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TITLE: Kinetics and Biomarkers of Severe Cytokine Release Syndrome after CD19 Chimeric Antigen Receptor-modified T Cell Therapy

AUTHORS:

Kevin A Hay[1,2], Laïla-Aïcha Hanafi[1], Daniel Li[3], Juliane Gust[4], W Conrad Liles[5], Mark M Wurfel[5], Jose A Lopez[5,6], Junmei Chen[6], Dominic Chung[6], Susanna Harju-Baker[5], Sindhu Cherian[7], Xueyan Chen[7], Stanley R Riddell[1,5], David G Maloney[1,5], Cameron J Turtle[1,5]

Corresponding Author:

Kevin A. Hay

Clinical Research Division, Fred Hutchinson Cancer Research Center

1100 Fairview Ave N

Seattle WA 98109

Email: khay@fredhutch.org ; Phone: 206 667 7449; Fax: 206 667 7983

Affiliations:

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA; [2]Department of Medicine, University of British Columbia, Vancouver, BC, Canada; [3]Juno Therapeutics, Seattle, WA; [4]Seattle Children's Hospital, Seattle, WA; [5]Department of Medicine, University of Washington, Seattle, WA; [6]Bloodworks Northwest, Seattle, WA; [7]Department of Laboratory Medicine, University of Washington, Seattle, WA.

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KEY POINTS

1. Characterization of the kinetics and risk factors for severe CRS after CD19 CAR-T cells will facilitate preemptive therapy and management.
2. Severe CRS is characterized by endothelial activation.

ABSTRACT

Lymphodepletion chemotherapy followed by infusion of CD19-specific chimeric antigen receptor-modified T cells (CAR-T) has produced impressive antitumor responses in patients with refractory $CD19^+$ B cell malignancies, but is often associated with cytokine release syndrome (CRS). Our understanding of CRS continues to evolve, and identification of the kinetics of CRS and predictive clinical and laboratory biomarkers of severity are needed to evaluate strategies to mitigate toxicity. We report the clinical presentation and identify biomarkers of severe CRS in 133 adult patients who received CD19 CAR-T cells. CRS developed in 70% of patients, including 62.5% with grade 1-3 CRS (grade 1, 26%; grade 2, 32%; grade 3, 4.5%), 3.8% with grade 4 CRS, and 3.8% with grade 5 CRS. A majority of cases of grade ≥4 CRS occurred during CAR-T cell dose-finding. Multivariable analysis of baseline characteristics identified high marrow tumor burden, lymphodepletion using cyclophosphamide and fludarabine, higher CAR-T cell dose, thrombocytopenia before lymphodepletion, and manufacturing of CAR-T cells without selection of $CD8^+$ central memory T cells as independent predictors of CRS. Severe CRS was characterized by hemodynamic instability, capillary leak, and consumptive coagulopathy. Angiopoietin-2 and von Willebrand Factor, which are biomarkers of endothelial activation were increased during severe CRS, and also before lymphodepletion in patients who subsequently developed CRS. We describe a classification-tree algorithm to guide studies of early intervention after CAR-T cell infusion for patients at high risk of severe CRS. These data provide a framework for early intervention studies to facilitate safer application of effective CD19 CAR-T cell therapy.

INTRODUCTION

Lymphodepletion chemotherapy followed by infusion of T cells that are engineered to express a CD19-targeting CAR (CD19 CAR-T cells) has shown remarkable efficacy in patients with relapsed and/or refractory (R/R) $CD19^+$ B cell malignancies, with reported complete response (CR) rates as high as 93% in B cell acute lymphoblastic leukemia (B-ALL), and overall response (OR) rates of 77% in chronic lymphocytic leukemia (CLL), and 82% in non-Hodgkin's lymphoma (NHL).[1-13] Durable CRs without subsequent anti-tumor therapy have been observed in a subset of patients who received CD19 CAR-T cell therapy, demonstrating the potential of this approach to improve survival in otherwise refractory patients.[1,2,8]

After adoptive transfer, CAR-T cells are activated by encounter with $CD19^+$ tumor or normal B cells, which results in proliferation of CAR-T cells, lysis of the target cell, and cytokine secretion that can be associated with the clinical evidence of cytokine release syndrome (CRS) and neurotoxicity. CRS after CD19 CAR-T cell therapy presents with fever, hypotension, coagulopathy and capillary leak, and has been reported to occur in 54–91% of patients, including severe CRS in 8.3–43%.[1,2,7-10,14-16] The increased availability of CD19 CAR-T cell therapies in multicenter trials highlight the need to provide clinicians treating B-ALL, NHL and CLL patients with a detailed description of the clinical syndrome of CRS.[17,18]

A comprehensive description of the time course of presentation and biomarkers of CRS in a large cohort of patients has not been reported. Here, we report the clinical and laboratory findings from 133 adult patients with $CD19^+$ R/R B-ALL, NHL, and CLL who received lymphodepletion chemotherapy followed by infusion of CD19 CAR-T cells. We identify risk factors before and after CAR-T cell infusion that are associated with the incidence and severity of subsequent CRS, allowing identification of patients at high risk of severe toxicity who might be candidates for early intervention studies. The data will facilitate recognition, diagnosis, and treatment of CRS.

METHODS

Study design

We enrolled patients with R/R CD19$^+$ B cell malignancies on a phase I/II clinical trial evaluating lymphodepletion chemotherapy followed by CD19 CAR-T cells.[1,2] The study is available at www.clinicaltrials.gov (NCT01865617), and was conducted according to the principles of the Declaration of Helsinki and approval of the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board. This manuscript reports clinical and laboratory data from 133 consecutively treated patients on the study receiving their first cycle of lymphodepletion and CAR-T cell infusion.

Lymphodepletion chemotherapy and CD19 CAR-T cell infusion

The design of the CAR transgene and CAR-T cell manufacturing from CD4$^+$ T cells and either bulk or central memory-enriched CD8$^+$ T cells have been previously described (**Supplemental data**).[1,2]. A truncated human epidermal growth factor receptor (EGFRt) was encoded in the lentiviral vector to allow precise enumeration of transduced CAR-T cells by flow cytometry.[19] Patients received lymphodepletion chemotherapy with a cyclophosphamide (Cy)-based regimen with or without fludarabine (Flu) (**Table S1**), followed 2-4 days later by infusion of CD19 CAR-T cells formulated in a 1:1 ratio of CD4$^+$:CD8$^+$ CAR-T cells and infused at one of three dose levels (DL; DL1, 2x10^5 EGFRt$^+$ cells/kg; DL2, 2x10^6 EGFRt$^+$ cells/kg; DL3, 2x10^7 EGFRt$^+$ cells/kg).

Grading of CRS and neurotoxicity

The severity of CRS was graded according to consensus criteria.[20] Neurologic adverse events (AEs) were graded according to the Common Terminology Criteria of Adverse Events (CTCAE) v4.0.3 and did not contribute to organ toxicity criteria for CRS grading.

Evaluation of clinical laboratory parameters, CAR-T cell counts, and serum biomarkers

Blood was collected before lymphodepletion, on day 0 before CAR-T cell infusion, and at intervals after CAR-T cell infusion for analyses of complete blood counts, renal function, hepatic function, coagulation, and serum cytokine concentrations. In a subset of patients, serum angiopoietin (Ang)-1, Ang-2, and von Willebrand Factor (VWF) concentrations were measured. $CD4^+$ and $CD8^+$ CAR-T cells were identified by flow cytometry as viable $CD45^+/CD3^+/CD4^+/CD8^-/EGFRt^+$ and $CD45^+/CD3^+/CD4^-/CD8^+/EGFRt^+$ events, respectively, in a lymphocyte forward/side scatter (FS/SS) gate. Additional details are provided in **Supplemental data**.

Statistical analyses

Statistical methods are reported in **Supplemental data**.

RESULTS

Patient and treatment characteristics

One hundred and thirty-three patients with relapsed or refractory B cell malignancies were included in the analyses (B-ALL, n=47; NHL, n=62; CLL, n=24). The median age was 54 years (range 20–73) and the median number of prior therapies was 4 (range 1–11; **Table 1**). Twenty-five patients (19%) had previously undergone allogeneic hematopoietic stem cell transplantation (HCT), 22 (17%) had undergone autologous HCT, and 3 (2%) had undergone both autologous and allogeneic HCT. The lymphodepletion regimens given prior to CAR-T cell infusion are shown in **Table S1**. A majority of patients (78%) received a regimen containing both Cy and Flu. Thirty-five (26%) patients received CAR-T cells at DL1, 86 (65%) received DL2, and 12 (9%) received DL3.

The incidence and kinetics of CRS and neurotoxicity

CRS of any grade developed in 93 of 133 patients (70%). The incidence, severity, and clinical presentation of CRS in B-ALL, CLL, and NHL patients were similar (**Table 1, Figure S1-2**). A majority of patients (123 of 133; 92.5%) had either no CRS (grade 0, 30%), or grade 1-3 CRS (grade 1, 26%; grade 2, 32%; grade 3, 4.5%). Ten patients (7.5%) developed grade ≥4 CRS (grade 4, 3.8%; grade 5, 3.8%) (**Figure 1**). Five of these 10 patients died within the first 30 days after CAR-T cell infusion as a result of complications associated with CRS and/or neurotoxicity. One additional patient died 4 months after CAR-T cell therapy due to irreversible neurotoxicity. Of the 10 patients (7.5%) with grade ≥4 CRS, 8 were enrolled and received CAR-T cells during the dose-escalation phase of the study. At the maximum tolerated dose (MTD) of CAR-T cells, grade ≥4 CRS was observed in 2 of 79 patients (2.5%).

Fever ≥38°C was the first objective sign of CRS with the exception of one patient who presented with hypotension without fever. Fever occurred a median of 2.2 days [interquartile range, IQR, 0.9–5.6] after CAR-T cell infusion and lasted for a median [IQR] of 3.0 [1.2–4.8]

days (**Table 2**). Compared to patients with grade 1-3 CRS, fever in patients with grade ≥4 CRS presented earlier after CAR-T cell infusion (*P*<.0001), peaked earlier (*P*=.001), reached a higher maximum temperature (*P*<.0001), and was of longer duration (*P*=.03, **Table 2, Figure 2A-B**). All patients who ultimately had grade ≥4 CRS were febrile within 25 hours after CAR-T cell infusion, and only 4 patients, all with grade ≤3 CRS, developed their first fever more than 12 days after CAR-T cell infusion (**Figure 2A**). Fifty-three of 133 patients (40%) had one or more grade ≥1 neurologic AEs (grade 1-2, 18%; grade ≥3, 21%), and the severity of neurotoxicity was associated with the severity of CRS (*P*<.0001; **Table S2**); all patients with grade ≥4 CRS also developed grade ≥3 neurotoxicity (**Figure 2C**). Neurotoxicity typically presented after CRS (*P*=.003), with the first neurologic AE of any grade presenting a median [IQR] of 4 [2–7] days after CAR-T cell infusion (**Figure 2D**). The first grade ≥3 neurologic AE presented 4.5 [3.2–6.2] days after the first fever.

One hundred and nine patients (82%) received both the lymphodepletion chemotherapy and CAR-T cell infusion in the outpatient setting. Outpatients were admitted at the first fever ≥38°C. Because the severity of CRS did not reach grade ≥3 until a median of 3.4 days after onset of fever (range 1.4–4.7), there was sufficient time for hospital admission and therapeutic interventions to mitigate CRS progression. The median [IQR] duration of hospitalization for all patients was 7 [3–14] days and was associated with the maximum severity of CRS (grade 0, median 0 days; grade 1-3, 9 days; grade ≥4, 18 days; *P*<.0001, **Table S2**). Twenty-six patients (20%) with CRS and/or neurotoxicity received tocilizumab and/or dexamethasone to treat CRS and/or neurotoxicity. Twenty patients received dexamethasone and tocilizumab, 5 received dexamethasone alone, and one received tocilizumab alone. All patients who received tocilizumab had grade ≥3 CRS and/or neurotoxicity, except 2 patients who had progressive grade 2 CRS. Fever resolved a median [IQR] of 0.4 [0.2–2.0] days following the first dose of tocilizumab or dexamethasone.

Severe CRS is associated with vascular instability and organ dysfunction

After CAR-T cell infusion, patients with severe CRS exhibited hemodynamic instability and capillary leak with hypotension, tachycardia, tachypnea, hypoalbuminemia, hypoproteinemia and weight gain (**Figure 3**). Seventeen of 133 patients (13%) required admission to the intensive care unit (ICU) for management of CRS and/or neurologic AEs, and the median [IQR] duration of care in the ICU was 3 [2–7] days. Eleven of 133 patients (8%) received vasopressor support. Only 2 patients with grade ≤3 CRS required vasopressor support. Ten patients (7.5%) required intubation to manage respiratory failure associated with severe neurotoxicity (n=5), management of pulmonary dysfunction (n=3), or disease progression (n=2).

All patients with grade ≥4 CRS also developed grade ≥3 non-neurologic organ toxicity, which resolved a median of 24 days (range 12–32) after resolution of fever. Nine of the 10 patients with grade ≥4 CRS developed hepatic dysfunction, manifest by elevated AST, ALT, ALP, and bilirubin, with 5 patients having grade ≥3 transaminase elevation (**Figure S3A-D**). The AST peaked between days 2-5, whereas the ALT, ALP and total bilirubin peaked later at day 6-8. One patient developed late hepatic dysfunction on day 20 associated with severe hypotension due to gastrointestinal hemorrhage. Three of the 10 patients with grade ≥4 CRS developed grade ≥3 acute kidney injury, with one patient requiring hemodialysis for 15 days until recovery of renal function (**Figure S3E-F**). Only 3 patients with grade ≤3 CRS developed grade 3 non-neurologic organ toxicity (2 hepatic, 1 cardiac), and these events resolved in 1-2 days.

Delayed hematopoietic recovery in patients with grade ≥4 CRS

We evaluated recovery of blood counts in all patients who had received lymphodepletion chemotherapy and CAR-T cell infusion. To ensure observed differences in hematopoietic

toxicity in patients with distinct grades of CRS were not due to differences in intensity of the lymphodepletion regimen, only patients who received Cy/Flu lymphodepletion (n=104) were included in this analysis. The absolute neutrophil count (ANC), hematocrit (HCT), hemoglobin concentration (Hb) and platelet count declined after Cy/Flu chemotherapy, reaching nadirs, between days 2 and 5 after CAR-T cell infusion (**Figure 4A-D**). The ANC, platelet and hematocrit nadirs were lower in patients with more severe CRS, and patients with grade ≥4 CRS received more platelet (*P*=.002) and red cell (*P*=.04) transfusions than those with grade ≤3 CRS (**Figure 4E**). Five of 10 patients with grade ≥4 CRS became refractory to platelet transfusion. Marrow tumor burden (*P*<.0001), the number of prior therapies (*P*=.02), and the occurrence of CRS (*P*=0.0002) were associated with longer hematologic recovery. The time to hematologic recovery was longer than expected in most patients with grade 4 CRS (**Table S3**), and was delayed in patients with grade 1-3 CRS (median [IQR] 13.5 [6.5–18.1] days compared to those without CRS (median [IQR], 4.1 [2.9–7.5] days, *P*=.0002).

CRS has been associated with macrophage activation syndrome.[20,21] Consistent with this, we observed higher ferritin and CRP levels, and more prolonged monocytopenia in blood of patients with grade ≥4 CRS compared to those with grade ≤3 CRS (**Figure S3G-I**). However, examination of bone marrow biopsies from patients with grade ≥4 CRS showed no evidence of increased hemophagocytosis that might contribute to delayed hematopoietic recovery. Rather, in 5 of 7 patients with grade ≥4 CRS and available marrow pathologic examination, the bone marrow was hypocellular without morphologic evidence of residual tumor, suggesting alternative mechanisms for delayed recovery (**Table S3**).

Consumptive coagulopathy in grade ≥4 CRS

We examined the prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer, and fibrinogen in patients at intervals after CAR-T cell infusion. Patients receiving therapeutic anticoagulation were excluded from the analyses (n=9). In the first week after CAR-

T cell infusion, patients with grade ≤3 CRS had normal or mildly elevated prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer, and fibrinogen. In contrast, those with grade ≥4 CRS developed early prolongation of the PT and aPTT, which peaked approximately 2-5 days after CAR-T cell infusion (**Figure 4F-G**). Increasing D-dimer and falling fibrinogen concentrations started at day 2-5, with hypofibrinogenemia occurring from days 9-12, consistent with disseminated intravascular coagulation (DIC; **Figure 4H-I**). Compared to patients with grade 1-3 CRS, those with grade ≥4 CRS received more cryoprecipitate transfusions to correct coagulopathy (*P*<.0001, **Figure 4E**), and had more severe and prolonged thrombocytopenia (**Figure 4D**). Grade ≥3 bleeding occurred in only 3 patients (2%), all of whom had grade ≥4 CRS. Red cell fragmentation was not a prominent feature on blood film morphology analysis. The findings were consistent with development of a consumptive coagulopathy in patients with severe CRS.

Biomarkers of endothelial activation are elevated in severe CRS

The presence of hemodynamic instability, capillary leak and a consumptive coagulopathy raised the possibility that endothelial activation might contribute to the clinical findings of severe CRS. Von Willebrand Factor (VWF) is released from Weibel-Palade bodies upon endothelial activation,[22] and plays a key role in the initiation of coagulation. To determine if *in vivo* endothelial activation was present in patients with severe CRS, we evaluated serum concentrations of VWF at the peak of CAR-T cell expansion in blood in a subset of 60 patients with different severities of CRS (grade 0 CRS, n=12; grade 1-3 CRS, n=39; grade ≥4 CRS, n= 9). We found that patients with grade ≥4 CRS had higher VWF concentrations compared to those with grade ≤3 CRS (**Figure 4J**). Serum concentrations of Ang-2, which is also released from Weibel-Palade bodies on endothelial activation and promotes capillary leak,[23,24] were also higher in patients with grade ≥4 CRS (**Figure S4A**). Ang-1 promotes endothelial stability and an increase in the Ang-2:Ang-1 ratio has been associated with morbidity and mortality in sepsis

and cerebral malaria.[25-30] At the peak of CAR-T cell expansion in blood, increasing severity of CRS was associated with lower Ang-1, higher Ang-2, and an increased Ang-2:Ang-1 ratio (**Figure 4K; Figure S4A**). Of note, before both lymphodepletion and CAR-T cell infusion, and on day 1 after CAR-T cell infusion, increasing serum VWF concentration was associated with increased severity of subsequent CRS (**Figure S4B**). Furthermore, before lymphodepletion and on day 1 after CAR-T cell infusion there was an association between increased Ang-2:Ang-1 and severity of CRS (**Figure S4C**). Together, these data indicate that biomarkers of endothelial activation are elevated during severe CRS, and that endothelial activation even prior to commencing lymphodepletion and CAR-T cell therapy may increase the risk of subsequent development of severe CRS.

Patient and treatment characteristics associated with the development and severity of CRS

To identify patients at risk of developing CRS, we performed univariate analyses of the impact of baseline clinical and laboratory characteristics on the development of any grade of CRS. Patients with higher marrow tumor burden (P<.0001), a higher percentage of $CD19^+$ cells in the marrow (P=.0001), and more severe thrombocytopenia (P=.002) were at higher risk of developing CRS (**Table 1**). Manufacturing of CAR-T cells using bulk $CD8^+$ T cells without selection of the central memory subset (P=.001) and the infused CAR-T cell dose (P=.002) were associated with increased risk of CRS. Despite our previous observation that addition of Flu to Cy in lymphodepletion enhanced *in vivo* CAR-T cell expansion,[1,2] this was not associated with increased occurrence of CRS in univariate analysis. However, analysis of the interaction between CAR-T cell dose and Cy/Flu lymphodepletion showed that addition of Flu at any given CAR-T cell dose increased the risk of CRS (P=.03). Stepwise multivariable analysis showed that higher bone marrow $CD19^+$ tumor burden (P<.0001), more severe thrombocytopenia (P=.05), bulk $CD8^+$ T cell selection (P=.03), Cy/Flu lymphodepletion (P=.02), higher CAR-T cell

dose (*P*=.003), and the interaction effect of CAR-T cell dose and Cy/Flu lymphodepletion (*P*=.009) were independently associated with development of CRS (**Table 1**). Risk factors for CRS within each disease cohort are presented in **Tables S4A-C**.

We then examined risk factors for the occurrence of any grade of CRS that were identified in the multivariable model to see if these factors also impacted the severity of CRS (**Table 3**). Univariate pairwise analysis showed that only higher CAR-T cell dose (*P*=.0003) and Cy/Flu lymphodepletion (*P*=.03) were associated with the development of grade ≥4 compared to grade 1-3 CRS.

Mitigation of toxicity by reduction in peak CAR-T cell counts in blood will be associated with reduced response rates

Consistent with the observation that a high CAR-T cell dose and Cy/Flu lymphodepletion were associated with severity of CRS, we found earlier and higher peaks in absolute CAR-T cell numbers in blood of patients with grade ≥4 CRS compared to grade 1-3 or no CRS (**Figure 5A-D**). To identify a therapeutic window of absolute CAR-T cell numbers that would minimize the risk of CRS and neurotoxicity while retaining a high probability of anti-tumor activity in each disease, we modeled the relationship between the peak CAR-T cell counts in blood and the occurrence of toxicity or disease response using logistic regression (**Figure 5E-H**). B-ALL patients that achieved a peak of 10 $CD8^+$ CAR-T cells/μL had an estimated probability of MRD^- CR of 95%, and the estimated probabilities of grade ≥2 CRS and grade ≥3 neurotoxicity were 37% and 15%, respectively. Similar findings were noted for patients with 5 $CD4^+$ CAR-T cells/μL (MRD- CR, 94%; grade ≥2 CRS, 42%; grade ≥3 neurotoxicity, 19%). Reduction of the infused CAR-T cell dose in B-ALL patients with high marrow tumor burden resulted in consistent targeting of peak CAR-T cell counts in the ranges associated with high efficacy without undue toxicity (**Table S5**);[2] however, the therapeutic window was narrow. The probabilities of marrow

response and toxicity in CLL patients were similar to those in B-ALL. In NHL patients, a therapeutic window with high efficacy and low toxicity could not be established. These data suggest that CAR-T cell dose reduction as a sole strategy to mitigate toxicity will reduce efficacy, and that early intervention approaches that do not involve reduction in the peak CAR-T cell counts in blood should be investigated.

Early identification of patients at high risk of severe CRS

We investigated whether patients who would subsequently develop life-threatening CRS could be identified early after CAR-T cell infusion to allow institution of intervention strategies that might prevent progression of CRS. All patients who developed grade ≥4 CRS had fever ≥38.9°C within the first 36 hours after CAR-T cell infusion; however, using fever ≥38.9°C within 36 hours as the only indication for intervention would have resulted in unnecessary treatment of 20 patients with grade ≤3 CRS (sensitivity 1.00, specificity 0.84). Patients who developed grade ≥4 CRS also exhibited higher concentrations of IFN-γ, IL-6, IL-8, IL-10, IL-15, MCP-1, TNFRp55, and MIP-1β within 36 hours after CAR-T cell infusion, compared to those with grade ≤3 CRS (*P*<.0001; **Figure 6A-H**). These cytokines were elevated before the onset of grade ≥3 CRS and demonstrated similar kinetics in ALL, NHL and CLL patients (**Figure S5**), suggesting that one or more of these cytokines could be useful as predictive biomarkers for grade ≥4 CRS (**Figure 6A-H**). We performed classification tree modeling and found that in patients with fever ≥38.9°C within 36 hours of CAR-T cell infusion, a serum MCP-1 concentration ≥1343.5 pg/mL performed better than CRP, ferritin, or other cytokines, and enhanced identification of patients who developed grade ≥4 CRS (sensitivity 1.00, specificity 0.95) (**Figure 6I**). Using this approach 6 of 133 patients (4.5%) were misclassified as being at high risk of grade ≥4 CRS, 4 of whom developed grade ≥2 CRS and/or neurotoxicity, indicating that unnecessary early intervention

would uncommonly be employed for patients who did not develop moderate or severe CRS and/or neurotoxicity.

DISCUSSION:

The success of CD19-specific CAR-T cell immunotherapy in the treatment of patients with relapsed/refractory B cell malignances has resulted in an increase in the number of clinicians who are required to manage the novel toxicities associated with this therapy. We describe the clinical presentation, laboratory findings, and correlative and predictive biomarkers of CRS in a large cohort of patients who received lymphodepletion chemotherapy and CD19 CAR-T cells to guide physicians caring for these patients. CRS was a frequent event after CAR-T cell immunotherapy, occurring in 70% of patients; however, in a majority of patients it was mild-moderate and resolved within days without a requirement for tocilizumab or dexamethasone intervention. Life-threatening CRS was uncommon (7.5%) and mainly occurred during the CAR-T cell dose escalation phase of our study. At the CAR-T cell MTD, grade ≥4 CRS was rare (2.5%). Despite the potential for severe toxicity, a strategy of hospital admission at the first fever made outpatient lymphodepletion chemotherapy and CAR-T cell infusion a feasible approach, even in heavily pretreated patients with advanced B cell malignancies.

The risk of CD19 CAR-T cell therapy could potentially be reduced by identifying patients who are at high risk of developing severe CRS before therapy and modifying the treatment regimen, or early after CAR-T cell infusion when preventative interventions could be instituted. Multivariable analysis identified baseline and treatment related risk factors for CRS, including those associated with more robust CAR-T cell expansion, such as higher marrow tumor burden, Cy/Flu lymphodepletion, and higher CAR-T cell dose. Other pretreatment factors that were associated with CRS, such as thrombocytopenia and manufacturing of CAR-T cells from bulk $CD8^+$ T cells may be a reflection of the higher tumor burden in these patients; however, distinct mechanisms cannot be excluded.

Because *in vivo* CAR-T cell expansion is driven by recognition of cells expressing CD19, a logical approach to reducing the risk of severe CRS is to reduce the CAR-T cell dose in patients with a high tumor burden. This strategy was effective in mitigating toxicity in B-ALL

patients without impairing efficacy.[2] However, our analyses indicate that the therapeutic window is narrow and that a reduction in CAR-T cell dose that results in peak $CD8^+$ CAR-T cells <10 cells/μL and $CD4^+$ CAR-T cells <5/μL will likely result in reduced efficacy. This is particularly true in NHL, in which the probabilities of CR, grade ≥2 CRS, and grade ≥3 neurotoxicity were similar at any given peak CAR-T cell count.

The risk of impaired efficacy with CAR-T cell dose reduction suggests that the optimal strategy might enable delivery of an adequate CAR-T cell dose, followed by early intervention in those patients exhibiting clinical or laboratory findings associated with a high risk of subsequent toxicity. We previously reported that increases in distinct serum cytokines within the first day after CAR-T cell infusion were associated with subsequent requirement for ICU care.[1,2] An association of these cytokines with subsequent grade ≥4 CRS was confirmed in the current larger cohort of ALL, NHL and CLL patients. A predictive algorithm based on evaluating multiple cytokine concentrations, as reported in pediatric ALL[21] may be complex and expensive to implement. Therefore, we used classification-tree modeling to design a simple two-step algorithm to predict grade ≥4 CRS, in which a single serum cytokine concentration was measured only in the small subset of patients with fever ≥38.9°C within 36 hours of infusion. The model was designed to identify patients who would develop life-threatening CRS, despite appropriate intervention with tocilizumab and/or dexamethasone for grade 2-3 CRS. The best sensitivity and specificity was obtained by testing serum MCP-1 in patients with fever ≥38.9°C within 36 hours of infusion. Fever and CRP evaluation have previously been used to identify those at risk of severe toxicity;[9] however, in our study MCP-1 evaluation was superior to CRP testing. The optimal pre-emptive therapy of high-risk patients is unknown. While widely used approaches to treat severe CRS (e.g. tocilizumab 8 mg/kg I.V. and dexamethasone 8 mg/kg b.i.d. I.V.) might be suitable, these and other strategies to modify cytokine signaling should be studied in suitably designed clinical trials.

The presentation of vascular instability, capillary leak, and consumptive coagulopathy suggested that endothelial activation or dysfunction coincides with severe CRS. This was confirmed by demonstrating that severe CRS was accompanied by high serum concentrations of VWF and Ang-2, which are released from Weibel-Palade bodies on endothelial activation. The mechanisms that lead to endothelial activation in CRS have not been characterized; however, the high serum concentrations of endothelium activating cytokines, such as IL-6 and IFN-γ observed in patients with severe CRS suggest that these cytokines may contribute. Additional studies will be required to determine whether regulation of endothelial activation, for example by modification of the Ang-1/2 axis, could be employed to treat patients with severe CRS, as proposed for patients with infection-related microvascular dysfunction.[32] We also found that serum VWF and the Ang-2:Ang-1 ratio were higher prior to commencing CAR-T cell immunotherapy in patients who subsequently developed more severe CRS, suggesting that pre-existing endothelial activation might be a previously unrecognized risk factor for severe CRS. It is noteworthy that thrombocytopenia before lymphodepletion chemotherapy was also associated with subsequent severe CRS. Platelets are one of the few sources of the endothelial stabilizing cytokine, Ang-1, suggesting that patients with severe thrombocytopenia might be prone to endothelial activation.[26]

CRS is a common AE after CAR-T cell therapy, but is well tolerated in most patients who receive an optimized lymphodepletion regimen and CAR-T cell dose. The safety profile supports delivery of this treatment in the outpatient setting, and effective therapies are available for most patients who develop severe CRS. Additional understanding of the risk factors and mechanisms that lead to severe CRS will facilitate testing of interventions to prevent or reverse toxicity, and improve the safety of CD19 CAR-T cells, and potentially of CAR-T cells targeting other malignancies.[33]

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AUTHORSHIP:

KAH and JG collected and analyzed research data; SC and XC collected research data; LAH, WCL, MMW, JAL, JC, DC, and SH-B designed and performed experiments; DL performed statistical analyses. KAH, SRR, DGM and CJT wrote and edited the manuscript. All authors reviewed the final version of the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST:

KAH, LAH, MMW, JAL, JC, DC, SC, XC, and SH-B have no conflicts of interest to disclose. CJT, SRR and DGM receive research funding from Juno Therapeutics, Inc. DL is an employee of, and has equity interests in, Juno Therapeutics, Inc. SRR has equity interests in Juno Therapeutics, Inc. CJT, SRR, WCL, and DL are named as inventors on one or more patents or patent applications related to this work. FHCRC receives research funding from Juno Therapeutics.

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TABLES:

Table 1: Univariate and multivariable analysis of baseline and therapy-related characteristics by severity of CRS.

CRS Grade	0	1-3	4-5	Total	Univariate Analysis *P* value [a]	Multivariable Analysis *P* value [b]
Number of Patients, n	40	83	10	133		
Age, years					.55	-
Median [IQR]	56 [44, 65]	54 [43, 61]	53.5 [43, 62]	54 [43, 62]		
Range	27, 70	20, 73	20, 70	20, 73		
Sex, n (%)					.79	-
Male	28 (30)	59 (63)	6 (7)	93 (70)		
Female	12 (30)	24 (60)	4 (10)	40 (30)		
Karnofsky Performance, n (%)					.30	-
60 – 70	2 (14)	10 (71)	2 (14)	14 (10)		
80 – 90	32 (30)	67 (63)	7 (7)	106 (80)		
100	6 (46)	6 (46)	1 (8)	13 (10)		
Disease Type, n (%)					.30	-
ALL	12 (25)	31 (66)	4 (9)	47 (35)		
CLL	4 (17)	18 (75)	2 (8)	24 (18)		
NHL	24 (39)	34 (55)	4 (6)	62 (47)		
Prior Lines of Therapy, n					.13	-
Median [IQR]	3 [2, 5]	4 [3, 5]	5 [3, 7]	4 [3, 5]		
Range	1, 11	1, 11	2, 9	1, 11		
Prior Transplant, n (%)					.38[c]	-
Allogeneic only	3 (12)	21 (84)	1 (4)	25 (19)		
Autologous only	9 (41)	11 (50)	2 (9)	22 (17)		
Both	0 (0)	3 (100)	0 (0)	3 (2)		
Marrow Disease Burden by Flow Cytometry, %					**<.0001**	**<.0001**
Median [IQR]	0 [0, 1.3]	20 [0, 65]	21 [3.6, 40]	1.3 [0, 42]		
Range	0, 79	0, 97	0, 89.8	0, 97		
Not involved, n (%)	23 (47)	25 (51)	1 (2)	49 (37)		
CD19+ Cells in Marrow by Flow Cytometry, %					**.0001[d]**	**-**
Median [IQR]	3.6 [1.3, 6.6]	22 [3.0, 66]	22 [11, 40]	8.8 [2.2, 48]		
Range	0, 79	0, 99	0.3, 90	0, 99		
Platelet Count, 1000/µl					**.002**	**.05**
Median [IQR]	98 [58, 159]	69 [38, 119]	32 [19, 85]	77 [40, 133]		
Range	11, 265	1, 553	5, 162	1, 553		
$CD8^+$ Selection Method, n (%)					**.001**	**.03**
Bulk $CD8^+$	9 (15)	47 (77)	5 (8)	61 (46)		
Central Memory Enriched	31 (43)	36 (50)	5 (7)	72 (54)		
Lymphodepletion, n (%)					.67	**.02**
Cy/Flu based	30 (29)	65 (62)	9 (9)	104 (78)		
Non-Cy/Flu based	10 (35)	18 (62)	1 (3)	29 (22)		
CAR-T Cell Dose, n (%)					**.002**	**.003**
2×10^5 $EGFRt^+$ cells/kg	10 (29)	25 (71)	0 (0)	35 (26)		
2×10^6 $EGFRt^+$ cells/kg	27 (31)	54 (63)	5 (6)	86 (65)		
2×10^7 $EGFRt^+$ cells/kg	3 (25)	4 (33)	5 (42)	12 (9)		
Lymphodepletion/CAR-T Cell Dose Interaction Effect[e]					**.03**	**.009**

[a] Two-sided *P*-values calculated based on Kruskal-Wallis test for continuous variables, and Fisher's Exact test for categorical variables.

[b] Step-wise multivariable proportional odds models were performed to assess impact of baseline factors on the occurrence of CRS (Grade 0 vs 1-3 vs 4-5), where $\log_{10}$ values were used to transform data as appropriate, with 0.001 substituting for values of 0.

[c] Any transplant type versus no transplant.
[d] Since marrow disease burden and total $CD19^+$ cells in marrow have a strong correlation (r = 0.99, *P* <.0001), only marrow disease was included in the multivariable analysis.
[e] The interaction effect demonstrates that at increasing CAR-T cell dose levels the incorporation of Flu into the lymphodepleting regimen has a greater association with CRS.

Table 2: Characterization of fever in patients who develop CRS

CRS Grade	1-3	4-5	Total	*P* value[a]
Number of Patients, n	83	10	92	
Fever Onset (days after CAR-T cell infusion)				<.0001
Median [IQR]	3.9 [0.8, 5.6]	0.4 [0.3, 0.9]	2.2 [0.9, 5.6]	
Range	0.1, 19	0.2, 1.0	0.1, 19	
Time to Peak Temperature (days after CAR-T cell infusion)				.001
Median [IQR]	5.7 [4.3, 7.6]	2.8 [1.3, 3.2]	5.3 [3.4, 7.3]	
Range	0.2, 30	0.4, 11	0.2, 30	
Maximum Temperature (°C)				<.0001
Median [IQR]	39.4 [39.2, 30.6]	40.4 [40.1, 40.6]	39.5 [39.2, 39.8]	
Range	37.7, 41.3	39.9, 40.9	37.7, 41.3	
Fever Duration (days after first fever)				.03
Median, [IQR]	2.5 [1.2, 4.7]	4.4 [3.6, 5.4]	3.0 [1.2, 4.8]	
Range	0.02, 15	3.1, 6.8	0.02, 15	

[a]Two-sided *P*-values calculated based on Wilcoxon test.

Table 3: Univariate pairwise analysis of factors significant in the multivariable proportional odds model

CRS Grade	Univariate pairwise P values		
	0 vs. 1-3	0 vs. 4-5	1-3 vs. 4-5
Marrow burden of disease %	<.0001	0.0001	0.8
Platelet count	0.01	0.005	0.06
CAR-T cell dose level	0.7	0.005	0.0003
Bulk $CD8^+$ T cell selection	0.0005	0.12	0.7
Flu/Cy stratified by dose level	0.8	0.4	0.03

FIGURE LEGENDS:

Figure 1: Presentation, management, and outcomes of patients with grade ≥4 CRS. Colors on the swimmer plot indicate the CRS grade on each day through 28 days after CAR-T cell infusion in all patients who developed grade ≥4 CRS. The duration of grade ≥3 neurotoxicity and interventions with tocilizumab and/or corticosteroids are indicated in the figure. ALL-2 developed dialysis-dependent acute kidney injury (AKI) through day 26 followed by resolution of CRS-associated organ toxicity (grade 0) on day 37. ALL-3 died 4 months after CAR-T cell infusion with irreversible neurotoxicity, despite resolution of fever and hypotension associated with CRS on day 13 after CAR-T cell infusion. NHL-1 had ongoing grade 1 AKI at last available laboratory value on day 83. Doses of medications: dexamethasone 10mg intravenous (IV) or oral, methylprednisolone 1g IV, tocilizumab 4-8 mg/kg IV. NT, neurotoxicity.

Figure 2: Kinetics of presentation of CRS and neurotoxicity. (**A**) Cumulative incidence curve for first fever ≥38°C in patients with grade 1-3 (n=82) or grade ≥4 CRS (n=10). (**B**) Mean ± SEM of the maximum temperature after CAR-T cell infusion. Kruskal-Wallis test, *** P<.0001, ** .0001<P<.001, *.001<P<.005. (**C**) Incidence and grading of neurotoxicity within each CRS grade. (**D**) The median time of onset of fever ≥38°C (red, n=92) or neurotoxicity (blue, n=53) after CAR-T cell infusion. One patient with grade 2 CRS who developed hypotension without fever is not included. NT, neurotoxicity. Pre-chemo, prior to the start of lymphodepletion chemotherapy; Pre-infusion, before CAR-T cell infusion; h, hours; d, days after CAR-T cell infusion.

Figure 3: Hemodynamic instability and clinical capillary leak in grade ≥4 CRS. (**A-G**) Mean ± SEM of the minimum systolic and diastolic blood pressure (A-B), maximum heart and respiratory rates (C-D), minimum serum protein and albumin concentration (E-F), and weight

gain from the start of lymphodepletion (G) are shown at the indicated times after CAR-T cell infusion. Kruskal-Wallis test, ***P<.0001, **.0001<P<.001, *.001<P<.005. Pre-chemo, prior to the start of lymphodepletion chemotherapy; Pre-infusion, before CAR-T cell infusion; h, hours; d, days after CAR-T cell infusion. Grey shading indicates the normal range.

Figure 4: Hematopoietic toxicity, laboratory coagulopathy, and endothelial injury in grade ≥4 CRS. (**A-D**) The minimum ANC (A), hematocrit (B), hemoglobin (C) and platelet count (D) are shown for patients receiving Cy/Flu lymphodepletion at the indicated times after CAR-T cell infusion (n=104). (**E**) Total transfused units of packed red blood cells (pRBC), platelets (Plt), and cryoprecipitate (Cryo) in the first 28 days after CAR-T cell infusion. (**F-I**) The maximum PT (F) and aPTT (G), minimum fibrinogen (H), and maximum d-dimer (I) concentrations are shown at the indicated times after CAR-T cell infusion. (**J**) The fold change in VWF concentration in serum from a subset of patients at the peak of CAR-T cell expansion (n=60; grade 0, n=12; grade 1-3, n=39; grade ≥4 CRS, n=9) compared to the VWF concentration in pooled normal plasma (12.2 μg/mL; CRYOcheck, Precision Biologic, Dartmouth, NS, Canada). (**K**) The Ang-2:Ang-1 ratio at the peak of CAR-T cell expansion (n=60; grade 0, n=12; grade 1-3, n=39; grade ≥4 CRS, n=9). For (A-D) and (F-I): Data represent the mean +/- SEM. *P* values were determined using the Kruskal-Wallis test, *** P<.0001, **.0001<P<.001, *.001<P<.005. Pre-chemo, prior to the start of lymphodepletion chemotherapy; Pre-infusion, before CAR-T cell infusion; h, hours; d, days after CAR-T cell infusion. Grey shading indicates the normal range. For (E, J, K): Each point represents data from one patient. The median and interquartile range [IQR] are shown. *P* values were determined using the Wilcoxon test, Gr, grade.

Figure 5: CAR-T cell counts in blood and estimated probabilities of response or toxicity. (A-D) The absolute number (A-B) and percentage (C-D) of $CD8^+$ (left) and $CD4^+$ (right) CAR-T cells in blood. The mean ± SEM of the maximum values are shown; *P* values were determined

using the Kruskal-Wallis test, ***P<.0001, **.0001<P<.001, *.001<P<.005. h, hours; d, days after CAR-T cell infusion. (**E-F**) Estimated probabilities by logistic regression of grade ≥2 CRS and grade ≥3 neurotoxicity (NT) at peak $CD8^+$ (E) and $CD4^+$ (F) CAR-T cell counts in blood. (**G-H**) Estimated probabilities by logistic regression of bone marrow complete response (CR) in ALL and CLL patients by flow cytometry, and CR or overall response (OR) in NHL patients according to Cheson imaging criteria (2014) at peak $CD8^+$ (G) and $CD4^+$ (H) CAR-T cell counts in blood. Lymph node CR in CLL patients is not depicted due to the limited cohort size available for analysis. *P* values are color-coded to indicate the association between the CAR-T cell peak counts and outcomes.

Figure 6: Biomarkers for early prediction of grade ≥4 CRS. (A-H) Concentrations of listed cytokines in serum obtained from patients at the indicated time points. Pre-chemo, prior to the start of lymphodepletion chemotherapy; Pre-infusion, before CAR-T cell infusion; h, hours; d, days after CAR-T cell infusion. *P* values were determined using the Kruskal-Wallis test, ***P<.0001, **.0001<P<.001, *.001<P<.005. (**I**) An algorithm for early identification of patients at high risk of grade ≥4 CRS using classification tree modeling. Early high fever (≥38.9°C) within the first 36 hours after CAR-T cell infusion triggers evaluation of serum MCP-1 concentration. Patients with fever ≥38.9°C and serum MCP-1 ≥1343.5 pg/mL are at high risk for subsequent development of grade ≥4 CRS. Gr, grade.

Figure 1

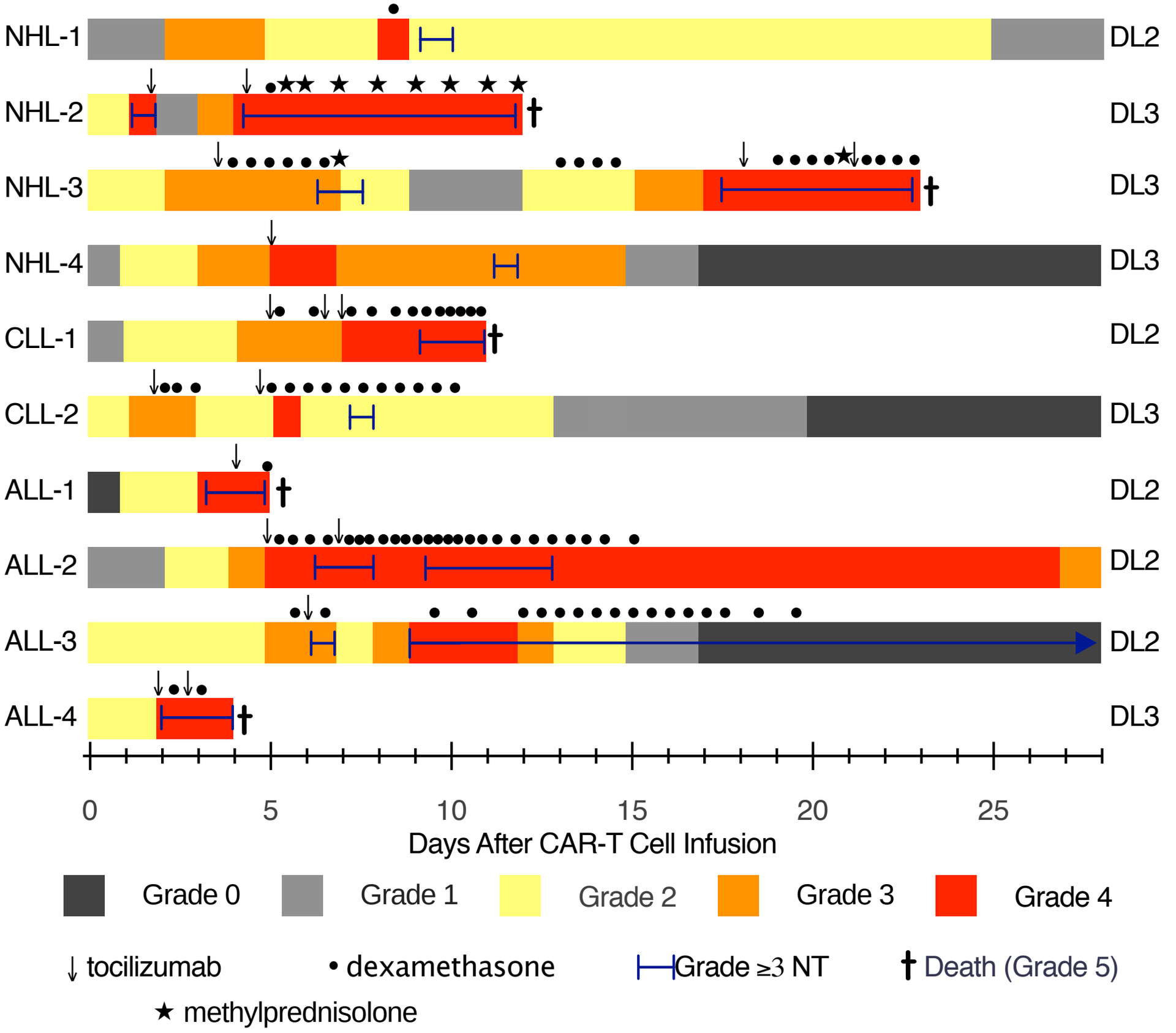
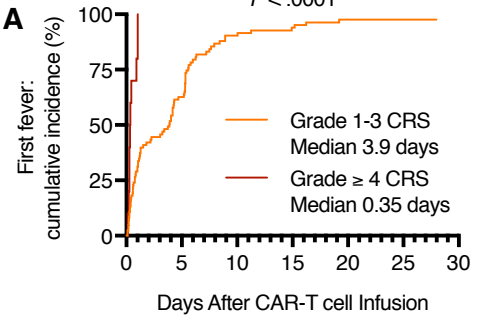
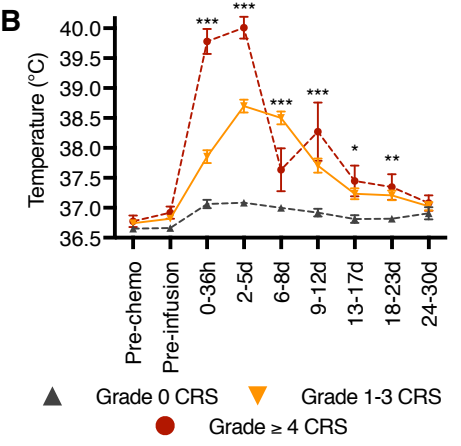
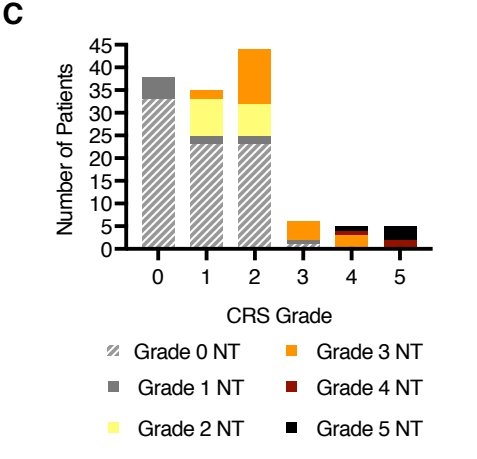

NHL-1
DL2
NHL-2
DL3
NHL-3
DL3
NHL-4
DL3
CLL-1
DL2
CLL-2
DL3
ALL-1
DL2
ALL-2
DL2
ALL-3
DL2
ALL-4
DL3
0
5
10
15
20
25
Days After CAR-T Cell Infusion
Grade 0
Grade 1
Grade 2
Grade 3
Grade 4
tocilizumab
dexamethasone
Grade ≥3 NT
Death (Grade 5)
methylprednisolone

Figure 2


A
P < .0001
First fever: cumulative incidence (%)
100
75
50
25
0
0 5 10 15 20 25 30
Days After CAR-T cell Infusion
Grade 1-3 CRS
Median 3.9 days
Grade ≥ 4 CRS
Median 0.35 days


B
Temperature (°C)
40.0
39.5
39.0
38.5
38.0
37.5
37.0
36.5
***
***
***
*
**
Pre-chemo
Pre-infusion
0-36h
2-5d
6-8d
9-12d
13-17d
18-23d
24-30d
Grade 0 CRS
Grade 1-3 CRS
Grade ≥ 4 CRS


C
Number of Patients
45
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5
CRS Grade
Grade 0 NT
Grade 1 NT
Grade 2 NT
Grade 3 NT
Grade 4 NT
Grade 5 NT

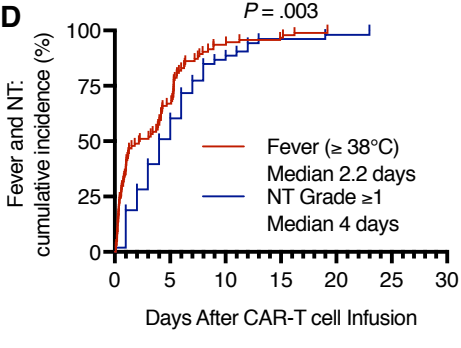

D
P = .003
Fever and NT: cumulative incidence (%)
100
75
50
25
0
0 5 10 15 20 25 30
Days After CAR-T cell Infusion
Fever (≥ 38°C)
Median 2.2 days
NT Grade ≥1
Median 4 days

Figure 3

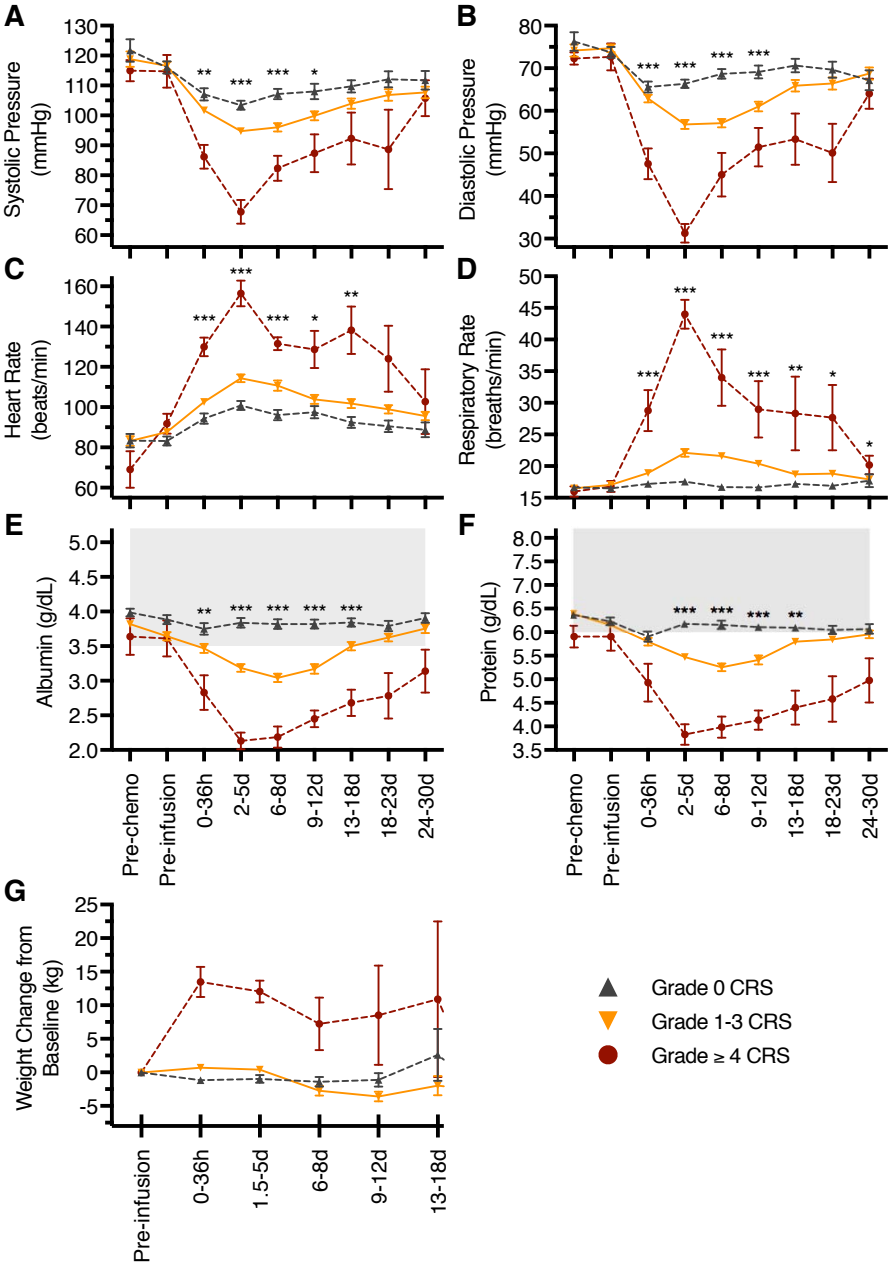

A
Systolic Pressure (mmHg)
B
Diastolic Pressure (mmHg)
C
Heart Rate (beats/min)
D
Respiratory Rate (breaths/min)
E
Albumin (g/dL)
F
Protein (g/dL)
G
Weight Change from Baseline (kg)
Pre-chemo
Pre-infusion
0-36h
2-5d
6-8d
9-12d
13-18d
18-23d
24-30d
1.5-5d
Grade 0 CRS
Grade 1-3 CRS
Grade ≥ 4 CRS

Figure 4

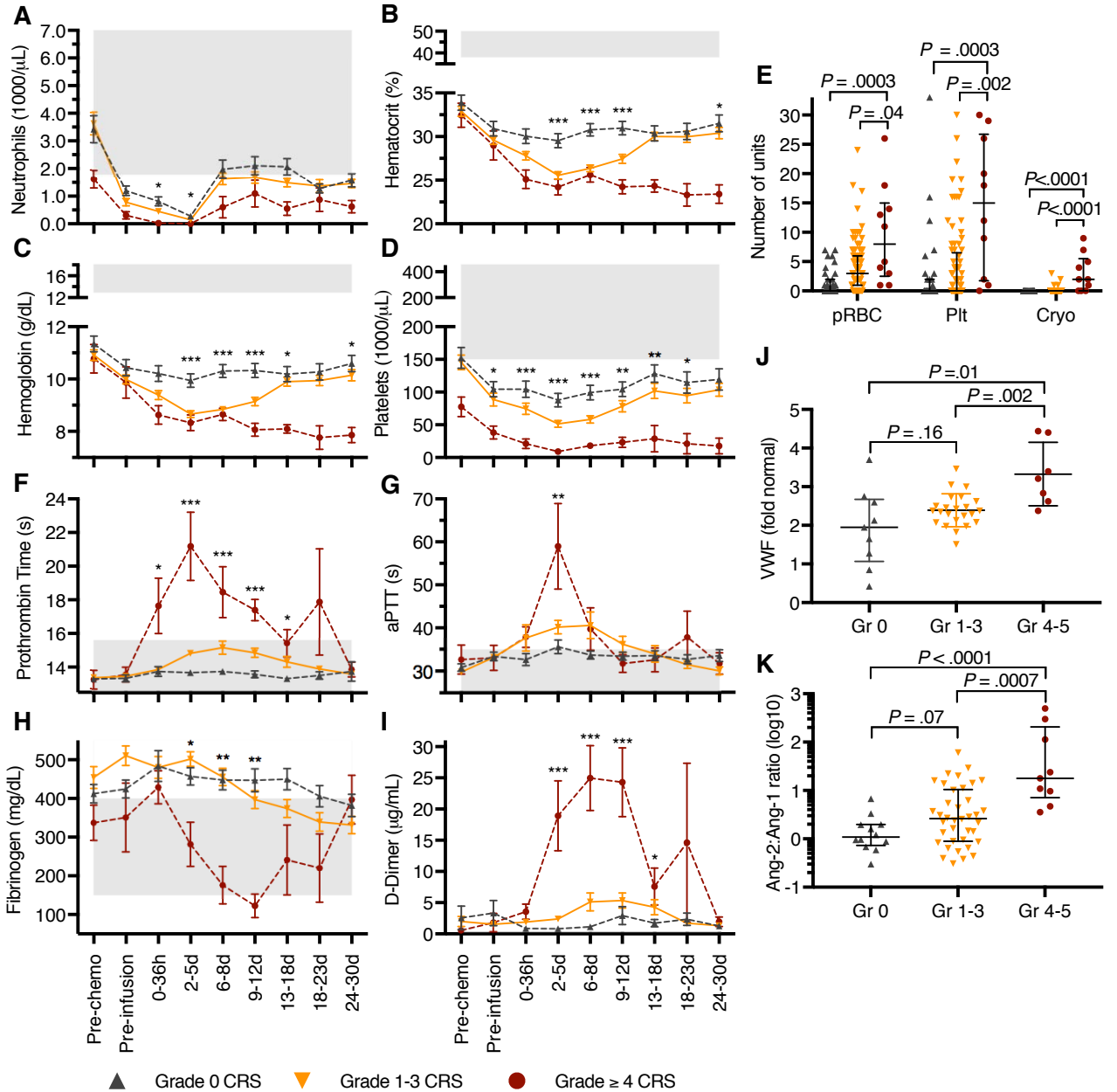
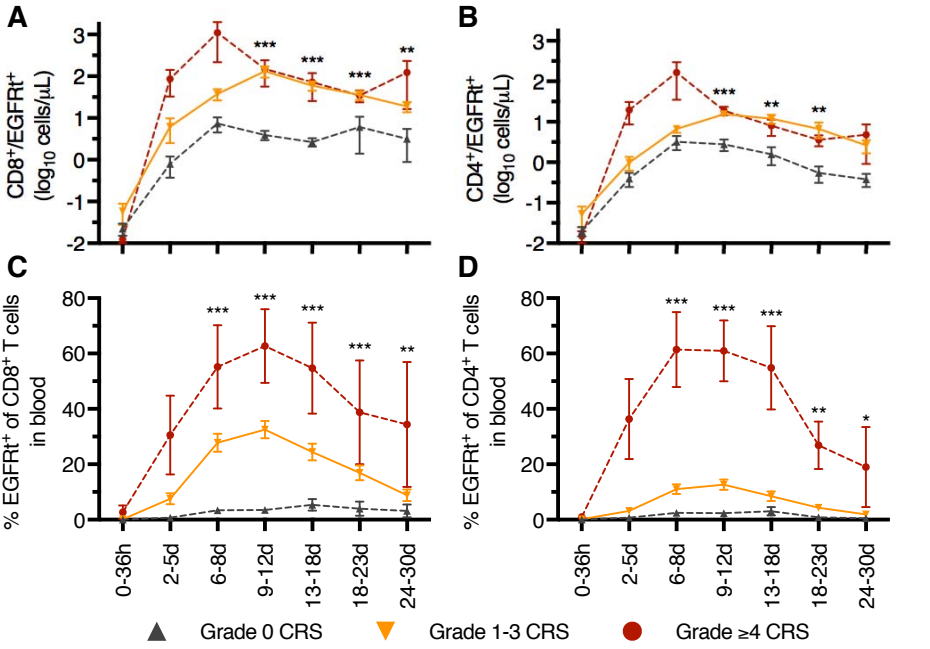

A
Neutrophils (1000/μL)
B
Hematocrit (%)
C
Hemoglobin (g/dL)
D
Platelets (1000/μL)
E
Number of units
P = .0003
P = .0003
P = .002
P = .04
P<.0001
P<.0001
pRBC
Plt
Cryo
F
Prothrombin Time (s)
G
aPTT (s)
H
Fibrinogen (mg/dL)
I
D-Dimer (μg/mL)
J
VWF (fold normal)
P = .01
P = .002
P = .16
Gr 0
Gr 1-3
Gr 4-5
K
Ang-2:Ang-1 ratio (log10)
P < .0001
P = .0007
P = .07
Gr 0
Gr 1-3
Gr 4-5
Pre-chemo
Pre-infusion
0-36h
2-5d
6-8d
9-12d
13-18d
18-23d
24-30d
Grade 0 CRS
Grade 1-3 CRS
Grade ≥ 4 CRS

Figure 5


A
B
C
D
CD8+/EGFRt+ (log10 cells/μL)
CD4+/EGFRt+ (log10 cells/μL)
% EGFRt+ of CD8+ T cells in blood
% EGFRt+ of CD4+ T cells in blood
0-36h
2-5d
6-8d
9-12d
13-18d
18-23d
24-30d
Grade 0 CRS
Grade 1-3 CRS
Grade ≥4 CRS

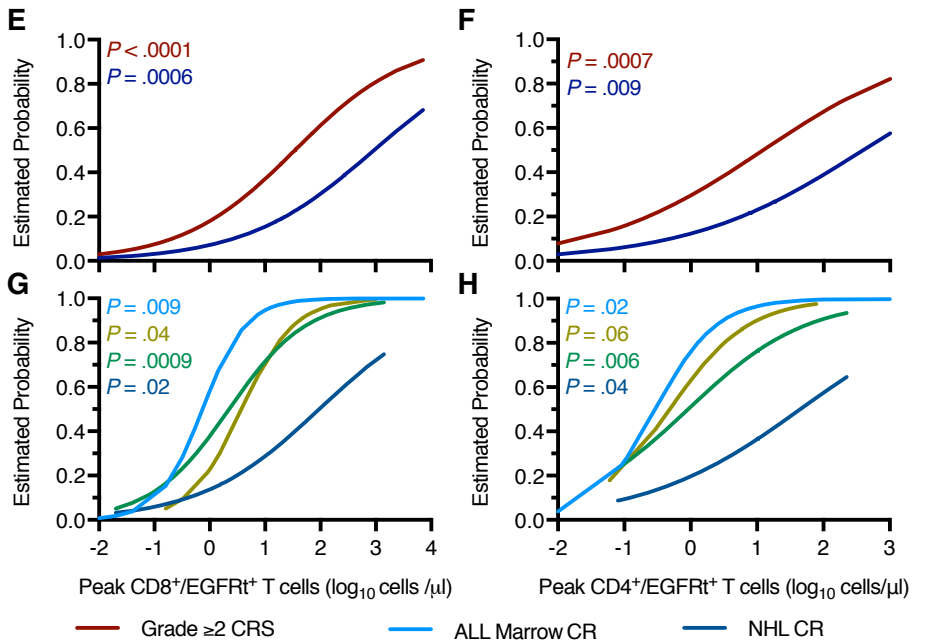
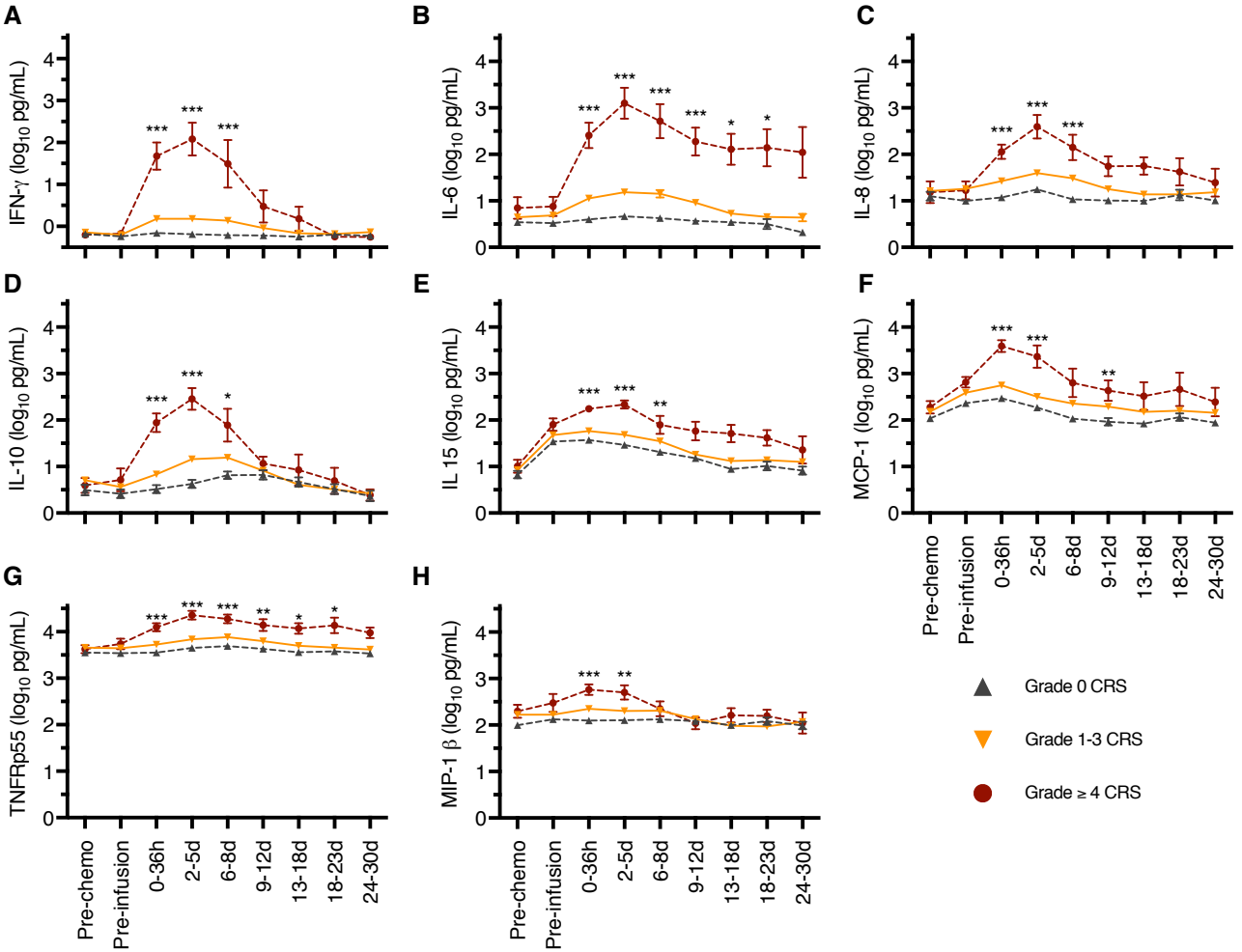
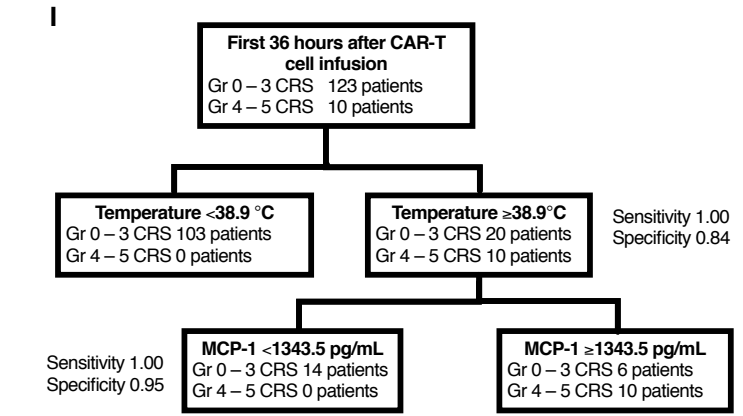

E
F
G
H
Estimated Probability
P < .0001
P = .0006
P = .0007
P = .009
P = .009
P = .04
P = .0009
P = .02
P = .02
P = .06
P = .006
P = .04
Peak CD8+/EGFRt+ T cells (log10 cells /μl)
Peak CD4+/EGFRt+ T cells (log10 cells/μl)
Grade ≥2 CRS
Grade ≥3 NT
ALL Marrow CR
CLL Marrow CR
NHL CR
NHL OR

Figure 6


A
IFN-γ (log10 pg/mL)
B
IL-6 (log10 pg/mL)
C
IL-8 (log10 pg/mL)
D
IL-10 (log10 pg/mL)
E
IL 15 (log10 pg/mL)
F
MCP-1 (log10 pg/mL)
G
TNFRp55 (log10 pg/mL)
H
MIP-1 β (log10 pg/mL)
Pre-chemo
Pre-infusion
0-36h
2-5d
6-8d
9-12d
13-18d
18-23d
24-30d
Grade 0 CRS
Grade 1-3 CRS
Grade ≥ 4 CRS


I
First 36 hours after CAR-T cell infusion
Gr 0 – 3 CRS 123 patients
Gr 4 – 5 CRS 10 patients
Temperature <38.9 °C
Gr 0 – 3 CRS 103 patients
Gr 4 – 5 CRS 0 patients
Temperature ≥38.9°C
Gr 0 – 3 CRS 20 patients
Gr 4 – 5 CRS 10 patients
Sensitivity 1.00
Specificity 0.84
Sensitivity 1.00
Specificity 0.95
MCP-1 <1343.5 pg/mL
Gr 0 – 3 CRS 14 patients
Gr 4 – 5 CRS 0 patients
MCP-1 ≥1343.5 pg/mL
Gr 0 – 3 CRS 6 patients
Gr 4 – 5 CRS 10 patients

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Kinetics and Biomarkers of Severe Cytokine Release Syndrome after CD19 Chimeric Antigen Receptor-modified T Cell Therapy

Kevin A. Hay, Laïla-Aïcha Hanafi, Daniel Li, Juliane Gust, W. Conrad Liles, Mark M. Wurfel, José A. López, Junmei Chen, Dominic Chung, Susanna Harju-Baker, Sindhu Cherian, Xueyan Chen, Stanley R. Riddell, David G. Maloney and Cameron J. Turtle

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