REGULAR ARTICLE

blood advances

Single-cell dynamics of breakthrough toxicities after anakinra prophylaxis for axicabtagene ciloleucel in lymphoma

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Key Points

- Daily subcutaneous anakinra is insufficient to prevent grade ≥2 CRS and NT in patients receiving axicabtagene ciloleucel.
- Single-cell RNA sequencing of immune cells identified IFN-γ as a potential mechanism for breakthrough toxicity despite anakinra prophylaxis.

Chimeric antigen receptor (CAR) T-cell (CAR-T) therapy is limited by cytokine release syndrome (CRS) and neurotoxicity (NT). We sought to use once-daily prophylactic anakinra, an interleukin-1 (IL-1) receptor antagonist, to prevent CRS/NT that would require hospitalization (grade ≥2) in patients receiving axicabtagene ciloleucel for large-cell lymphoma, with the goal of facilitating outpatient therapy and management. Our study, in line with others, demonstrates that once-daily prophylactic anakinra is insufficient to prevent the development of toxicities that would require hospitalization in most patients. As part of the initial study design, we prospectively incorporated single-cell RNA sequencing to gain insight into the molecular immune signaling associated with breakthrough CRS and NT despite anakinra prophylaxis. In patients who developed breakthrough CRS or NT, we found that interferon gamma (IFN-γ) pathways and ligand-receptor activities were significantly enriched, as were cytokine levels of IFN-γ and CXCL10 in $CD14^+$ monocytes. This correlated with increased IFN-γ and other cytokines in the peripheral blood. In infused CAR-T products, IL-4 and IL-10 anti-inflammatory pathways were negatively associated with grade ≥2 toxicities, regardless of anakinra treatment. These data identify IFN-γ as a potential key mechanism in CAR-T–associated toxicities, which is not inhibited by anakinra but may be otherwise targetable. This trial was registered at www.ClinicalTrials.gov as #NCT04150913.

Introduction

Chimeric antigen receptor (CAR) T cells (CAR-Ts) targeting CD19 are a revolutionary therapy for B-cell leukemias and large B-cell lymphomas (LBCLs), with a high frequency and duration of responses. Axicabtagene ciloleucel (axi-cel) was first approved by the US Food and Drug Administration (FDA) in

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Gene expression data for the single-cell RNA-sequencing data are available from the Gene Expression Omnibus (accession number GSE246490). The raw data have been deposited in the Database of Genotypes and Phenotypes (accession number phs003655).

All other data generated in this study are available upon reasonable request from the corresponding author, Marcela V. Mau (mvmaus@mgh.harvard.edu).

The full-text version of this article contains a data supplement.

2017 for patients with relapsed/refractory (R/R) disease, based on data from the ZUMA-1 study,[1] and expanded to second-line treatment of large-cell lymphoma in 2022.[2] The primary acute toxicities with axi-cel (and most CD19-directed CAR-Ts) include cytokine release syndrome (CRS) and neurotoxicity (NT), also referred to as immune cell–associated neurotoxicity syndrome (ICANS). In real-world use, CRS and NT occurred in 96% and 76% of patients, respectively,[3] requiring specialized inpatient monitoring and management and proximity to available intensive care unit facilities. Thus, approaches to prevent toxicities to allow for outpatient treatment would be clinically meaningful, especially to facilitate access to this lifesaving therapy.

CRS and NT are syndromes associated with robust cytokine production, ranging from mild symptoms (such as fever) to severe and life-threatening consequences. CRS, which shares many pathogenic features of macrophage activation syndrome,[4] is managed with the anti–interleukin-6 (IL-6) receptor (IL-6R) antibody, tocilizumab, alone or in combination with corticosteroids.[5] NT is thought to be mediated by a similar inflammatory cascade involving endothelial activation and increased blood-brain barrier permeability and is predominantly managed with corticosteroids.[6,7] Tocilizumab has been studied in the prophylactic setting but did not prevent NT. In fact, patients experienced significantly worse neurologic outcomes despite improvement in CRS incidence and severity.[8] Prophylactic corticosteroids have also been tested (ZUMA-1, cohort 6), and although the incidence of CRS was decreased, the incidence of NT was unchanged.[9] This approach has not been widely adopted in the United States because of concerns about impairing CAR-T proliferation and antitumor effects,[7,10,11] although we, and others, have demonstrated that CAR-T pharmacokinetics are not impacted by steroids except with extensively prolonged use.[12-14]

Anakinra, a human IL-1R antagonist, has demonstrated efficacy in the clinical treatment of rheumatoid and sepsis-related macrophage activation syndrome[15,16] and in mouse models of CAR-T–induced CRS and NT.[17] We, and others, have reported on the role of anakinra in treating acute toxicities and demonstrated decreased inflammatory cytokines, faster time to CRS/NT resolution, and decreased overall steroid use.[18,19] These data suggest that IL-1R blockade may be critical in the pathophysiology of CRS and NT without dampening CAR-T efficacy. Furthermore, signaling through IL-1R potentiates IL-6 production and, therefore, blockade of IL-1R may also block IL-6 production and its downstream consequences.[17,20,21] We hypothesized that blocking IL-1R signaling would prevent NT and CRS development.

Concurrent with our clinical study, investigators at other centers also initiated trials of prophylactic anakinra, although at higher doses and frequency of administration.[22,23] We focused on identifying a potential patient-friendly outpatient regimen (preloaded doses administered once daily, subcutaneously, for 1-2 weeks), rather than administering regimens that still would require hospitalization (such as IV and/or frequent dosing based on physician-level real-time assessments). Because anakinra already has a safety label and did not require phase 1 testing, we performed a phase 2 clinical trial studying prophylactic anakinra for the prevention of axi-cel–related NT and CRS in patients with R/R large-cell lymphoma per the product label at FDA's request. We started anakinra on the day of axi-cel infusion during our standard inpatient admission, and our primary objective was to determine whether this regimen would allow most patients to avoid toxicities to the level that would require specialized hospitalization for their management (such as rapid administration of tocilizumab or high-dose steroids). We hypothesized that some patients would still experience breakthrough CRS and/or NT; we, therefore, performed a prospective, single-cell transcriptional analysis of peripheral blood immune cells to identify pathways beyond the IL-1R that could potentially serve as alternate targets for future prevention of CRS and/or NT.

Methods

Clinical trial design

In November 2019, we opened a phase 2 clinical trial (ClinicalTrials.gov identifier: NCT04150913) combining axi-cel with 200 mg of daily subcutaneous anakinra, starting on the day of CAR-T infusion and continuing for a total of 7 days, with the option to continue for up to 14 days (Figure 1). The primary end point was the incidence of grade ≥2 NT and CRS, which was selected to reflect severity necessitating clinical intervention with immunomodulatory agents such as steroids or tocilizumab. Patients who experienced grade ≥2 CRS or NT despite anakinra prophylaxis were termed "breakthrough" because they necessitated intervention. We chose to use the same toxicity grading scales for CRS and NT, and toxicity treatment as were used in the original ZUMA-1 study (cohorts 1 and 2). Namely, we used the "Lee et al 2014"

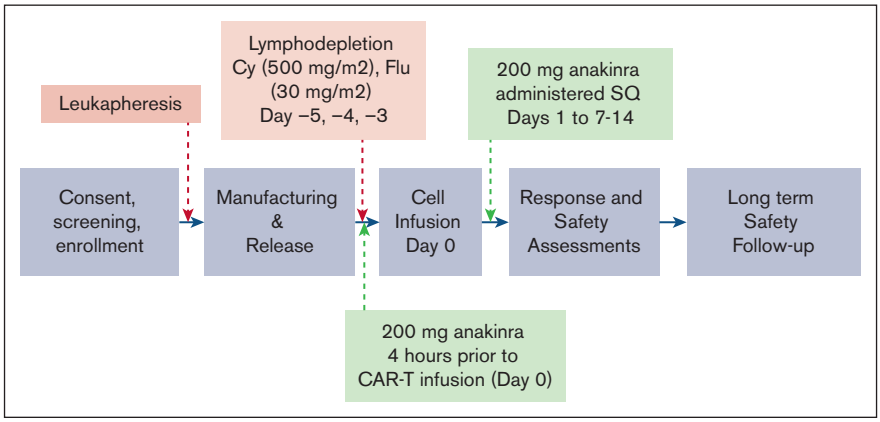


Figure 1. Study schema. After consent, screening, and enrollment, patients were apheresed for CAR-T manufacturing. After release of the CAR-T product (axi-cel), patients received standard cy/flu lymphodepletion per axi-cel label. Four hours before CAR-T infusion, patients received their first dose of 200 mg anakinra subcutaneously. Dosing continued through day 7 (first 6 patients) or 14 (subsequent protocol amendment). Patients were assessed for toxicity during the first 30 days and response starting 1 month after axi-cel infusion. Cy, cyclophosphamide; flu, fludarabine; SQ, subcutaneous.

criteria[24] for CRS, the National Cancer Institute's Common Terminology Criteria for Adverse Events version 4.03[25] for NT grading, and tocilizumab and/or steroids treatment for grade ≥2 CRS/NT only. Secondary end points included response rate and progression-free survival. Exploratory end points included the assessment of the function, expansion, and phenotype of axi-cel, and comparison of the Lee et al grading system relative to the American Society for Transplantation and Cellular Therapy (ASTCT) consensus grading system (Lee et al[26]), which is more commonly used now. We were able to compare cytokine levels and engraftment of axi-cel with those in the ZUMA-1 trial by using Kite's validated internal platforms via a collaborative research agreement. This also provided the most complete clinical data sets regarding the grading of toxicities, particularly CRS and NT, within the pivotal cohort, which currently has the longest follow-up to date.[27]

Statistics

The rate of grade ≥2 NT was evaluated using a Simon 2-stage design. Patients were observed for CAR-T–associated NT and CRS per Lee et al[24] up to day 30 after infusion. CRS/NT management was per ZUMA-1,[25] meaning patients only received tocilizumab and dexamethasone for grade ≥2 CRS/NT. The protocol was approved by the Dana-Farber/Harvard Cancer Center institutional review board and the trial was performed in accordance with the principles of the Declaration of Helsinki. Additional clinical trial design details and the full clinical protocol are included in the supplemental Materials.

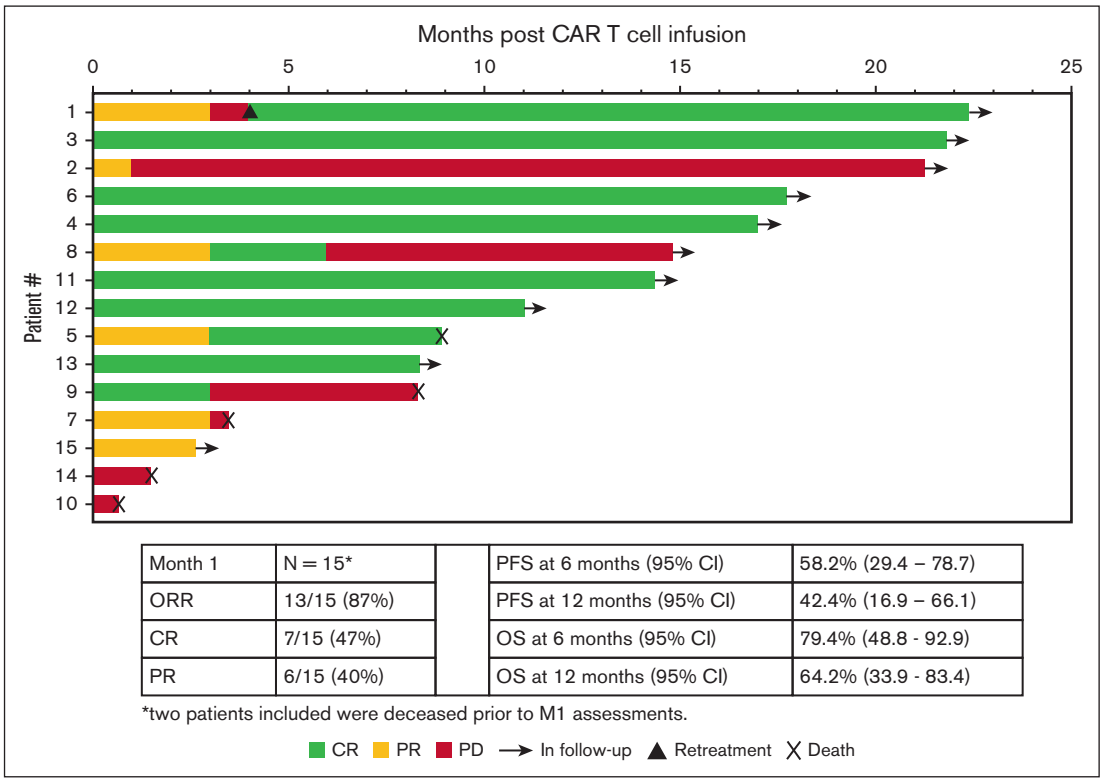
Treatment and patient samples

After providing written informed consent, patients were enrolled and underwent leukapheresis. Anakinra (200 mg) was subcutaneously dosed daily, starting 4 hours before cell infusion. Patients received a dose of 2×10^6 CAR$^+$ cells per kg up to 2×10^8 CAR$^+$ T cells, per the axicabtagene ciloleucel FDA label. All patients had repeat baseline assessments before lymphodepleting chemotherapy (LDC) and had additional scans performed at standard response assessment intervals. Blood samples were collected at varying time points after CAR-T infusion for measuring CAR-T expansion, serum cytokines, and performing single-cell RNA (scRNA) sequencing as described in the supplemental Materials.

Results

CAR-T antitumor efficacy and expansion were not affected by anakinra prophylaxis

A concern underlying any immunomodulatory approach for CRS or NT management in CAR-T recipients is reduced antitumor efficacy. In our study, the overall response rate was 87%, with 47% of patients achieving a complete response and 40% achieving a partial response (Figure 2). Baseline patient characteristics are provided in Table 1. With a median follow-up of 11 months (range, 2.6-22.4 months), the 6- and 12-month progression-free survival was 58.2% (29.4%-78.7%) and 42.4% (16.9%-66.1%),



Month 1	N = 15*		PFS at 6 months (95% CI)	58.2% (29.4 – 78.7)
ORR	13/15 (87%)		PFS at 12 months (95% CI)	42.4% (16.9 – 66.1)
CR	7/15 (47%)		OS at 6 months (95% CI)	79.4% (48.8 - 92.9)
PR	6/15 (40%)		OS at 12 months (95% CI)	64.2% (33.9 - 83.4)

*two patients included were deceased prior to M1 assessments.

Figure 2. Swimmers plot of positron emission tomography/computed tomography responses in patients treated with anakinra prophylaxis. Response per patient as assessed per the revised International Working Group criteria for malignant lymphoma.[28] Response rates are reported as the number of patients per total number of patients and percent. Survival is reported per survival category as a percent of patients available to assess at that time point with the 95% CI. CI, confidence interval; CR, complete response; ORR, overall response rate; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response.

Table 1. Patient characteristics, N = 15

	Value
Age, median (range), y	64 (42-79)
Sex, female, n (%); male, n (%)	7 (47); 8 (53)
ECOG PS score, n (%)	
0	5 (33)
1	10 (67)
Histology, n (%)	
tFL	5 (33)
DLBCL NOS	9 (60)
HGBCL	1 (7)
No. of prior regimens, median (range)	3 (2-4)
Primary refractory disease, n (%)	3 (20)
Prior autologous HSCT, n (%)	3 (20)
Tumor burden, median (range)	
Baseline LDH, U/L	223 (165-2 022)
Baseline SPD, mm^2	2 239 (542-13 807)
IPI at baseline, median (range)	3 (0-4)

DLBCL NOS, diffuse large B-cell lymphoma not otherwise specified; ECOG PS, Eastern Cooperative Oncology Group performance status; HGBCL, high-grade B-cell lymphoma; HSCT, hematopoietic stem cell transplant; IPI, International Prognostic Index; LDH, lactate dehydrogenase; SPD, sum of the products of the longest perpendicular diameters; tFL, transformed follicular lymphoma.

respectively, which is in line with historical controls of ~50% and 45%, respectively.[1] Importantly, CAR-T expansion was noted in all patients by both flow cytometry for CAR^+ cells and quantitative polymerase chain reaction (PCR). Median CAR-T expansion by flow cytometry and PCR were estimated as 31.6 CAR-Ts per μL blood (range, 0.42-98.9μL blood) and 36.6 CAR-T cells per μL blood (range, 1.37-208.88μL blood), respectively. Peak CAR-T expansion was significantly higher in patients who experienced either grade ≥2 breakthrough CRS and NT when measured by PCR (Figure 3). The 2 methods presented comparable results. Consistently, there is strong correlation between the PCR and flow cytometry assessments (supplemental Figure 1).

Prophylactic anakinra did not reduce CRS/NT rates but demonstrated differing cytokine profiles when comparing CRS and NT events

Despite receiving prophylactic anakinra, the rate of any CAR-T–associated CRS and NT were 93% and 66%, respectively, with (grade ≥2) CRS occurring in 53% (8/15) of patients and NT in 53% (8/15; Table 2). We did not notice a difference in rates when comparing Lee et al and ASTCT (supplemental Table 1). Interestingly, we observed a delay in toxicity onset and a shortened duration of toxicity (Figure 4) in the breakthrough toxicity that occurred after anakinra prophylaxis compared with the onset and duration of CRS and NT during the ZUMA-1 cohorts 1 and 2.[1] The median time to onset was 4 days (range, 0-7 days) after infusion for CRS, and 9 days (range, 5-14 days) after infusion for NT (vs 2 and 5 days in ZUMA-1) and the median durations were 5 days (range, 2-14 days) for CRS and 6 days (range, 1-19 days) for NT (vs 8 and 12 days in ZUMA-1).

We observed distinct patterns of inflammatory cytokines/molecules that were significantly elevated in both CRS and NT as well as in either CRS or NT alone when comparing patients with and without breakthrough grade ≥2 CRS or NT. In patients with grade ≥2 breakthrough CRS and NT, interferon gamma (IFN-γ), granulocyte-monocyte colony-stimulating factor, granzyme B, IL-4, IL-6, IP-10, macrophage inflammatory protein 1α, and tumor necrosis factor-α peaked at statistically higher levels (Figure 5A), consistent with previously reported data.[6] In patients who experienced grade ≥2 breakthrough CRS alone, different analytes, namely C-reactive protein, IL-2Ra, IL-6Ra, vascular cell adhesion molecule-1, IL-16, macrophage inflammatory protein 1β, and programmed death-ligand 1 were significantly higher at peak (Figure 5B), suggesting potential differences in the roles that these cytokines play in CRS compared with NT. Only ferritin was significantly elevated in patients with grade ≥2 breakthrough NT and not with grade ≥2 CRS, although a similar trend was observed for CRS (Figure 5C). Additional cytokines such as IL-2, IL-7, IL-8, IL-10, IL-12 (P40 and P70), IL-15, IL-17α, monocyte chemoattractant protein-1, granzyme A, and perforin showed no statistically significant difference when comparing grade ≤1 vs grade ≥2 CRS and/or NT, possibly because of the limited number of patients in the study (supplemental Figure 2).

We also analyzed cytokine levels over time and compared pre-infusion vs postinfusion levels across the entire study cohort (supplemental Figures 3-6). Cytokines associated with CRS and NT or CRS alone tended to peak at day 7 after CAR-T infusion (supplemental Figure 3). IL-1Ra was quantified at maximal assay detection levels in all patients on day 7 (supplemental Figures 4 and 5B). A few cytokines (Fas ligand [FasL], IL-12p70, Macrophage-derived chemokine [MDC], Vascular endothelial growth factor [VEGF], perforin, and RANTES) significantly decreased at day 7 after infusion vs before infusion (supplemental Figures 4 and 5). When comparing the entire cohort before and after CAR-T infusion, the serum markers that were significantly different varied from those we identified when analyzing expression at peak in grade ≤1 vs ≥2 (supplemental Figures 5 and 6), demonstrating the importance of distinguishing between patients who did and did not have breakthrough CRS and/or NT with anakinra prophylaxis.

Single-cell transcriptional analysis of CAR-Ts and other immune cells before and after infusion and with or without anakinra prophylaxis

For the first 10 patients enrolled on the anakinra prophylaxis study, we performed 10× scRNA sequencing on preinfusion products (IPs) derived from residual patient infusion bags and peripheral blood mononuclear cells (PBMCs) collected on day +7 after axi-cel infusion, both with and without sorting for CAR^+ cells (Figure 6A). To include as a comparator, we generated data with identical processing from 4 patients receiving axi-cel as standard of care but without prophylactic anakinra (Figure 6B). For increased power to identify cell types and signaling pathways relevant to toxicities in absence of anakinra, we collected CRS and NT grading for our previously published cohort of 19 patients who had received axi-cel as their standard of care with sampling at identical time points[29] (of note, the toxicity grading for this cohort was not previously published). The samples available for scRNA-sequencing analysis

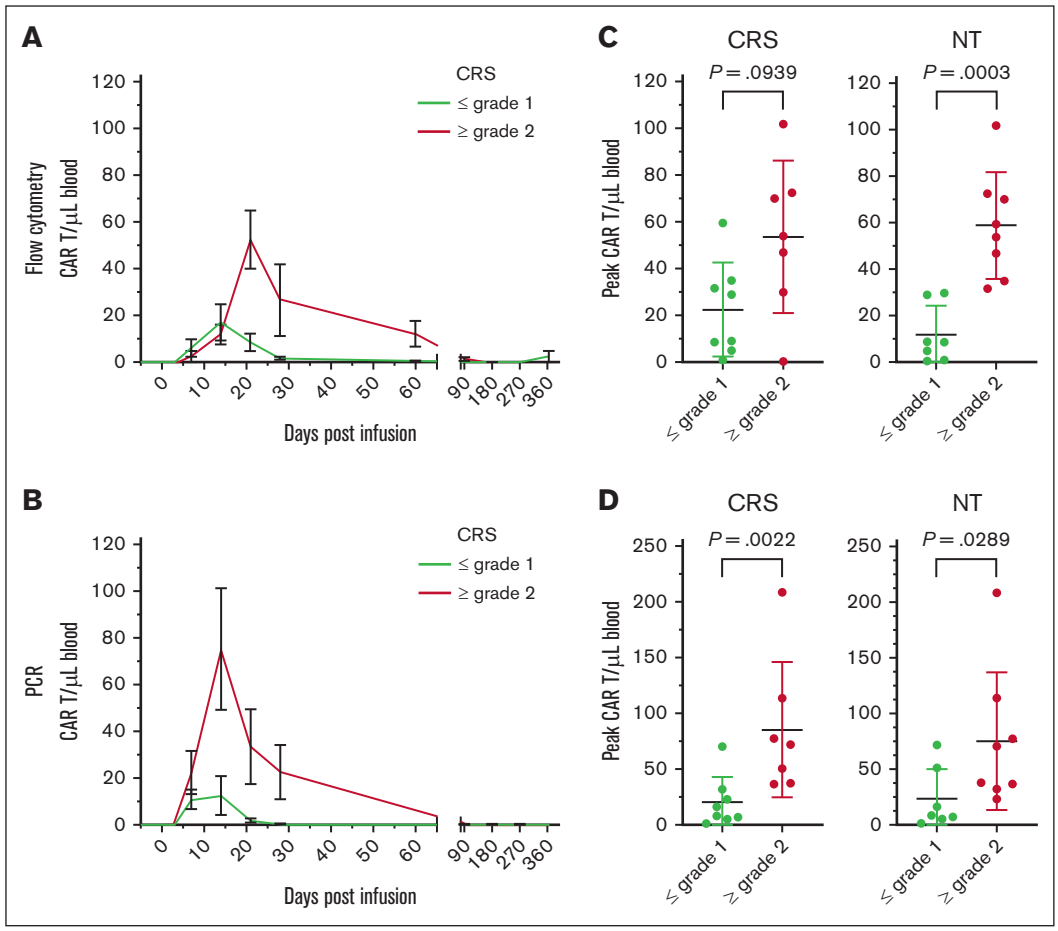


Figure 3. CAR-T expansion after infusion as measured by flow cytometry and PCR. CAR-T expansion was measured in patient peripheral blood by (A) flow cytometry or (B) PCR for percent CAR$^+$ cells among PBMCs. The number of CAR-Ts per μL of blood was calculated using the patient's absolute lymphocyte count. Data are presented as the time course from infusion (day 0), with pre–CAR-T (day −5) values also shown, and separated by patients with (red) or without (green) breakthrough grade ≥2 CRS. (C-D) Peak CAR-T expansion for each patient, separated by those who did and did not experience grade ≥2 CRS or NT by (C) flow cytometry or (D) PCR. *P* values indicate significance as determined by a 2-tailed Mann-Whitney *U* test.

along with the toxicity grading is summarized in supplemental Table 2 and listed by individual patient in supplemental Table 3).

We obtained 125 386 cells (28 132 cells from the pre-IP; mean, 2.5 × 10^3 per patient; and 97 254 cells from day +7 PBMCs; mean, 5.7 × 10^3 per patient), which we combined with the 80 487 cells (20 831 cells from the pre-IP; mean, 1 × 10^3 per patient; and 59 656 cells from day +7 PBMCs; mean, 3.3 × 10^3 per patient) from the prior cohort, which gave a total of 205 873 cells for analysis (supplemental Table 4). Clustering and cell type identification based on canonical markers of all IP and day +7 cells identified T cells, monocytes (both classical and nonclassical), natural killer cells, plasmacytoid dendritic cells, and megakaryocyte-generating platelets (Figure 6C; supplemental Figure 7A-E). We further subdivided T cells based on expression of canonical markers, and CAR-Ts were defined by detection of the axi-cel transcript ("Methods"; Figure 6D).

Pre-IPs consisted of primarily T cells but also comprised 2 myeloid-like clusters1 of which had expression characteristic of "ICANS-associated cells" previously defined in a prior single-cell sequencing study analyzing axi-cel IPs[30] including *LYZ* and *IL1B*, and another with expression of *LAMP3* typically found on dendritic cells although lacking other characteristic markers (supplemental Figure 8A-C). Frequencies of ICANS-associated cells were low (only >1% in 2 patients), and showed no clear enrichment for high-grade NT as was seen in the prior data in the absence of anakinra prophylaxis, although statistical power to draw conclusions is limited by the rarity of cases with detectible levels of this population (supplemental Figure 8D).

Cell composition differences related to toxicities and anakinra prophylaxis

The distribution of CAR-T subsets over time followed similar trends to those we previously described in patients without anakinra prophylaxis (supplemental Figure 9A-C). Reanalysis of our prior data set[29] for toxicity-associated composition changes identified increased proportions of regulatory T cells in the day +7 CAR$^-$ population (q = 0.019) and decreased CD8$^+$ T cells (q = 0.051) in patients with grade ≥2 compared with ≤1 CRS, and similar trends were seen with anakinra prophylaxis (supplemental Figure 9D). Curiously, these effects were most pronounced in cases of grade ≥2 CRS that did not have

Table 2. Rates of CAR-T toxicities and management, N = 15

	Value
CRS	
Maximum grade (Lee et al[24]), any, n (%)	14 (93)
Grade 1	6 (40)
Grade 2	6 (40)
Grade 3	1 (7)
Grade 4	1 (7)
Median onset, (range), d	4 (1-8)
Median duration, (range), d	5 (1-14)
NT	
Maximum grade (CTCAE), any, n (%)	11 (73)
Grade 1	3 (20)
Grade 2	2 (13)
Grade 3-4	5 (34)
Grade 5	1 (7)
Median onset, (range), d	8 (5-13)
Median duration, (range), d	4 (1-16)
CRS/NT interventions, n (%)	
Tocilizumab	8 (53)
Steroids	9 (60)
Siltuximab	1 (7)
Cyclophosphamide	1 (7)

CTCAE, Common Terminology Criteria for Adverse Events.

concurrent grade ≥2 NT. We did not observe significant differences in cell composition directly comparing NT grade ≥2 vs grade ≤1 (supplemental Figure 9D).

IL-4 and IL-10 pathway activities are associated with CRS and anakinra prophylaxis

Performing gene set enrichment analysis of a library of T-cell pathways for IP T-cell populations ("Methods"; supplemental Table 5), we found anti-inflammatory IL-10 and IL-4 signaling pathways negatively associated with grade ≥2 CRS (Figure 6E). Expression of IL-4 signaling genes such as *IL-13* and *IL-4L1* were drastically reduced (38- to 180-fold lower) at day +7 (supplemental Figure 9F; supplemental Table 8) whereas IL-10 signaling pathways were increased (supplemental Figure 9F). These pathways were not found to be associated with objective response to axi-cel therapy (supplemental Table 7).

IL4 and *IL10* expression were highly specific to the $CD4^+$ cells with a cytotoxic phenotype at day 7 after treatment ($CD4^+$ cytotoxic T lymphocytes; Figure 6F). Performing a head-to-head pseudobulk differential expression (DE) comparison of this population from patients who did or did not receive anakinra prophylaxis ("Methods"; supplemental Table 9), we found a significant enrichment of *IL10* expression (q value = 0.0481) in the presence of anakinra prophylaxis (Figure 6G-H; supplemental Figure 10A). By gene set enrichment analysis, anakinra prophylaxis was associated with the enrichment of IL-4 and IL-13 pathways (supplemental Figure 10B-D; "Methods"), along with upregulation of several proinflammatory pathways such as IL-18 (supplemental Table 10).

CRS and NT are associated with increased CAR-T and innate T-cell proliferation at day +7

In our comparisons between grade ≤1 and grade ≥2 NT, we identified the most differentially expressed genes to come from the day +7 $CD8^+$ innate (CAR^-) T-cell subsets (352 genes with $q < 0.1$ and more than twofold change). This difference was largely driven by proliferation-related genes such as *CCBB2*, *TUBB6*, and *RAD51* (supplemental Figure 9G). Scoring cells for S and G2M cell cycle gene sets,[31] we found patients receiving anakinra with either grade ≥2 NT or CRS had significantly increased fractions of cycling cells for both innate (false discover rate–corrected Wilcoxon rank-sum test: q = 0.0079) and CAR^+ cells (q = 0.016; Figure 6I). This observation was particularly striking for innate T cells (median, 6.1% vs 22% cycling cells in patients with grade ≤1 vs ≥2 breakthrough CRS or NT). In patients without anakinra prophylaxis, no significant changes were observed (q = 0.92 and q = 0.15 for CAR^+ and CAR^- subsets, respectively). The clinical significance and biologic mechanism underlying this increase in cell cycling will require future confirmation.

Breakthrough toxicities in the presence of anakinra harbors a stronger proinflammatory phenotype characterized by interferon signaling pathway and resistin *(RETN)* expression

Because monocyte signaling is believed to have a critical mechanistic role in CRS, we performed a DE analysis comparing transcriptional changes of patients with grade ≥2 vs grade ≤1 CRS and NT across both cohorts (Figure 7A; supplemental Table 11). Subsets of genes were associated with grade ≥2 CRS regardless of anakinra prophylaxis (Figure 7A; supplemental Figure 11A); for example upregulation of components of the complement pathway (*C1QA/B/C*), macrophage mannose receptor 1 (*MRC1*), a marker for M2 macrophages,[32] and transmembrane protein members (*TMEM176A/B*), previously reported to inhibit the activation of the inflammasome and IL-1β secretion.[33] IL-1 pathway genes including *IL-1B* and CC-chemokine ligands (*CCL3/4*) were downregulated in grade ≥2 CRS.

We further identified a set of proinflammatory genes associated with grade ≥2 CRS exclusively in the presence of anakinra prophylaxis. These included interferon-induced production 35 (*IFI35*), interferon-induced transmembrane protein 1 (*IFITM1*), and *IFIT2*, which are leading edge genes to the interferon signaling pathway, and C-X-C motif chemokine ligand 10 (*CXCL10*) and *RETN*, with functions related to interferon signaling[34] (Figure 7A; supplemental Figure 11B-C). Similar trends were observed comparing grade ≥2 vs grade ≤1 NT, although statistical significance was not reached (supplemental Figure 11D). This was consistent with the results from our peripheral blood cytokine analysis showing increased IFN-γ and IP10 (CXCL10) in patients with CRS and NT across the whole study cohort (Figure 5A) as well as this subset of 10 patients (Figure 7B-C). Notably, the 1 patient with grade ≥2 NT but only grade 1 CRS showed an early peak of these cytokines during days 3 to 5, which was declining day +7, which may explain the lower gene expression associations seen overall with NT in our day +7 RNA-sequencing data. We found that IFN-γ response gene set scores were the highest in monocytes whereas *IFNG* itself was expressed in cytotoxic CAR-Ts (both CD8 and CD4 cytotoxic T lymphocytes) and, thus, the signaling likely directly stems from cross talk between these populations (supplemental Figure 11E-F).

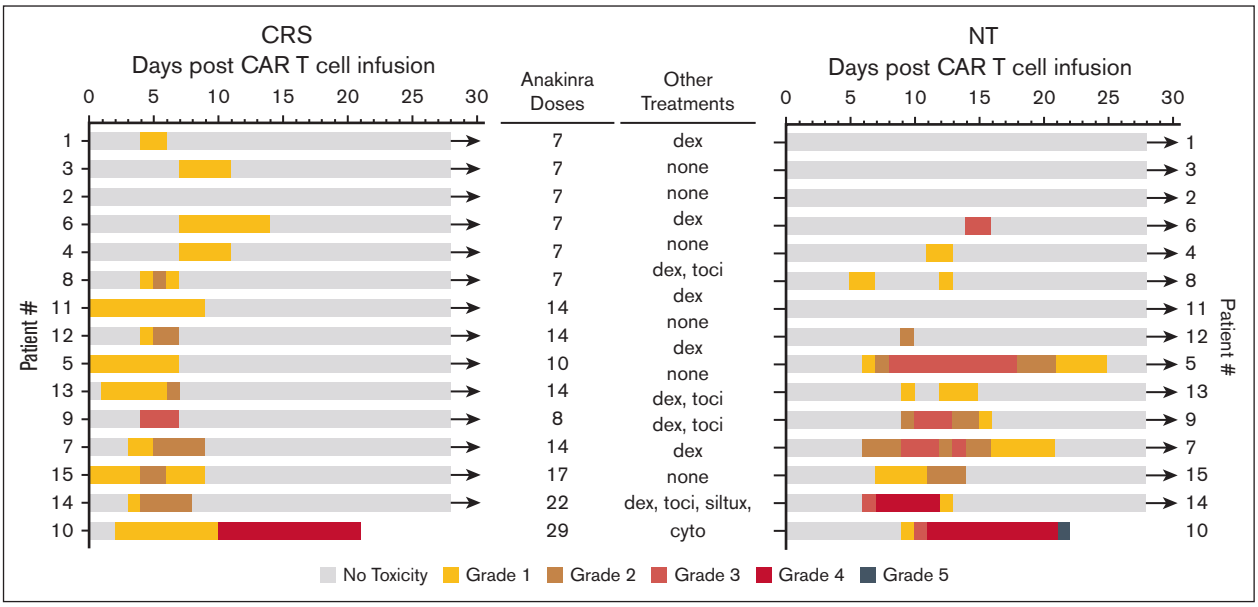


Figure 4. Breakthrough CRS and NT course of patients treated with anakinra prophylaxis. The grade and duration of CRS and NT within the first 30 days after axi-cel treatment reported per patient based on the day of onset. Grade coloring indicates the highest grade of CRS or NT experienced during the toxicity duration. CRS graded per Lee et al[24] and NT graded per Common Terminology Criteria for Adverse Events version 4.03 in line with historical ZUMA-1 cohort.[25] cyto, cyclophosphamide; dex, dexamethasone; siltux, siltuximab; toci, tocilizumab.

DE of genes involved in ligand-receptor interactions reveal changes in cellular cross talk associated with anakinra prophylaxis

To elucidate potential cross talk between monocytes and CAR-Ts related to breakthrough toxicities, we compiled a list of 80 candidate ligand receptor (LR) pairs using the methods CellPhoneDB and CellChat DB in which at least 1 ligand or receptor subunit was previously identified as anakinra- or toxicity-associated in our DE analyses (see "Methods"). Twenty-nine LR pairs had at least 1 pair of cell types that were significantly different between patients with and without breakthrough grade ≥2 CRS (false discover rate–corrected *U* test *P* value <.1; supplemental Figure 11G). We observed the interaction between *CXCL10* from day 7 CD14 monocytes and *CXCR3*, dipeptidyl peptidase 4 (*DPP4*) from various CAR^- and CAR^+ T cells were differentially upregulated in samples from patients with breakthrough CRS as well as NT under anakinra prophylaxis (Figure 7D). We also observed the upregulation of an interaction between *RETN* from day +7 CD14 monocyte and adenylyl cyclase–associated protein 1 (*CAP1*) across T-cell types (Figure 7D), which is a known receptor for *RETN* in the proinflammatory signaling cascade.[35,36] Similar associations with toxicity for these cell-cell interactions were not significant in the absence of anakinra prophylaxis (Figure 7E).

Discussion

We did not observe a significant improvement in the rate or severity of CRS or NT with daily subcutaneous dosing of 200 mg of prophylactic anakinra in patients treated with axi-cel for R/R LBCL. Despite the small number of patients, we did not observe a detrimental effect of anakinra prophylaxis on CAR-T efficacy, because the overall and complete response rates were also similar to historical controls and CAR-Ts expanded after infusion in the peripheral blood. When making this comparison, differences between "chemotherapy-refractory disease" as defined in ZUMA-1 vs R/R disease as defined by the product label, should be acknowledged. As previously reported, this expansion was similar to the ZUMA-1 cohort when normalized for sum of the products of the longest perpendicular diameters.[37]

Our goal was to choose a prophylaxis regimen that would allow for outpatient management of potential toxicities and reduce hospitalizations after CAR-T administration. However, our dose, timing, and/or frequency may have been inadequate to achieve the optimal area under the curve for effective prophylaxis against CRS and NT by anakinra. Two recent clinical trials also examined anakinra prophylaxis to prevent NT in patients with R/R LBCL treated with CD19-targeted CAR-T therapy, with the major difference being their dosing regimens.[22,23] Strati[23] treated with 200 mg anakinra subcutaneously either daily or every 12 hours starting 6 hours before CAR-T infusion and for a duration of 7 days. Park et al[22] treated with 100 mg anakinra subcutaneously every 6 or 12 hours starting on day 2 after CAR-T infusion or upon grade 1 CRS for a duration of 10 days or until the resolution of symptoms, whichever came first. Both studies analyzed CRS and ICANS according to the ASTCT guidelines. In line with our study, Strati et al did not see an improvement in the incidence, grade, or duration of CRS with anakinra prophylaxis compared with the ZUMA-1 trial, but did report a lower incidence of all grades of ICANS compared with ZUMA-1 (35% vs 53%). Park et al observed a slight decrease in the incidence of CRS among patients treated with axi-cel compared with ZUMA-1 (74% vs 93%) and a larger decrease in the incidence of all grades of ICANS (22% vs 53%) and grade ≥3 ICANS (9% vs 28%). Although these studies used a lower dose, the frequency was increased up to 2 to 4 times daily vs once daily in our study. Strati et al also compared cohorts treated

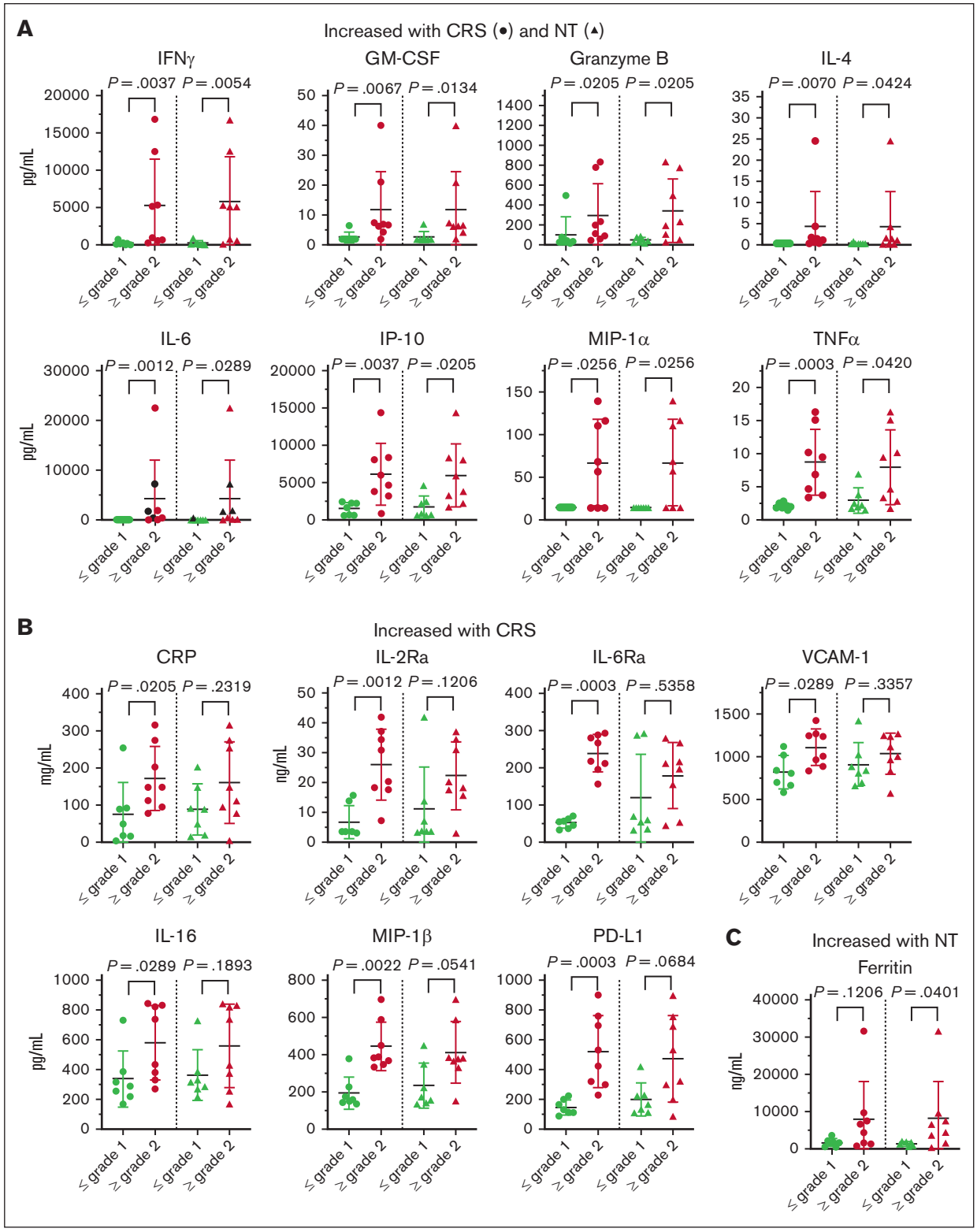


Figure 5. Serum markers associated with breakthrough grade ≥2 CRS and/or NT in patients treated with anakinra prophylaxis. Serum proteins were analyzed via V-PLEX in serum collected from patient peripheral blood prior to CAR-T infusion; on the day of CAR-T infusion (day 0); and on days 1, 3, 5, 7, 14, and 28 after CAR-T infusion. Symbols represent the peak cytokine concentration detected for each patient during that timeframe. Lines indicate mean ± standard deviation. Black symbols in the IL-6 plot indicate patients treated with tocilizumab as part of their toxicity management. Data are separated by patients with (red) or without (green) grade ≥2 CRS (circles) or NT (triangles). *P* values indicate significance as determined by a 2-tailed Mann-Whitney *U* test. Cytokines shown are significantly increased in (A) patients with grade ≥2 CRS and patients with grade ≥2 NT, (B) only patients with grade ≥2 CRS, or (C) only patients with grade ≥2 NT. CRP, C-reactive protein; GM-CSF, granulocyte-monocyte colony-stimulating factor; ICAM, intercellular adhesion molecule; IP-10, INF-γ inducible protein (also known as CXCL10); MIP, macrophage inflammatory protein; PD-L, programmed death-ligand; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

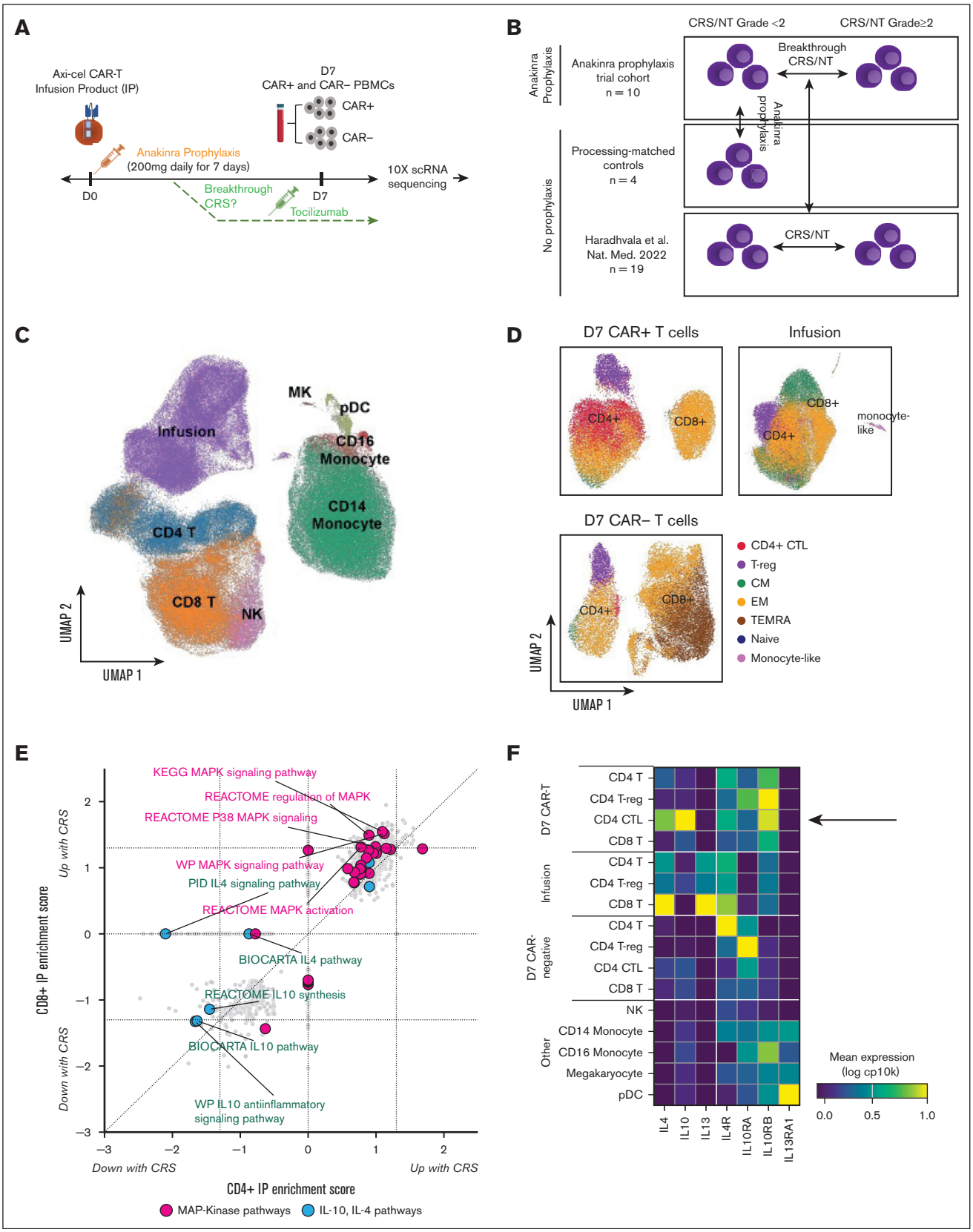


Figure 6. Transcriptomic landscape of CAR-T under treatment with anakinra prophylaxis. (A) Schematic of sample collection from anakinra prophylaxis and nonanakinra prophylaxis cohorts along with data analysis design. Samples from patient IP or PBMCs collected on day 7 after CAR-T infusion underwent scRNA-sequencing and were analyzed for signatures of CRS development and differential gene expression and differential cell-cell interactions between patients receiving anakinra prophylaxis vs no prophylaxis.

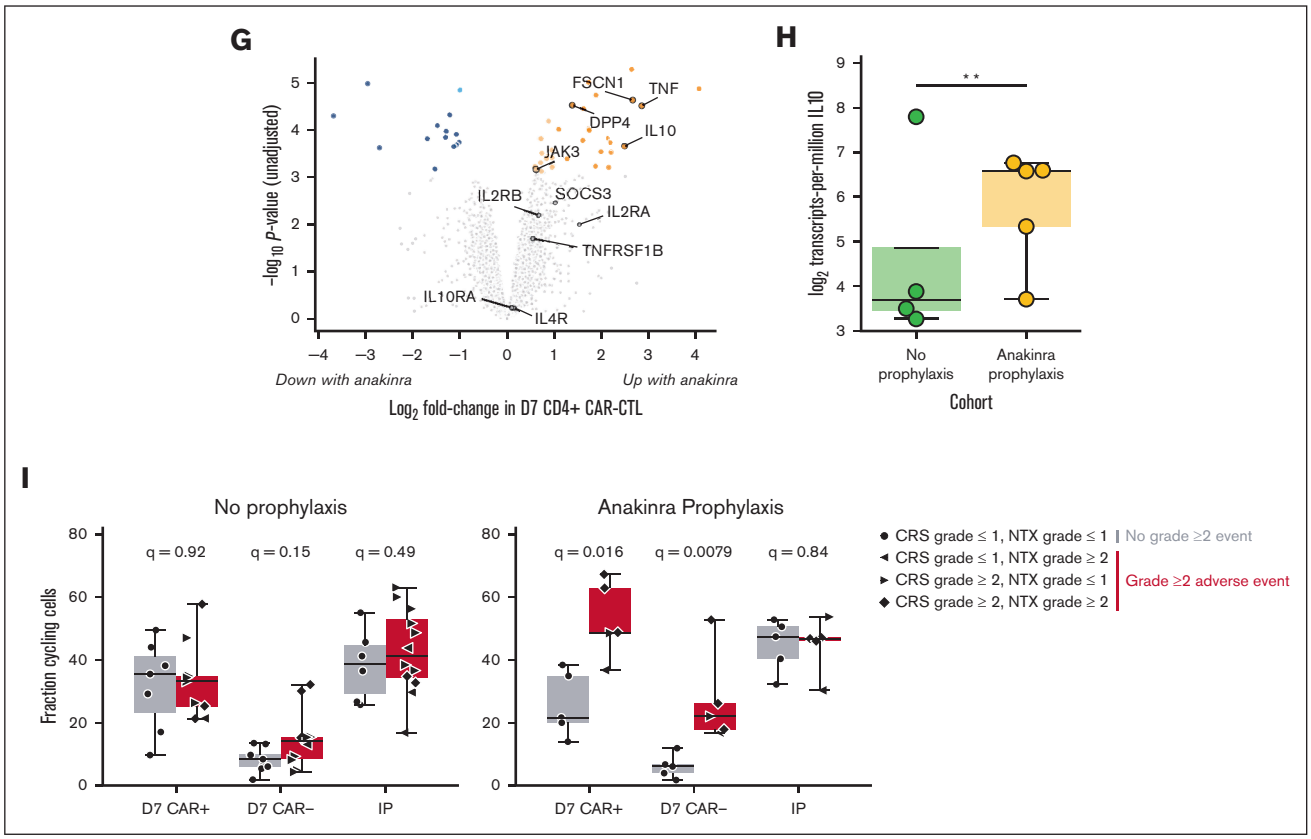


Figure 6 (continued) (B) Illustration of the different samples and comparisons being used in this study. Associations with breakthrough CRS/NT under anakinra prophylaxis in this cohort are identified and compared with CRS/NT associations seen in absence of anakinra prophylaxis using samples published in Haradhvala et al[29]. An additional n = 4 samples from patients without anakinra prophylaxis were generated as part of this study to control for potential between-study differences in sample processing. (C) Harmony batch-corrected based uniform manifold approximation and projection (UMAP) representation for all cells analyzed in the study, colored by cell type. The cells from the IPs cluster together and that cluster is labeled "Infusion." (D) UMAP of IP and day 7 CAR^+ and CAR^- cells colored by assigned T-cell subtype. (E) Scatterplot of normalized enrichment score for gene sets associated with CRS (enriched in patients with grade ≥2 CRS) in $CD4^+$ and $CD8^+$ IPs in the nonanakinra prophylaxis cohort from Haradhvala et al[29]. (F) Average expression of IL-4, IL-10, and IL-13 genes and their receptors across different cell subsets and time points. (G) Volcano plot of DE analysis in day 7 CAR^+ CD4 CTL cells from patients in this study, without grade ≥2 CRS, with (n = 5) vs without (n = 4) anakinra prophylaxis. (H) Distribution of the $\log_2$ pseudobulk IL-10 expression in day 7 CAR^+ CD4 CTL cells from panel G. (I) Fraction of cells in the cell cycle across different T-cell subsets in patients with and without anakinra prophylaxis. False discover rate (FDR)–corrected Mann-Whitney *U* q values are shown comparing patients with grade ≥2 CRS or NT to those with grade ≤1 CRS or NT. CM, Central Memory; CTL, cytotoxic T lymphocyte; EM, Effector Memory; MK, megakaryocyte; NK, natural killer cell; pDC, plasmacytoid dendritic cell; T-reg, T-regulatory; TEMRA, Terminal effector memory cells that re-express the CD45RA molecule.

daily vs twice daily but did not see numerical differences between these groups in terms of CRS or ICANS. These data suggest that increasing the dosing to 4 times daily may be necessary for preventing toxicities, although this approach would need to be evaluated in future trials. Even if successful, such frequent dosing would be much more difficult to perform in an outpatient setting. Regardless, anakinra use for CRS and ICANS may still play an important role in clinical management.

One of the strengths of this study was our evaluation of the differences in cytokines and cell populations between patients who had breakthrough toxicities despite anakinra prophylaxis and comparison to our previously published scRNA-sequencing cohort,[29] which we updated here with toxicity grading. We found IL-4 and IL-10 gene set enrichment in CAR-T IPs without breakthrough CRS development, and anakinra prophylaxis in cases without breakthrough CRS to associate with reduced posttreatment IL-10 messenger RNA. Although these genes were upregulated, they are likely protective but not sufficient to prevent CRS. Additionally, there are no known data showing that exogenous IL-4 or IL-10 would not negatively impact CAR-T function. With anakinra prophylaxis, IFN-γ-regulated genes expressed by monocyte populations and serum cytokine levels were associated with breakthrough CRS. We have previously demonstrated that serum from patients treated with CD19 CAR-Ts is sufficient to activate production of IL-1a and IL-6 and that this activation was prevented when including anti-IFN-γ but not anti-IL-6 or anti-ILRa.[38] Thus, targeting IFN-γ or early administration of IL-10 may be methods of preventing breakthrough toxicity with or without anakinra prophylaxis. One limitation of our cytokine analysis was that some of these patients were treated with tocilizumab (as per the standard of care for CRS) before the predetermined sample collection time points, which would affect their serum cytokine levels. The predetermined time points selected for scRNA sequencing also limited our ability to draw associations with peak CRS or ICANS. Finally, the generalizability of our findings is also limited by the small number of patients analyzed.

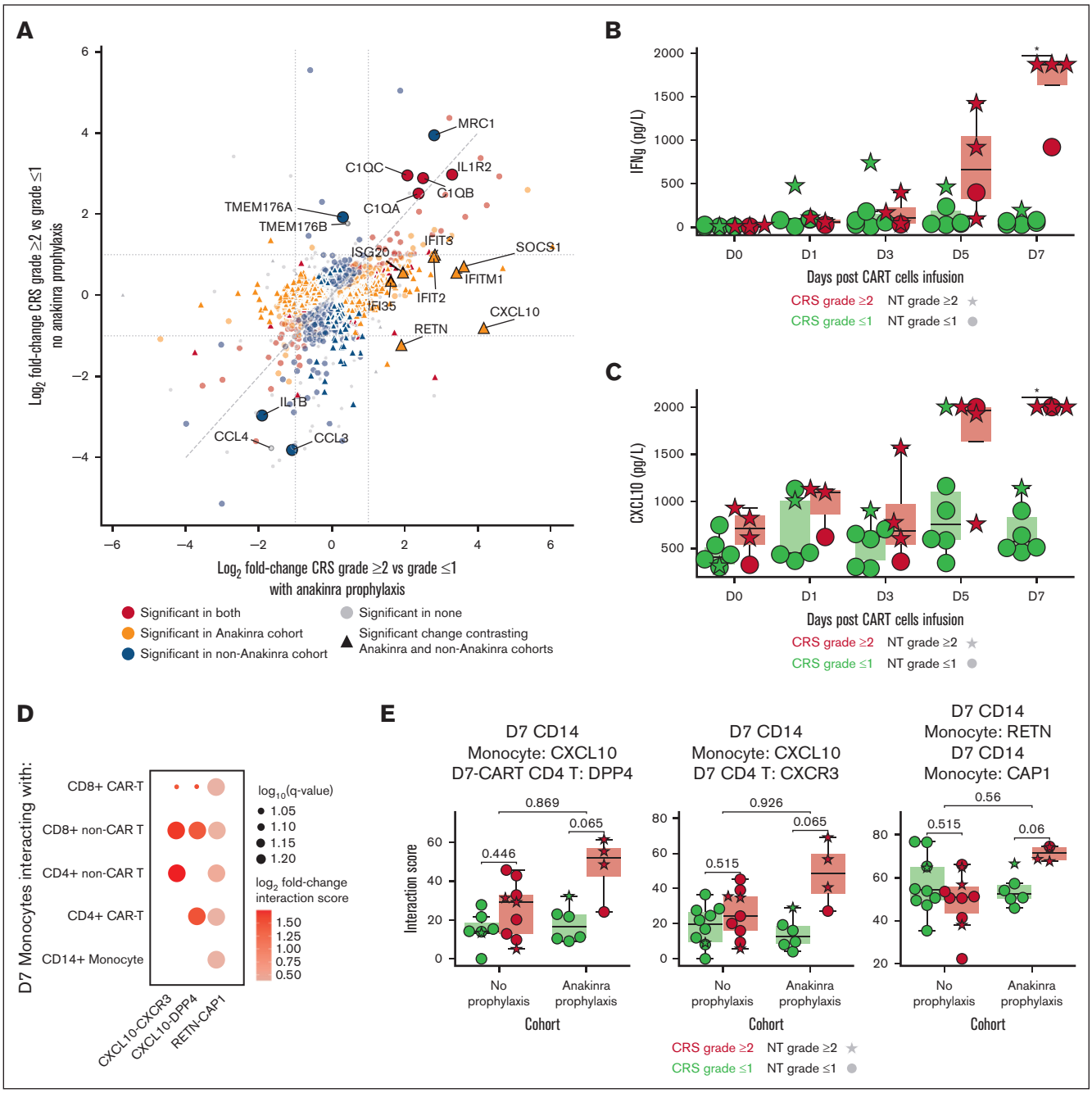


Figure 7. INF-γ signaling pathway and *RETN* ligand-receptor interaction upregulated in CD14 monocytes in patients with breakthrough CRS to anakinra. (A) Comparisons between the $\log_2$-fold change of differentially expressed genes associated with high- (grade ≥2) vs low-grade CRS (grade ≤1) in patients with anakinra prophylaxis (x-axis, n = 10 patients from this study: 6 grade ≤1 and 4 grade ≥2) and without anakinra prophylaxis (y-axis, n = 4 low-grade patients from our biobanked samples and n = 16 patients from Haradhvala et al[29]: 6 grade ≤1 and 10 grade ≥2) among day 7 $CD14^+$ monocytes. Colors indicate DE significance levels, setting Benjamini-Hochberg-corrected *P* value <.1 as significant. Triangles represent genes that had a significant contrast (Benjamini-Hochberg-corrected *P* value <.1) between the effects observed with or without anakinra prophylaxis. (B) IFN-γ and (C) CXCL10 cytokine levels between patients with grade ≥2 and grade ≤1 CRS and NT who received anakinra prophylaxis (n = 10 from this study) across 5 time points after CAR-T infusion as measured by Luminex. Colors indicate the grade of CRS, and shape indicates the grade of NT for each patient. (D) Dot plot of significantly interacting ligand-receptor (LR) pairs with corresponding cell type combinations. Each dot is colored by $\log_2$ change in interaction score and sized by $-\log_{10}$ *U* test FDR-adjusted *P* value. (E) Distribution of interaction scores for each LR pair between samples from patients in this study who did or did not develop grade ≥2 CRS and/or NT in the presence and absence of anakinra. Colors indicate the grade of CRS, and shape indicates the grade of NT for each patient.

Our findings are consistent with previous data showing that high levels of IFN-γ are predictive of severe CRS.[38] In preclinical models, IFN-γ release from CAR-T activates macrophages to release proinflammatory cytokines, leading to CRS and ICANS.[17,21] We have demonstrated that knocking out IFN-γ in CD19 CAR-Ts or using an IFN-γ-blocking antibody suppresses macrophage production of proinflammatory cytokines without inhibiting the antitumor activity of the CAR-Ts in preclinical models.[39] Manni et al confirmed these findings in a humanized mouse model of CD19 CAR-T treatment, demonstrating that IFN-γ neutralization with the clinically approved antibody emapalumab reduced proinflammatory cytokines, prevented brain injury, and increased survival.[40] Emapalumab has also been used to successfully manage CRS in a patient treated with tisagenlecleucel who developed grade 4 CRS that was refractory to tocilizumab and glucocorticoids.[41] Together, these data suggest that IFN-γ blockade could be used to treat or prevent CRS and ICANS without influencing CD19 CAR-T efficacy. Prospective clinical trials are needed to determine whether emapalumab alone could treat CRS and ICANS, whether it should be combined with anakinra and or tocilizumab, or whether it can be used more effectively in the prophylactic setting.

Our single-cell data highlighted a distinct CRS-associated cytokine gene signature in monocytes from patients with breakthrough CRS after anakinra prophylaxis. One gene in this signature was *RETN*, a cytokine upregulated during macrophage differentiation and elevated in a number of inflammatory conditions.[36,42] Its binder, *CAP1*, was identified in our receptor-ligand analysis as a candidate partner expressed on both monocytes and T cells. Upon binding *RETN*, CAP1 activates NF-κB signaling and leads to expression of the proinflammatory cytokines IL-1β, tumor necrosis factor α, and IL-6 in vitro.[35] Our results raise the question of whether *RETN* signaling may be rendering macrophages more resistant to IL-1R blockade. These data present candidates for further proteomic and functional validation that may ultimately be targetable interactions for blocking breakthrough CRS signaling.

In conclusion, more studies are needed to determine the optimal prevention strategy for CRS and NT in patients receiving CAR-T therapy. The results of our study in comparison to other recently published trials of anakinra prophylaxis suggest that the dose, schedule, and route of administration of anakinra affect its efficacy as a prophylactic agent. Increased doses, more frequent scheduling, intravenous routes of administration,[43,44] and/or adaptive administration may improve these results across patients but may not improve the overall feasibility of administering and managing the majority of patients in the outpatient or community settings. Additionally, our data suggest that targeting additional cytokine pathways, such as IFN-γ or IL-10, may be necessary for preventing and/or treating CAR-T toxicities.

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Authorship

Contribution: M.J.F. and M.V.M. conceived the study; M.J.F., N.Y., T.R.B., N.J.H., and M.V.M. wrote the manuscript; M.J.F., C.A.J., Y.-B.C., M.B.L., Z.D., A.R.E.-J., P.C.J., A.K., J.C., R.W.M., D.C., M.T., H.C., and R.J. III performed the clinical trial; N.Y., K.M.E.G., M.W., and N.J.H. performed the experiments with patient samples; M.J.F., N.Y., K.M.E.G., T.R.B., J.N., G.G., N.J.H., M.W., and M.V.M. analyzed the data; and all authors contributed intellectually to the experiments, as well as editing and approving of the final version of the manuscript.

Conflict-of-interest disclosure: M.J.F. receives research funding from Kite and Arcellx; and receives consulting fees from Sobi, Novartis, Bristol Myers Squibb (BMS), Kite/Gilead, Iovance, and Johnson & Johnson/Legend. C.A.J. reports receiving consulting fees from Kite/Gilead, Novartis, BMS/Celgene, ImmPACT Bio, Caribou Bio, Instil Bio, Miltenyi, Ipsen, Morphosys, ADC Therapeutics, AbbVie, AstraZeneca, Sana, Synthekine; and reports research funding from Kite/Gilead and Pfizer. Y.-B.C. consults for Incyte, Takeda, Novartis, Novo Nordisk, Editas, Pharmacosmos, and Vor. Z.D. receives research support from Incyte Corp, REGiMMUNE Corp, and Taiho Oncology, Inc; and has received consulting fees from Sanofi, Incyte Corp, MorphoSys AG, Inhibrx, PharmaBiome AG, and ONO PHARMA. A.R.E.-J. consults for GlaxoSmithKline, Novartis, Incyte, AIM Pathway, and Tuesday Health. P.C.J. has served as a consultant for Seagen, ADC Therapeutics, AbbVie, Incyte, and AstraZeneca; and reports research funding from AstraZeneca. R.W.M. served on the advisory boards for Genmab, Adaptive Biotechnologies, BMS, AbbVie, Inteillia, Epizyme, and Seattle Genetics; consults for Alphasights; and has received institutional research funding from Merck, BMS, Genmab, and Genentech/Roche. Authors R.S., S.F., and J.N.Kite report employment with Kite, a Gilead company, and stock or other ownership in Gilead Sciences. G.G. receives research funds from IBM, Pharmacyclics, and Ultima Genomics; is an inventor on patent applications related to MSMuTect, MSMutSig, MSIDetect, POLYSOLVER, SignatureAnalyzer-GPU, and MinimuMM-seq; is a founder, consultant, and holds privately held equity in Scorpion Therapeutics; is a founder and holds privately help equity in Predicta Biosciences; and received travel support from Caris Life Sciences. M.V.M. is an inventor on patents related to adoptive cell therapies, held by Massachusetts General Hospital and the University of Pennsylvania (some licensed to Novartis); holds equity in 2seventybio, Genocea, Oncternal, and Neximmune; serves on the board of directors of 2seventybio; and has served as a consultant for multiple companies involved in cell therapies (M.V.M.'s interests were reviewed and are managed by Massachusetts General Hospital, and Mass General Brigham in accordance with their conflict-of-interest policies). The remaining authors declare no competing financial interests.

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