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ARTICLE

Early and late hematologic toxicity following CD19 CAR-T cells

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Abstract

Autologous T cells transduced with CD19-directed chimeric antigen receptors have recently been approved by several regulatory agencies for the treatment of relapsed and refractory leukemia and lymphoma, after demonstrating remarkable remission rate in advanced patients. The most common adverse events reported are cytokine-release syndrome (CRS), neurotoxicity, and hematologic toxicity. Here, we focus on early and late cytopenia occurring after CD19 CAR-T cells in 38 patients treated with CD19 CAR-T cells. Neutropenia, thrombocytopenia, and anemia occur frequently (94, 80, and 51%, respectively) after CAR-T cell infusion, and are associated with a biphasic nature, as in 93% of patients hematologic toxicity occurs after 21 days from cell infusion. Late hematologic toxicity was more common in patients with high grade CRS and in patients treated after a recent stem cell transplantation. Interestingly, since these events occur late after the lymphodepleting chemotherapy and after resolution of CRS, we found perturbations in SDF-1 levels to correlate with events of late neutropenia, likely associated with B-cell recovery.

Introduction

T cells expressing a chimeric antigen receptor targeting CD19 (CD19 CAR-T cells) are a form of adoptive cellular therapy leading to very high response rates and durable remissions in patients with refractory B cell malignancies, including acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma (NHL), and chronic lymphocytic leukemia (CLL) [1, 2]. To enhance cellular efficacy, adoptive cell transfer is performed after a lymphodepleting regimen [3], with the combination of fludarabine and cyclophosphamide being a common safe regimen leading to better outcomes compared to other regimens [4–7]. Clinical success is associated with unique toxicity rarely observed after chemotherapeutic regimens, namely cytokine release syndrome (CRS), neurotoxicity, and B-cell aplasia [8–13]. These toxicities have been reported after immune-effector cell therapies and following allogeneic hematopoietic stem cell transplantation (HSCT).

Toxicity of the hematopoietic system, including neutropenia, anemia, and thrombocytopenia, has been reported after CAR-T cell therapy, and attributed mostly to the lymphodepleting chemotherapy regimen or CRS. Here, we systematically analyzed the hematologic toxicity of pediatric and adult patients participating in a phase 1b/2 clinical trial using CD19 CAR-T cells to treat relapsed and refractory B-cell malignancies.

Methods

Patients

A total of 38 patients with relapsed or refractory B cell malignancies were enrolled on a phase 1b/2 study of locally produced CD19 CAR-T cells: 14 children under 18 years of age and 21 adults. Patients were eligible if they had a CD19-expressing B cell ALL or lymphoma, relapsed or

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refractory after receiving at least two line of standard therapy. The study was conducted according to the principles of the Declaration of Helsinki and was approved by the local institutional review board (IRB) of Sheba Medical Center and by the Israeli Ministry of Health.

All patients received a lymphodepleting preparative regimen of cyclophosphamide (900 mg/m^2, day −2) and fludarabine (25 mg/m^2 per dose, 3 doses, days −4 to −2), followed by intravenous infusion of autologous CD19 CAR-T cells with a CD28 costimulatory domain. Dosing of CAR-T cells was 1–1.5 million CAR+ cells per kg. Clinical response was determined at 28 days following cell administration. Responding patients were referred to an allogeneic HSCT 2 months after CAR-T cell infusion. This study was registered at clinicaltrials.gov (NCT#02772198).

Definitions of hematologic toxicity

Neutropenia was defined as absolute neutrophil count lower than 1500/μL peripheral blood; severe neutropenia was defined as neutrophil count lower than 500/μL. Thrombocytopenia was defined as a platelets count lower than 150×10^3/μL; severe thrombocytopenia was defined as platelets count lower than 50×10^3/μL. Severe anemia was defined as anemia that required a packed red blood cells (PRBC) infusion. Data was collected till admission of further therapy, including for allogeneic HSCT. The severity of CRS was graded according to the NCI criteria [8].

Sample isolation

Peripheral blood was collected from all patients at predetermined time points. DNA and RNA were extracted using ALLPrep DNA/RNA Mini Kit (Qiagen). qPCR for determination for CAR copy number was performed as previously published [14]. Serum was separated by centrifugation and was cryopreserved in −80 °C. Cytokine analysis of serum was performed on thawed serum, using Milliplex Map, human cytokine/chemokine panel II (Millipore Corporation, 2013) according to the manufacturer's protocol.

Statistics

Due to the small sample size we used non-parametric testing for statistics. For continuous variables, median and range were used for descriptive statistics, and Mann–Whitney analysis was performed to compare between groups. Categorical variables were analyzed with Fisher's exact test and Chi Square, accordingly. Statistical analysis was preformed using Prism Graphpad version 7.

Table 1 Demographic and clinical characteristics of patients[a]

	Total ($n = 35$)[a]	ALL ($n = 19$)	NHL ($n = 16$)
Age, years (range)	27 (3.5–55)	14 (5–48)	35 (3.5–55)
Sex: male	25 (71.4%)	12 (63.2%)	13 (81.3%)
Prior HSCT	13 (37%)	7 (37%)	6 (37%)
CRS			
None	8 (22.8%)	3 (36.8%)	5 (31.1%)
G1	16 (45.7%)	8 (42.1%)	8 (50%)
G2	6 (17.1%)	4 (21.1%)	2 (12.5%)
G3-4	5 (14.3%)	4 (21.1%)	1 (6.3%)
Response			
CR	24 (68.6%)	18 (94.7%)	6 (37.5%)
PR	5 (14.3%)	0 (0%)	5 (31.3%)
NR	6 (17.1%)	1 (5.3%)	5 (31.3%)
Pre-lymphodepletion			
ANC <1500/μL	7 (20%)	7 (36.8%)	0 (0%)
PLT <150,000/μL	16 (45.7%)	8 (42.1%)	8 (50%)
Post CAR			
Neutropenia	33 (94.3%)	18 (94.7%)	15 (93.8%)
Thrombocytopenia	28 (80%)	16 (84.2%)	12 (75%)
Anemia	18 (51.4%)	10 (52.6%)	8 (50%)

ALL acute lymphoblastic leukemia, *NHL* non-Hodgkin's lymphoma, *HSCT* hematopoietic stem cell transplantation, *CRS* cytokine release syndrome, *G* grade, *CR* complete remission, *PR* partial remission, *NR* no response, *ANC* absolute neutrophil count, *PLT* platelet count

[a]Data here represents the entire cohort of patients and not only responding patients

Results

Between July 2016 and March 2018, 38 patients were enrolled on the trial, and 35 patients who received CAR-T cells and survived more than 21 days were included in this analysis. Three patients were not included: one did not receive cells due to production failure, and two died of disease—one prior to lymphodepletion and one after lymphodepletion. The indication for CD19 CAR-T cells was ALL in 19 patients and NHL in 16 (Table 1). The clinical outcome of the patients with ALL has been previously reported [14]. Altogether, 29 patients (83%) had a complete or partial response by 30 days. Thirty four patients developed hematologic toxicity following CAR-T cell therapy: 33 had neutropenia, 28 thrombocytopenia, and 18 severe anemia (Fig. 1a).

Since bone marrow involvement of leukemia may impact count recovery in non-responding patients, we further analyzed the hematologic toxicity only in the 29 responding patients. All responding patients were referred to an allogeneic HSCT 2 months after CAR-T cell administration.

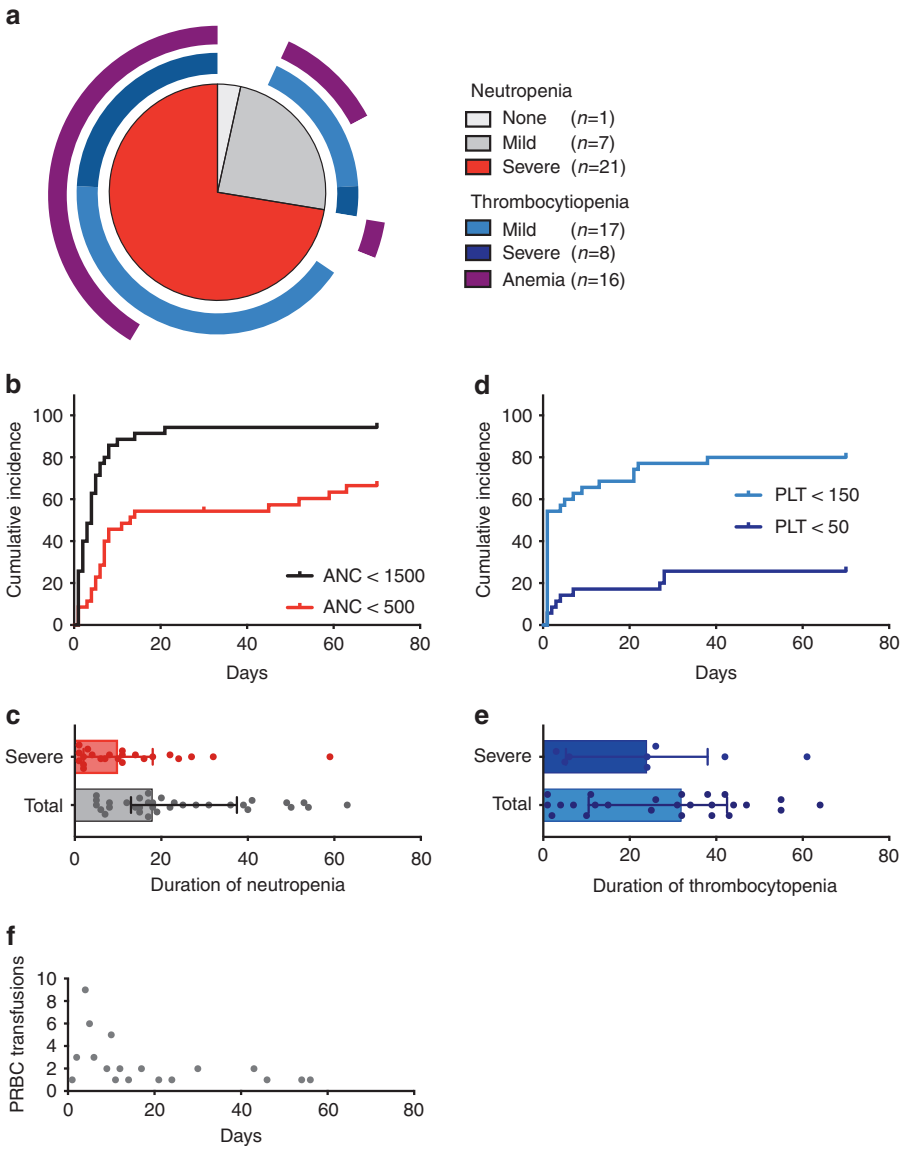

Fig. 1 Hematologic toxicity following CAR-T cells. **a** Pie chart depicting the proportional incidence of neutropenia (inner pie), thrombocytopenia (interim donut), and anemia (outer donut) in responding patients after CAR-T cell infusion ($n = 29$). **b** Cumulative incidence of neutropenia (black line) and severe neutropenia (red line) after CAR-T cell infusion in the entire cohort of patients ($n = 35$). **c** Duration of neutropenia (gray dots) and severe neutropenia (red dots). Bar represents median time and whiskers represent interquartile range. **d** Cumulative incidence of thrombocytopenia (light blue line) and severe thrombocytopenia (dark blue line) after CAR-T cell infusion. **e** Duration of thrombocytopenia (light blue dots) and severe thrombocytopenia (dark blue dots). Bar represents median time and whiskers represent interquartile range. **f** Incidence of PRBC transfusion in the cohort of patients on each day after CAR T cells. In all graphs day 0 represents day of CAR-T cell infusion. ANC absolute neutrophil count, PLT platelet, PRBC packed red blood cell

Hematologic toxicity following CAR-T cells

Neutropenia was observed in 7 (20%) of 35 patients at baseline, prior to chemotherapy (range, 830–1480 neutrophils/μL). Following cell therapy, 28 responding patients (97%) developed neutropenia, 21 (72%) of them had severe neutropenia (Fig. 1a). The median time to onset of neutropenia was 3 days from cell infusion (range, 0–21), and the median time to onset of severe neutropenia was 7 days (range 0–63, Fig. 1b). The median duration of neutropenia, when occurred, was 19.5 days (range 0–63), and the median duration of severe neutropenia, when occurred, was 10 days (range 0–59, Fig. 1c). Twenty five (86%) responding patients had thrombocytopenia following CAR-T cell administration, and 16 of them had low platelet counts following lymphodepletion and prior to cellular therapy (median platelet count 133, range 22–525). Only one patient had a platelet count lower than 50/μL at baseline. Severe thrombocytopenia developed in eight patients (28%) following CAR-T cells (Fig. 1a), and appeared in a biphasic form: five within the first week, and 3 at after a month (27–28 days following the treatment, Fig. 1d). The median time to onset of thrombocytopenia was 0 days (range 0–38), and its median duration was 32 days (range 1–64). Severe thrombocytopenia occurred within a median of 5.5 days (range 0–28), and its duration was 24 days (range 3–61, Fig. 1d, e). Sixteen responding patients (55%) have received packed red-blood cell (PRBC) transfusion during the study (Fig. 1a). The median number of PRBC per patient was two (range 1–7), and the peak transfusion demand was on day +4 (Fig. 1f).

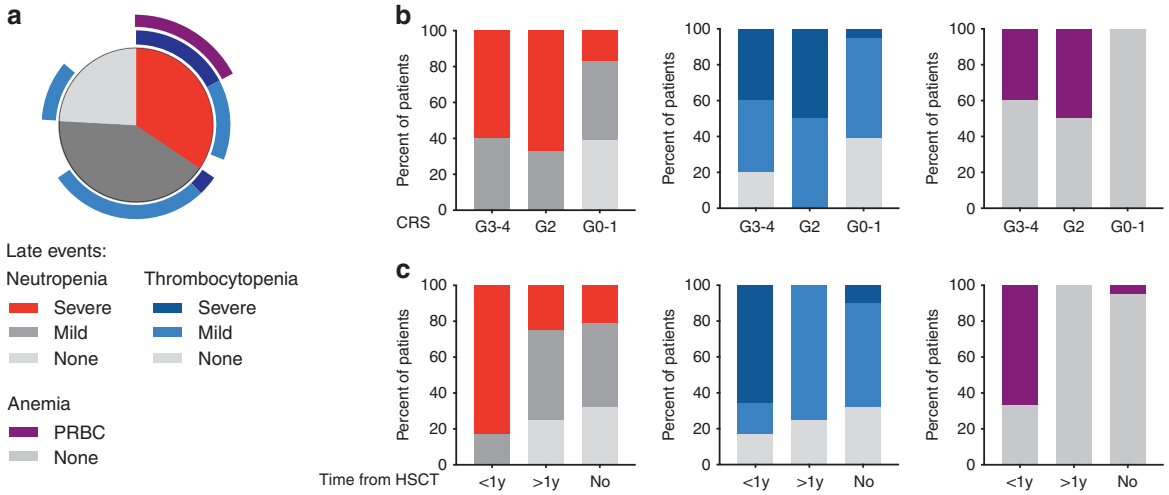

Fig. 2 Late hematologic toxicity following CAR-T cells. **a** Pie chart depicting the proportional incidence of neutropenia (inner pie), thrombocytopenia (interim donut) and anemia (outer donut) in responding patients occurring >21 days after CAR-T cell infusion. **b** and **c** Incidence of neutropenia (left), thrombocytopenia (center), and anemia (right) as a function of CRS grade (**b**) and prior HSCT (**c**). PRBC packed red blood cell unit, CRS cytokine release syndrome, HSCT hematopoietic stem cell transplantation

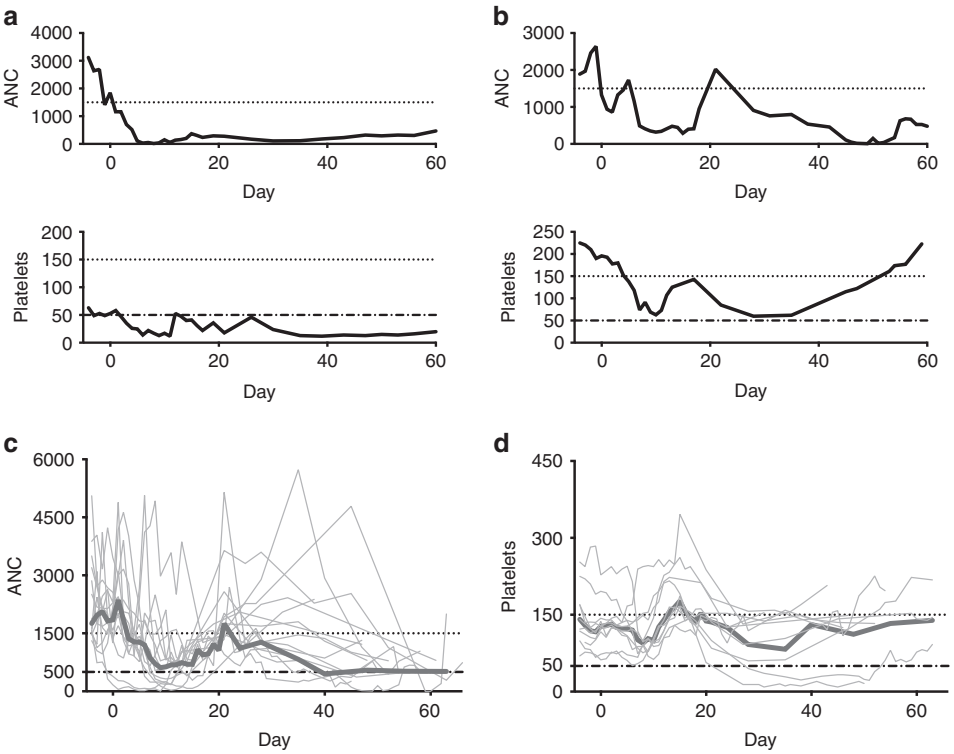

Fig. 3 Patterns of prolonged cytopenia: **a** Neutrophil and platelet count of patient C015 who developed prolonged severe neutropenia and thrombocytopenia. **b** Neutrophil and platelet count of patient C033, who had biphasic cytopenia with interim recovery of neutrophil and platelet levels. **c** Individual absolute neutrophil counts (ANC, thin lines) and median ANC (think line) in patients with late neutropenia following a biphasic pattern. **d** Individual platelet levels and median platelet levels (thick line) and in patients with late thrombocytopenia following a biphasic pattern. Day 0 represents the day of CAR-T cell administration.

On univariate analysis, the only risk factor associated with severe thrombocytopenia and anemia that reached statistical significance was an HSCT within 1 year prior to CAR-T cell infusion (supplementary table 1). Although 10 of the 11 patients with grade 2 or higher CRS and six our of seven patients with baseline cytopenia had severe hematologic toxicity, this did not reach statistical significance, likely due to the small cohort.

Biphasic nature of post CAR hematologic toxicity

Twenty seven of the 29 responding patients (93%) experienced late hematologic toxicity beyond day 21 following CAR-T cells, well after the period expected to be related to lymphodepleting chemotherapy preparation. Late neutropenia occurred in 22 patients (76%), and was severe in 10 (34%). Late thrombocytopenia occurred in 22 patients

(76%), and was severe in six (21%). Five patients (17%) required PRBC transfusion in the late post-CAR period (Fig. 2a). Neutropenia, thrombocytopenia, and anemia occurring later than 42 days from CAR-T cell therapy, a period considered in most CAR-T cell trials as a hematologic severe adverse event, was noted in 18, 13, and 5 patients, respectively (62%, 44, and 17%).

We identified a strong correlation between the late hematologic toxicities (thrombocytopenia and neutropenia, $p = 0.018$, thrombocytopenia and anemia, $p < 0.0001$, anemia and neutropenia $p = 0.05$). Other factors affecting late cytopenia were prior HSCT ($p = 0.0015$, 0.0083, and 0.02 for anemia, thrombocytopenia, and neutropenia, respectively) and higher CRS grade $p = 0.003$, 0.018, and 0.04 for late anemia, thrombocytopenia and anemia (Fig. 2b-c and supplementary table 2).

Despite several patients having prolonged cytopenia (Fig. 3a), the majority of late events occurred following a biphasic pattern, characterized by two trough levels with an intermediate recovery, seen in 15 patients (52%) with neutropenia and 10 (34%) with thrombocytopenia (Fig. 3b-d). Interestingly, despite a strong correlation with CRS, the second trough of cytopenia was after resolution of CRS and after patients have been discharged from the inpatient service. Bone marrow aspirations obtained from patients with ALL as disease assessment at day 28 showed, in case of remission, relatively normal cellularity without paucity of progenitors.

Clinically, only one patient was admitted for fever while experiencing late neutropenia, with negative blood cultures. Two patients received G-CSF during this period: the first received a single dose of GCSF on day +20 and had a rapid recovery of neutrophil count. The second was a patient treated 3 months following an autologous HSCT for DLBCL, and during the second phase of post-CAR neutropenia received 3 days of GCSF, with partial recovery of neutrophil count prior to allo-HSCT. Of 20 responding patients evaluable at allo-HSCT following CAR-T cells, 10 (50%) had an ANC below 1500 on admission, and four (20%) had an ANC below 500 when admitted for the transplant conditioning regimen. No events of significant bleeding were recorded while patients had early or late thrombocytopenia.

Changes in SDF-1 are related to late neutropenia post CAR

Lack of symptoms related to CRS or hemophagocytic lymphohistiocytosis (HLH) after 21 days from cell administration led us to explore other hypotheses for the late neutropenia following CAR-T cells. Median persistence of the CAR-T cells in patients' blood was 23 days [14], similar to other CD28-based CARs, followed by relatively early

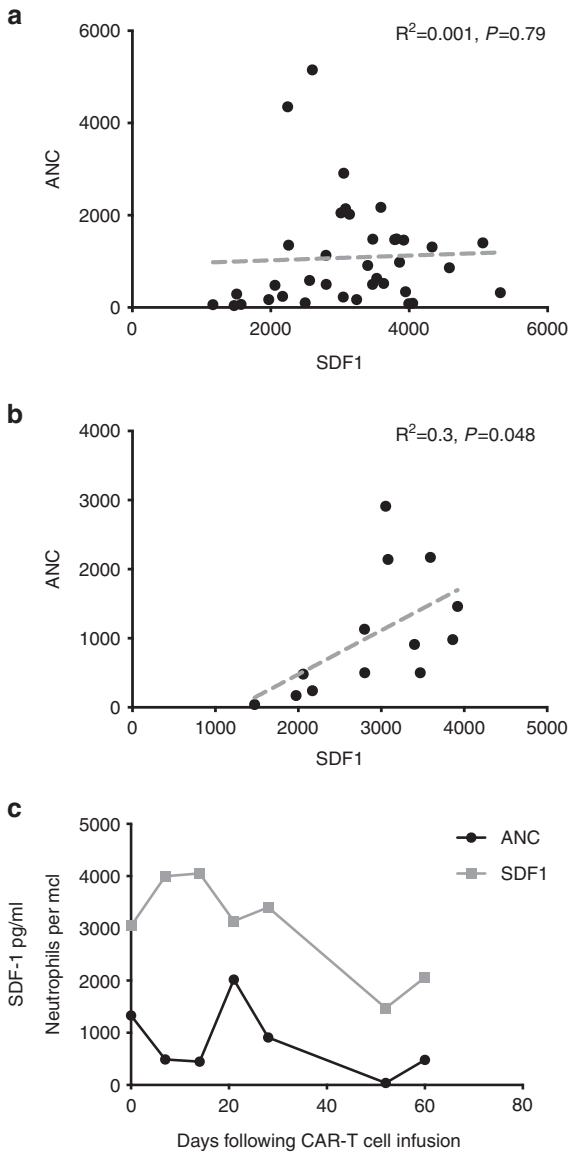

Fig. 4 Alterations in SDF-1 levels are related to late neutropenia post CAR. **a**, **b** Absolute neutrophil count (ANC) as a function of serum SDF-1 levels in 16 patients at different time-points throughout the post-CAR observation period (**a**), and limited to 28 days or later after CAR infusion (**b**). **c** pattern of neutrophil count and SDF-1 levels in a patient with early and late neutropenia following CD19 CAR-T cells

B-cell recovery. B-cell recovery after the use of rituximab, a CD20-targeting monoclonal antibody, was correlated with late onset neutropenia, through perturbations in the levels of SDF-1, a chemokine essential for B-cell development and for trafficking of neutrophils as well as hematopoietic stem cells [15]. Thus, we measured the levels of serum SDF-1 at different time points, in 16 responding patients: seven with late neutropenia and nine with normal neutrophil count beyond 21 days. No correlation was seen between SDF-1 levels in the serum and neutrophil count in the entire cohort (Fig. 4a). However, when accounting solely for late events,

levels of SDF-1 were correlated with absolute neutrophil count (Fig. 4b, c).

Discussion

Chimeric antigen receptor T-cell targeting CD19 following lymphodepleting regimen may induce long-term and durable remissions in patients with previously highly resistant B-cell malignancies. The main toxicities of CAR-T cells include CRS, neurotoxicity, and hematologic toxicity. This is the first comprehensive look at early and late hematologic toxicities, which are common following CD19 CAR-T cells.

We identified CRS and prior HSCT as risk factors for hematologic toxicity. A prior HSCT, mostly less than 1 year before patients received CAR-T cells, is reflective of the overall treatment intensity and lack of complete marrow recovery, which in turn may lead to insufficient myeloid function under hematopoietic stress. CRS was previously correlated with hematologic toxicity, mostly of the acute type. Neutropenia, thrombocytopenia, and anemia were seen in greater than 80, 50, and 60%, respectively, following CD28-based CAR-T cells for B-cell malignancies [16–18], with the majority being grade 3 and 4 events. CAR-T cells bearing a 41BB costimulatory domain may have different kinetics, but also have similar high rates of cytopenia [11, 19, 20]. Patients with grade 4 CRS were shown to develop cytopenia more rapidly, have lower trough levels, and longer time to recovery compared to patients with a lower grade of CRS [11]. CRS was previously related to HLH [21, 22], a syndrome in which cytopenia is one of the main diagnostic criteria [23], and is a result of macrophage activation. CRS, which involves activation of both T-cell and macrophages [22, 24] may be a part of the HLH spectrum, and entail severe related cytopenia especially in its severe form.

The most unique aspect of the hematologic toxicities following CD19 CAR-T cells is their prolonged duration, which is unrelated to the myelotoxic effect of the lymphodepleting regimen. This occurred after resolution of CRS, in absence of symptoms related to HLH, as seen here. In fact, a bi-phasic form of neutropenia (mostly) and thrombocytopenia may arise from two distinct mechanisms: the first event occurs following the lymphodepleting therapy and in conjunction with the peak of CRS/HLH. The second event seems unrelated to these. There may or may not be an interim recovery between these two phases. Late hematologic toxicities were found to be related to prior HSCT, likely representing poor marrow reserve, and to CRS grade. Delayed neutrophil recovery was noted in several other studies: in the ELIANA study, 41 and 53% of 75 patients had thrombocytopenia and neutropenia following tisagenlecleucel, respectively, that did not resolve by day +28 from cell therapy, of which 12 and 11%, respectively, were ongoing throughout the follow-up period [19]. In an adult population treated with 41BB-based CD19 CAR-T cells for various B-cell malignancies, 15% of patients did not have neutrophil recovery before day 28 or censoring [20]. Later follow-up of patients with NHL and CLL from this group showed that 20% of patients who received no further therapy had ongoing cytopenia at 90 days following CAR infusion [25]. Following CD28-based CAR-T cells for ALL, 33% of patients had ongoing severe neutropenia more than 14 days following the infusion, and an event of late onset of grade 4 neutropenia (day +47 after cellular therapy) was reported in one patient [16]. Of patients treated with axicabtagene ciloleucel for DLBCL, 11, 7, and 3% had neutropenia, thrombocytopenia, and anemia, respectively, 3 months following infusion [26]. Our study confirms that these late events are common, and relates these to the status of prior HSCT and the grade of CRS. Interestingly, prolonged cytopenia have also been seen after CD22 CAR-T cells: four of 18 patients had severe neutropenia following 30 days from cell infusion, and the median time to platelet recovery was 36 days from CAR infusion [27].

We sought for alternative mechanisms of late neutropenia events. A similar finding, of late onset neutropenia (LON), was previously reported after B-cell depletion following treatment with rituximab, a CD20-targeting monoclonal antibody. Rituximab was also associated with thrombocytopenia events [28]. Several explanations for rituximab-associated LON have emerged, including polymorphism in the IgG Fc-Receptor-gamma-IIIa [29] and perturbations of stromal-derived factor 1 (SDF-1)/CXC-ligand 12 (CXCL12) [15]. SDF-1 is a chemokine that regulates hematopoietic stem cell migration and survival, neutrophil migration, and pro-pre-B cell development [30, 31], through its receptor CXCR4. Importantly, only very-early B-cell precursors are dependent on SDF-1 levels. Thus, recovery of early B-cells after CD19 CAR-T cell depletion may lead to alterations in SDF-1 levels in the marrow microenvironment. We report a correlation between serum SDF-1 levels and neutrophil count only later than 21 days after CAR-T cell infusion, after resolution of CRS and expected recovery from lymphodepleting chemotherapy. This observation may be consistent with the hypothesis regarding rituximab-associated LON, that disrupted SDF-1 concentrations during rapid B-cell expansion result in reduced neutrophil egress from the bone marrow [15]. One limitation of our study is lack of serial B-cell counts, not required in our trial, thus we cannot correlate these with the serum levels of SDF-1. Generally, B-cell recovery occurs within 1–2 months following CD28-based CAR-T cells, and later (if at all) following 41BB-based CARs. Ongoing depletion of B-cells in 41BB-based CAR-T cells, which

persist longer, may explain increased incidence of prolonged cytopenia in these patients. Kinetics of CAR expansion and subsequent contraction, which will lead to B-cell recovery, are related to CRS grade [11], and may explain its correlation of late cytopenia events.

No major infectious events were seen in our series following late neutropenia, and no major bleeding events following late thrombocytopenia were observed. In a large series of adults treated with CAR-T cells, Hill et al. report that most infections (including late ones) following CAR-T cells occurred during neutropenia [20]. In contrast, the significant neutropenia following CD22 CAR-T cells was not associated with increased risk of bacterial or fungal infections [27], similar to our report. Current guidelines do not specifically comment on incidence or causality, but offer the use of growth factors such as G-CSF for CAR-related neutropenia [10]. Our data, linking SDF-1 to CAR-related neutropenia, suggests that indeed growth factors may enhance resolution of neutropenic events, after resolution of CRS.

Overall, this report focuses on a common complication of CD19 CAR-T cells in the hematopoietic system. Since CAR therapies are FDA approved and in clinical practice, our observations should help clinicians in detection and management of post CAR cytopenia. Currently, monitoring of blood counts and appropriate support are essential following these therapies. Additional studies in large patient populations are needed to identify factors that could be associated with the risk of prolonged cytopenia.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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