

[ORIGINAL ARTICLE]

Activated CD4⁺ T Cell Proportion in the Peripheral Blood Correlates with the Duration of Cytokine Release Syndrome and Predicts Clinical Outcome after Chimeric Antigen Receptor T Cell Therapy

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Abstract:

Objective Chimeric antigen receptor (CAR) T cell therapy is an emerging and effective therapy for relapsed or refractory diffuse large B cell lymphoma (R/R DLBCL). The characteristic toxicities of CAR T cell therapy include cytokine release syndrome (CRS) and prolonged cytopenia. We investigated the factors associated with these complications after CAR T cell therapy by analyzing lymphocyte subsets following CAR T cell infusion.

Methods We retrospectively analyzed peripheral blood samples on days 7, 14, and 28 after tisagenlecleucel (tisa-cel) infusion by flow cytometry at our institution between June 2020 and September 2022.

Patients Thirty-five patients with R/R DLBCL who received tisa-cel therapy were included.

Results A flow cytometry-based analysis of blood samples from these patients revealed that the proportion of CD4⁺CD25⁺CD127⁺ T cells (hereafter referred to as “activated CD4⁺ T cells”) among the total CD4⁺ T cells on day 7 after tisa-cel infusion correlated with the duration of CRS ($r=0.79$, $p<0.01$). In addition, a prognostic analysis of the overall survival (OS) using time-dependent receiver operating characteristic curves indicated a significantly more favorable OS and progression-free survival of patients with a proportion of activated CD4⁺ T cells among the total CD4⁺ T cells <0.73 ($p=0.01$, and $p<0.01$, respectively).

Conclusion These results suggest that the proportion of activated CD4⁺ T cells on day 7 after tisa-cel infusion correlates with the CRS duration and predicts clinical outcomes after CAR T cell therapy. Further studies with a larger number of patients are required to validate these observations.

Key words: chimeric antigen receptor T cell therapy, diffuse large B cell lymphoma, flow cytometry, cytokine release syndrome, prolonged cytopenia

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Introduction

Chimeric antigen receptor (CAR) T cell therapy targeting CD19 has demonstrated high efficacy against relapsed or refractory diffuse large B cell lymphoma (R/R DLBCL),

which is resistant to multiple lines of chemotherapy (1-3). Characteristic toxicities, such as cytokine release syndrome (CRS) or prolonged cytopenia, occur after CAR T cell therapy (4, 5). CRS can be a severe life-threatening complication after CAR T cell therapy, with 2-22% of patients reportedly developing grade ≥ 3 CRS that requires multidisci-

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plinary treatment and the median duration of CRS lasting approximately 1 week (1-3). In addition, 38-93% of patients reportedly develop cytopenia 3-4 weeks after CAR T cell therapy (6-12), which predisposes patients to infection and reduces their quality of life.

Several risk factors are associated with CRS severity after CAR T cell therapy, including a high disease burden, infused CAR T cell number, peak number of expanded CAR T cells, thrombocytopenia, and endothelial activation prior to CAR T cell infusion (13-15). However, an association between CRS and prolonged cytopenia has also been reported (6, 8, 10, 12).

The mechanism underlying the development of CRS after CAR T cell therapy is presumed to be as follows: CAR T cells engage with the corresponding target antigen cells and are activated, triggering the release of inflammatory cytokines and chemokines, such as interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), granulocyte macrophage colony-stimulating factor, interleukin (IL)-2, IL-8, and IL-10, which in turn activate bystander monocytes and macrophages, resulting in the release of IL-1, IL-6, and nitric oxide (16-19). A recent study using single-cell RNA sequencing of CAR T cell products and cell-free DNA sequencing of patient plasma reported that cellular and molecular features of CAR T cell infusion products representing the functional status of CAR T cells (e.g. exhausted CD8⁺ or CD4⁺ cells) are associated with the development of high-grade CRS (20). Although these studies have provided key molecular insights into complications after CAR T cell infusion, clinically feasible methods of predicting these complications are required.

The present study therefore investigated the factors associated with CRS severity and duration, prolonged cytopenia, and clinical outcomes after CAR T cell therapy by analyzing lymphocyte subsets in the blood of R/R DLBCL patients after CAR T cell infusion.

Materials and Methods

Patient selection

Patients over 20 years old who received tisagenlecleucel (tisa-cel) for R/R DLBCL, including *de novo* DLBCL and transformed DLBCL from follicular lymphoma, at Okayama University Hospital between June 2020 and September 2022 were included in this study. Patient data were retrospectively collected from the medical records. Informed consent was obtained via an opt-out approach, and the protocol was approved by the Institutional Review Board of Okayama University Hospital.

Definitions

The performance status was graded according to the Eastern Cooperative Oncology Group (ECOG) guidelines (21). DLBCL cells of origin were classified using Hans algorithm (22). Patients who did not achieve a complete re-

sponse (CR) after any treatment, including autologous hematopoietic stem cell transplantation (auto-HSCT), were defined as “primary refractory.” The clinical stage, treatment response before tisa-cel infusion, and disease status on day 28 after tisa-cel infusion were assessed according to the Lugano classification (23).

CRS and immune effector cell-associated neurotoxicity syndrome (ICANS) were graded according to the consensus grading criteria defined by the American Society of Transplantation and Cellular Therapy (ASTCT) (24). The duration of CRS was defined as the period from the first day when the patient’s axillary temperature was $\geq 38^{\circ}\text{C}$ (onset) to the day before the first of 2 consecutive days when the patient’s axillary temperature was $< 37.5^{\circ}\text{C}$ without treatment for hypotension and/or hypoxia and use of antipyretics. Cytopenia was graded according to the National Cancer Institute’s Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 and classified into three categories: granulocyte-colony stimulating factor (G-CSF) dependence, red blood cell (RBC) transfusion dependence, and platelet concentrate (PC) transfusion dependence. G-CSF dependence on day 28 was defined as neutropenia (neutrophils $< 0.5 \times 10^9/\text{L}$) from days 22 to 28 after tisa-cel infusion. RBC or PC transfusion dependence on day 28 was defined as anemia (hemoglobin $< 7.0 \text{ g/dL}$) or thrombocytopenia (platelet count $< 20 \times 10^9/\text{L}$) from day 22 to 28 after tisa-cel infusion, respectively.

The overall survival (OS) was defined as the time between tisa-cel infusion and the last follow-up appointment or death. The progression-free survival (PFS) was defined as the time interval from tisa-cel infusion to relapse [patients with complete response (CR) after tisa-cel infusion], disease progression [patients with partial response (PR) or less after tisa-cel infusion], or death in remission, whichever occurred first.

Flow cytometry

Flow cytometric analyses were performed on peripheral blood samples on days 7, 14, and 28 after tisa-cel infusion. All products were purchased from Beckman Coulter (Brea, USA) and were used according to the manufacturer’s instructions. RBCs were lysed with VersaLyse Lysing Solution and stained with the following fluorochrome-conjugated antibodies: anti-CD3 (UCHT1), anti-CD4 (13B8.2), anti-CD8 (B9.11), anti-CD25 (B1.49.9), anti-CD45 (J33), anti-CD127 (R34.34), anti-CD45RA (2H4LDH11LDB9), anti-CD62L (DREG56), and anti-programmed cell death-1 (PD-1) (PD1.3). The samples were analyzed using a NAVIOS™ flow cytometer (Beckman Coulter, Brea, USA), and the data were analyzed using the FlowJo software program (TreeStar, Ashland, USA).

Statistical analyses

The Mann-Whitney U test was used to compare continuous variables, and Fisher’s exact test was used to compare categorical variables. Spearman’s rank correlation was used to determine the associations between continuous variables.

Table 1. Baseline Patient and Disease Characteristics before Tisa-cell Infusion.

		Total (35)
Age (years), median		58 (45-73)
Sex (%)	Male	22 (62.8)
	Female	13 (37.2)
ECOG-performance status* (%)	0-1	30 (85.7)
	2-4	5 (14.3)
Disease (%)	DLBCL	30 (85.7)
	Transformed FL	5 (14.3)
Cell of origin† (%)	GCB type	11 (31.4)
	Non-GCB type	12 (34.3)
	NA	12 (34.3)
Past clinical course (%)	Primary refractory‡	11 (31.4)
	Relapse	24 (68.6)
Previous chemotherapy and/or radiation therapy (lines), median		4 (2-6)
Previous auto-HSCT (%)	No	21 (60.0)
	Yes	14 (40.0)
Clinical stage before lymphodepleting chemotherapy§ (%)	1-2	13 (37.1)
	3-4	15 (42.9)
	NA (3rd CR)	7 (20.0)
Cytopenia (CTCAE grade ≥2) before lymphodepleting chemotherapy (%)	Neutrophil<1.5 (×10 ⁹ /L)	12 (34.3)
	Hemoglobin<10.0 (g/dL)	23 (65.7)
	Platelet<75 (×10 ⁹ /L)	6 (17.1)

*Graded according to the ECOG guidelines (21). †Defined according to the Hans algorithm (22). ‡Defined as a patient who did not achieve complete response after several treatments.

§Defined according to the Lugano classification (23).

auto-HSCT: autologous hematopoietic stem cell transplantation, CR: complete response, CTCAE: common terminology criteria for adverse events, DLBCL: diffuse large B-cell lymphoma, ECOG: Eastern Cooperative Oncology Group, FL: follicular lymphoma, GCB: germinal center B-cell-like, NA: not available, tisa-cel: tisagenlecleucel

Time-dependent receiver operating characteristic curve analyses for the OS were performed to determine the optimal cutoff for the proportion of lymphocyte subsets using a previously described approach (25). The probabilities of the OS and PFS were assessed using the Kaplan-Meier method, and the groups were compared using the log-rank test. All statistical tests were two-tailed, and statistical significance was set at $p < 0.05$.

All statistical analyses were performed using the GraphPad Prism 9 software program (GraphPad Software, San Diego, USA).

Results

Patient characteristics

Patient characteristics before tisa-cel infusion are summarized in Table 1. A total of 35 patients were included in the study, with a median age of 58 (range: 45-73) years old. All patients were diagnosed with R/R DLBCL, among which five had transformed from follicular lymphoma. The median number of prior lines of chemotherapy and/or radiation ther-

apy was 4 (range: 2-6), and 40% of the patients had received auto-HSCT. All patients received cyclophosphamide (250 mg/m²) and fludarabine (25 mg/m²) as lymphocyte-depleting chemotherapy (LDC) on days -5, -4, and -3, followed by tisa-cel infusion on day 0. Eighty percent of the patients had non-CR at tisa-cel infusion. CTCAE grade ≥2 neutropenia, anemia, and thrombocytopenia before LDC were observed in 12 (34.3%), 23 (65.7%), and 6 (17.1%) patients, respectively.

CAR T cell dose, cytokine release syndrome, prolonged cytopenia, and treatment response

The characteristics of patients after tisa-cel infusion are summarized in Table 2. The median CAR T cell dose was 3.3×10^8 (range: 1.2 - 5.2×10^8). Most patients (82.9%) developed grade 1 or 2 CRS, and 1 patient (2.9%) developed grade 4 CRS requiring mechanical ventilator support and hemodialysis. All but 1 patient who developed CRS were treated with tocilizumab, and approximately 60% of them required corticosteroids to control CRS. The median time from tisa-cel infusion to corticosteroid initiation was 4 days (range: 0-6 days). The median onset of CRS was 3 days

Table 2. Patient Characteristics after Tisa-cell Infusion.

		Total (35)
Infused CAR T cell dose ($\times 10^8$)		3.3 (1.2-5.2)
CRS grade*	0	5 (14.3)
	1-2	29 (82.9)
	≥ 3	1 (2.9)
Onset of CRS (days after tisa-cel infusion), median		3 (0-8)
Duration of CRS (days), median†		5 (0-33)
ICANS grade*	0	33 (94.3)
	1-2	2 (5.7)
	≥ 3	0 (0.0)
Use of tocilizumab (%)		29 (82.9)
Use of corticosteroids (%)		18 (51.4)
Time from tisa-cel infusion to corticosteroids initiation (n=18)		4 (2-6)
Peak ferritin level from day 0 to 28 after tisa-cel infusion (ng/mL), median		1,056 (224-11,276)
Prolonged cytopenia on day 28 after tisa-cel infusion (%)	G-CSF dependence‡	22 (62.9)
	RBC transfusion dependence‡	8 (22.9)
	PC transfusion dependence‡	12 (34.3)
Disease status on day 28 after tisa-cel infusion§ (%)	CR or PR	26 (74.3)
	SD or PD	9 (25.7)
Follow-up (days), median		219 (28-760)

*Graded according to the consensus grading criteria defined by the American Society of Transplantation and Cellular Therapy (24). †Defined as the period from the first day when the axillary temperature was $\geq 38^\circ\text{C}$ (onset) to the day before the first of two consecutive days when the axillary temperature was $<37.5^\circ\text{C}$ without treatment for hypotension and/or hypoxia and use of antipyretics. ‡Defined as a patient with neutropenia (neutrophils $<0.5 \times 10^9/\text{L}$), anemia (hemoglobin $<7.0 \text{ g/dL}$), and thrombocytopenia (platelet count $<20 \times 10^9/\text{L}$) from days 22 to 28 after tisa-cel infusion. §Defined according to the Lugano classification (23).

CAR: chimeric antigen receptor, CR: complete response, CRP: C-reactive protein, CRS: cytokine release syndrome, G-CSF: granulocyte-colony stimulating factor, ICANS: immune effector cell-associated neurotoxicity syndrome, NA: not available, PC: platelet concentrate, PD: progressive disease, PR: partial response, RBC: red blood cell, SD: stable disease, tisa-cel: tisagenlecleucel

(range: 0-8 days) after tisa-cel infusion, and the median duration of CRS was 5 days (range: 0-33 days). Only one patient developed ICANS. On day 28 after tisa-cel infusion, 22 (62.9%), 8 (22.9%), and 12 (34.3%) patients were dependent on G-CSF use, RBC transfusion, and PC transfusion, respectively. The overall response rate [ORR (CR+PR)] on day 28 after tisa-cel infusion was 74.3%, and the median follow-up period was 219 (range: 28-760) days.

Association of the proportion of activated CD4⁺ T cells among total CD4⁺ T cells on day 7 with the CRS duration

The absolute number of T cells in the blood on day 7 after tisa-cel infusion (median: 599/ μL , range: 67-5,053/ μL) was not significantly different between patients with and without CRS (Supplementary material 1A). Thus, we hypothesized that specific lymphocyte subsets might be associated with CRS in patient T cells.

First, we analyzed whether or not the CD4⁺/CD8⁺ T cell ratio on day 7 after tisa-cel infusion was associated with CRS. Although the CD4⁺/CD8⁺ T cell ratio on day 7 after

tisa-cel infusion was comparable between patients with and without CRS (Supplementary material 1B), the value was positively correlated with the duration of CRS (Fig. 1A, $r=0.34$, $p=0.046$), suggesting that a cell population associated with the CRS duration might be present in CD4⁺ T cells. CD25, also known as the IL-2 receptor (IL-2R) α chain, is expressed on the surface of activated T cells and regulatory T cells (Tregs) (26) and can be distinguished by their expression of CD127, also known as the IL-7 receptor α chain (27). Therefore, we defined CD4⁺CD25⁺CD127⁺ T cells as “activated CD4⁺ T cells” (Fig. 1B). We compared the absolute number of activated T cells on day 7 in patients with and without CRS and found no significant differences between the two groups (Supplementary material 1C).

Next, we analyzed the proportion of activated CD4⁺ T cells among the total CD4⁺ T cells in patient blood on day 7 after tisa-cel infusion. Although the number of patients who did not develop CRS was limited (five patients), patients with CRS had a significantly higher proportion of activated CD4⁺ T cells among the total CD4⁺ T cells and a significantly lower proportion of CD4⁺CD25⁺CD127^{low} T cells

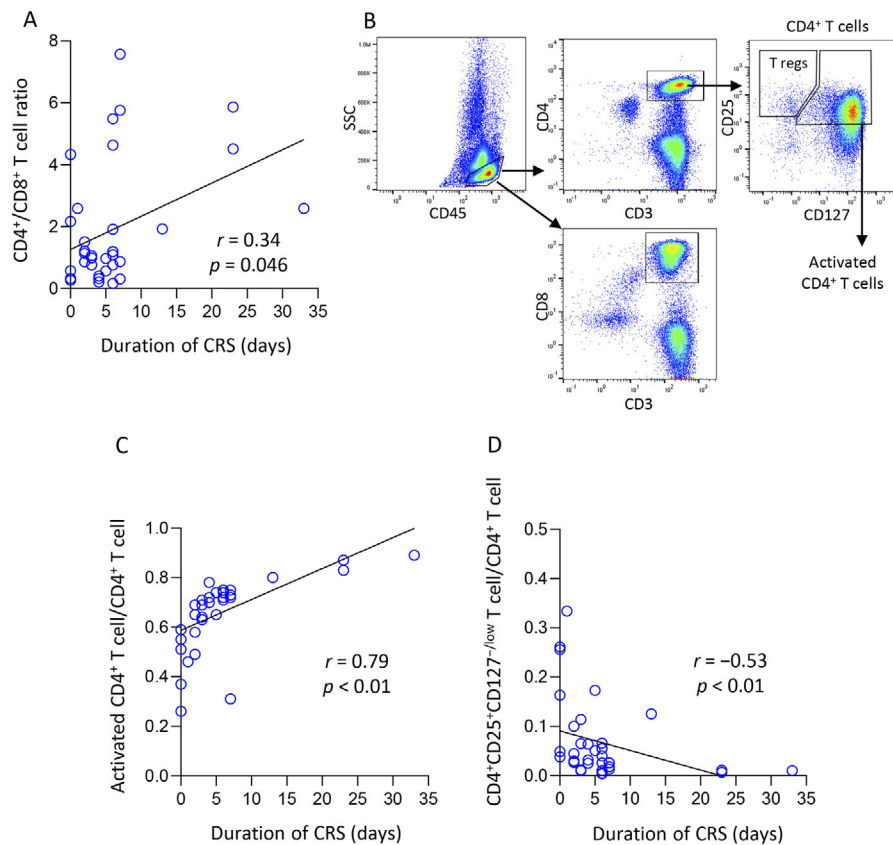


Figure 1. The CD4⁺/CD8⁺ T cell ratio and proportion of activated CD4⁺ T cells among total CD4⁺ T cells on day 7 after tisa-cel infusion predicts the duration of cytokine release syndrome (CRS). (A) Correlation between CD4⁺/CD8⁺ T cell ratio on day 7 after tisa-cel infusion and the CRS duration. (B) Gating strategy defining activated T cells by the flow cytometric analysis of peripheral blood. Correlations between the proportion of (C) activated CD4⁺ T cell or (D) CD4⁺CD25⁺CD127^{low} CD4⁺ T cells among total CD4⁺ T cells and the CRS duration.

among the total CD4⁺ T cells than patients without CRS (Supplementary material 1D, E). Furthermore, the proportion of activated CD4⁺ T cells on day 7 was more strongly correlated with the CRS duration than with the absolute number of activated CD4⁺ T cells (Fig. 1C, $r = 0.79$, $p < 0.01$, Supplementary material 1F). Conversely, the proportion of CD4⁺CD25⁺CD127^{low} T cells among total CD4⁺ T cells was negatively correlated with the CRS duration (Fig. 1D; $r = -0.53$, $p < 0.01$).

In our study, corticosteroids were initiated on day 6 in patients who received corticosteroid therapy for CRS (Table 2). Therefore, it is possible that the use of corticosteroids might have influenced the proportion of activated CD4⁺ T cells on day 7. Thus, we analyzed whether or not corticosteroid administration influenced the proportion of activated T cells in 30 patients who developed CRS (30 patients). The proportion of activated CD4⁺ T cells among the total CD4⁺ T cells on day 7 was significantly higher in patients who received corticosteroid therapy than in those who did not (Supplementary material 1G). Next, as activated T cells express IL-2R on their cell surface, and the soluble portion of IL-2R is known to be released extracellularly as soluble IL-2R (sIL-2R) (28), we investigated the relationship

between sIL-2R levels and the proportion of activated CD4⁺ T cells among total CD4⁺ T cells on day 7 after tisa-cel infusion. Despite the limited number of patients (eight patients), sIL-2R levels and the proportion of activated T cells showed a strong correlation (Supplementary material 1H).

To further explore the relationship between specific subsets of T cells and CRS, we analyzed memory or exhausted CD4⁺ T cells or CD8⁺ T cells (29, 30) in patient blood on day 7 after tisa-cel infusion (Fig. 2A). Neither the proportion of memory or exhausted CD4⁺ T cells among the total CD4⁺ T cells nor the proportion of memory or exhausted CD8⁺ T cells among the total CD8⁺ T cells was associated with the CRS duration (Fig. 2B-E). The proportion of memory or exhausted CD4⁺ T cells among the total CD4⁺ T cells and memory CD8⁺ T cells among the total CD8⁺ T cells did not differ markedly between patients with and without CRS (Supplementary material 2A-C). The proportion of exhausted CD8⁺ T cells among total CD8⁺ T cells tended to be higher in patients who developed CRS than in those who did not (Supplementary material 2D).

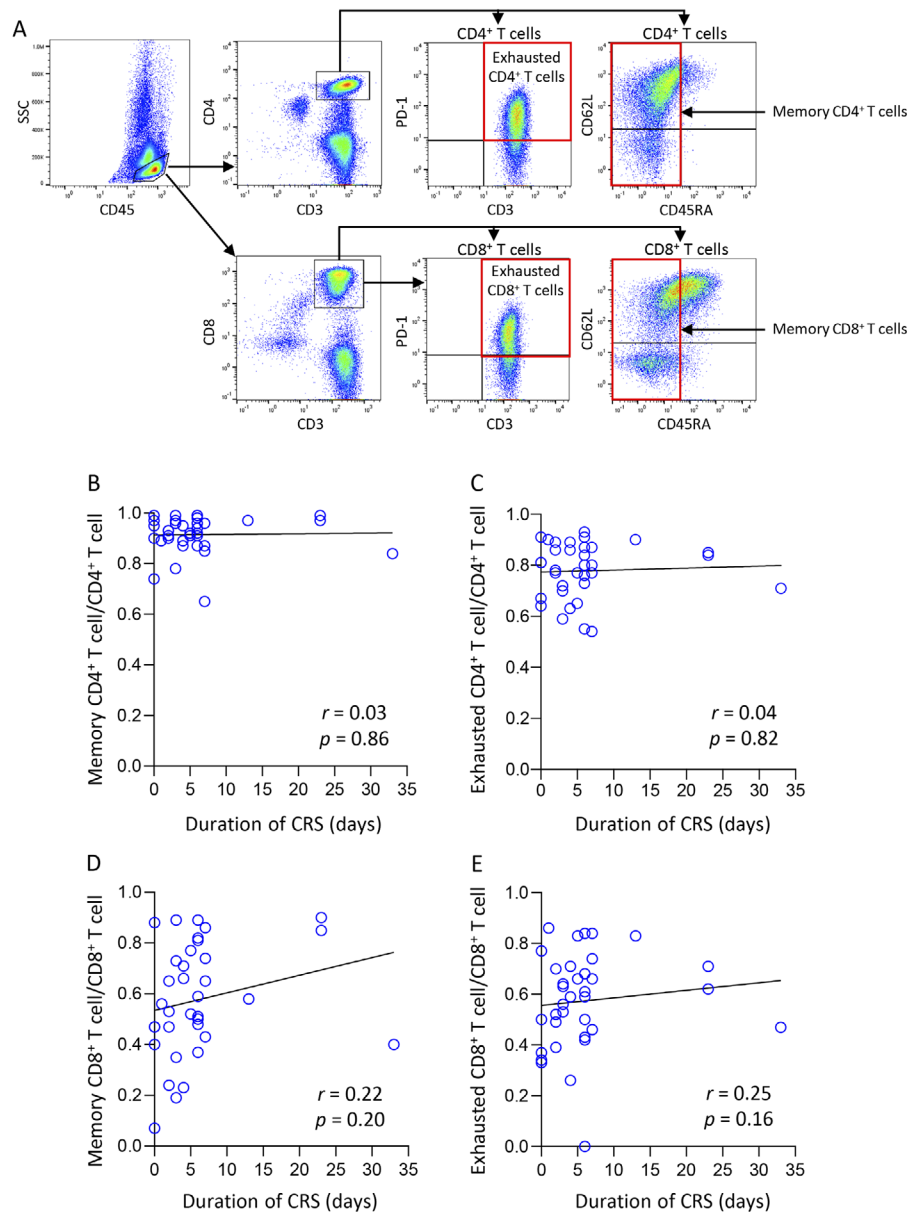


Figure 2. Proportion of memory or exhausted CD4⁺ T cells among total CD4⁺ T cells and those CD8⁺ T cells among total CD8⁺ T cells does not correlate with the duration of cytokine release syndrome (CRS). (A) Gating strategy defining memory or exhausted T cell subsets by the flow cytometric analysis of peripheral blood. Correlations between the proportion of (B) memory or (C) exhausted CD4⁺ T cells among total CD4⁺ T cells on day 7 after tisa-cel infusion and the CRS duration. Correlations between the proportion of (D) memory or (E) exhausted CD8⁺ T cells among total CD8⁺ T cells on day 7 after tisa-cel infusion and the CRS duration.

Association of the proportion of activated CD4⁺ T cells among CD4⁺ T cells on day 7 with the clinical outcome

Next, we evaluated whether or not the proportion of activated CD4⁺ T cells among CD4⁺ T cells on day 7 predicted the clinical outcomes in patients treated with tisa-cel. To verify the optimal cutoff value for the proportion of activated CD4⁺ T cells, we calculated time-dependent receiver operating characteristic curves for the OS. A cutoff value of 0.73 for the proportion of activated CD4⁺ T cells (area under the curve: 0.787) distinguished 22 patients (62.9%) with a

low proportion of activated CD4⁺ T cells (<0.73) and 13 patients (37.1%) with a high proportion of activated CD4⁺ T cells (≥0.73) (Table 3). Patients with a high proportion of activated CD4⁺ T cells had significantly higher lactate dehydrogenase (LDH) levels before LDC and a longer duration of CRS than those with a low proportion of these cells. In addition, the dose of infused CAR T cells, the proportion of patients who developed CRS (grade ≥1) after tisa-cel infusion, the proportion of patients who accordingly used tocilizumab, and the peak ferritin level from day 0 to 28 after tisa-cel infusion tended to be higher in patients with a high proportion of activated CD4⁺ T cells. The 6-month OS and

Table 3. Relationship between Activated CD4⁺ T Cell/CD4⁺ T Cell Ratio on Day 7 after Tisa-cel Infusion and Clinical Outcome.

		Activated CD4 ⁺ T cell/CD4 ⁺ T cell		p value
		Low (22) (<0.73)	High (13) (≥0.73)	
Lymphocyte count before apheresis (×10 ⁹ /L), median		1.59 (0.49-4.91)	1.85 (0.15-9.79)	0.47
Clinical stage before lymphodepleting chemotherapy* (%)	NA (3rd CR)	6 (27.3)	1 (7.7)	0.36
	1-2	8 (36.4)	5 (38.5)	
	3-4	8 (36.4)	7 (53.8)	
Platelet count before lymphodepleting chemotherapy (×10 ⁹ /L), median		175 (49-252)	165 (49-394)	0.84
LDH before lymphodepleting chemotherapy (IU/L), median		208 (136-340)	257 (163-1,250)	0.03
Present of bulky mass		0 (0.0)	0 (0.0)	1.00
Infused CAR T cell dose (×10 ⁸), median		3.0 (1.2-5.2)	3.9 (2.5-4.8)	0.06
CRS grade [‡]	0	5 (22.7)	0 (0.0)	0.09
	1-2	17 (77.3)	12 (92.3)	
	≥3	0 (0.0)	1 (7.7)	
Duration of CRS (days), median [‡]		3 (0-7)	6 (4-33)	<0.01
Use of tocilizumab (%)		16 (72.7)	13 (100.0)	0.06
Use of corticosteroids (%)		9 (40.9)	9 (69.2)	0.16
Peak lymphocyte count (×10 ⁹ /L) [§]		1.31 (0.59-7.18)	1.32 (0.65-3.97)	0.81
Peak ferritin level (ng/mL) [§]		914 (224-8,207)	1,821 (237-11,276)	0.09
Disease status on day 28 after tisa-cel infusion [†] (%)	CR or PR	18 (81.8)	8 (61.5)	0.24
	SD or PD	4 (23.5)	5 (38.5)	
Follow-up (days), median		272 (28-760)	198 (33-526)	0.21

*Defined according to the Lugano classification (23). [‡]Graded according to the consensus grading criteria defined by the American Society of Transplantation and Cellular Therapy (24). [‡]Defined from the first day when the axillary temperature was ≥38°C (onset) to the day before the first of two consecutive days when the axillary temperature was <37.5°C without treatment for hypotension and/or hypoxia and use of antipyretics. [§]Time from day 0 to 28 after tisa-cel infusion.

CAR: chimeric antigen receptor, CR: complete response, CRS: cytokine release syndrome, LDH: lactate dehydrogenase, NA: not available, PD: progressive disease, PR: partial response, SD: stable disease, tisa-cel: tisagenlecleucel

PFS were significantly higher in patients with a low proportion of activated CD4⁺ T cells than those with a high proportion of these cells (Fig. 3A, B, $p=0.01$, and $p<0.01$, respectively), indicating that the proportion of activated CD4⁺ T cells on day 7 was not only correlated with the duration of CRS but also effectively predicted clinical outcomes. Interestingly, despite the limited number of patients, the proportion of activated CD4⁺ T cells on days 14 and 28 among CD4⁺ T cells was not associated with the OS but was significantly associated with the PFS (Supplementary material 3A-D).

Association of the proportion of activated CD4⁺ T cells among CD4⁺ T cells on day 7 with prolonged cytopenia after CAR T cell therapy

Several studies have suggested an association between prolonged cytopenia and the CRS grade (6, 8, 12); however, the effect of CRS duration on prolonged cytopenia has not been reported. Thus, we evaluated the association between the proportion of activated CD4⁺ T cells on day 7 and prolonged cytopenia on day 28 after tisa-cel infusion. The proportion of activated CD4⁺ T cells on day 7 was not related to dependence on G-CSF, RBC transfusion, or PC transfu-

sion on day 28 (Supplementary material 4A-C). Among the factors reportedly associated with prolonged cytopenia (7, 8, 10-12), the neutrophil count before LDC was significantly associated with G-CSF dependence on day 28 (Supplementary material 4D). In contrast, neither the hemoglobin level nor the platelet count before LDC was associated with RBC or PC transfusion dependence (Supplementary material 4E, F). Peak ferritin levels from day 0 to 28 after tisa-cel infusion were associated with RBC and PC transfusion dependence but not with G-CSF dependence on day 28 (Supplementary material 4G-I). These results concurred with those of a previous study in which peak ferritin and ferritin levels higher than the normal upper limit on day 30 were associated with prolonged hematological toxicity (10).

Sensitivity analyses

To assess whether or not the above results could be reproduced even when the absolute number of T cells was low, we divided patients into two groups based on the median absolute number of T cells on day 7 after tisa-cel infusion and performed sensitivity analyses exclusively in the 18 with a low absolute number of T cells. The results were similar

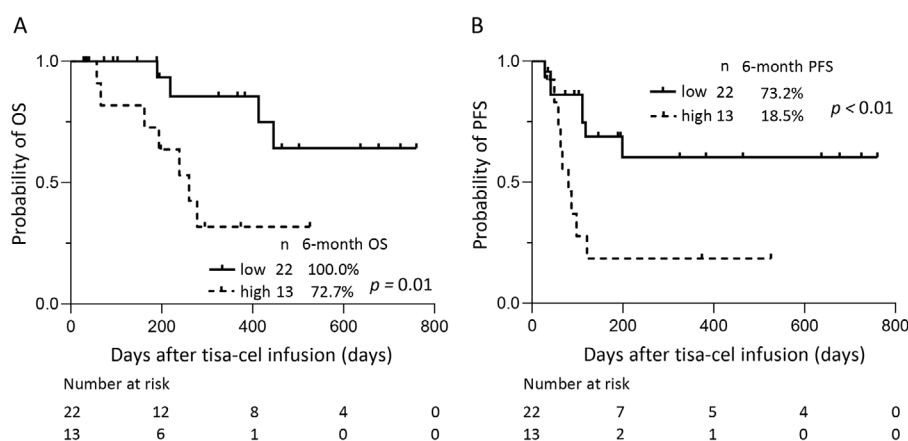


Figure 3. Relationship between the proportion of activated CD4⁺ T cells among total CD4⁺ T cells on day 7 and the clinical outcome. (A) The overall survival and (B) the progression-free survival. The solid line indicates a low proportion of activated CD4⁺ T cells (<0.73) (n=22). The broken line indicates a high proportion of activated CD4⁺ T cells (≥0.73) (n=13).

to those derived from the primary analyses (Supplementary material 5A-E).

Discussion

Our results illustrate the potential effectiveness of using the proportion of activated CD4⁺ T cells among total CD4⁺ T cells on day 7 after tisa-cel infusion, which was correlated with the duration of CRS, to predict clinical outcomes. In our study, the proportion, rather than the absolute number, of T cells or activated T cells on day 7 was more strongly associated with the development and duration of CRS. These results might be due, in part, to the large variation in T cell number among patients on day 7 after tisa-cel infusion (median: 599/μL, range: 67-5,053/μL). In line with our observations, previous studies have indicated that the proportion of T cell subsets in CAR T cell products or peripheral blood is associated with the efficacy or complications of CAR T cell therapy (31-35).

A previous report suggested that tocilizumab did not affect T cell proliferation, whereas corticosteroids did, in cases of CRS following bispecific antibody therapy (36). In addition, corticosteroids have been reported to suppress the IL-2R expression and induce T-cell apoptosis in a dose-dependent manner (37, 38). Based on these considerations, the increase in the proportion of activated T cells on day 7 in patients receiving corticosteroids may be underestimated.

To date, several studies have attempted to determine the predictive factors for severe CRS after CAR T cell infusion; however, precise factors associated with the CRS duration have not yet been reported. Since CRS is a consequence of excessive cytokine release from immune cells triggered by CAR T cell expansion, previous studies have evaluated inflammatory cytokines in the blood of patients (13-15, 39, 40). Macrophages and monocyte lineage cells activated by CAR T cells are major sources of IL-6, a signature mediator of CRS (13, 18, 19). The roles of specific lymphocyte subsets

in CRS after CAR T cell therapy have not been clarified. However, CD4⁺ T cells are known to differentiate into several subsets with distinct effector cell functions, including type 1 helper T cells (Th1), which produce large amounts of IFN-γ and activate macrophages involved in the cytokine storm (41, 42). Thus, our finding that the proportion of activated CD4⁺ T cells was strongly correlated with the duration of CRS suggests that Th1, an activated CD4⁺ T cell subset, may be associated with CRS pathogenesis. In contrast, Tregs, whose role in CRS remains unclear, are characterized by their ability to suppress immunity and prevent the deleterious effects of helper T cells, including Th1. In addition, Tregs are known to be important in preventing late-phase expansion of CAR T cells and ameliorating the pathophysiology of virus-associated acute lung injury by inhibiting the cytokine storm (43-45). Thus, our finding of a negative correlation between the CD4⁺CD25⁺CD127^{low} T cell/CD4⁺ T cell ratio and the CRS duration suggests that this T cell population might possess a function similar to that of Tregs (26, 27).

Regarding clinical outcomes, the proportion of activated CD4⁺ T cells was not associated with ORR on day 28 after tisa-cel infusion; however, the OS and PFS at six months were significantly better in patients with a low proportion of activated CD4⁺ T cells than those with a high proportion of these cells. The JULIET trial reported that patients who maintained PR or better for three months after tisa-cel infusion had an improved PFS (1). In the current study, among 26 patients with PR or better on day 28 after tisa-cel infusion, 8 relapsed, and 0 of 3 and 4 of 5 patients relapsed by 3 months in the low and high activated CD4⁺ T cell proportion groups, respectively. This result suggests that the proportion of activated CD4⁺ T cells on day 7 may be a useful predictor for identifying patients with a poor prognosis among those who achieve PR or better on day 28 after tisa-cel infusion. LDH levels before LDC represent the tumor volume, and a lower LDH level before LDC has been

shown to be associated with durable remission after CAR T cell therapy (46, 47). In our cohort, patients with a higher proportion of activated CD4⁺ T cells exhibited significantly higher LDH levels before LDC and worse outcomes than those with a low proportion of these cells, consistent with previous reports.

Prolonged cytopenia is another undesirable event after CAR T cell therapy, which is hypothesized to be the result of inflammatory damage to the bone marrow induced by CRS combined with a low bone marrow stem cell reserve due to prior chemotherapy (7, 48, 49). In our analysis, neither neutrophil nor hemoglobin nor platelet recovery on day 28 after tisa-cel infusion was associated with the proportion of activated CD4⁺ T cells among CD4⁺ T cells. Previous studies have reported that severe CRS (grade ≥ 3) is an independent predictive factor for cytopenia following CAR T cell therapy (6, 8, 12). The proportion of activated CD4⁺ T cells among CD4⁺ T cells on day 7 was higher in patients who developed CRS than in those who did not develop CRS. However, whether this proportion is related to the severity of CRS or cytopenia following severe CRS is unclear, since our study included only one patient with severe CRS (grade 3-4).

Several limitations associated with the present study warrant mention. First, it was conducted retrospectively on a limited number of patients at a single institution. Second, this analysis only included patients who received tisa-cel, and the toxicity of CAR T cell therapies may vary depending on the CAR T cell construct (1-3). Thus, the application of the findings of this study to patients treated with other CAR T cell products warrants further investigation. Finally, we did not analyze the lymphocyte subsets of CAR T cell products. The cellular composition of CAR T cell products is heterogeneous, and the frequency of CD4⁺ and CD8⁺ T cell subsets in patient blood varies markedly among individuals (39, 50). Recent reports have suggested that the heterogeneity of CAR T cell products can affect the treatment response and toxicity (20, 31, 32, 46). However, we were unable to assess the direct association between lymphocyte composition in CAR T cell products and the proportion of activated CD4⁺ T cells in patient blood on day 7 after tisa-cel infusion.

In conclusion, our results suggest that a high proportion of activated CD4⁺ T cells among total CD4⁺ T cells on day 7 after CAR T cell infusion, as detected by a standard flow cytometric analysis, is useful for the early detection of patients who develop prolonged CRS after CAR T cell therapy and may be a marker for predicting the subsequent clinical course. Further studies with a larger number of patients are warranted to validate these results and to advance our understanding of the efficacy of CAR T cell therapy.

Author's disclosure of potential Conflicts of Interest (COI).

Noboru Asada: Honoraria, Novartis. Daisuke Ennishi: Honoraria, Novartis. Keiko Fujii: Honoraria, Novartis. Nobuharu Fujii: Honoraria, Novartis. Ken-ichi Matsuoka: Honoraria, Novartis.

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