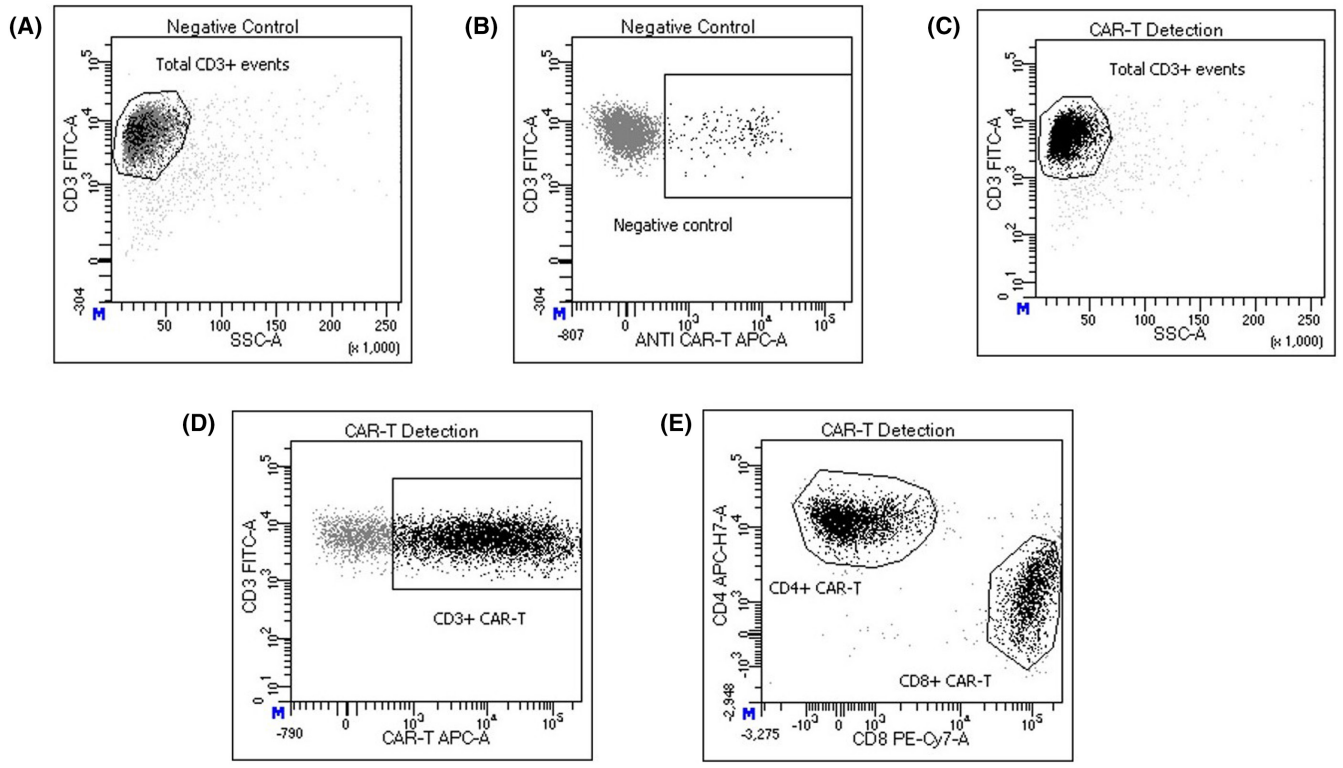

FIGURE 1 Determination of CAR-T populations by flow cytometry. Total $CD3^+$ T lymphocytes in negative control tube (A) and in CAR-T detection tube (C). (B) CAR-T negative control (FMO). (D) $CD3^+$ positive CAR-T events are represented in the plot. (E) Total $CD3^+$ CAR-T events are split into the two subpopulations of $CD4^+$ CAR-T cells (upper left) and $CD8^+$ CAR-T cells (lower right). [Colour figure can be viewed at wileyonlinelibrary.com]

test, *t*-test or Mann–Whitney rank sum test, as applicable to each variable type. In cases where we aimed to explore the correlation between a categorical event (e.g. the response at M3) and one or multiple continuous variables, logistic regression was employed. To determine cut-off points, receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was analysed. Progression-free survival (PFS) analysis was investigated using the Kaplan–Meier log-rank test. Statistical analyses were performed using the NCSS 2020 Statistical Software by NCSS, LLC. Kaysville, Utah, USA (ncss.com/software/ncss).

The study was conducted in adherence to the Helsinki criteria and received approval from the local ethics committee (ID 4879 Prot 0020777/22). Patients provided informed signed consent for biobanking of samples and the anonymized use of their data in the study.

RESULTS

Leftovers from CAR-T bags were available for 31 out of 36 patients with aggressive B-cell lymphoma treated with CAR-T in our centre: the patients received tisa-cel (13), axi-cel (13) or brexu-cel (5). The selection of the CAR-T product was made in accordance with the histological subtype of the lymphoma. Specifically, for mantle cell lymphoma, brexu-cel was chosen, while for primary mediastinal B-cell lymphoma, axi-cel was selected. In cases of aggressive B-cell lymphomas, where a choice had to be made between two commercially available CAR products (axi-cel and tisa-cel), the final decision made by the clinician was influenced by both the availability of a production slot and the distinctions in the risk profiles of the products. It is important to note that these decisions did not result in any noteworthy variations in patient characteristics between the two chosen products (Table 1).

The median age of the patients was 52 years, and they had received a median of two previous lines of therapy. There were no significant differences in age, gender, number of previous lines or disease-related characteristics, such as lactate dehydrogenase (LDH) and disease status assessed immediately before CAR-T infusion, among the three groups, as shown in Table 1.

Regarding the T-cell composition of infused bags, tisa-cel products contained a significantly lower proportion of CAR+ cells among $CD3^+$ cells compared to axi-cel and brexu-cel (median 45% vs. 71% vs. 79%, $p = 0.022$). Additionally, tisa-cel had the lowest proportion (median 26%) of $CD8^+$ cells among CAR+ cells compared to axi-cel (median 38%) and particularly brexu-cel (median 60%, $p = 0.03$).

The CAR+$CD4^+$/$CD8^+$ ratio varied from 0.18 to 7.86, with a median of 1.44. Brexu-cel appeared to have the lowest CAR+$CD4^+$/$CD8^+$ ratio (median 0.54, range 0.49–0.99); the median of CAR+$CD4^+$/$CD8^+$ ratio in axi-cel products was 1.54 (range 0.18–5.57), and in tisa-cel products, it was 2.56

TABLE 1 Characteristics of the population, overall and by infused CAR-T product.

	All, *n*	Axi-cel, *n* (%)	Brexu-cel, *n* (%)	Tisa-cel, *n* (%)	*p*-value
	31	13	5	13	
Age					
Median (years)	52	50	62	50	0.49
Gender					
Males	14 (45)	4 (31)	4 (80)	6 (46)	0.17
Previous lines					
Median	2	2	2	2	0.80
Status at infusion					
CR	3 (10)	2 (15)	0	1 (8)	0.65
PR	9 (30)	3 (23)	3 (60)	3 (23)	
SD	6 (20)	3 (23)	1 (20)	2 (16)	
PD	13 (40)	5 (39)	1 (20)	7 (53)	
LDH at infusion					
Median	226	210	226	271	0.31

Abbreviation: LDH, lactate dehydrogenase.

(range 0.97–7.76), without however reaching statistical significance ($p = 0.126$). The CAR+CD4$^+$/CD8$^+$ ratio showed a strong correlation to the CD4$^+$/CD8$^+$ ratio in the total CD3$^+$ population in the infused bags ($p < 0.001$).

Examining the factors influencing the CAR+CD4$^+$/CD8$^+$ ratio in the infused product, we found no correlation between the CD4$^+$CD8$^+$ ratio in PB at the time of apheresis and the overall CAR+CD4$^+$/CD8$^+$ ratio in the CAR-T product at the time of infusion ($p = 0.950$).

In linear regression analysis, only LDH levels at the time of apheresis ($p = 0.0002$) impacted the CAR+CD4$^+$/CD8$^+$ ratio in the final product, while other characteristics such as age ($p = 0.528$), number of previous lines of therapy of therapies or previous autologous stem cell transplantation ($p = 0.191$) did not show a significant influence.

Regarding the impact of the CAR+CD4$^+$/CD8$^+$ ratio on toxicities, most patients (29/31) experienced some grade of cytokine release syndrome (CRS), which was mild (Grade 1) in 6 patients, moderate (Grade 2) in 20 patients and more severe (Grade 3) in only 3 patients. The median onset of CRS occurred on Day 1 and the median duration was 4 days. Nine patients developed immune effector cell-associated neurotoxicity syndrome (ICANS), with (Grade 1 observed in three cases, Grade 2 in two cases, Grade 3 in three cases and Grade 4 in one case). The median onset of ICANS was observed at Day 5, and the median duration was 3 days. While the CAR+CD4$^+$/CD8$^+$ ratio did not show an association with the development of CRS graded 2 or higher ($p = 0.352$), patients who experienced ICANS exhibited lower CAR+CD4$^+$/CD8$^+$ ratios compared to those patients without ICANS (median ratio 0.83 vs. 2.03, $p = 0.015$). Using the cut-off ratio of 1.12, a lower CAR+CD4$^+$/CD8$^+$ ratio predicted the onset of ICANS of any grade with an odds ratio of 3.00 (95% CI, 0.16–2.03; $p = 0.021$).

The impact of infused CAR+CD4$^+$/CD8$^+$ ratio on outcomes was assessed. Response evaluation at 3 and 6 months following CAR-T infusion is highly predictive of the final outcome of CAR-T therapy. Patients classified as M3-responders had received bags containing higher percentages of CAR+CD8$^+$ cells compared to M3-non-responders (56% of CAR+CD8 vs. 27% $p = 0.02$). This difference was also observed between M6 responders and non-responders (55.4% vs. 27.3%, $p = 0.034$). Similarly, M3-responders had a lower CAR+CD4$^+$/CD8$^+$ ratio in the infused CAR-T products compared to non-responders (ratio 0.74 vs. 2.47, $p = 0.011$) (Figure 2A).

We next performed a ROC analysis to determine a cut point of 1.12 for the CAR+CD4$^+$/CD8$^+$ ratio to predict response at M3, which showed a 100% sensitivity and 70% specificity in identifying M3 responses (AUC 82%, $p = 0.001$) (Figure 2B).

Logistic regression analysis revealed that a CAR+CD4$^+$/CD8$^+$ ratio higher than the cut point of 1.12 was associated with an increased risk of treatment failure at M3 (odds ratio 23.3, 95% CI 1.99–273.29, $p = 0.012$) and M6 (odds ratio 10, 95% CI 4.35–10, $p = 0.028$). In the Kaplan–Meier curves of PFS, patients with a higher CAR+CD4$^+$/CD8$^+$ ratio exhibited a median PFS of only 76 days, whereas patients with a lower ratio did not reach the median PFS within 1 year of follow-up ($p = 0.047$). This resulted in a 6-month PFS of 76% for patients with a lower ratio versus 31%, for those with a higher ratio. (Figure 2C).

Given the observed differences in CAR+CD4$^+$/CD8$^+$ ratios across different CAR-T products, we further investigated the potential impact of the costimulatory domain (CD28 vs. 4-1BB) on the prognostic value of CAR+CD4/CD8 ratio. In a multivariate logistic regression analysis, we found that the CAR+CD4/CD8 ratio remained an independent predictor of response at M3 (OR 16.41, 95% CI 1.10–243.32, $p = 0.041$) regardless of the CAR-T costimulatory domain used (Table 2).

LDH measured at the time of apheresis was above the upper limit of normal (ULN) in 15 (48%) patients: those

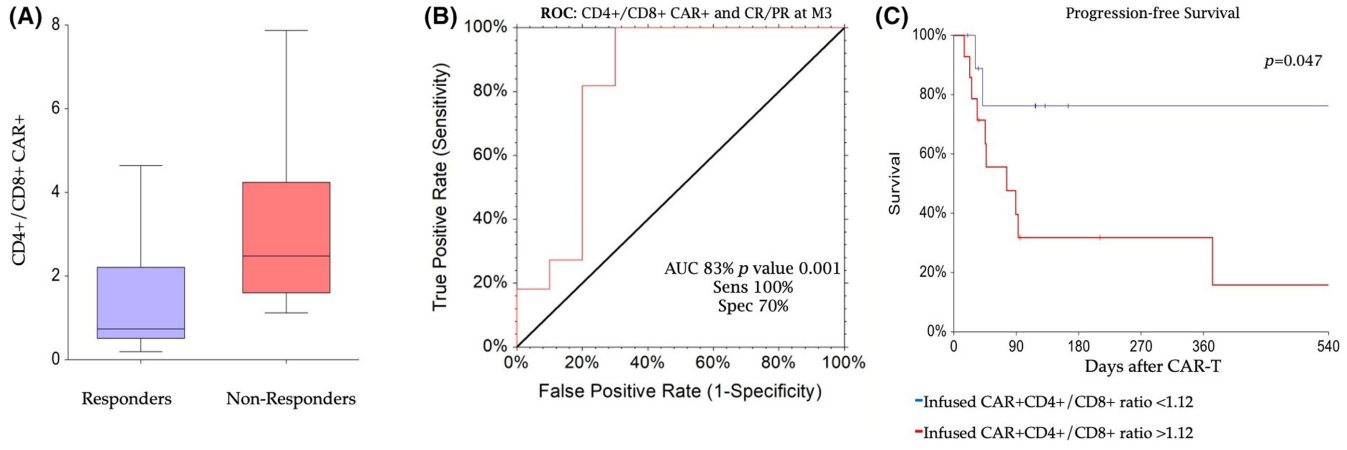

FIGURE 2 Impact of $CD4^+/CD8^+$ ratio among infused CAR-T on efficacy outcomes. (A) The difference of CAR+ $CD4^+/CD8^+$ ratio among responders (blue) and non-responders (red) at Month 3 (M3). (B) A ROC analysis identifies in 1.12 the best cut-off value of CAR+$CD4^+/CD8^+$ ratio in predicting response at M3. (C) progression-free survival of patients with infused CAR+$CD4^+/CD8^+$ ratio lower (blue) or higher (red) than the cut-off value of 1.12.

TABLE 2 Multivariate logistic regression: risk factors for M3 failure.

	Standard error/ (95% CI)	*p* value	Odds ratio
4-1BB (vs. CD28)	1.41/(−0.43–5.11)	0.098	10.35
CAR+CD4/CD8 ratio >1.12	1.37/(0.10–5.49)	0.041	16.41 (1.10–243.32)

patients had an inferior outcome of response at M3 ($p = 0.017$, OR 13.33, 95% CI 2.18–81.22), and at M6 ($p = 0.004$, OR 18.33, 95% CI 2.53–133.25). When compared in multivariate analysis, LDH at apheresis was not independent from the CAR+$CD4^+/CD8^+$ in predicting M3, nor M6, but showed only a trend (data not shown).

Furthermore, we monitored the CAR+ T cells in PB at multiple time points following infusion (see Supplementary Material). On Days 7 (D7) and 14 (D14), the median count of CAR+ T cells per microlitre peaked at 24 and 29, respectively. Subsequently, the median count declined, reaching 10 at M1 and 5 at M3.

Notably, the median CAR+$CD4^+/CD8^+$ ratio did not exhibit significant fluctuations across the diverse time points (0.43 at D7, 0.76 at D14, 0.63 at M1, and 0.47 at M3).

In linear regression analysis, the CAR+$CD4^+/CD8^+$ in the infused product did not influence the ensuing CAR+$CD4^+/CD8^+$ ratio measured at D7 ($p = 0.401$), D14 ($p = 0.632$) or M1 ($p = 0.746$). Notably, the ratios at these various time points did not exhibit the capability to predict disease response at M3 (data not shown).

DISCUSSION

The major finding of our study highlights the association between a lower CD4/CD8 ratio in the CAR product and a better response to CAR-T therapy. Notably, the effect remained independent of the type of CAR T construct and product used. Our results emphasize the significance of the CAR+/$CD8^+$ population in driving clinical responses, as these cells play a crucial role as major effector cells with potent cytotoxic functions. The unique immunological synapse formed by $CD8^+$ T cells enables faster engagement with the target and a swift release of granzyme and perforin, resulting in leading to efficient cell-to-cell contact-mediated killing.[9]

Our data are consistent with previous reports that underscore the importance of the CAR+/$CD8^+$ population in predicting clinical response to CAR therapy. For instance, Locke et al. analysed data from the ZUMA1 trial, utilizing axi-cel, and found that the number of $CD8^+$ cells in the infusion bag significantly correlated with durable responses.[11] They also observed that the number of infused CCR7+CD45RA+ T cells was associated with objective responses, suggesting that the presence of a more immature T-cell population, such as T naïve and T memory stem cells ($T_{N/SCM}$) cells, could be predictive of response. Cuffel et al. analysed the impact of the composition of the starting apheresis product for CAR-T-cell generation using tisa-cel.[4] They reported that CD4/CD8 ratio in the starting material had no impact on clinical response, while they observed a trend for a better PFS for patients with a lower CD4/CD8 ratio among the $T_{N/SCM}$ subset.[4] In addition, early responders had a higher proportion of CD8 $T_{N/SCM}$ in the starting apheresis product. Lamure et al. compared the T-cell composition between apheresis and infusion bags in 60 patients treated with either axi-cel or tisa-cel and found a significant increase in the proportion of $CD4^+$ cells during the culture, while the proportion of $CD8^+$ cells did not significantly change. These authors reported that increased percentages of both CD4 and CD8 CAR+ effector memory cells in the infused bags were associated with complete metabolic responses.[12] Monfrini et al. found that patients with a better in vivo expansion of CAR-T cells postinfusion, which was associated with a better clinical response, had a higher proportion of $CD8^+$ T central

memory cells in their final product.[13] In this study, the authors compared the CD4/CD8 ratio between tisa-cel and axi-cel and found a significantly higher CD4/CD8 ratio in tisa-cel products, which is in line with our finding.

However, our study has several limitations. We were unable to analyse the impact of the absolute number of T-cell subsets since the manufacturers mostly did not provide the absolute cell counts in the final products. Additionally, the data on T-cell maturation subsets were incomplete for comprehensive analysis. We found a correlation between LDH levels at the time of apheresis and a higher CAR+CD4$^+$/CD8$^+$ ratio, which led to a worse prognosis. Although the multivariate analysis did not demonstrate an independent effect of the two variables, this association suggests that higher disease activity not only predicts unfavourable outcomes by itself but also affects the quality and potential of manufactured CAR-T cells. While an assessment of tumour burden using PET imaging has not been conducted formally in our study, this aspect remains a potential avenue for exploration in future investigations.

CONCLUSION

The findings from our study provide valuable insights into the T-cell composition and its impact on outcomes in patients receiving CAR-T therapy for aggressive B-cell lymphoma. A higher percentage of CAR+CD8$^+$ cells in the infused CAR-T bags is a significant predictor for response at 3 and 6 months following treatment with CD19-CAR-T. Furthermore, a CAR+CD4$^+$/CD8$^+$ ratio in infused bags above the defined cut-off of 1.12 predicts unfavourable PFS and M3 response, regardless of the specific costimulatory domain of the CAR-T product used. These findings provide valuable support for the critical role played by CD8-mediated cytotoxic mechanisms of CAR-T cells in achieving therapeutic efficacy, importantly, these results suggest that strategies aimed at optimizing the composition of manufactured CAR-T products, such as balancing the CAR+ CD4$^+$/CD8$^+$ ratio, may hold potential for enhancing treatment outcomes.

AUTHOR CONTRIBUTIONS

Eugenio Galli, Silvia Bellesi and Stefan Hohaus conceived the paper and prepared the manuscript. Silvia Bellesi, Camilla Iacovelli, Giacomo Di Cesare, Rosalia Malafronte and Elena Maiolo performed laboratory testing and analysed data. Eugenio Galli performed statistical analysis. Ilaria Pansini and Eugenio Galli collected clinical data of patients. Federica Sorà and Simona Sica provided reagents and statistical software. Eugenio Galli, Silvia Bellesi, Elena Maiolo, Rosalia Malafronte, Federica Sorà, Patrizia Chiusolo, Simona Sica and Stefan Hohaus clinically managed patients. All authors critically reviewed the paper.

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CONFLICT OF INTEREST STATEMENT

The authors declare no relevant conflict of interest.

DATA AVAILABILITY STATEMENT

For data availability, please contact the corresponding author.

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