Single-cell profiling of CD19-directed CAR T-cell phenotypes and immune system dynamics in pediatric B-cell acute lymphoblastic leukemia

Tisagenlecleucel is the only chimeric antigen T-cell (CAR T) therapy approved by the US Food and Drug Administration and the European Medicines Agency for pediatric B-cell precursor acute lymphoblastic leukemia (B-ALL). Tisagenlecleucel demonstrated remarkable efficacy in relapsed/refractory B-ALL[1] and diffuse large B-cell lymphoma (DLBCL).[2]

Significant heterogeneity in CAR$^+$ T-cell infusion products (IP) has been demonstrated in LBCL.[2,3] It remains unclear whether these phenotypic variations impact clinical outcomes.[3] While pre-infusion composition of tisagenlecleucel products and their post-infusion behavior in children with B-ALL has not been previously studied in depth, the persistence of CAR$^+$ T cells in peripheral blood post-infusion may play a crucial role in therapy outcomes.[1,4,5]

We analyzed relationships between clinical outcomes, pre-treatment patient status, and prior therapies in a cohort of relapsed/refractory B-ALL patients (N=19, aged 2-16 years) receiving CAR T therapy with tisagenlecleucel in Poland between September 2022, and August 2024, with follow-up until February 2025. All patients provided informed consent. The study was approved by the Human Research Ethics Committee of the Medical University of Lodz (approval number RNN/93/22/KE). Participants gave informed consent to participate in the study before taking part, according to the Declaration of Helsinki and its subsequent amendments. No personally identifiable information has beens included in the manuscript. Treatment response was assessed using polymerase chain reaction-based minimal residual disease (PCR-MRD) in bone marrow biopsies, and toxicity was evaluated using the Penn scale for CRS[6] and ICANS scale for neurotoxicity.[7] This study was conducted in accordance with the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines.[8] We used mass cytometry to characterize the phenotype of CAR$^+$ T cells in infusion products and tracked their persistence in peripheral blood on days 7 and 28. Additionally, we profiled T cells and non-CAR T mononuclear cells, specifically natural killer (NK) cells and monocytes, to assess broader immune compartment changes post-infusion (*Online Supplementary Figure S1*).

At the time of tisagenleucel infusion, eight patients (42%) had positive measurable residual disease (MRD), as detected by PCR-based assessment in the bone marrow (BM) ($<1\times10^{-4}$: negative; $\geq1\times10^{-4}$: positive). Among these, only one patient had a high disease burden (88% BM blasts) prior to infusion, whereas the remaining PCR-MRD-positive patients had less than 1% blasts by morphology. Fourteen patients (74%) received CAR T therapy for first relapse, either after allogeneic hematopoietic stem cell transplantation (allo-HSCT) or without complete remission (CR) after one cycle of re-induction therapy. Four patients were treated for second relapse and one patient for fourth relapse. Prior to CAR T therapy, nine patients (47%) had received allo-HSCT, two blinatumomab, and six inotuzumab ozogamicin. Cytokine release syndrome (CRS) occurred in 74% of patients, with a maximum severity of grade 2. Seven patients received tocilizumab, all within 7 days post-infusion. Immune effector cell-associated neurotoxicity syndrome (ICANS) was observed in three patients, with two experiencing grade 2 and one experiencing grade 4 neurotoxicity. Complete remission with B-cell aplasia for longer than 6 months was achieved in 14 patients (73%); however, four subsequently relapsed within 1 year. One patient experienced disease progression, and two patients died due to leukemia relapse or resistance, with a median follow-up of 1 year (Figure 1; *Online Supplementary Table S1*).

Nineteen patients contributed 77 samples across several time points as shown in Figure 2A. All data shown for T-cell subsets are gated on CAR$^+$ T cells, unless otherwise specified. Tisagenleucel infusion products were primarily composed of MAIT/NKT (7.04%), CD4$^+$ central memory (16.6%), and predominantly regulatory T-cell (Treg) memory (63.3%) cells, while other subsets comprised less than 2% of CAR$^+$ T cells (Figure 2B).

CAR$^+$ Treg memory cells, decreased significantly by day 7 (<0.5%; $P<0.0001$) and day 28 ($P<0.001$) compared to IP (Figure 2C). Other CAR$^+$ T cell subsets also declined, including CD4 central memory (day 7: $P<0.001$; day 28: $P<0.05$), CD4$^-$ MAIT/NKT (day 7: $P<0.01$).

Conversely, the proportion of CD8-naïve cells increased post-infusion (day 7: $P<0.0001$; day 28: $P<0.01$), with further expansion between day 7 and 28 ($P<0.01$). Similar trends were observed for CD8 central memory (day 7: $P<0.01$; day 28: $P<0.05$), CD8 effector memory (day 7: $P<0.0001$; day 28: $P<0.001$), and CD8 terminal effector cells (day 7: $P<0.0001$; day 28: $P<0.001$). CD4 naïve, CD4 effector memory, and CD4$^-$CD8$^-$ $\gamma\delta$ T cells also increased ($P<0.01$), with further expansion between day 7 and 28 ($P<0.05$) (Figure 2D). Further analysis of activation and exhaustion markers in CAR$^+$ T cells revealed an increased expression of interleukin (IL)-7Rα (CD127) on days 7 and 28 post-infusion compared to the IP, particularly in CAR$^+$ CD4$^+$ central memory and effector memory subsets (*Online Supplementary Figure*

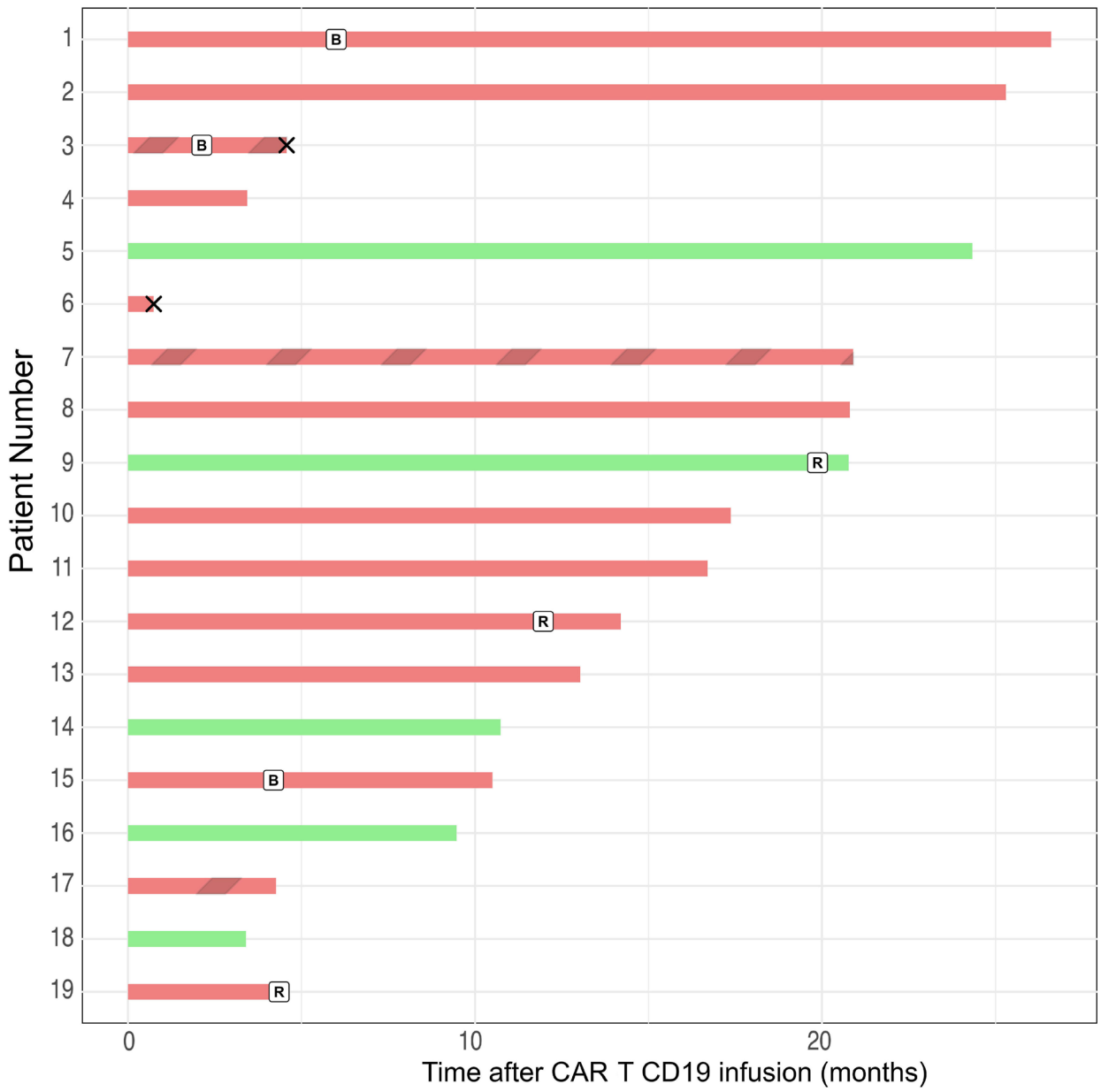

Figure 1. Patient characteristics and clinical outcomes. The figure displays a summary of patient characteristics and clinical outcomes. The swimmer plot portion illustrates key events over time, including relapse (R), loss of B-cell aplasia (B), and death (X). Patients who experienced cytokine release syndrome are indicated by a red bar (graded according to the Penn scale), while those who experienced immune effector cell-associated neurotoxicity syndrome are marked with a patterned bar (assessed using the ICE score). CAR: chimeric antigen receptor.

S2A, B). CTLA-4 expression was elevated on days 7 and 28 post-infusion compared to IP, especially in CAR$^+$ CD4$^-$CD8$^-$ γδ T cells (*Online Supplementary Figure S2C*).

To characterize host immune system changes, we analyzed peripheral blood mononuclear cells (PBMC) from 16 patients at day 0 (pre-infusion), day 7, and day 28 post-infusion. Monocytes, as well as early and late NK cells, significantly increased within 7 days post-infusion (P<0.01; P<0.05 and P<0.05, respectively) and remained elevated at day 28 compared to day 0 (P<0.01; P<0.001 and P<0.01, respectively) (Figure 3A-C).

Despite increasing monocyte counts, expression of activation markers (CD16, CD66b) on monocytes decreased significantly by day 7 (P<0.01; P<0.001) before partially recovering by day 28 (P<0.05; P<0.05) (*Online Supplementary Figure S2D*). In contrast, activation markers on early and late NK cells increased over time. CD16 expression on NK cells rose between day 0 and 28 (P<0.05) and between day 7 and 28 (P<0.05). Additionally, CD161 expression on early NK cells increased between day 0 and 28 (P<0.05) (*Online Supplementary Figure S2E*).

No associations were observed between CAR$^+$ T-cell subset composition and patient risk group, disease burden, prior treatment with blinatumomab, inotuzumab ozogamicin, or HSCT. Furthermore, CAR$^+$ T-cell composition was not correlated with treatment outcomes or the development of ICANS (*Online Supplementary Table S2*). However, patients who developed CRS (grade ≥1, Penn scale[6]) had a higher frequency of CAR$^+$ CD4$^-$ MAIT/NKT and CAR$^+$ CD8 terminal effector cells in infusion products compared to those without CRS (P<0.05) (Figure 3D, E). At day 0 pre-infusion, patients who later developed CRS had fewer early and late NK cells (P<0.05) (Figure 3F, G) compared to those without CRS. At day 7 post-infusion, patients who experienced CRS had fewer classical monocytes and lower expression of IL-7RA (CD127) (P<0.05) on these cells compared to those without CRS (Figure 3H; *Online Supplementary Figure S2F*). By day 28, CRS patients had lower counts of transitional monocytes (P<0.05) (*Online Supplementary Figure S2G*).

In this study we characterized CAR$^+$ T-cell subsets in IP and tracked their evolution post-infusion, providing insights into treatment response and immune system changes (*Online Supplementary Figure S3*).

IP were predominantly composed of CAR$^+$ CD4$^+$ T cells, with a significant fraction exhibiting a central memory phenotype. Notably, CAR$^+$ Treg memory cells constituted the largest

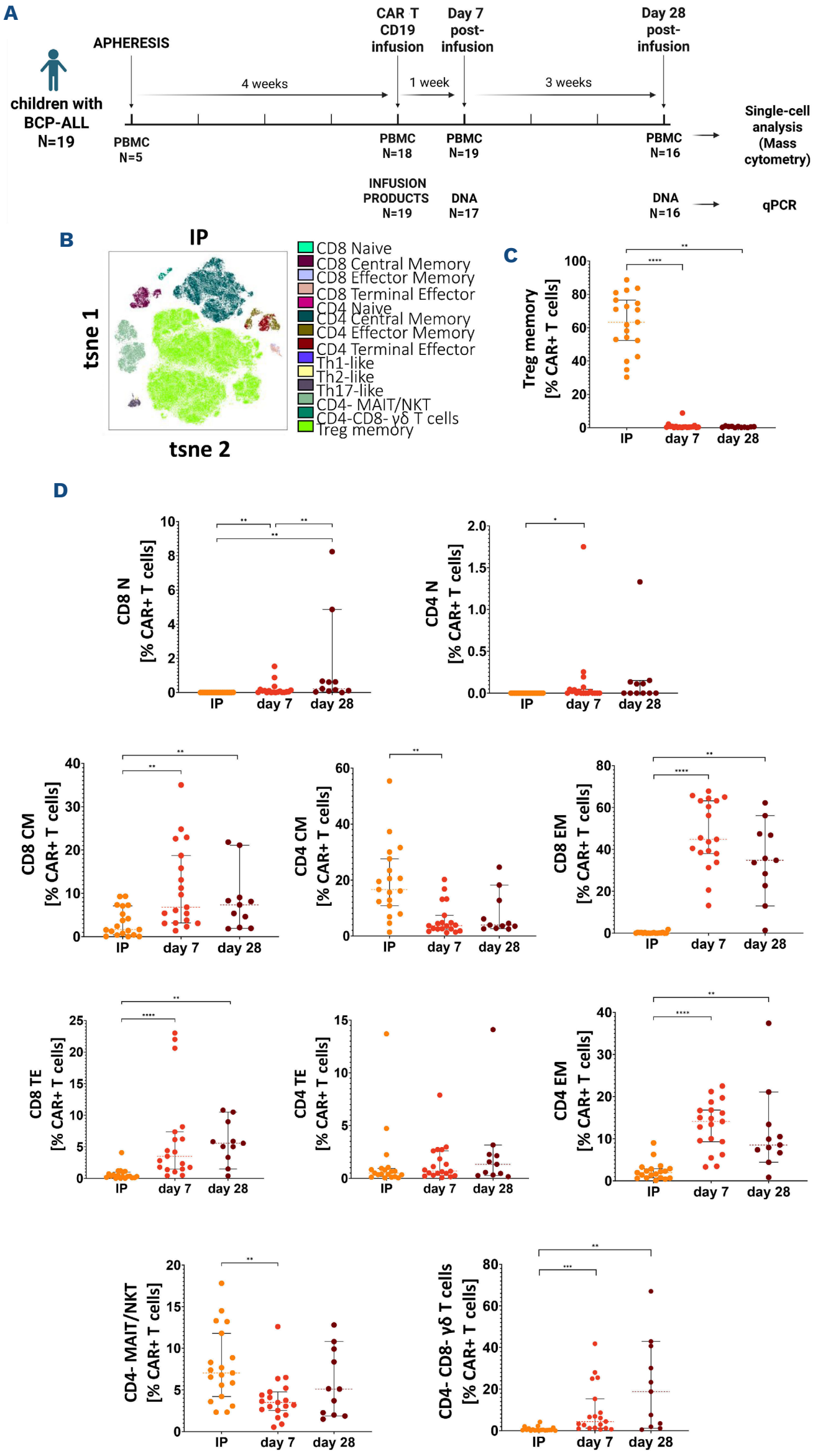

Figure 2. Phenotypic diversity and post-infusion dynamics of CAR⁺ T-cell subpopulations in CD19 CAR T-cell therapy. (A) Schematic of sample collection and analysis, including mass cytometry and quantitative polymerase chain reaction (qPCR) for validation from pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients. Correlation between chimeric antigen receptor-positive (CAR⁺) cell percentages in peripheral blood mononuclear cell (PBMC) by mass cytometry and CAR⁺ transgene expression at day 7 (R^2=0.9043; *P*<0.0001) and day 28 (R^2=0.9825; *P*<0.0001). Figure created using BioRender.com. (B) T-cell subsets within CAR⁺ T cells in infusion products (IP) using Mahalanobis distance. (C) Longitudinal analysis of CAR⁺ regulatory T cells (Treg) memory from infusion to day 7 and day 28 (**P*<0.05; ***P*<0.01; ****P*<0.001; ****P*<0.0001, Wilcoxon test, false discovery rate [FDR]-adjusted). (D) Longitudinal analysis of CAR⁺ T-cell subsets from infusion to day 7 and day 28 (**P*<0.05; ***P*<0.01; ****P*<0.001; ****P*<0.0001, Wilcoxon test, FDR-adjusted).

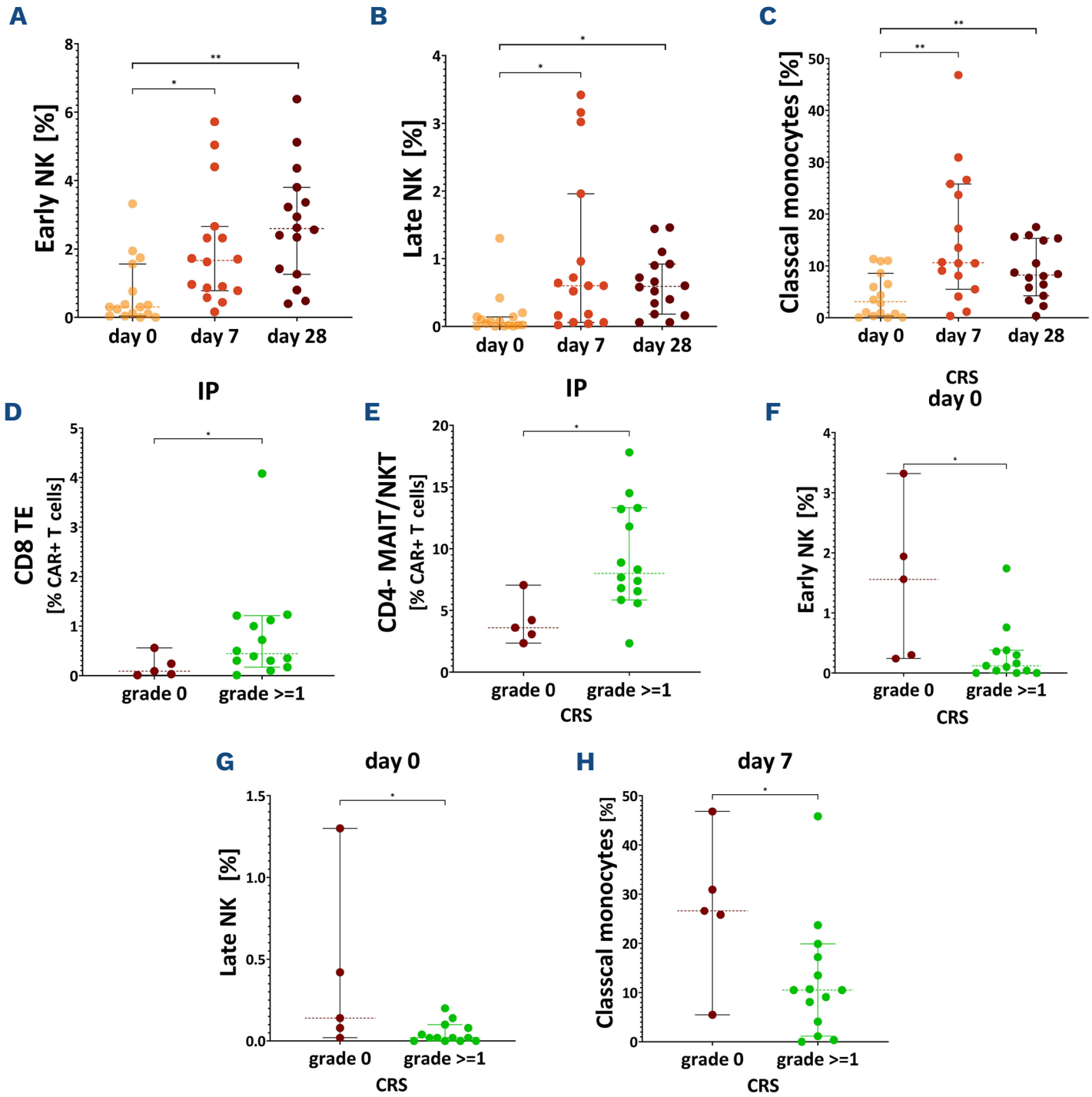

Figure 3. Immune system dynamics and association with cytokine release syndrome. (A) Longitudinal analysis (day 0, 7, 28) of early natural killer (NK) cells (*P<0.05; **P<0.01; ***P<0.001). (B) Longitudinal analysis (day 0, 7, 28) of late NK cells (*P<0.05; **P<0.01; ***P<0.001). (C) Longitudinal analysis (day 0, 7, 28) of classical monocytes (*P<0.05; **P<0.01; ***P<0.001). (D) Abundance chimeric antigen receptor-positive (CAR^+) CD8 terminal effector cells in infusion products (IP) based on development of cytokine release syndrome (CRS) (*P<0.05). (E) Abundance CAR^+ $CD4^-$ MAIT/NKT cells in IP based on development of CRS (*P<0.05). (F) Pre-infusion peripheral blood mononuclear cell (PBMC) composition at day 0 indicates higher levels of early NK cells in patients who did not develop CRS (*P<0.05; **P<0.01). (G) Pre-infusion PBMC composition at day 0 indicates higher levels of late NK cells in patients who did not develop CRS (*P<0.05; **P<0.01). (H) Abundance of classical monocytes at day 7 based on CRS incidence (*P<0.05).

subset, while naïve $CD4^+$ and $CD8^+$ subsets were largely absent. These findings are consistent with previous reports in DLBCL patients treated with tisagenlecleucel, although those adult studies did not characterize CAR^+ Treg memory cells, $CD4^-CD8^-$ γδ T cells, $CD4^-$ MAIT/NKT cells, or T-helper cells.[3] Post-infusion, the proportion of CAR^+ Treg memory cells declined by day 7 post-infusion, while $CD8^+$ naïve and effector subsets expanded, indicating a shift toward a more cytotoxic phenotype. This was accompanied by changes in activation and exhaustion markers in DLBCL, high CAR^+ Treg numbers in IP associated with tumor growth due to suppression of cytotoxic CAR^+ T cells, correlating with disease progression and reduced neurotoxicity.[1] However, in our pediatric B-ALL cohort, CAR^+ Treg memory cells were absent in PBMC at days 7 and 28 post-infusion, suggesting a transient effect.

Our data also revealed a post-infusion decline in CAR^+ $CD4^+$ central memory cells and an expansion of CAR^+ $CD8^+$ subsets, consistent with previous studies.[9]

Beyond CAR^+ T-cell dynamics, we document expansion and activation in the NK and myeloid compartments, which may be key determinants of CAR T efficacy and toxicity. Our findings challenge the assumption that low absolute neutrophil count uniformly enhances CAR T-cell expansion,

as previously suggested.[10] Instead, we observed that immune recovery, particularly the presence of monocytes and neutrophils, correlates with improved clinical outcomes. This aligns with previous reports linking neutropenia to B-cell recovery,[11] suggesting that hematologic reconstitution may play a role in sustained remission.

The higher frequency of CAR$^+$ CD4$^-$ MAIT/NKT and CAR$^+$ CD8$^+$ terminal effector cells in IP correlated with CRS development. This aligns with evidence that highly differentiated effector T cells exhibit impaired *in vivo* function despite strong *in vitro* activity and that cytokine signaling can influence T-cell efficacy.[12]

While tocilizumab has proven effective in managing CRS,[13] its impact on the immunological profile of CAR$^+$ T cells remains insufficiently studied. Our findings suggest that baseline immune profiles and monocyte dynamics may serve as predictors of CRS. Patients who developed CRS exhibited lower pre-infusion levels of NK cell.[14] By day 7, these patients displayed reduced classical monocyte numbers and IL-7RA expression, suggesting altered immune recovery, potentially influenced by tocilizumab.[15] These findings suggest that immune profiling could aid in identifying high-risk patients and refining CRS management strategies.

This study has several limitations, including a relatively small cohort, interpatient variability, and retrospective data collection. Despite these constraints, our study is the first to characterize the phenotypic composition of tisagenlecleucel CAR$^+$ T-cell subsets in pediatric B-ALL, revealing a significant number of Treg in IP that do not persist post-infusion. These findings suggest that the same CD19 CAR T product may exhibit different behaviors in B-ALL compared to lymphoma, as well as in pediatric *versus* adult patients. Furthermore, our data indicate that early changes in the immune system, particularly monocytes and NK cells, are associated with CAR T treatment outcomes, underscoring the potential value of comprehensive immune profiling in pediatric B-ALL.

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Contributions

AO conceived the study and secured funding. KLD and WM conceptualized the study and supervised the project. AO, BP, NCP, JCC, SJ, MP and MPV developed methods. AO, BP, NCP and MM performed all experiments. AO and AK developed the software. AO, AK and JCC performed formal data analysis for all of the data

generated. PM, MRP, MMS, KK and JS treated patients and/or acquired clinical samples and data. AO, MP, KLD and WM interpreted the results and wrote the first draft of the paper. All authors critically reviewed the manuscript.

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Data-sharing statement

Data are available upon request to the corresponding author.

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