

**Cellular phenotypes correlated with immune effector cell-associated neurotoxicity
syndrome severity in CD19 CAR T cell therapy**

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Abstract

Cellular phenotypes correlated with immune effector cell-associated neurotoxicity syndrome severity in CD19 CAR T cell therapy

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CD19-directed chimeric antigen receptor (CAR) T cell therapy has emerged as a transformative treatment for patients with relapsed or refractory B cell malignancies. Since gaining FDA approval in 2017, CAR T cell therapy has become increasingly implemented in both pediatric and adult populations. Despite its remarkable efficacy, treatment-related toxicities remain common, most notably cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). While CRS is often mitigated through IL-6 pathway blockade, the underlying mechanisms driving ICANS remain incompletely understood.

In this thesis, I leverage high-dimensional mass cytometry to characterize immune phenotypic heterogeneity across multiple stages of CD19 CAR T cell therapy. As ICANS is thought to arise from dysregulated cellular phenotypes, I hypothesize that cellular phenotypes present before and during therapy reflect underlying inflammatory programs that drive toxicity. By resolving these phenotypes at high dimensionality, I aim to identify cellular states that both inform mechanisms of ICANS pathogenesis and serve as predictive biomarkers.

First, I define differences in T cell composition within leukapheresis samples and corresponding infusion products generated at distinct institutions, evaluating how patient age and manufacturing

practices contribute to phenotypic variability. I then assess whether pre-infusion cellular features serve as predictive biomarkers of severe ICANS.

Finally, I conduct longitudinal immune profiling of adult patients with diffuse large B cell lymphoma undergoing CD19 CAR T cell therapy, integrating cellular and cytokine data to identify dynamic immune shifts associated with severe ICANS compared to patients without severe ICANS to define the temporal immune dynamics that precede and accompany toxicity and gain insight into mechanisms driving ICANS. Collectively, this work provides a systems-level framework linking pre-infusion immune composition and post-infusion immune dynamics to neurotoxicity risk in CAR T cell therapy.

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Chapter 1. Introduction

B cell overview, malignant transformation, and therapeutic targeting in hematologic malignancies

B cells play a central role in adaptive immunity, primarily through the production of antibodies that recognize and neutralize foreign pathogens¹. B cells arise from hematopoietic stem cells in the bone marrow, where they undergo immunoglobulin heavy- and light-chain VDJ recombination during early development². This tightly regulated process generates antigen receptor diversity but also introduces genomic vulnerability. B cell development proceeds through defined stages—including pro-B, pre-B, immature B, and mature naïve B cells—before cells migrate to peripheral lymphoid tissues³.

Malignant transformation can occur at virtually any stage of B cell development, giving rise to diverse hematologic malignancies including leukemias and lymphomas⁴. Among lymphomas—which also include T cell and NK cells—B cell lymphomas represent the most common subtype⁴. The intrinsic susceptibility of B cells to malignant transformation is closely linked to the very mechanisms that generate immunoglobulin diversity. Processes such as V(D)J recombination and class switch recombination, while essential for adaptive immunity, can become dysregulated and result in oncogenic mutations, chromosomal translocations, or aberrant signaling pathways⁵.

In pediatric populations, acute lymphoblastic leukemia (ALL) is the most common malignancy, and B cell acute lymphoblastic leukemia (B-ALL) accounts for approximately 85% of ALL cases^{6,7}. In contrast, diffuse large B-cell lymphoma (DLBCL) represents the most prevalent B cell malignancy in adults, with a median age at diagnosis of approximately 70 years^{8,9}. This age-dependent shift in disease predominance reflects fundamental differences in B cell biology across the lifespan. During early childhood, the bone marrow contains abundant, rapidly proliferating B cell precursors, predisposing pediatric patients to precursor B cell leukemias. In

adults, malignant transformation more commonly arises in mature peripheral B cells, resulting in lymphomas¹⁰.

For decades, standard treatment for aggressive B cell lymphomas relied on combination chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)¹¹. In the early 2000s, the addition of the anti-CD20 monoclonal antibody rituximab established the R-CHOP regimen, which significantly improved clinical outcomes¹². Rituximab targets CD20, a B cell surface antigen expressed during later stages of B cell maturation but absent on early B cell precursors and terminally differentiated plasma cells¹³. Since its introduction, R-CHOP has achieved approximately 58% five-year overall survival in certain aggressive B cell lymphoma populations¹⁴. Despite these advances, relapse and antigen escape remain major clinical challenges, highlighting the limitations of both cytotoxic chemotherapy and CD20-directed therapy.

CD19 has emerged as a compelling alternative therapeutic target due to its broad expression across the B cell lineage. CD19 is expressed from early pro-B cells through mature naïve and memory B cells, plasmablasts, and in some subsets of plasma cells¹¹⁵. This near-pan-B cell distribution provides broader lineage coverage compared with CD20-targeted strategies. Consequently, CD19-directed therapies—including chimeric antigen receptor (CAR) T cell approaches—have demonstrated substantial efficacy in multiple B cell malignancies. The consistent expression of CD19 across developmental stages, coupled with its restricted expression outside the B cell compartment, has established CD19 as one of the most effective and widely adopted targets in modern cellular immunotherapy.

History and Overview of CAR T cell therapy

Chimeric antigen receptor (CAR) T cell therapy has transformed the treatment landscape for relapsed and refractory B cell malignancies over the past decade, producing unprecedented response rates in diseases previously considered incurable. The concept of engineering T cells to express synthetic chimeric antigen receptors was first described in 1987¹⁶. These early constructs laid the foundation for what would later become a clinically transformative platform.

In the context of B cell malignancies, first-generation CARs were designed using a single-chain variable fragment (scFv) derived from a CD19-specific monoclonal antibody fused to the intracellular signaling domain of CD3 ζ . This design enabled major histocompatibility complex (MHC)-independent recognition of malignant B cells. Unlike conventional T cell receptor (TCR)-mediated antigen recognition, which requires peptide presentation on human leukocyte antigen (HLA) molecules, CARs directly bind surface antigens. This MHC-independent targeting is particularly advantageous in cancer, as tumors frequently evade immune surveillance through downregulation of HLA expression¹⁷. CARs typically exhibit higher intrinsic binding affinity for target surface antigens compared with TCR–peptide–MHC interactions¹⁸. However, TCR signaling benefits from serial triggering and co-receptor amplification, rendering T cells highly sensitive to low antigen densities¹⁹. In contrast, CAR T cells generally require higher antigen density for robust activation.²⁰ This greater binding affinity results in stronger downstream activation, cytokine secretion, and overall immune response and cytolytic killing²¹. However, first-generation CAR T cells demonstrated limited *in vivo* expansion and persistence following infusion.²² To address these limitations, second-generation CARs incorporated intracellular co-stimulatory domains, most commonly CD28 or 4-1BB, to enhance T cell expansion, survival, and persistence.^{23,24} CD28-based CAR T cells demonstrate rapid expansion kinetics, increased IL-2 secretion, and robust effector differentiation, often accompanied by a more glycolytic metabolic profile and earlier exhaustion²⁵. In contrast, 4-1BB–containing CARs promote mitochondrial biogenesis, enhanced oxidative metabolism, and the development of central memory–like phenotypes associated with prolonged persistence²⁶. To this day, most commercial CAR T cell products are second generation CAR T cells. In 2017, tisagenlecleucel became the first CAR T cell therapy approved by the U.S. Food and Drug Administration (FDA) for the treatment of relapsed or refractory B-ALL.²⁷ Since then, CAR T cell therapy has been commercially utilized for other hematologic malignancies, namely, diffuse large B cell lymphoma, mantle cell lymphoma, and multiple myeloma²⁸. Ongoing efforts are focused on developing future generations of CAR T cells with on/off switches to minimize on-target, off-tumor toxicities, and other adverse events that commonly arise in CD19 CAR T cell therapy²⁹. For many clinicians, the goal is to eventually adapt CAR T cell therapy beyond B-cell malignancies for implementation in solid tumor treatment and autoimmunity^{30,31}.

CAR T cell manufacturing

Beyond CAR design, therapeutic outcomes are highly dependent on CAR T cell manufacturing.

Ultimately, the priorities in CD19 CAR T cell therapy are to generate sufficient numbers of functional CAR T cells in a timely and cost-effective manner. Currently, all FDA-approved CAR T cell therapies are manufactured from autologous patient-derived cells; however, this approach is limited by manufacturing delays, high cost, and variability in starting material due to patient health and prior treatments. As a result, alternative strategies are being explored to overcome these challenges, including allogeneic off-the-shelf CAR T cells derived from healthy donors.^{32,33}. An additional advantage of transitioning towards a universal allogeneic cell line is the reduction in heterogeneity within infusion products due to inter-patient variability³⁴. These manufacturing considerations underscore the importance of understanding how variability introduced at the earliest stages of manufacturing may determine the composition and functionality of the final product. The first step of CAR T cell manufacturing involves the collection and cryopreservation of PBMCs from leukapheresis samples³⁵. T cells are then isolated and enriched via bead-based activation to proliferate and expand until therapeutic dosage numbers are met³⁶. Following activation and expansion, T cells undergo lentiviral transduction with a CAR-encoding construct. The CAR construct encodes (1) an extracellular scFv antigen-binding domain, (2) a hinge and transmembrane region, (3) a co-stimulatory domain and 4) an intracellular CD3 ζ activation domain.^{37,38}. Cytokine stimulation follows post CAR transduction in which cytokines are introduced in order to expand the newly generated CAR T cells³⁹. Finally, the expanded and purified CAR T cells are cryopreserved until infusion⁴⁰.

CAR T cell signaling and mechanism of action

Upon CD19 engagement, CAR molecules cluster within a **non-classical immunological synapse distinct from the canonical TCR synapse**, bringing intracellular CD3 ζ domains into close proximity⁴¹. Following dimerization, immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 ζ are phosphorylated by the Src-family kinase Lck, mimicking TCR signaling pathways⁴². ITAM phosphorylation, in turn, results in a downstream signaling cascade leading to ZAP70 phosphorylation and activation⁴³. ZAP70 activation initiates downstream signaling

cascades involving LAT, PLC γ 1, and MAPK pathways, culminating in transcriptional activation of NFAT, NF- κ B, and AP-1 and driving T cell proliferation, cytokine secretion, and cytolytic function⁴⁴. Core effector molecules produced following CAR activation include IL-2, IFN- γ , TNF- α , and GM-CSF, in addition to cytolytic granule proteins such as perforin and granzymes⁴⁵. Depending on whether CAR T cells were generated with either a CD28 or 4-1BB costimulatory domain, additional signaling pathways activated will differ. Upon CD19 engagement, CD28-based CD19 CAR T cells will activate PI3K, AKT and mTOR signaling while 4-1BB based CD19 CAR T cells will activate LKB1-AMPK pathways⁴⁶. The difference in pathway activation due to differences in costimulatory domain contribute to differences in glycolytic and mitochondrial processes within CAR T cells that ultimately drive differences in cytokine secretion capacity and persistence⁴⁷.

Efficacy

Advances in manufacturing practices have led to remarkable clinical results, particularly with respect to CAR T cell response and persistence. Response rates to CD19 CAR T cell therapy have been substantial, with complete remission achieved in approximately 50–70% of patients with non-Hodgkin lymphoma⁴⁸. In addition to high initial response rates, durable remissions have been observed in patients, with long-term persistence of CAR T cells correlating with sustained disease control^{49,50}.

Efforts to improve therapeutic efficacy have led to studies defining optimal lymphodepletion regimens administered prior to CAR T cell infusion. Standard lymphodepletion includes the combination of fludarabine and cyclophosphamide to eliminate host immune cells, reduce cytokine sinks, and create a permissive environment that supports CAR T cell expansion following infusion^{51,52}. Recent studies have emerged highlighting the importance of lymphodepletion timing and dosage to optimize CAR T cell efficacy^{48,53,54}.

In addition to lymphodepletion strategies, combination approaches incorporating immune checkpoint blockade have emerged as a potential means to enhance CAR T cell efficacy. Tumor cells frequently exploit checkpoint signaling pathways to suppress T cell receptor-mediated cytotoxicity, most notably through upregulation of inhibitory receptors such as PD-1⁵⁵. However, clinical trials combining CAR T cell therapy with PD-1 blockade have not demonstrated improved response rates compared with CAR T cell therapy alone, suggesting that alleviation of

inhibitory signaling not be sufficient in achieving complete remission⁵⁶. Efforts to improve therapeutic efficacy; including methods to address antigen escape and CAR T cell exhaustion, must be balanced against the risk of immune-mediated toxicities that accompany enhanced CAR T cell activation and persistence^{57,58}.

Safety

Although CAR T cell therapy has certainly proven to be efficacious relative to conventional chemotherapy and radiation therapy, significant immune-mediated toxicities remain a major limitation. Cytokine release syndrome (CRS) and immune effector cell–associated neurotoxicity syndrome ICANS are both characterized by systemic hyperinflammatory responses that occur following CAR T cell infusion. CRS most commonly manifests within the first week following infusion, whereas ICANS frequently emerges shortly after the onset of CRS. Mild CRS symptoms include fever, fatigue, headache, and nausea, while severe cases can progress to severe hypotension, respiratory failure, multi-organ dysfunction, or death. ICANS presents with a range of neurological manifestations, from mild confusion, aphasia, and lethargy to seizures, coma, cerebral edema, and death. The severity of both syndromes is graded from 0 (no symptoms) to 5 (death), with grades ≥ 2 or ≥ 3 commonly used to define clinically significant toxicity depending on institutional criteria.

Mechanisms of Immune Effector Cell-Associated Neurotoxicity Syndrome

Adverse event development has been categorized into five stages: 1) CAR T cell infusion and trafficking to tumor site for antigen recognition, 2) CAR T cell activation and cytokine release, resulting in CRS onset, 3) systemic inflammatory response, vascular leakage and organ dysfunction, 4) cytokine diffusion and CAR T cell migration into damaged blood-brain barrier and neurotoxicity onset, 5) CAR T cell apoptosis, inflammation resolution and decreased serum cytokine levels^{59,60}. While CRS is largely driven by systemic cytokine amplification and is increasingly manageable with IL-6 blockade, the cellular and molecular drivers of ICANS remain incompletely defined. Emerging evidence implicates myeloid cell activation, endothelial dysfunction, and blood–brain barrier disruption, yet the precise immune signatures that predict

severe neurotoxicity remain unclear^{61,62}. Recent studies have identified elevated inflammatory cytokine signatures, namely IFN- γ , GM-CSF, and IL-6 within cerebrospinal fluid and serum as biomarkers of ICANS⁶³. Regarding cellular associations with ICANS, more recent studies have focused on leukocyte-endothelial cells lining the blood brain barrier through cytokine induced upregulation of endothelial adhesion molecules; namely ICAM-1 and VCAM-1⁶⁴. Of the leukocytes that may contribute to ICANS onset, we are interested in neutrophil populations in particular. Recent studies have characterized molecular neutrophil signatures that precede CRS onset⁶⁵. Elevated peripheral neutrophil abundances have also been described in patients who develop inflammatory disease in contexts such as sepsis and COVID-19^{66,67}. Furthermore, the abundance of immature CD10⁺ neutrophil populations quantified at early timepoints (within 4 hours of CAR T cell infusion) have been associated with poor efficacy and prognosis in CD19 CAR T cell therapy^{65,68}.

We hypothesize that profiling the immune cell landscapes of patients who develop ICANS will help elucidate the mechanisms driving its onset and severity. In particular, the contribution of neutrophils to ICANS is of growing interest, as recent studies have linked neutrophil-associated signatures to CRS and therapeutic efficacy, but their role in neurotoxicity remains poorly defined. This dissertation employs multi-modal analytical approaches to delineate cellular and molecular features that contribute to the onset of severe ICANS in patients undergoing CD19 CAR T cell therapy.

In Chapter 2, I describe our approach to characterizing leukapheresis T cells and CAR T cell infusion products from pediatric and adult patients treated for B-ALL, highlighting general manufacturing- and cohort-specific phenotypic differences. In Chapter 3, I present ongoing work utilizing a broad mass cytometry panel to profile immune correlates in peripheral blood following CAR T cell infusion, with a particular focus on monocyte and neutrophil populations associated with severe ICANS. In addition, we employ Meso Scale Discovery (MSD) and ELISA assays to quantify longitudinal cytokine dynamics and identify soluble mediators associated with the development of neurotoxicity.

In summary, this research establishes a foundation for identifying and characterizing potential drivers of adverse immune responses that arise during CD19 CAR T cell therapy, with a

particular focus on ICANS. The biomarkers identified in this study may inform the development of safer, next-generation cell-based immunotherapies and guide strategies to mitigate toxicity across adoptive immune cell platforms.

Chapter 2. Factors associated with pre-infusion cellular heterogeneity

2.1 Introduction

CD19-directed CAR T cell therapy has substantially improved outcomes for patients with relapsed or refractory B-cell malignancies. Despite its clinical success, many determinants of efficacy and toxicity remain incompletely understood. Outcomes following CD19 CAR T cell therapy are shaped not only by post-infusion immune dynamics but also by the intrinsic properties of the T cells used during manufacturing.

Prior to CAR transduction, leukapheresis T cell populations comprise a complex and heterogeneous mixture of subsets whose phenotypic and functional states influence downstream CAR T cell expansion, activation potential, and in vivo persistence⁶⁹. Variability in these pre-manufacturing cellular features may arise from multiple factors, including patient age, disease history, prior therapies, and institutional manufacturing protocols. Additionally, manufacturing practices used for different CAR T cell products also drive biological differences of the infused T cells which may ultimately impact patient outcomes.

We hypothesized that both donor age and institutional manufacturing practices independently contribute to phenotypic heterogeneity within CAR T cell products and may influence risk of immune effector cell-associated neurotoxicity syndrome (ICANS).

To address this, we analyzed two distinct CD19 CAR T-treated cohorts: pediatric patients enrolled in the Pediatric Leukemia Adoptive Therapy trials (PLAT-02 and PLAT-06) at Seattle Children's Research Institute (SCRI), and adult patients treated at the Fred Hutchinson Cancer Center (FHCC).

By integrating high-dimensional mass cytometry profiling with detailed clinical metadata, we sought to:

1. Define age-associated differences in leukapheresis T cell composition
2. Characterize manufacturing-driven phenotypic divergence in infusion products
3. Identify pre-infusion cellular features associated with severe ICANS

We demonstrated that:

1. Age is a primary driver of leukapheresis T cell heterogeneity.
2. Institutional manufacturing practices generate distinct CAR T infusion phenotypes.
3. *In vitro* CD19 priming during manufacturing promotes an activated CD8⁺ phenotype.
4. In pediatric cohorts, elevated activation signatures—particularly CD38 expression—are associated with severe ICANS.

Together, these findings suggest that both intrinsic immune composition and ex vivo manufacturing cues shape CAR T cell phenotypes prior to infusion and may influence neurotoxicity risk.

2.2 Results

2.2.1 Donor Age Drives Variability in Leukapheresis T Cell Phenotypes Prior to CAR T Cell Manufacturing

We hypothesized that intrinsic phenotypic differences exist within leukapheresis T cells collected from pediatric and adult patients undergoing CD19 CAR T cell therapy. Furthermore, we speculate that these intrinsic differences within leukapheresis T cells can drive variation within CAR T cell phenotypes. We analyzed frozen peripheral blood mononuclear cells (PBMCs) collected at the pre-lymphodepletion timepoint, along with CAR T cell infusion products, from pediatric B-ALL patients enrolled in the PLAT-02 and PLAT-06 trials at Seattle Children's Research Institute (SCRI) (n = 61; 17 with grade 3–5 ICANS), and from adult B-ALL patients treated at the Fred Hutchinson Cancer Center (FHCC) (n = 52; 12 with grade 3–5 ICANS). (**Figure 1A**).

Two complementary mass cytometry antibody panels were developed to characterize these samples: (1) a phenotype panel to broadly assess T cell maturity, activation, memory, migration, and proliferative status; and (2) a functional panel to evaluate cytokine secretion profiles following stimulation with PMA and ionomycin (**Figure 1B**).

Our primary objective was to identify pre-infusion cellular biomarkers associated with the development of severe ICANS. To this end, we performed regression and correlation analyses linking resolved T cell cluster abundances to subsequent toxicity onset. Additionally, we looked

for correlations specifically with PLAT-06, a clinical trial that was discontinued due to extreme cases of severe ICANS, namely incidents of cerebral edema (**Figure 1C**).

With our phenotype panel, we resolved 24 distinct pre-manufacture CD8⁺ T cell populations defined by differential expression of surface and intracellular markers (**Figure 2A**). Certain clusters were cohort-specific, with PBMC CD8 Cluster 8 predominantly detected in pediatric patients and PBMC CD8 Cluster 2 enriched in adult FHCC patients (**Figure 2B**). Cluster 8 is characterized by elevated CCR7, CD27, and CD45RA expression, suggesting a naïve T cell phenotype, while Cluster 2 is characterized as CD45RA⁺CD38^{mid}KLRG^{mid}. A comparison of cluster abundances between cohorts revealed pronounced differences in CD8⁺ T cell composition (**Figure 2C**). While multiple clusters showed statistically significant differences between SCRI and FHCC cohorts, our analysis focused on those with the highest proportional differences. Notably, naïve-like CD8⁺ T cell populations such as Cluster 8 were substantially enriched in the pediatric cohort (**Figure 2D**).

When correlating pre-manufacture CD8⁺ cluster abundance with severe ICANS onset within our pediatric and adult cohorts separately, we found that a higher frequency of Cluster 8 was associated with severe ICANS in pediatric patients (**Figure 2E**). This relationship was not observed in the adult cohort, suggesting an age-specific link between pre-infusion naïve CD8⁺ T cell abundance and neurotoxicity risk.

Next, we stained pre-manufacture CD8⁺ T cells with our functional panel following 4 hour PMA/ionomycin stimulation and resolved 27 functional CD8⁺ clusters (**Figure 3A**). As with the phenotype analysis, functional clusters exhibited cohort-specific enrichment patterns (**Figure 3B**). Substantial differences existed in cluster proportions between SCRI and FHCC samples, as we observed elevated proportions of Cluster 1 and 4 both characterized a CD45RA⁺IL-2^{mid}IL-5^{low}, within our pediatric cohort (**Figure 3C,D**). Notably, Clusters 1 and 2 are correlated with severe ICANS onset within our pediatric cohort while Cluster 4, another CD8⁺ population capable of secreting IL-5 upon PMA/ionomycin stimulation, demonstrated only a weak association with severe ICANS onset (**Figure 3E**). Interestingly, these same clusters are not associated with severe ICANS onset within our adult cohort.

2.2.2 CD19 Priming During Manufacturing Results in an Activated Phenotype within CAR T Cell Infusion Products

Following our phenotypic and functional characterization of pre-manufacture T cells, we phenotype CAR T cell infusion products to identify manufacturing-related factors contributing to cellular heterogeneity. Notably, manufacturing protocols differed between Seattle Children's Research Institute (SCRI) and the Fred Hutchinson Cancer Center (FHCC). At FHCC, CD19 CAR T cells underwent *in vitro* stimulation through co-culture with a CD19-expressing cell line during manufacturing (**Supplemental Figure 1**). We hypothesized that this additional antigen exposure prior to cryopreservation and infusion would drive CAR T cells toward a more activated phenotypic state.

It is also important to note that CAR construct designs differed across clinical cohorts. Patients in the PLAT-02 trial received products incorporating a murine anti-human FMC63 single-chain variable fragment (scFv), whereas those in PLAT-06 received products manufactured with a humanized anti-CD19 scFv. Likewise, among adult cohorts, trial X2639 utilized FMC63-based CAR T cells, while X9346 employed a humanized construct. These design distinctions stemmed from the hypothesis that humanized CARs might improve cellular engraftment and persistence by minimizing immune rejection. Regarding our pediatric cohort, we observed that four of ten PLAT-06 patients experienced severe toxicity, motivating us to identify PLAT-06-specific biomarkers that could signal an elevated risk of severe ICANS onset. To characterize phenotypic and functional heterogeneity within CAR T cell infusion products, we applied the same mass cytometry phenotype and functional panels used for leukapheresis T cell characterization.

Regarding our phenotypic analysis of CD8 cells, which included both patient infusion products and PBMC from healthy donors, 25 phenotypically distinct clusters were identified, 16 of which are CAR T cells and 9 were healthy donor T cell clusters. (**Figure 4A**). Naïve-like populations expressing CD27⁺CCR7⁺ (Clusters 2 and 12) were enriched in the pediatric cohort, whereas the dominant CD8⁺ clusters in adults (Clusters 6 and 8) lacked high expression of CCR7 and CD27, indicative of more differentiated or activated subsets (**Figure 4B-D**). However, no significant correlations were observed between the abundance of these clusters of interest and severe ICANS onset in either cohort.

Functional analysis of CD8⁺ CAR T cells and CD8⁺ T cells derived from healthy donors identified 23 subpopulations (16 CAR T CD8⁺ cell populations and 7 healthy donor CD8⁺ cell populations) (**Figure 5A**). Across both pediatric and adult cohorts we observe predominant CD4 clusters (Clusters 1,2,5,8, and 20) within our patients. Pediatric-associated clusters (Clusters 1, 2, and 5) were defined by CD45RA⁺CD27⁺IL-2⁺ expression and lacked elevated HLA-DR following PMA/ionomycin stimulation (**Figure 5B-D**). Among these clusters, Cluster 2—characterized by elevated CD25 expression—was significantly associated with severe ICANS onset in the pediatric cohort ($p = 0.025$), suggesting that primed, IL-2–responsive subsets may contribute to neuroinflammatory toxicity risk (**Figure 5E**). In contrast, no CD8⁺ T cell clusters were associated with severe ICANS in the adult cohort.

We hypothesize that differences in CAR T CD8 cells manufacturing between SCRI and FHCC may have contributed towards differences in functional capacity, phenotypes, and ultimately cellular-ICANS associations. Regarding functional differences associated with differences in manufacturing, we observed that CAR T CD8s manufactured at the FHCC secreted a greater abundance of IFN- γ , TNF- α , and IL-2, among other inflammatory cytokines, upon PMA/ionomycin stimulation (**Figure 6A**). Furthermore, both CD4 and CD8 CAR T cells produced at FHCC exhibited a distinctly more activated phenotype than those generated at SCRI. We observe elevated chemokine receptor expression in CAR T CD8s manufactured at the FHCC (**Figure 6B**). We also observed elevated expression of exhaustion markers such as TIM3 and TBET within CAR T CD8⁺ cells generated from the FHCC in addition to activation markers upregulated (**Figure 6B**).

We looked for correlations between biomarkers on CD4 and CD8 CAR T cells manufactured at Fred Hutch and SCRI cohorts with severe ICANS onset (**Figure 7A**). We observed that within both CD4 and CD8 CAR T cells manufactured at SCRI, CD38 was elevated in patients that developed severe ICANS. Interestingly, CD38 expression within CD4 and CD8 CAR T cells from the FHCC did not correlate with severe ICANS onset. Notably, CD38 expression was elevated in approximately 90% of CD4⁺ CAR T cells derived from FHCC manufacturing processes, consistent with prior CD19 antigen priming and enhanced activation (**Figure 7B**). In contrast, CD38 expression was far more heterogeneous across CD4 CAR T cells manufactured at SCRI. We observe that CD38 expression infusion products manufactured at SCRI, in particular;

CD8 CAR T cells, was associated with ICANS (**Figure 7C,D**). In particular, we observe a correlation between CD38 expression in both CD4 and CD8 CAR T cells and the development of cerebral edema (**Figure 7E**). These findings collectively indicate that *in vitro* CD19 priming during CAR T cell manufacturing drives activation signatures and contributes to phenotypic differences among infusion products. In FHCC-manufactured products, near-uniform CD38 upregulation following CD19 priming reduced inter-patient variability, potentially obscuring correlations with clinical toxicity. In contrast, greater heterogeneity in CD38 expression within SCRI-manufactured products may have preserved its predictive association with ICANS.

Together, these findings highlight that differences in manufacturing protocols—particularly *in vitro* CD19 priming during manufacturing—can substantially influence the activation state and functional potential of CAR T cell infusion products. The observed enrichment of activated phenotypes, especially within CD8⁺ subsets, underscores how manufacturing decisions may shape toxicity profiles. These results emphasize the importance of standardizing and optimizing CAR T cell production methods to balance cellular activation with long-term persistence and safety in clinical applications.

2.3 Discussion

It is not unexpected that *in vitro* stimulation of CAR T cells with a CD19-expressing cell line promotes an activated phenotype. Similarly, the association between activated CD8⁺ CAR T cells and severe ICANS aligns with links between hyperactivated effector T cells and inflammatory cytokines found in serum (GM-CSF, IFN γ , TNF)⁷⁰. Unexpectedly, however, CD38 expression correlated with severe ICANS only in pediatric patients. We hypothesize that CD19 antigen exposure during adult manufacturing may mask intrinsic CD38 upregulation and its relationship to neurotoxicity. In contrast, CD38 upregulation in pediatric CAR T cells may reflect endogenous activation pathways accompanied by unique transcriptional programs not induced by CD19 stimulation in adults.

Beyond its canonical role as an activation marker, CD38 also functions as a receptor for CD31 (PECAM-1), a key endothelial adhesion molecule. This interaction supports the hypothesis that intrinsic CD38⁺ CAR T cells may preferentially traffic toward the blood–brain barrier, which is lined by CD31⁺ endothelial cells. Such directed migration could facilitate CAR T cell entry into

the central nervous system and contribute to ICANS pathogenesis. These findings suggest that both intrinsic and manufacturing-driven activation states intersect to shape CAR T cell phenotypes and neurotoxicity risk.

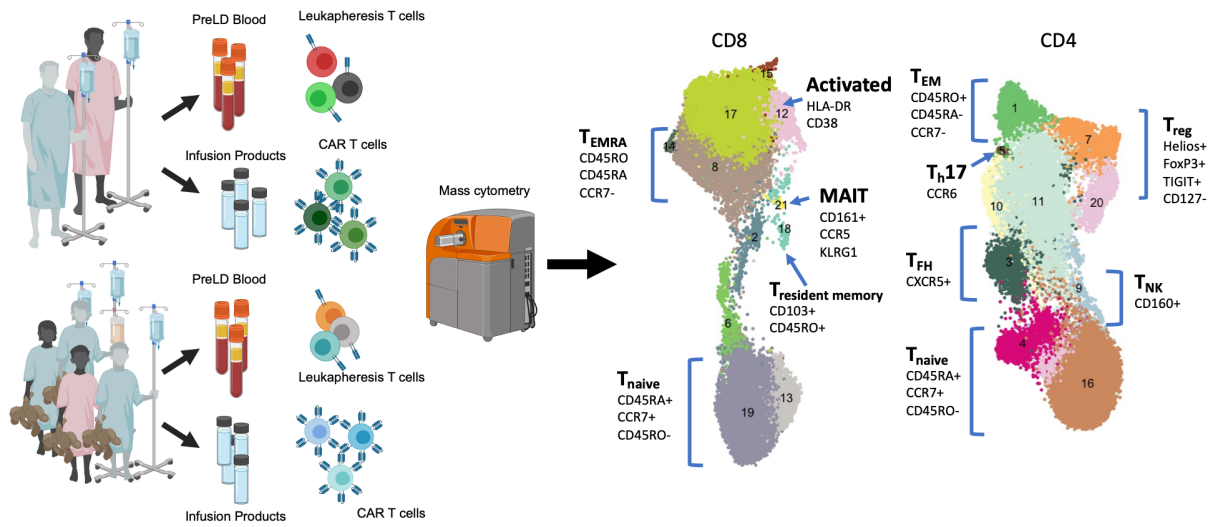
In addition to patient age and manufacturing protocol differences, confounding variables of this study include differences in marrow disease burden at the time of CAR T cell infusion and infused cellular dosage.

Together, these results underscore that both patient-intrinsic factors (such as age and baseline immune composition) and manufacturing-related variables (such as *in vitro* antigen priming) play critical roles in defining CAR T cell phenotypes prior to infusion. The interplay between these determinants influences activation states, trafficking potential, and toxicity risk. These findings reinforce a central principle in adoptive cellular therapy: the infusion product is not simply a living drug, but a dynamic biological entity whose phenotype reflects both the immunologic history of the donor and the artificial signals imposed during manufacturing. Understanding how pre-infusion cellular and manufacturing heterogeneity contributes to adverse outcomes such as ICANS will be essential for refining next-generation CAR T cell design and developing safer, standardized manufacturing practices for adoptive cellular therapies.

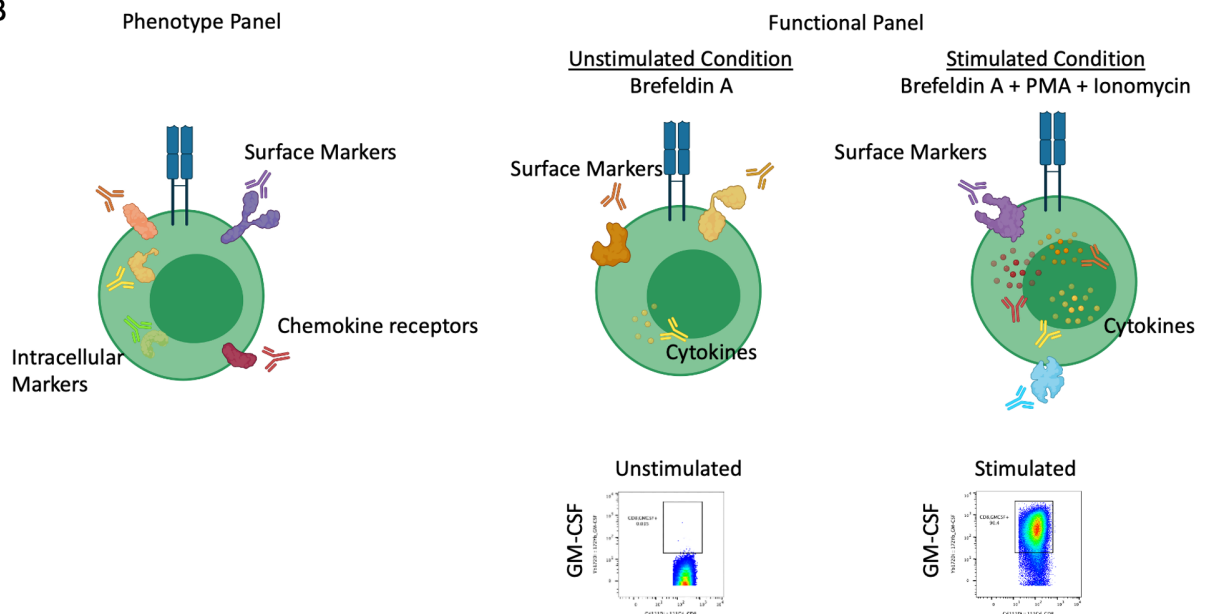
2.4 Figures

Figure 1

A



B



C

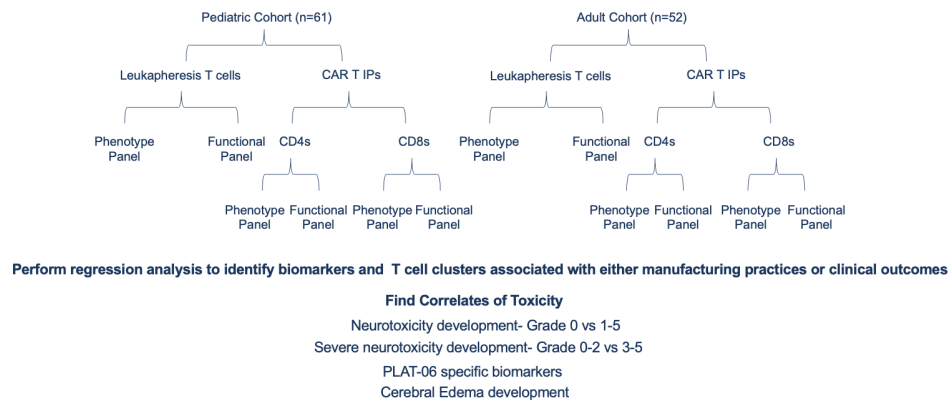


Figure 1.

- A. Sample collection of leukapheresis T cells and infusion products manufactured at either Fred Hutchinson Cancer Center or Seattle Children's Research Institute for adult and pediatric patients undergoing CD19 CAR T cell therapy for relapsed B cell acute lymphoblastic leukemia. Samples were processed via mass cytometry to characterize T cell phenotypes
- B. Phenotypic and functional characterization of T cell populations via mass cytometry. A 40 marker phenotype panel was designed to characterize memory, activation, proliferation, migration, and exhaustion profiles. A 40 marker functional panel was designed to characterize secretive capacity upon PMA/Ionomycin stimulation.
- C. Analysis strategy to correlate T cell phenotypes from leukapheresis samples and infusion products with neurotoxicity onset

Figure 2

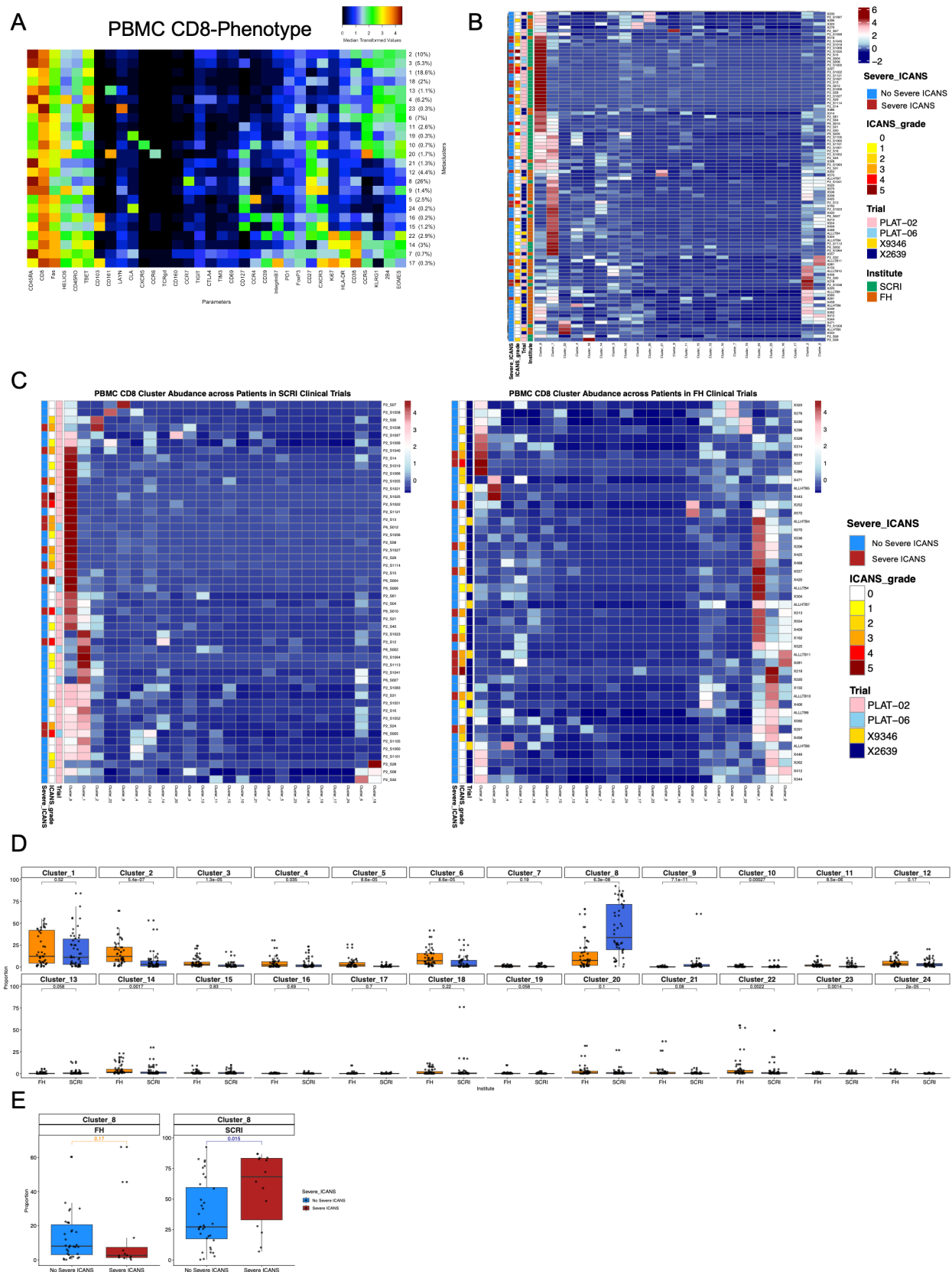


Figure 2

- A. Heatmap of marker expressions characterizing each leukapheresis CD8 population based off mass cytometry phenotype panel
- B. Heatplots of overall leukapheresis CD8 cluster abundances in patients enrolled in both FHCC and SCRI clinical trials
- C. Heatplots of cluster abundances in separate FHCC and SCRI clinical trials
- D. Box and whisker plots comparing cluster abundances in FHCC (orange) vs SCRI (blue) patients
- E. Box and whisker plots of Cluster 8 within FHCC and SCRI patients separately, grouped by severe ICANS status.

Figure 3

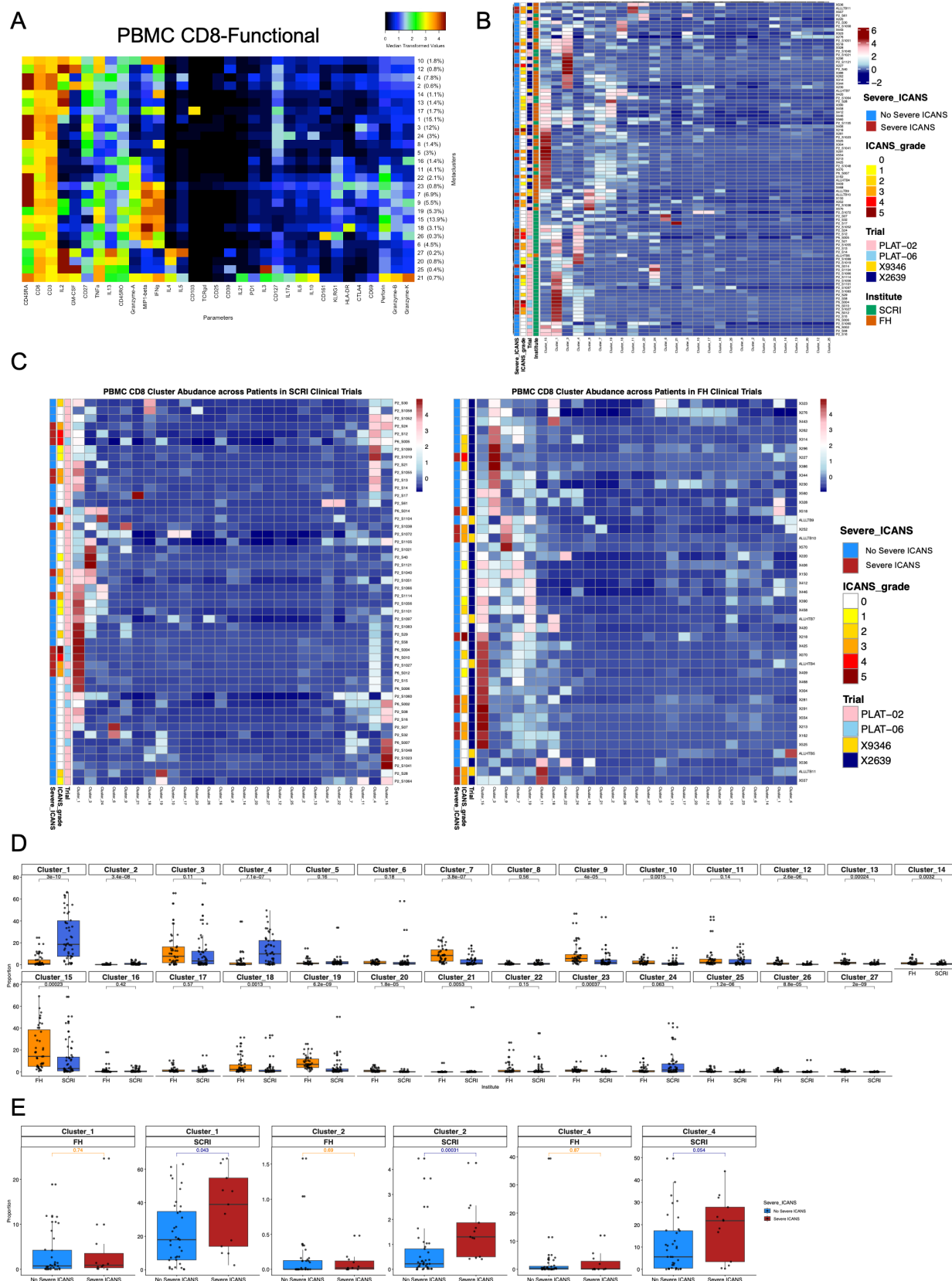


Figure 3

- A. Heatmap of marker expressions characterizing each leukapheresis CD8 population based off mass cytometry functional panel
- B. Heatplots of overall leukapheresis CD8 cluster abundances in patients enrolled in both FHCC and SCRI clinical trials
- C. Heatplots of cluster abundances in separate FHCC and SCRI clinical trials
- D. Box and whisker plots comparing cluster abundances in FHCC (orange) vs SCRI (blue)
- E. Box and whisker plots of Clusters 1,2, and 4 within FHCC and SCRI patients separately, grouped by severe ICANS status.

Figure 4

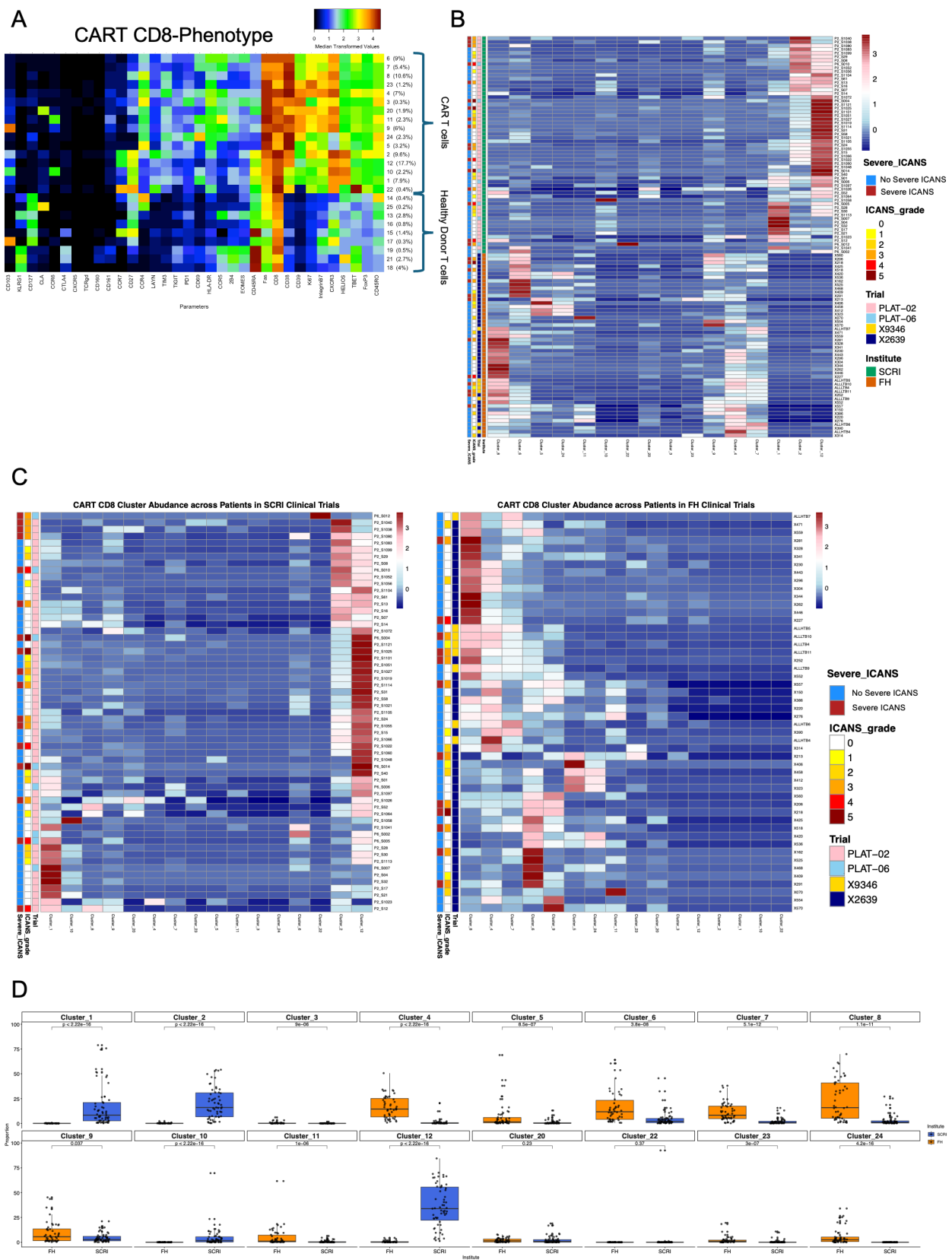


Figure 4

- A. Heatmap of marker expressions characterizing each CART CD8 and HD CD8 population based off mass cytometry phenotype panel
- B. Heatplots of overall CART CD8 and HD CD8 cluster abundances in patients enrolled in both FHCC and SCRI clinical trials
- C. Heatplots of cluster abundances in separate FHCC and SCRI clinical trials
- D. Box and whisker plots comparing CART CD8 cluster abundances in FHCC (orange) vs SCRI (blue)

Figure 5

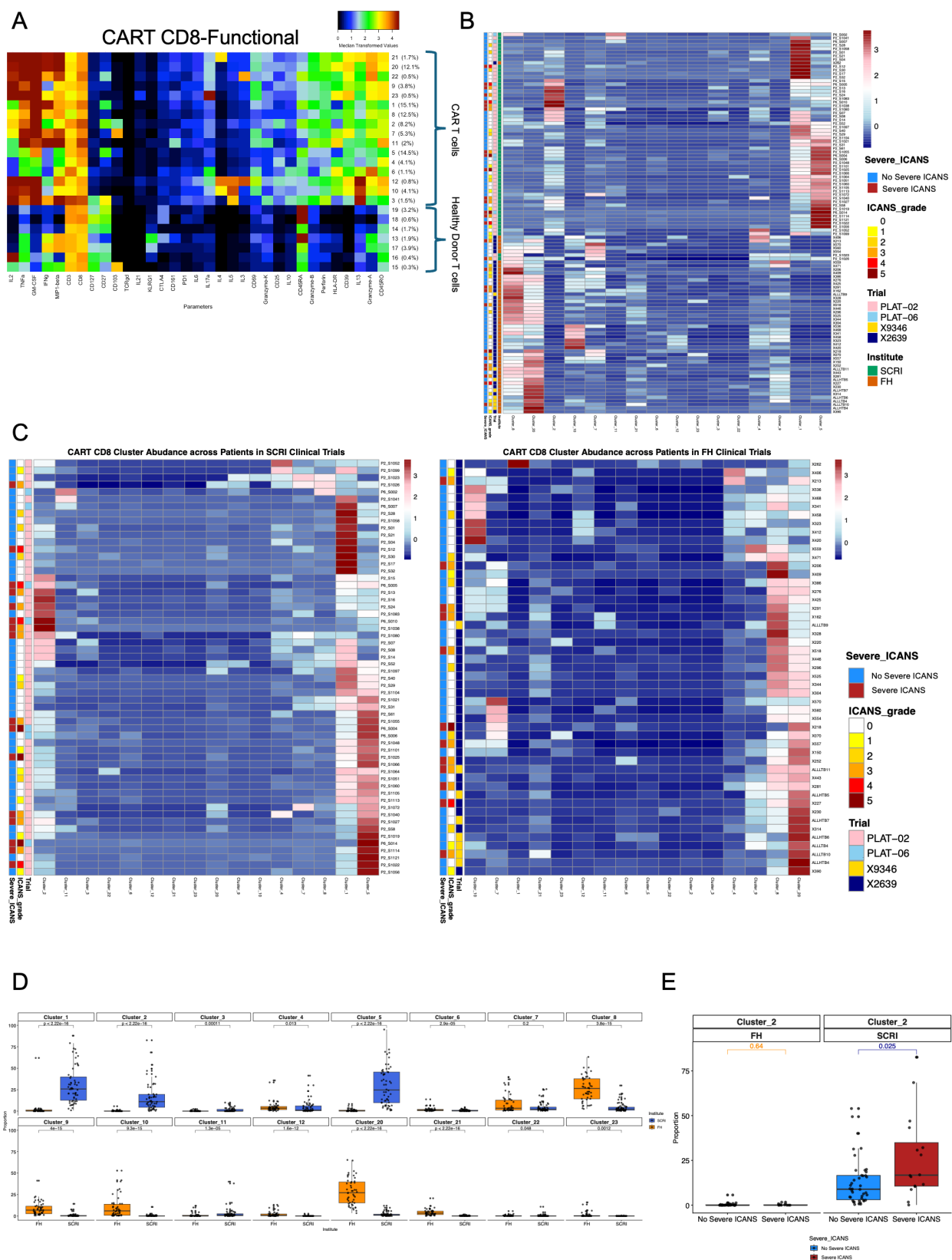
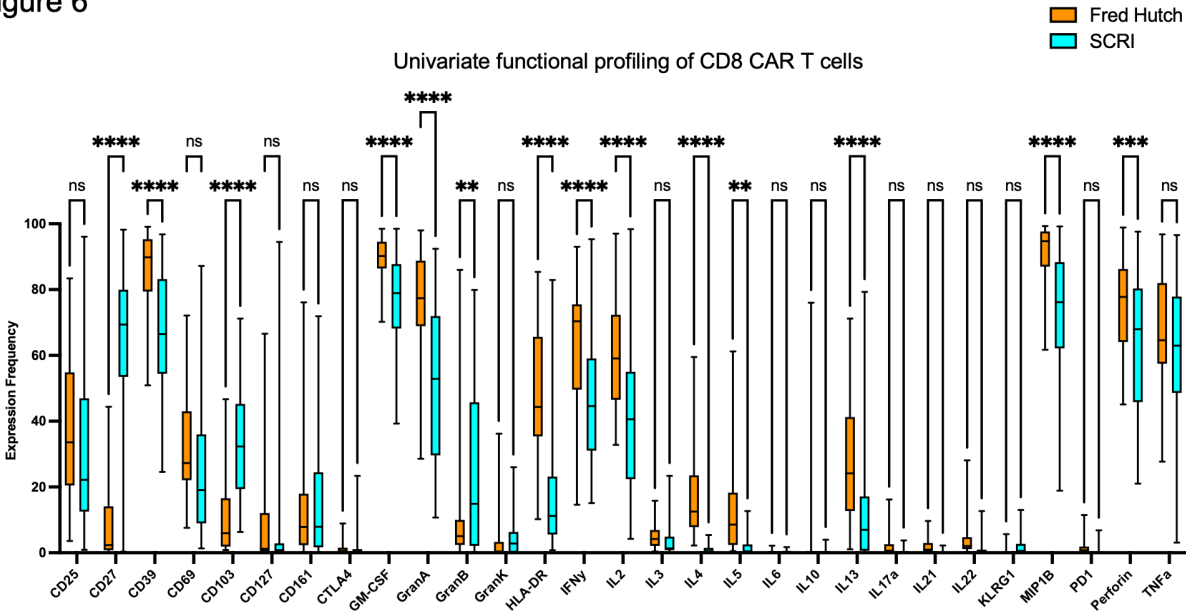


Figure 5

- A. Heatmap of marker expressions characterizing each CART CD8 and HD CD8 population based off mass cytometry functional panel
- B. Heatplots of overall CART CD8 and HD CD8 cluster abundances in patients enrolled in both FHCC and SCRI clinical trials
- C. Heatplots of cluster abundances in separate FHCC and SCRI clinical trials
- D. Box and whisker plots comparing CART CD8 cluster abundances in FHCC (orange) vs SCRI (blue)
- E. Box and whisker plot of Cluster 2 abundance in SCRI and FHCC patients grouped by severe ICANS status

Figure 6

A



B

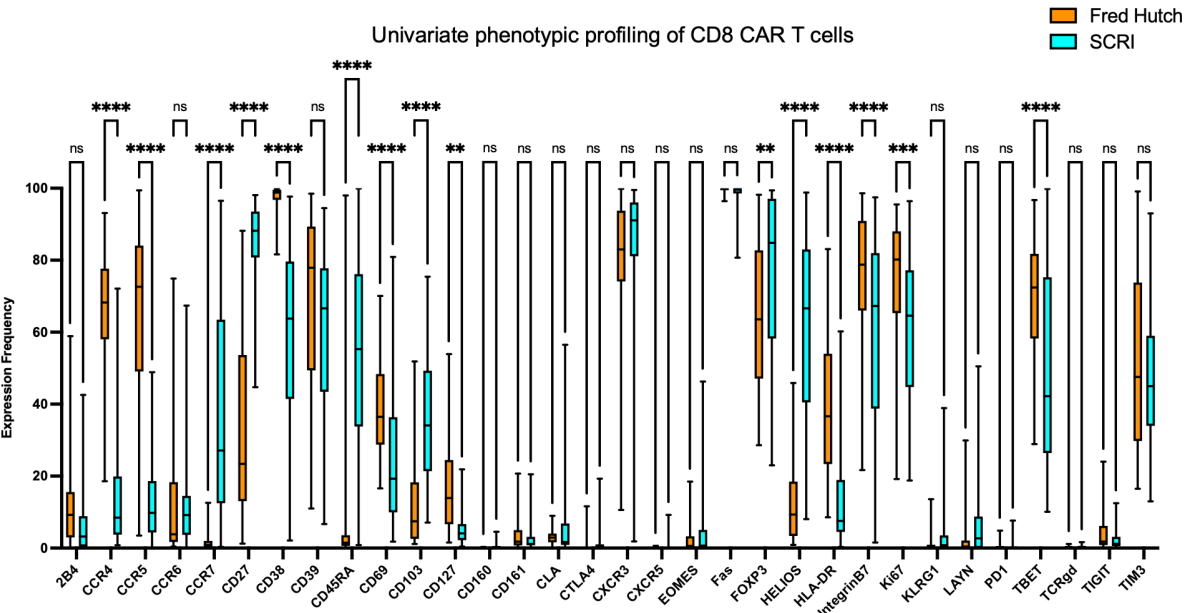


Figure 6.

- A. Univariate profiling of CD8 CAR T cells from SCRI and FHCC based off markers from mass cytometry functional panel after 4 hour stimulation with PMA and Ionomycin
- B. Univariate profiling of CD8 CAR T cells from SCRI and FHCC based off markers from mass cytometry phenotype panel

Figure 7

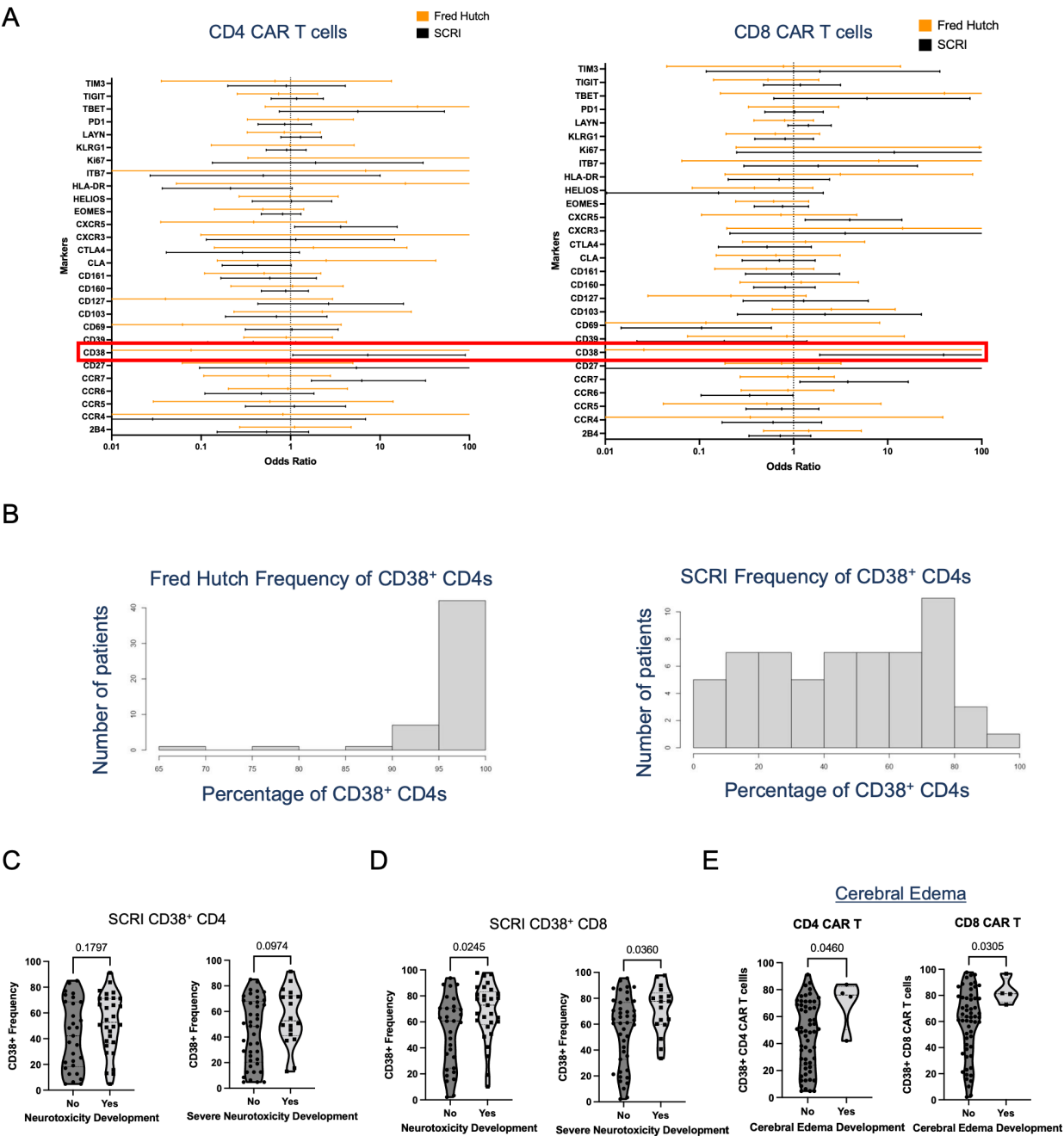


Figure 7.

- A. Odds ratio plot of phenotypic markers on CD4 and CD8 CAR T cells manufactured at SCRI and FHCC and association with severe ICANS
- B. Distribution of CD38 expression on CD4 and CD8 CAR T cells originating from SCRI and FHCC
- C. Correlation of CD38⁺ CD4 CAR T cells manufactured at SCRI with neurotoxicity
- D. Correlation of CD38⁺ CD8 CAR T cells manufactured at SCRI with neurotoxicity
- E. Correlation of CD38⁺ CD4 and CD8 CAR T cells manufactured at SCRI with cerebral edema onset.

2.5 Supplemental Figures

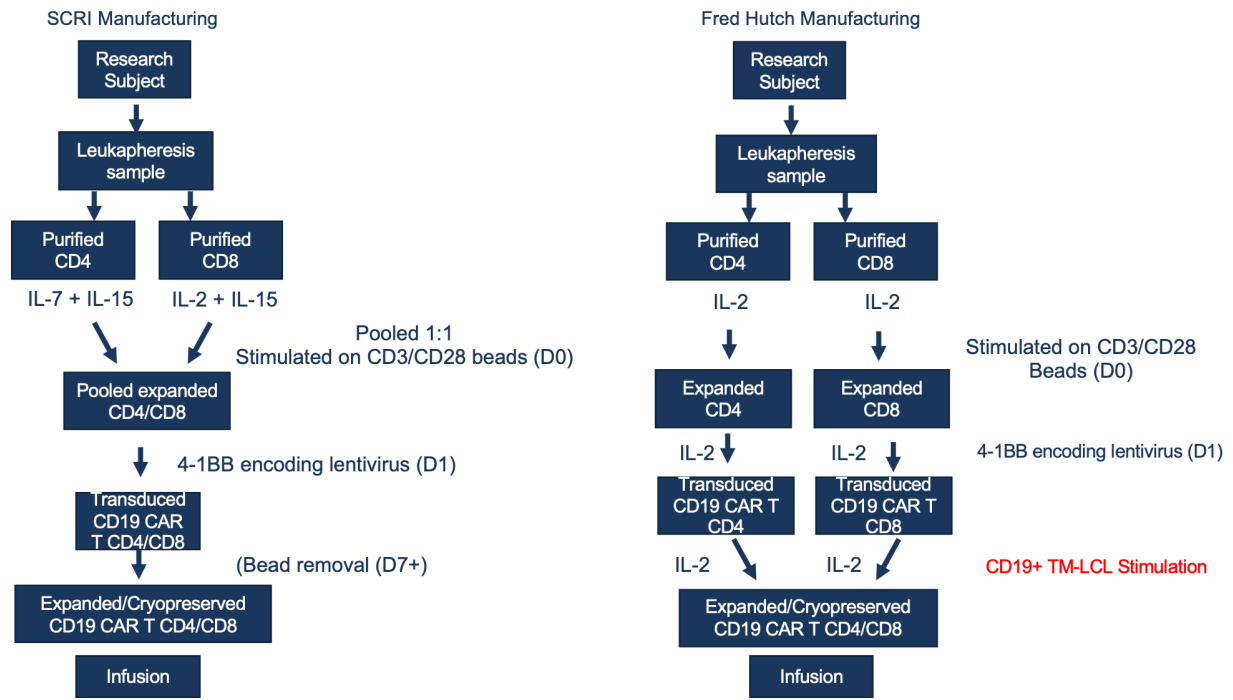
Supplemental Table 1

Target	Clone	Metal isotope	Source	Part number
CD45	HI3O	89 Y	Standard Biotools	3089003
CD4	RPA-T4	110 Cd	BioLegend	300502
CD8a	RPA-T8	111 Cd	BioLegend	301002
CD14	TuK4	112 Cd	Thermofisher	MHCD1400
CD19	HIB19	113 Cd	BioLegend	302202
CD56	REA196	114 Cd	Miltenyi Biotec	130-108-016
CLA	HAE-1f	141 Pr	BioLegend	336002
HLA-DR	L243	142 Nd	BioLegend	307602
IntegrinBeta7	FIB504	143 Nd	BD	321202
	MBSA-43 or			
TIGIT	MAB7898	144 Nd	eBio or R&D	16950082
CD69	FN50	145 Nd	BioLegend	310902
2B4	C1.7	146 Nd	BioLegend	329502
CD160	BY55	147 Sm	BioLegend	341202
CD45RO	UCHL1	148 Nd	BioLegend	304202
KLRG1	13F2F12	149 Sm	Thermofisher	16-9488-85
CD45RA	HI100	150 Sm	BioLegend	304102
CD27	LG.7F9	151 Eu	Thermofisher	14-0271-82
Ki-67	Ki-67	152 Sm	BioLegend	350502
CD103	B-Ly7	153 Eu	Thermofisher	14-1038-82
TCRgd-PE + anti-PE	5A6.E9 PE001	154 Sm	Thermofisher, Invitrogen	MHGD04,408102
FoxP3-biotin + SAV-155	PCH101	155 Gd	Thermo Fisher	13-4776-82
CD3	UCHT1	156 Gd	BioLegend	300402
Fas	DX2	158 Gd	BioLegend	305602
CD161	W18070C	159 Tb	BioLegend	339902
PD-1	eBioJ105	160 Dy	Thermofisher	14-2799-80
CD127	A019D5	161 Dy	BioLegend	351337
TIM3	F38-2E2	162 Dy	BioLegend	345002
LAYN	3F7D7E2	163 Dy	Sino Biological	MAB26371
CXCR3	G025H7	164 Dy	BioLegend	353702
CXCR5	RF8B2	165 Ho	BD	552032
CTLA4	BNI3	166 Er	BD	550405
anti-EGFR to ID CART cells		167 Er	in house	
	G043H7 or			
CCR7	150503	168 Er	BioLegend or R&D	353202
HELIOS	22F6	169 Tm	BioLegend	137202
TBET	eBio4B10	170 Er	eBio	14-5825-82
CCR5	HEK/1/85a	171 Yb	BioRad	MCA2175GA
Eomes	WD1928	172 Yb	Thermo Fisher	14-4877-82
CD39	A1	173 Yb	BioLegend	328202
CCR4	205410	174 Yb	R&D	MAB1567-100
CCR6	G034E3	175 Lu	BioLegend	353427
CD38	HIT2	176 Lu	BioLegend	303502
CD16	3G8	209 Bi	BioLegend	3209002B

Supplemental Table 2

Target	Clone	Metal isotope	Source	Part number
CD45	HI30	89 Y	Standard Biotools	3089003
CD4	RPA-T4	110 Cd	BioLegend	300502
CD8a	RPA-T8	111 Cd	BioLegend	301002
CD14	HCD14	112 Cd	Thermofisher	MHCD1400
CD19	HIB19	113 Cd	BioLegend	302202
CD56	HCD56	114 Cd	Miltenyi Biotec	130-108-016
GranzymeK	GM6C3	141 Pr	Santa Cruz Biotechnology	sc-56125
TNF-a	Mab11	142 Nd	BioLegend	502902
IFN-g	B27	143 Nd	BioLegend	506521
GranzymeB	2CF/F5	144 Nd	BioLegend	550558
CD69	FN50	145 Nd	BioLegend	310902
IL-6	MQ2-13A5	146 Nd	BioLegend	501101
IL-3	BVD8-3G11	147 Sm	BioLegend	500502
CD45RO	UCHL1	148 Nd	BioLegend	304202
KLRG1	13F2F12	149 Sm	Thermofisher	16-9488-85
CD45RA	HI100	150 Sm	BioLegend	304102
CD27	LG.7F9	151 Eu	Thermofisher	14-0271-82
CD25	M-A251	152 Sm		
CD103	B-Ly7	153 Eu	Thermofisher	14-1038-82
TCRgd-PE + anti-PE	MHGD04/PE001	154 Sm	Thermofisher, Invitrogen	MHGD04,408102
CD127	A019D5	155 Gd	BioLegend	351302
CD3	UCHT1	156 Gd	BioLegend	300402
IL-13	JES10-5A2	158 Gd	BioLegend	501902
CD161	W18070C	159 Tb	BioLegend	339902
PD-1	eBioJ105	160 Dy	Thermofisher	14-2799-80
IL-22	IL22JOP	161 Dy	Thermofisher	16-7222-82
IL-2	MQ1-17H12	162 Dy	BioLegend	500302
IL-8	E8N1	163 Dy	BioLegend	511402
Perforin	B-D48	164 Dy	Abcam	ab47225
HLA-DR	L243	165 Ho	BioLegend	307602
CTLA-4	BNI3	166 Er	BD	550405
GranymeA	CB9	167 Er	BioLegend	557449
MIP1beta	24006	168 Er	R&D Systems	MAB271-100
IL-4	MP4-25D2	169 Tm	BioLegend	500837
IL-5	JES1-39D10	170 Er	BioLegend	500902
IL-10	JES3-9D7	171 Yb	BioLegend	501423
GM-CSF	BVD2-21C11	172 Yb	BioLegend	502315
CD39	A1	173 Yb	BioLegend	328202
IL-17A	BL168	174 Yb	BioLegend	512331
IL-21	3A3-N2	175 Lu	BioLegend	513009
CD16	3G8	209 Bi	BioLegend	3209002B

Supplemental Figure 1



CAR T cell infusion product manufacturing methods at SCRI and FHCC

2.6 Methods

Study Design and Participants

This study included 50 adult patients (12 with grades 3-5 ICANS) treated at the Fred Hutchinson Cancer Center (FHCC) and 62 pediatric patients (17 with grades 3-5 ICANS) treated at Seattle Children's Research Institute (SCRI) for relapsed or refractory B cell acute lymphoblastic leukemia (B-ALL).

Among adults, 40 patients received a murine-derived anti-human CD19 CAR T cell product incorporating the FMC63 single-chain variable fragment (scFv), and 10 patients received a humanized anti-CD19 CAR construct. Among pediatric patients, 50 received a murine-derived anti-human CD19 CAR T cell product, and 10 received a humanized anti-CD19 construct.

Pediatric patients were enrolled in the PLAT-02 and PLAT-06 clinical trials at SCRI. Adult patients were treated under institutional CAR T cell protocols at FHCC. Clinical metadata, including ICANS severity grading, were obtained through chart review under approved institutional protocols.

Surface and Intracellular Staining

Cells were first washed with cytometry buffer (PBS containing 0.5% BSA and 0.02% sodium azide). For TCR $\gamma\delta$ exclusion, cells were incubated with PE-conjugated anti-TCR $\gamma\delta$ antibody for 15 minutes on ice, followed by washing. Samples were then stained with titrated cocktails of metal-conjugated antibodies targeting surface markers for 30 minutes on ice. After surface staining, cells were washed three times in cytometry buffer. For intracellular staining, cells were permeabilized using appropriate fixation/permeabilization buffers and stained with metal-conjugated antibodies targeting transcription factors and intracellular proteins. Following intracellular staining, cells were washed twice. Cells were fixed in 2% paraformaldehyde containing iridium DNA intercalator (1:4000 dilution) and stored at 4°C until acquisition.

Functional Characterization

Cryopreserved PBMCs and infusion products were thawed on ice and resuspended in 10% FBS + 90% RPMI. Following three washes and spin downs with RPMI, cell counts were performed to obtain 10M cells per sample to transfer onto 24 well plates for PMA/ionomycin stimulation and Brefeldin A blockade at 37°C for 4 hours. Following PMA/ionomycin stimulation, cells were transferred onto 96 well plates for functional staining. Afterward, cells were washed with cytometry buffer and stained with a cocktail of metal-conjugated surface marker antibodies at titrated concentrations and incubated on ice for 30 minutes. Following surface marker staining, cells were washed in cytometry buffer twice and permeabilized and stained with a cocktail of antibodies targeting cytokines. An additional two rounds of wash with cytometry buffer followed before incubating with 2% paraformaldehyde and DNA intercalator (1:4000) and stored at 4°C until the day of CyTOF acquisition.

CyTOF acquisition

On day of sample acquisition, cells were washed 3× with cytometry buffer and resuspended in 10% EQ beads (Catalog #201078) in MaxPar Cell Acquisition Solution prior to CyTOF processing.

Following CyTOF processing, the signal of each parameter was normalized based on EQ beads with Matlab software (<https://github.com/nolanlab/bead-normalization/releases/tag/v0.3>).

Clustering analysis

Repeated clustering at the single cell level – typically necessary to identify and factor out technical noise, or putative batch effect -- is computationally intractable at the scale of this study. Hence, to arrive at globally defined clusters across all batches, we first ‘over’-clustered within each batch by clustering then subclustering each cluster⁷¹. Next we computed the centroids of all clusters in each batch, then (meta)clustered these centroids (or ‘metacells’ or ‘supercells’). At each stage we used the Rphenograph algorithm, with Leiden clustering of shared nearest-neighbor graphs⁷².

To address moderate drift across batches in some parameters, we aligned the repeated control median values across batches for each parameter and applied the corresponding batch- and parameter-specific shifts to the samples before computing metacells for global metaclustering⁷³.

We used Uniform Manifold Approximate Projection (UMAP) for dimension reduction and data visualization⁷⁴.

Statistical Analysis

Rank-sum Wilcoxon tests were applied to identify statistically significant differences in median cluster abundances between severe and non-severe ICANS patient groups.

Chapter 3: Longitudinal profiling of cellular populations and cytokines post CAR T cell infusion

3.1 Introduction

Over the past decade, CAR T cell therapy has demonstrated its efficacy as a potent treatment option for relapsed B cell-lineage malignancies, including leukemia, lymphomas, and multiple myeloma⁷⁵. Despite the demonstrated efficacy in terms of remission, adverse effects commonly arise post CAR T cell infusion⁷⁶. Approximately 70% of patients receiving CAR T cell therapy develop cytokine release syndrome (CRS) of any grade (1–5) and, an overlapping 40% experience immune effector cell-associated neurotoxicity syndrome (ICANS) across grades 1–5⁷⁷. Many factors account for the different rates of CRS and ICANS incidents including disease and CAR T cell product. While CRS is known to correlate with elevated inflammatory proteins such as IL-6 and GM-CSF in the serum, we sought to explore potential immune mechanisms underlying these adverse events⁷⁸.

To elucidate the cytokine and cellular contributions to the development of ICANS post CAR T cell infusion, we collected serum and fixed whole blood at pre- and post-CAR T cell infusion timepoints from patients who have undergone CD19 CAR T cell therapy for DLBCL. We employed high-dimensional profiling platforms, including mass cytometry, to characterize shifts in cellular distributions. In parallel we also used meso-scale discovery assays and ELISAs to quantify serum cytokine levels throughout CD19 CAR T cell therapy. While most studies have focused on quantifying tumor burden alongside CAR T cell infusion product abundance and persistence post infusion as key correlates of efficacy and safety, recent studies have drawn attention to the roles of monocytes, macrophages and neutrophils in CRS and ICANS^{79,65,80,81}. Recently, neutrophil subpopulations have emerged as a key contributor in various inflammatory conditions, namely COVID-19 and sepsis^{67,82}. Based on these observations, we hypothesized that neutrophil heterogeneity may contribute to ICANS pathogenesis and accordingly we characterized neutrophil populations in CAR T cell-treated patients to determine their association with severe ICANS.

Neutrophil populations have been challenging to characterize in comprehensive single-cell studies due to their fragile nature (flow cytometry) and non-robust RNA counts (single-cell

RNA-seq)⁸³. We utilize SMARTube Proteomic Stabilizer to preserve intact CD66b⁺ cells, facilitating high-dimensional analysis of neutrophils to assess heterogeneity and monitor longitudinal changes within patient samples over the course of CAR T cell therapy⁸⁴.

From our mass cytometry analysis, we observed an elevated proportion of CD163⁺ monocytes along with a reduced proportion of non-classical CD16⁺ monocytes in patients that developed moderate to severe ICANS (grade 2-5) compared to patients that did not develop severe ICANS (grades 0-1), when looking within 3 days of peak ICANS. Within this peak ICANS time window, we also observed an increased proportion of immature CD10⁺ neutrophils in patients that developed moderate to severe ICANS (grade 2-5). Concurrently, in the serum of moderate to severe ICANS patients, we observed elevated levels of ST2, IL2RA, IL-6 and Eotaxin 3 within three days of peak ICANS.

3.2 Results

3.2.1 Immune composition of patients undergoing CD19 CAR T cell therapy is vastly heterogeneous compared to healthy donors

Thirty-three patients with relapsed diffuse large B cell lymphoma (DLBCL) receiving commercially available CD19-directed CAR T cell therapy were prospectively enrolled in a correlative study to collect longitudinal biospecimens for analysis of CRS and ICANS (**Supplemental Table 1**). We profile the immune composition of patients who developed adverse effects throughout the course of CD19 CAR T cell therapy, including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). The severity of CRS and ICANS development within CD19 CAR T cell patients was graded based on the Lee 2019 consensus criteria and the Common Terminology Criteria for Adverse Events (CTCAE) v4.03 criteria⁸⁵. In our cohort of 33 patients, 17 developed severe CRS, defined as grades 2-5, while 16 developed moderate to moderate to severe ICANS, also defined as grade 2-5 (**Figure 1A**). Within patients who developed ICANS, the median day for ICANS onset was 7 days post-CAR T cell infusion. For patients who developed CRS, the median onset day was 5 days post-CAR T cell infusion.

SMARTube-fixed whole blood samples and serum were collected at multiple timepoints: prior to CAR T cell infusion, at the onset of adverse events, and up to 20 days post-infusion throughout the course of therapy (**Figure 1B**). We developed a pipeline to quantify cellular abundances and serum concentrations across all sample collection timepoints. SMARTube-fixed whole blood samples contain lymphocytes, monocytes, neutrophils, and other immune populations that we profiled with mass cytometry to gain insight into cellular abundances, dynamics across CD19 CAR T cell therapy, and associations with CRS and ICANS (**Figure 1C**). Cytokine concentrations from serum were measured using ELISA and Meso Scale Discovery (MSD) assays to assess protein concentrations, fluctuations, and associations with CRS and ICANS across CD19 CAR T cell therapy (**Figure 1C**).

Regarding cellular populations, we designed a 40-marker antibody panel incorporating lineage, activation, memory, proliferation, phosphorylation, and migration markers to identify immune populations potentially involved in ICANS pathogenesis after CD19 CAR T cell therapy (**Supplemental Table 2**). Fixed whole blood samples at baseline and all post-treatment timepoints from the 33 patients and 4 healthy donors underwent red blood cell lysis, followed by staining of leukocytes with a high-dimensional mass cytometry panel to delineate immune cell subsets based on proteomic signatures. Using mass cytometry, we broadly profiled leukocyte populations, including CD4⁺, CD8⁺, and $\gamma\delta$ T cells, MAIT cells, B cells, classical monocytes, non-classical monocytes, inflammatory macrophages, anti-inflammatory macrophages, dendritic cells, basophils, natural killer cells, and neutrophils in both study participants and healthy donors (**Figure 1D, Supplemental Figure 1**). We observed striking differences in monocyte and neutrophil subpopulations between CD19 CAR T cell recipients and healthy controls (**Figure 1D**). Among mononuclear cells, we observed a difference in the abundance of CD4 and CD8 populations between our healthy donor and patient populations, likely due to the infusion of CAR T CD4 and CD8s absent in our healthy donor samples (**Figure 1E**). Furthermore, we observed significant heterogeneity within the mononuclear populations in patients compared to the healthy donors (**Figure 1F**). Neutrophil and eosinophil populations preserved intact using the SMARTube fixation buffer allowed us to separate out CD66b⁺ populations from our bulk mass cytometry dataset for a second stream of in-depth clustering analysis (**Supplemental Figure 1**). CD66b⁺ neutrophil and eosinophil clusters within healthy donors showed major compositional

differences compared to patients from our clinical trial (**Figure 1G**). A narrow population of neutrophils dominated healthy donor profiles, while at baseline pre-lymphodepletion collection timepoints, substantial population heterogeneity was observed within patient neutrophil samples (**Figure 1H**). Although the underlying reasons for these differences remain unclear, it has been suggested that the combination of prior chemotherapy regimens and G-CSF administered to CAR T cell recipients may contribute to distinct neutrophil phenotypes released from the bone marrow into the bloodstream⁸⁶.

3.2.2 Distinct monocyte populations demonstrate varying correlations with peak ICANS development

To comprehensively characterize the diverse mononuclear populations within our patient cohort, we delineated mononuclear subsets based on the differential expression of surface markers identified through our mass cytometry panel (**Supplemental Table 2**). We gated on CD66b⁻ cells and clustered cells into 24 phenotypically distinct immune cell subsets (**Figure 2A of CD4**, CD8, NK cells, B cells, monocytes, and dendritic cells (**Figure 2B**).

We applied generalized estimating equations (GEE) to model the longitudinal abundances of mononuclear clusters after CD19 CAR T cell infusion. GEE identified several mononuclear clusters associated with severe CRS and/or ICANS (**Figure 2C**).

Among the 24 mononuclear clusters identified, seven were associated (FDR < 0.05) with either any grade CRS and ICANS or the development of severe CRS and moderate to severe ICANS. These seven populations include dendritic cell, basophil, non-classical CD16⁺ monocyte, CD14⁺ monocyte, and CD8 subpopulations.

To focus on cellular differences at peak ICANS and peak CRS, we synchronized data using each patient's estimated day of peak ICANS (**Figure 2D**, **Supplemental Figure 2A,B**). Specifically, for patients with ICANS, we discretized time into four bins:

1. **Baseline pre-lymphodepletion**—cells derived from samples collected prior to lymphodepletion and CAR T cell infusion;
2. **Peak ICANS**—a reference period centered on the day of peak ICANS severity, encompassing three days preceding and following;

3. **Pre-peak ICANS**—from the day of CAR T infusion to three days preceding peak ICANS onset; and
4. **Post-peak ICANS**—timepoints beyond three days following peak ICANS onset.

For patients without ICANS, we used day 7 post-infusion in lieu of Peak ICANS date; hence, the four time bins are baseline pre-lymphodepletion, 0 to 3 days post infusion, 4-10 days after infusion, and more than 10 days after infusion. When patients had multiple samples in a given time bin, we averaged respective cluster proportions. We observed that a CD163⁺ monocyte population (Monocyte 19) exhibits an upward trend throughout the course of CD19 CAR T cell therapy and remains pronounced in moderate to severe ICANS patients post-peak ICANS development relative to patients who do not experience severe ICANS (**Figure 2E,F**). This CD163⁺ monocyte population (Monocyte 19) is the cellular population most abundant in patients who develop moderate to severe ICANS compared to patients who do not develop severe ICANS within the comparable time window (**Figure 2G**). Within patients that develop moderate to severe ICANS we observe a statistically significant elevation in Monocyte cluster 19 proportion within the blood mononuclear composition from the baseline PreLD levels to Peak ICANS onset levels (**Figure 2H**). Manual gating onto CD163⁺ populations within our CD14⁺ monocytes verifies that CD163⁺ monocytes are elevated in patients who develop moderate to severe ICANS at the time of peak ICANS onset (**Figure 2I**). Not only is there a statistically significant difference in CD163⁺ in patients who develop moderate to severe ICANS at the time of peak ICANS relative to patients who do not develop severe ICANS, we also observe an upward trend of CD163⁺ expressing monocytes over the course of CAR T cell therapy until peak ICANS onset in moderate to severe ICANS patients (**Figure 2J**). CD163⁺ monocytes are conventionally associated with anti-inflammatory responses and induced by IL-6 and IL-10 in inflammatory conditions⁸⁷. More recent studies have identified CD163⁺ cells as perivascular macrophages that contribute to vasculature permeability during peripheral inflammation¹⁵. CD163⁺ perivascular macrophages are known to limit the passage of molecules >10kDA through the blood-brain barrier and recruit neutrophils⁸⁸. Furthermore, we also observe a rise in CD163⁺ monocytes in patients who develop severe CRS within the timeframe of peak CRS relative to other mononuclear populations (**Supplemental Figure 3 A,B**). Combined with our findings, this represents a promising step towards identifying CD163⁺ as a biomarker of adverse event onset in CD19 CAR T cell therapy.

In addition to the CD163⁺ monocyte population associated with moderate to severe ICANS, we observed a consistent negative correlation between moderate to severe ICANS and the abundance of monocyte and monocyte-derived dendritic cells during the peak and post-peak ICANS timeframes (Monocyte-15 and DC2-23) (**Figure 2D**). Further comprehensive blood count analysis depicts a global reduction in lymphocyte, mononuclear and neutrophil populations within the lymphodepletion and CAR T cell infusion timeframe (**Supplemental Figure 4A**). Specifically, overall monocyte counts among patients who develop moderate to severe ICANS remain diminished compared to patients who do not develop severe ICANS at the time of peak ICANS onset. (**Supplemental Figure 4B,C**).

3.2.3 Immature Neutrophil are positively correlated with the peak development of ICANS

Similarly, for neutrophils, we profiled CD66b⁺ cells to identify any subsets associated with the development of ICANS. Our clustering analysis resolved 17 distinct CD66b⁺ populations, including one eosinophil population and 16 neutrophil subsets (**Figure 3A,B**). Neutrophil subpopulations exhibit continuous, gradient-like marker expression in contrast to discrete lineage-defined expression observed in mononuclear populations (**Supplemental Figure 5A,B**). Again, we used generalized estimating equations to test for clusters associated with CRS and/or ICANS severity over all post-infusion timepoints, centered at peak ICANS. We tested CRS and ICANS severity grades, as well as dichotomized (grade ≥ 1 or not) CRS and ICANS severity. Of the 17 CD66b⁺ populations, three neutrophil clusters (clusters 2, 7, and 10) were significantly associated with moderate to moderate to severe ICANS (grades 2–5 vs. 0–1) (FDR < 0.05) (**Figure 3C**).

Neutrophil clusters 2, 7, and 10 have an overall association with moderate to severe ICANS occurrence. Notably, Neutrophil cluster 2 exhibited a positive association with moderate to severe ICANS at the time of peak onset, whereas Neutrophil clusters 7 and 10 demonstrated negative associations. (**Figure 3D**). Looking at each discrete time window, we observed the most pronounced difference in Neutrophil cluster 2 proportions at peak ICANS, with markedly higher levels in patients who developed severe ICANS compared with those who did not (**Figure 3E,F**). Although Neutrophil cluster 2 was the only neutrophil cluster to show a statistically

significant correlation with moderate to severe ICANS at peak ICANS, two additional clusters—Neutrophils 6 and 9—exhibited borderline-significant positive correlations with severe ICANS onset during this same period. **(Figure 3G)**. Interestingly, all three neutrophil clusters exhibit reduced CD10 and CD101 expression relative to other resolved neutrophil clusters. CD10 and CD101 are maturity status markers and in other inflammatory contexts, such as sepsis⁸⁹. Among patients that develop severe ICANS, we observed a statistically significant increase in the proportion of Neutrophil cluster 2 within the blood mononuclear compartment from the baseline (PreLD) to Peak ICANS onset **(Figure 3H)**. Alongside our clustering analysis, we quantified CD10⁺ mean fluorescence intensity (MFI) in bulk neutrophils across time bins. At peak ICANS, there is a statistically significant increase in immature neutrophils in patients who developed severe ICANS at peak onset compared with those who did not. Additionally, CD10⁺ and CD101⁺ neutrophil expression exhibited a progressive decline over the course of CAR T-cell therapy, reaching its lowest levels at peak ICANS onset. **(Figure 3I)**.

Furthermore, when examining the contribution of neutrophil clusters to the development of severe CRS, we observed a positive association between immature neutrophil clusters and severe CRS onset during the peak CRS toxicity window, although this association did not remain statistically significant after correction for multiple hypothesis testing. **(Supplemental Figure 8A)**. Among the three CD10⁺CD101⁺ neutrophil clusters associated with severe CRS, Neutrophil cluster 11 showed the largest difference in median proportions, comparing patients between patients who did and did not develop severe CRS during the peak CRS time window. **(Supplemental Figure 8A)**. Neutrophil cluster 11 is characterized by elevated HLA-DR expression and low CD10, distinguishing it from the other neutrophil clusters. **(Figure 3B)**. Neutrophil cluster 8 is the only other neutrophil cluster with elevated HLA-DR expression, but unlike Neutrophil cluster 11, this cluster has high CD14. We observed an increase in Neutrophil cluster 11 abundance around peak CRS onset in patients who developed severe CRS compared with those who did not, although this trend was not statistically significant **(Supplemental Figure 8B)**. Overall, Neutrophil cluster 11 abundance increased over the course of CAR T-cell therapy, rising from baseline Pre-LD levels to peak ICANS timeframe in patients who developed severe CRS **(Supplemental Figure 8C)**. To complement these clustering-based findings, we manually gated HLA-DR⁺CD14⁺ cells within the CD66b⁺ neutrophil compartment

(Supplemental Figure 8D). We found that HLA-DR⁺CD14⁻ neutrophils exhibited a gradual increase in proportion from Pre-LD baseline through peak adverse event onset, followed by a return toward baseline levels after peak CRS **(Supplemental Figure 8E).**

3.2.4 Patients who develop moderate to severe ICANS have distinct cytokine signatures at peak ICANS

In parallel with cellular profiling, we characterized serum proteomic signatures associated with moderate to severe ICANS development. To this end, we devised ELISA and multiplex MSD panels to measure a broad range of inflammatory cytokines pertinent to CD19 CAR T cell therapy, including GM-CSF and IL-6, members of the IL-1 cytokine family such as IL-18, interferon-gamma (IFN- γ), and multiple chemokines of interest **(Supplemental Table 3)**. From our cohort of 33 patients, we were able to obtain and quantified serum cytokine levels in 19 patients. Using generalized estimating equations, we identified proteins significantly associated with any grades of CRS and ICANS as well as severe CRS and moderate to severe ICANS.

(Figure 4A, Supplemental Figure 9A). We stratified patients by ICANS severity to longitudinally assess cytokines exhibiting positive or negative correlations at peak ICANS and peak CRS **(Figure 4B)**, again aligning patient timepoints relative to estimated peak ICANS/CRS dates or days 5 and 7 for patients without CRS or neurotoxicity, respectively. **(Supplemental Figure 9B).** At peak ICANS, serum cytokine concentrations were quantified from 7 patients who did not develop severe ICANS and 12 patients who developed moderate to severe ICANS. As expected, cytokine levels increased progressively following CAR T-cell infusion relative to pre-lymphodepletion time points. Among patients who developed moderate to severe ICANS, the most pronounced changes in cytokine concentrations from lymphodepletion to peak ICANS included increased IL-22, GM-CSF, IL-6, IL-5, IFN- γ , SAA, and CRP expression **(Figure 4C)**. Focusing specifically on the peak ICANS timeframe (n=15), inflammatory cytokines such as IL-6, Eotaxin-3, ST2, and IL2RA were significantly elevated in patients with moderate to severe ICANS **(Figure 4D)**. Notably, ST2 (log OR = 1.98, 95% CI .44 – 4.71, $p = .006$) and IL2RA (log OR = 1.94, 95% CI .48 – 4.42, $p = .004$) emerged as the cytokines most strongly associated with moderate to severe ICANS at peak neurotoxicity **(Figure 4E)**. Discrete-time analyses further demonstrated that IL2RA and ST2 levels increased from pre-lymphodepletion baseline to

peak ICANS in patients who developed severe ICANS and were significantly higher at the peak ICANS compared to patients who did not develop severe ICANS (**Figure 4F**).

Consistent with shared inflammatory features across toxicities, several cytokines enriched during peak ICANS were also elevated at non-statistically significant levels in patients who developed severe CRS at peak CRS, including Eotaxin-3 and GM-CSF (**Figure 4B; Supplemental Figure 10A,B**).

Immune modules elucidate biomarkers predictive of moderate to severe ICANS development in CD19 CAR T cell therapy

For a comprehensive multivariate perspective on the cellular and molecular dynamics driving severe ICANS development in CD19 CAR T cell therapy, we combined our clustering analysis with our cytokine analysis. We examined correlations between CD66b⁺ neutrophil clusters, CD66b⁻ mononuclear clusters, and circulating cytokines at peak ICANS (**Figure 5A**). We observe that the two cellular populations highlighted above, Monocyte 19 and Neutrophil 2, clustered together hierarchically, as patients with higher proportion of Monocyte 19 also tended to have higher Neutrophil 2 proportion ($\rho = .66, p = .006$) (**Figure 5B**).

To identify inflammatory mediators associated with CD163⁺ monocytes and immature neutrophils at peak ICANS, we performed Spearman correlation analyses of Mononuclear 19 and Neutrophil 2 cluster frequency and serum cytokine concentrations (**Supplemental Figure 11A,B**). Mononuclear 19 is most positively associated with IL-2RA ($\rho = 0.749, p = 0.001$) and ST2 ($\rho = 0.679, p = 0.005$). Similarly, Neutrophil 2 is also most positively correlated with peripheral ST2 ($\rho = 0.569, p = 0.026$) and IL-2RA ($\rho = 0.453, p = 0.089$), and is inversely associated with peripheral IL-12p70 ($\rho = -0.593, p = 0.019$). (**Figure 5C,D**). The three immature neutrophil populations – Neutrophil clusters 2,6, and 9 displayed distinct cytokine correlation profiles, underscoring functional heterogeneity within this compartment (**Figure 5A**).

Additionally, the two activated (HLA-DR⁺) neutrophil clusters (Neutrophil 11 and Neutrophil 8) correlate with different serum cytokines, suggesting differential inflammatory signaling. Cytokines more commonly associated with adverse effects arising from CAR T cell therapy including GM-CSF, IL-6, and IFN- γ , showed a stronger correlation with the proportion of Neutrophil 11 in addition to IL-22⁹⁰ (**Supplemental Figure 12A,B**).

To interpret these coordinated cellular and cytokine dynamics, we applied the immune modules computational framework to integrate our multi-omic data and assess systems-level interactions during peak ICANS⁹¹. This approach clusters correlated serum cytokines and cellular phenotypes at each time point into immune modules, summarizes their variance using PCA, and uses the summarized immune modules as inputs into a random forest classifier predicting patient ICANS status.

From the peak ICANS time bin, we resolved 40 immune modules across all features within our 15 patients and selected the most predictive immune modules for downstream analysis (**Figure 5E**). The number of immune modules resolved was based on maximizing the silhouette score (**Figure 5F**). From ranked predictive modules, IL2RA and ST2 were components of the top two immune modules most strongly associated with moderate to severe ICANS during the critical ICANS window (**Figure 5G**). With an anti-inflammatory signature, IL2RA and IL10 together formed the top module. Consistent with our earlier analysis, we observed that Monocyte 19 and Neutrophil 2 clusters were grouped together due to their high correlation. Conversely, we identified a module comprising non-classical CD16⁺ monocytes (nc_Monocyte_21) and DC2s (DC2_23) that exhibited an inverse association with severe ICANS. Taken together, these findings suggest a dynamic and interconnected cellular and proteomic network that shapes clinical outcomes following CD19 CAR T cell therapy.

3.3 Discussion

In this study, we identified cellular and serum biomarkers associated with severe neurotoxicity within a three-day window surrounding diagnosis of peak adverse events. Employing a multimodal approach, we longitudinally profiled immune cell dynamics and serum protein changes throughout CD19 CAR T cell therapy to uncover associations with moderate to severe ICANS onset. To focus temporal resolution and identify significant trends across time, we synchronized sample collection timepoints in relation to each patient's peak ICANS (or CRS) diagnosis.

A key strength of our study is the use of SMARTube Proteomic Stabilizer to keep typically fragile neutrophils intact for cellular profiling. Our novel approach achieved high-resolution neutrophil profiling and revealing dynamic changes in CD10⁻ and HLA-DR⁺ neutrophil subsets that coincided with CAR T-cell expansion and peaks in inflammatory cytokines.

In other inflammatory contexts, CD10 has been characterized as a maturity marker that is downregulated in patients who develop sepsis⁶⁶. Consistent with these observations, we found that at peak disease severity, CD10 expression was reduced in neutrophils from patients who developed severe ICANS. This provides novel insight into a potential effector role for immature neutrophils in ICANS pathogenesis.

HLA-DR⁺ neutrophil clusters were also enriched during peak CRS episodes in our cohort. Together, these findings suggest shared neutrophil-associated mechanisms across inflammatory disease contexts that may contribute to CAR T-cell therapy-associated toxicities. Interestingly, elevated frequencies of HLA-DR⁺ neutrophil clusters have been reported in the blood of pediatric patients with COVID-19 within 72 hours of hospital admission⁹².

Correlation analyses indicated a significant association between peak HLA-DR⁺ neutrophil abundance and elevated levels of IL-22, IL-6, GM-CSF and other inflammatory cytokines at peak neurotoxicity (**Supplemental Figure 12 A,B**). Consistent with recent findings, GM-CSF correlated with HLA-DR⁺ neutrophil abundance⁹³. Taken together, our results point to activated neutrophil as potential mediators of toxicity associated with CD19 CAR T cell therapy.

Along with the expansion of neutrophil subpopulations, we observed a significant enrichment of CD163⁺ monocytes at peak ICANS among patients who developed severe ICANS. Among the cytokines quantified at peak ICANS onset, ST2 and IL2RA were most correlated with CD163⁺ monocyte abundance. Notably, soluble ST2 and CD163 are established biomarkers of hemophagocytic lymphohistiocytosis (HLH), another adverse effect of CAR T cell infusion⁹⁴. Although we do not identify a definitive cellular source of ST2 and IL2RA, we aim to resolve their cellular origins using single-cell transcriptomic analysis. Elevated serum concentrations of soluble CD163 and IL2RA are also found in patients with HLH and SARS-CoV-2⁹⁵. Given that HLH and SARS-CoV-2 represent inflammatory conditions characterized by an over-activated and uncontrolled immune response that leads to headaches, fever, and multiple organ failure in severe cases, it is plausible that ICANS may share correlates and potential drivers of adverse effects^{96,97}. Previous studies have shown that elevated levels of soluble CD163, present in the blood of patients following traumatic injury, originate from peripheral monocytes and play a role in cross-communication among lymphocytes; ultimately suppressing the adaptive immune response⁹⁸. Our findings suggest that CD163⁺ monocytes may contribute to an anti-inflammatory

response following the initial onset of ICANS. We further hypothesize that these monocytes may have transitioned towards an anti-inflammatory phenotype in response to the elevated proportion of activated CD4 CAR T cells observed in severe ICANS patients. Previous studies have demonstrated that CD4 CAR T cells secrete IL-4 and IL-10 following B cell binding and activation, driving an anti-inflammatory monocyte polarization⁹⁹.

We also observed a reduced proportion of peripheral non-classical CD16⁺ monocytes in patients who developed severe ICANS, particularly after the peak ICANS. This observation is consistent with a recent study indicating an inverse correlation between peripheral blood CD16⁺ monocytes and COVID-19 severity¹⁰⁰. Specifically, pro-inflammatory nonclassical monocytes migrate from the peripheral blood during the onset of acute inflammatory response¹⁰¹. For patients who experience severe COVID, these nonclassical monocytes are enriched in the distal lung tissues, leading to severe acute respiratory ailments¹⁰². As such, low peripheral CD16⁺ monocyte population within our patients who develop moderate to severe ICANS may reflect a migration of CD16⁺ cells into the central nervous system and endothelium. Other studies have demonstrated the capacity of CD16⁺ monocytes to transverse the brain microvascular endothelial layer and enter the central nervous system under inflammatory conditions¹⁰³. A blood count analysis of our cohort revealed a general reduction in circulating monocyte counts among moderate to severe ICANS patients compared to those without severe ICANS. These findings suggest impaired or delayed monocyte recovery in severe ICANS cases following lymphodepletion.

Limitations of this study include relatively small sample sizes for several comparisons made, particularly for combined blood and serum samples at the peak ICANS timeframe (n=15). Also, several cytokines that failed to meet a 20% detection cutoff with our MSD assay were excluded from analysis. Of course, whether the emergence of CD163⁺ monocytes and immature neutrophils causally contributes towards severe ICANS or if these populations emerge because of severe ICANS remains an open question.

Additional mass cytometry-based cellular analysis on additional, larger cohorts of patients undergoing CAR T cell therapy is necessary to validate our findings. Mechanistic assays, including *in vitro* co-culture systems and blood-brain barrier models *in vivo*, are also needed to investigate the precise roles of neutrophils and monocytes in neurotoxicity. Lastly, probe-based

single-cell transcriptomic assays may reveal additional neutrophils and myeloid biomarkers myeloid cells that drive ICANS pathogenesis.

Our study has demonstrated the potential for longitudinal immune profiling in future CAR T clinical trials, which may refine therapeutic windows and guide biomarker-guided intervention strategies. Clinically, our findings could lead to the development of preventative treatments administered with CD19 CAR T cell therapy to reduce or altogether avoid neurotoxicity. Specifically, interventions focused on suppressing immature neutrophils populations may mitigate ICANS severity.

3.4 Figures

Figure 1

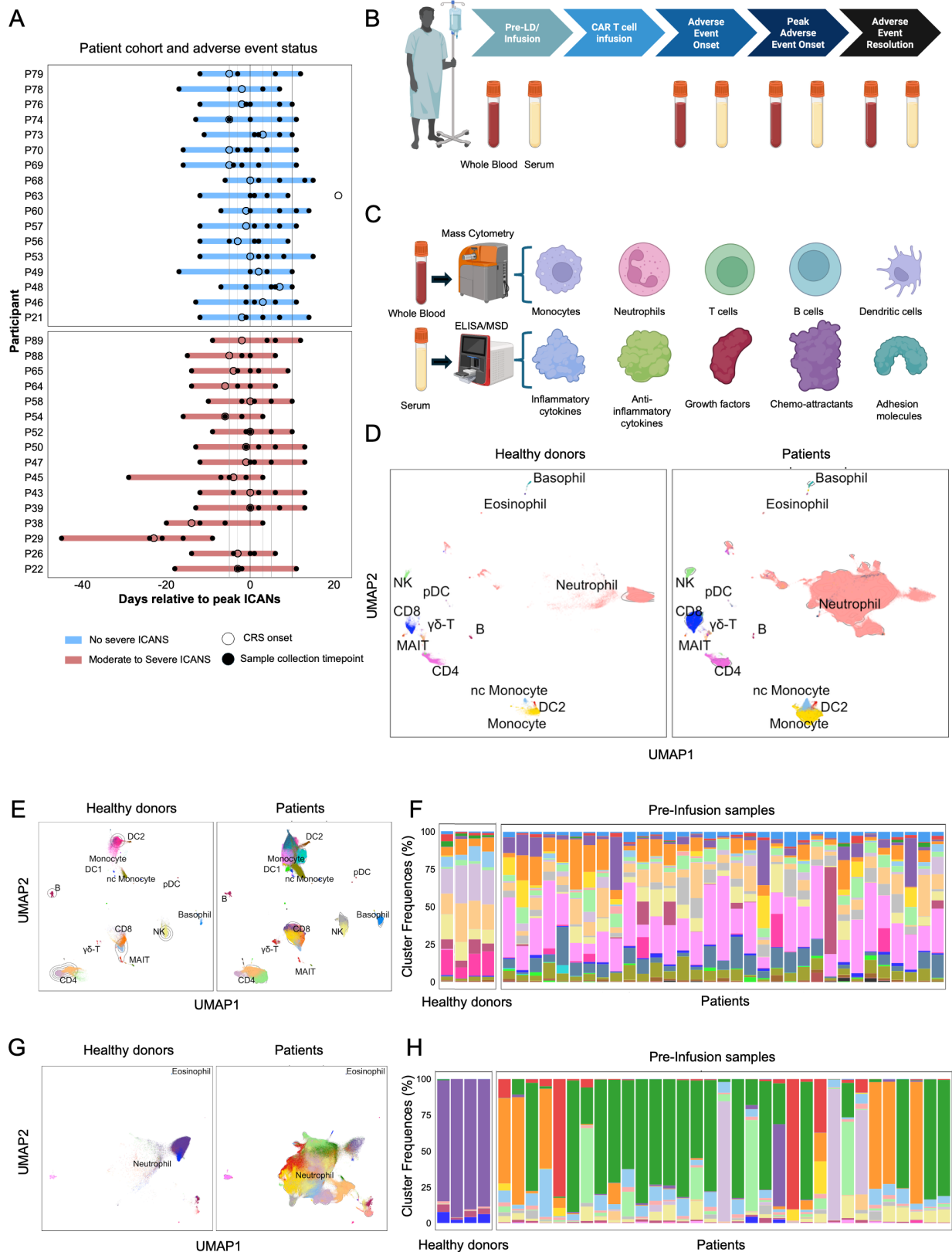


Figure 1. Sample collection and experimental outline

- A. Swimmer plot depicting ICANS severity status of each patient alongside sample collection timepoints relative to day of peak ICANS onset. For patients that do not develop ICANS, the median day of peak ICANS onset- determined to be day 7 post CAR T cell infusion was used (n=33).
- B. Diagram of sample collection (blood and serum) from patients undergoing CD19 CAR T cell therapy at select timepoints pre-lymphodepletion and post CAR T cell infusion
- C. Schematic of experimental outline to utilize mass cytometry to profile fixed cells from blood to identify innate and adaptive immune populations
- D. UMAP embedding depicting broad immune populations profiled from our healthy donor controls and patient samples
- E. UMAP embedding of CD66b⁻ clusters resolved between Healthy Donor control samples and patient samples
- F. Abundance plot highlighting CD66b⁻ cluster abundances between Healthy Donor control samples and patient samples
- G. UMAP embedding of CD66b⁺ clusters resolved between Healthy Donor control samples and patient samples
- H. Abundance plot highlighting CD66b⁺ cluster abundances between Healthy Donor control samples and patient samples

Figure 2.

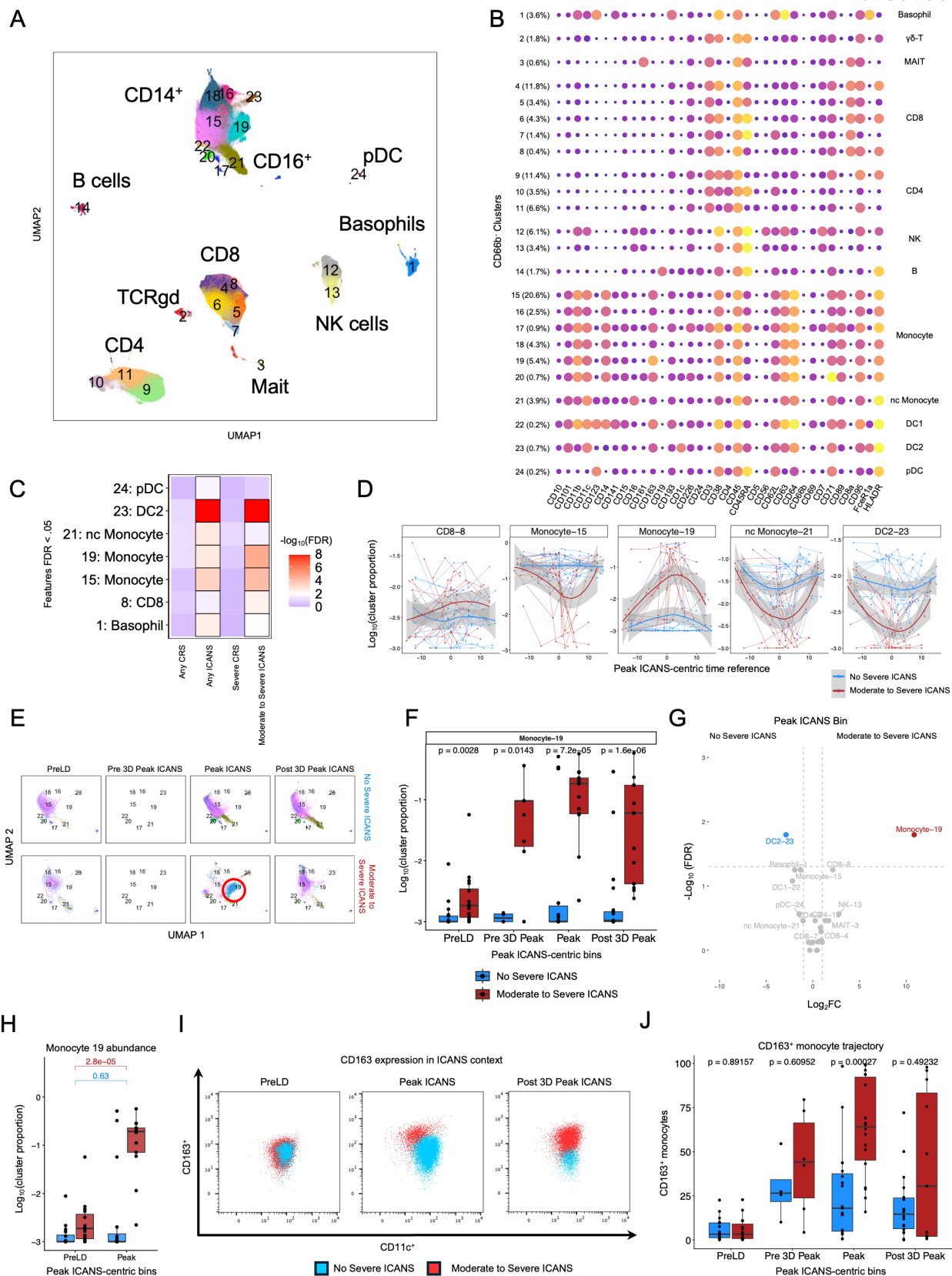


Figure 2. Longitudinal mononuclear profiling reveals CD163⁺ monocyte association with severe ICANS

- A. UMAP embedding and resolution of CD66b⁺ mononuclear clusters across all patients and healthy donor samples
- B. Bubble plot of surface marker expression profiles used for CD66b⁺ mononuclear cluster characterization
- C. Mononuclear clusters identified with generalized estimating equations to have an association with any gradation of CRS/ICANS or severe CRS/ICANS at peak ICANS onset. Outlined parameters are statistically significant correlation (FDR < .05)
- D. Mononuclear cluster abundances as a proportion of CD66b⁺ clusters relative to peak ICANS onset (set as Day 0, for patients that had grade 0, median peak ICANS onset day 7 was used). Lines connect samples collected from the same patient at different timepoints.
- E. Mononuclear clusters profiled within ICANS centric bins in a non-severe ICANS patient (top) and moderate to severe ICANS patient (bottom)
- F. Temporal shift in Mononuclear Cluster 19 in patients that develop moderate to severe ICANS vs patients that do not develop severe ICANS within ICANS-centric bins.
- G. Differentially abundant clusters between non-severe ICANS and moderate to severe ICANS patients based on proportion of CD66b⁺ clusters at peak ICANS onset. Colored clusters are noteworthy (FDR < 0.05) as determined by Wilcoxon signed rank test.
- H. Mononuclear Cluster 19 abundance shift from baseline PreLD levels to peak ICANS onset
- I. CD163⁺ CD11c⁺ populations within CD14⁺ cells in non-severe ICANS and moderate to severe ICANS patients
- J. CD163⁺ monocyte proportion in non-severe ICANS and moderate to severe ICANS patients over ICANS centric bins as quantified by manual gating

Figure 3.

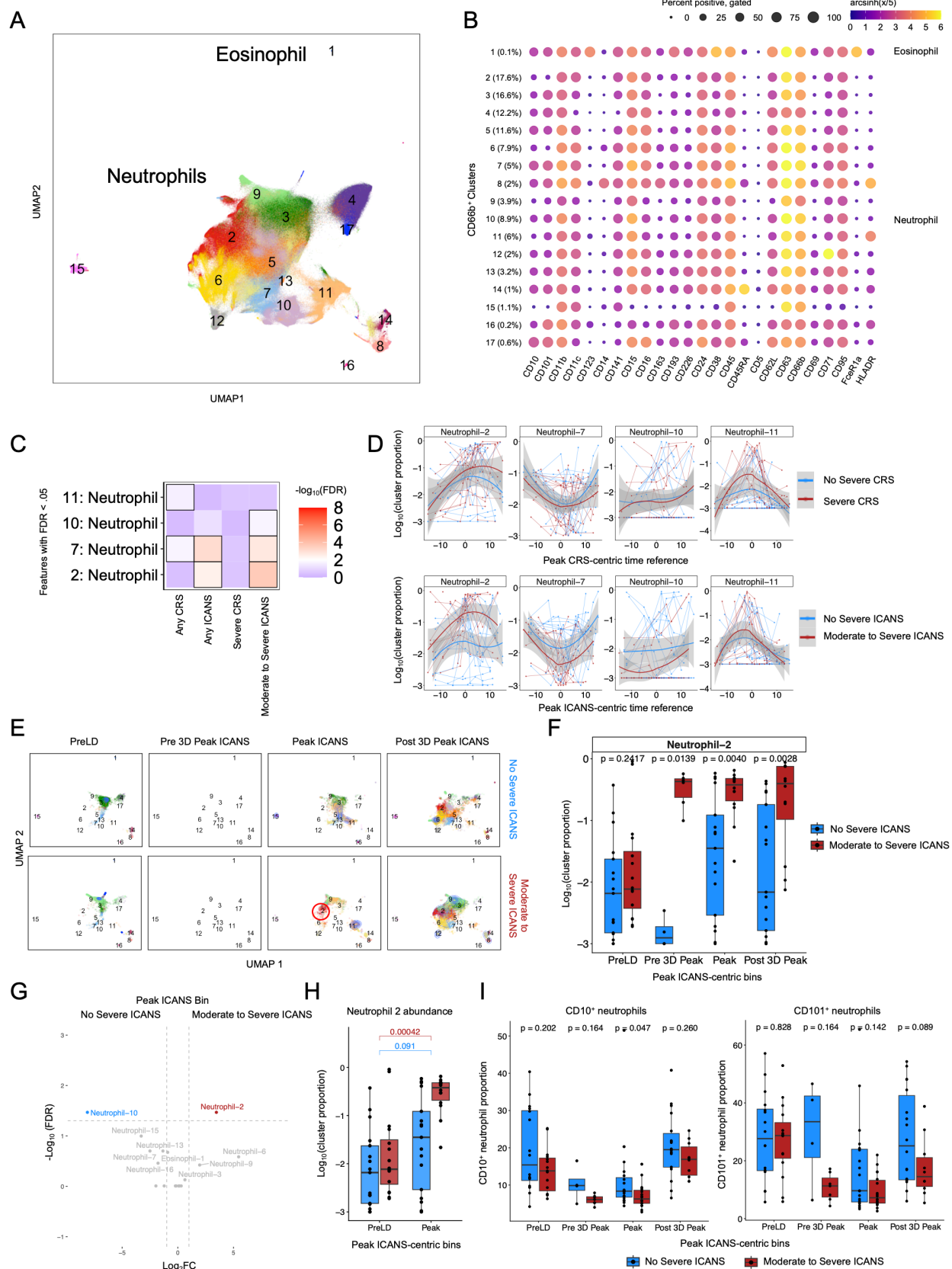
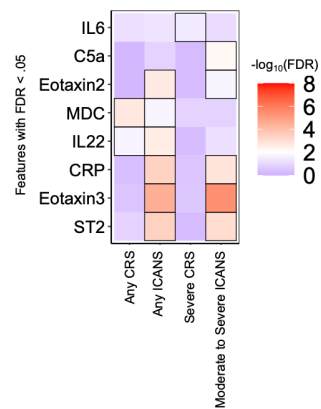


Figure 3. Longitudinal neutrophil profiling reveals CD10⁺ CD101⁺ neutrophil association with severe ICANS

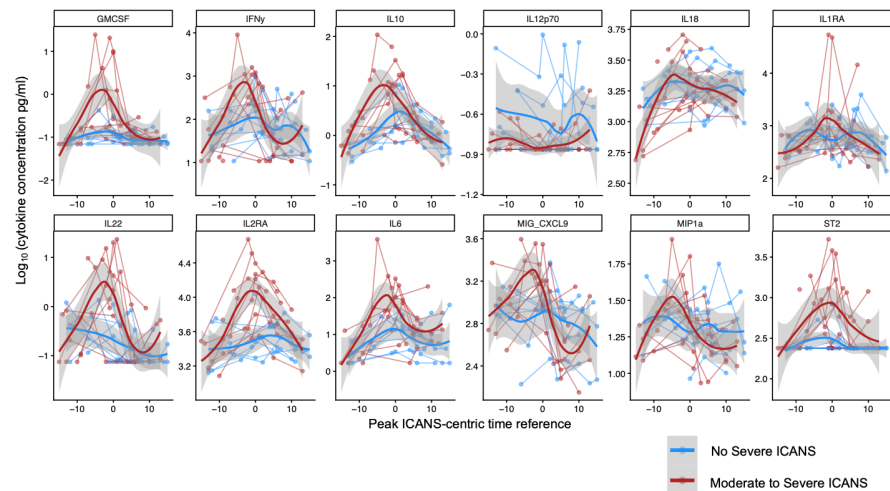
- A. UMAP embedding and resolution of CD66b⁺ neutrophil clusters across all patients and healthy donor samples
- B. Bubble plot of surface marker expression profiles used for CD66b⁺ neutrophil cluster characterization
- C. Neutrophil clusters identified with generalized estimating equations to have an association with any gradation of CRS/ICANS or severe CRS/ICANS at peak ICANS onset. Outlined parameters are statistically significant correlation (FDR<.05)
- D. Scatter plots of select neutrophil cluster abundances as a proportion of CD66b⁺ clusters relative to peak CRS and ICANS onset. Lines connect samples collected from the same patient at different timepoints. Patients whom developed severe CRS or moderate to severe ICANS were color-coded as red compared to patients that did not develop severe CRS or ICANS (blue). For patients that had grade 0 CRS, median peak CRS onset day 5 was used, for patients that had grade 0 ICANS, median peak ICANS onset day 7 was used.
- E. UMAP embedding of neutrophil clusters profiled within ICANS centric bins in a patient that did not develop severe ICANS (top) and a patient that developed moderate to severe ICANS (bottom)
- F. Box and whisper plot depicting Neutrophil 2 abundances in non-severe ICANS or severe ICANS patients at peak ICANS centric timebins
- G. Differentially abundant clusters between non-severe ICANS and severe ICANS patients based on proportion of CD66b⁺ clusters at peak ICANS onset. Colored clusters are noteworthy ($p < 0.1$) as determined by Wilcoxon signed rank test.
- H. Neutrophil Cluster 2 abundance shift from baseline PreLD to peak ICANS onset in non-severe ICANS and moderate to severe ICANS patients
- I. CD10⁺ and CD101⁺ populations within CD66b⁺ cells in non-severe ICANS and moderate to severe ICANS patients quantified by mean fluorescent marker intensity.

Figure 4.

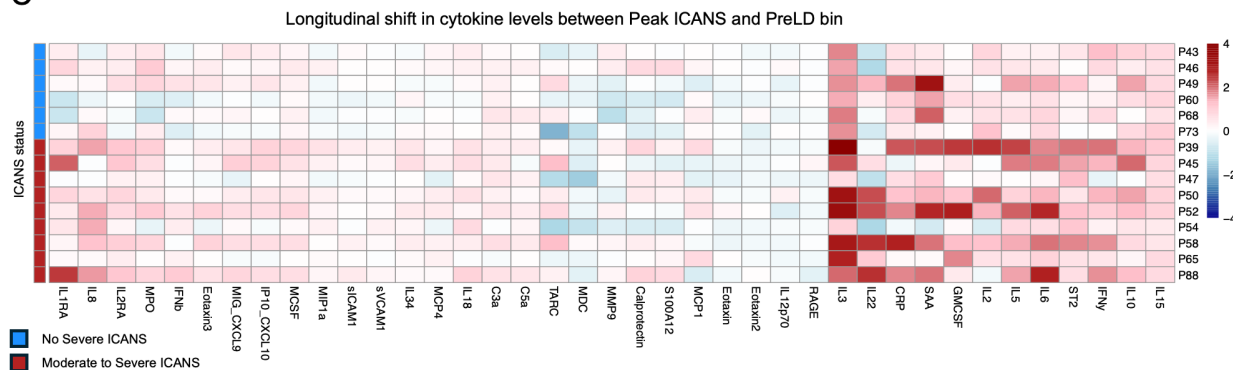
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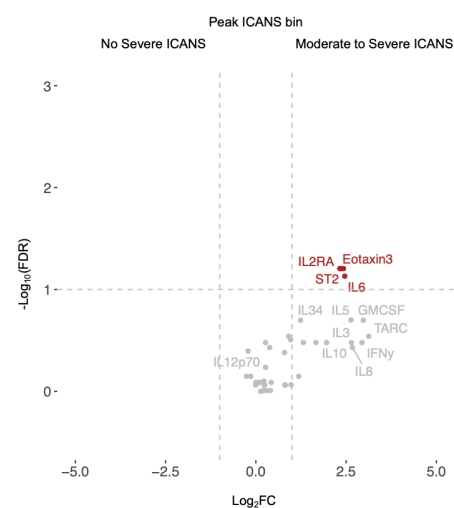
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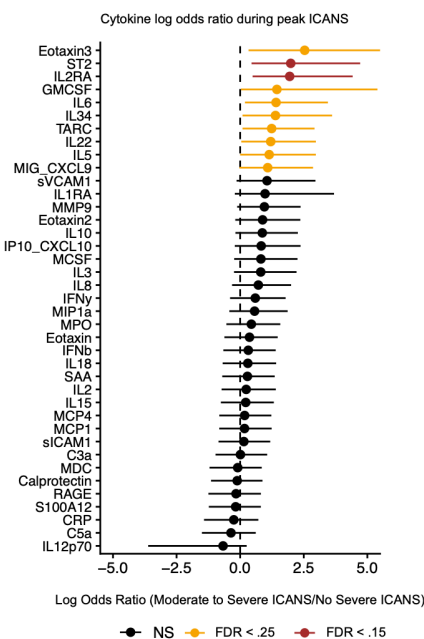
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D



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F

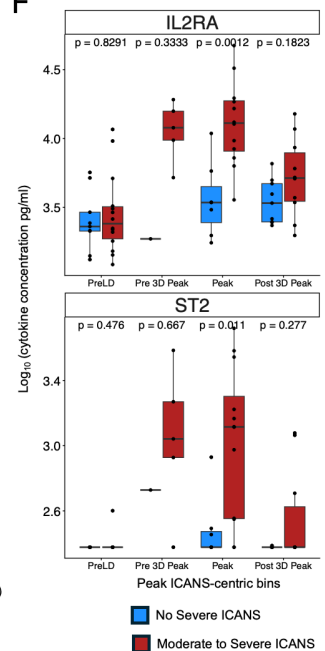
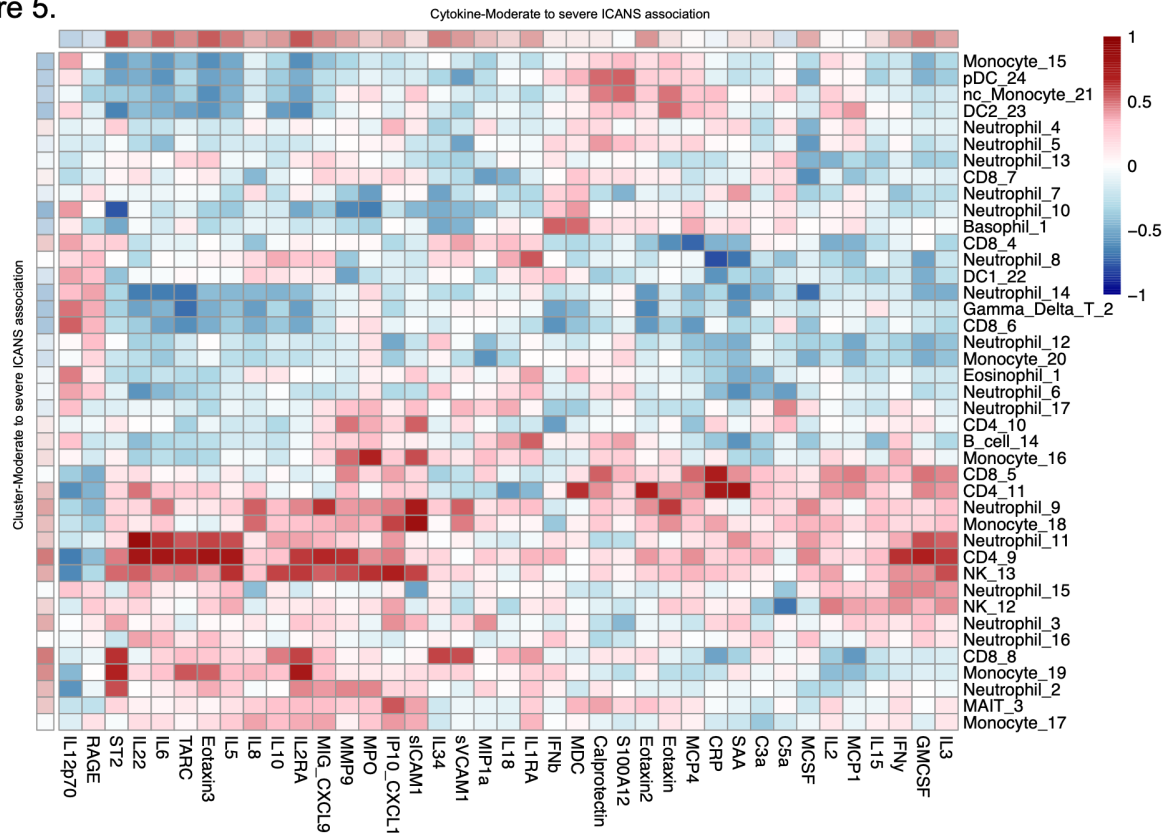


Figure 4. Longitudinal cytokine profiling highlights peripheral ST2 and IL2RA as correlates of severe ICANS

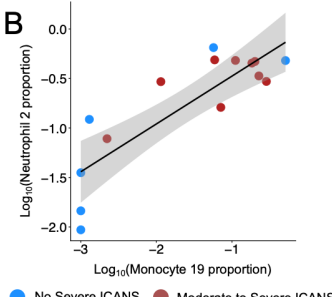
- A. Cytokines identified as having a correlation with CRS/ICANS gradation or severe CRS/ICANS at peak ICANS onset. Outlined parameters are statistically significant correlation (FDR <.05)
- B. Cytokine concentrations relative to peak ICANS onset (set as Day 0, for patients that had grade 0, median peak ICANS onset day 7 was used). Lines connect samples collected from the same patient at different timepoints.
- C. Difference in peak cytokine levels from the peak ICANS onset and preLD timebins
- D. Differentially abundant cytokines between non-severe ICANS and moderate to severe ICANS patients at peak ICANS onset as determined by Wilcoxon signed rank test (FDR < .1).
- E. Association strength of cytokines with moderate to severe ICANS at peak ICANS onset as determined by Firth's penalized logistic regression.
- F. Shift in cytokine concentrations across binned timepoints throughout CD19 CAR T cell therapy in no severe ICANS and moderate to severe ICANS patients

Figure 5.

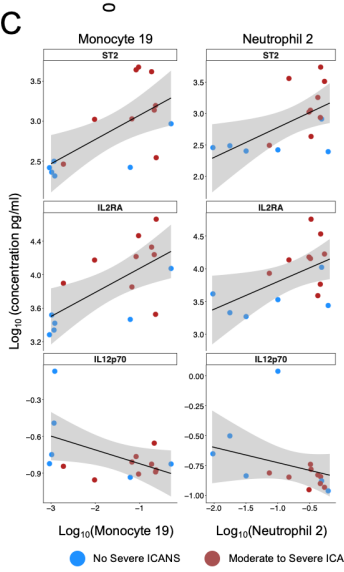
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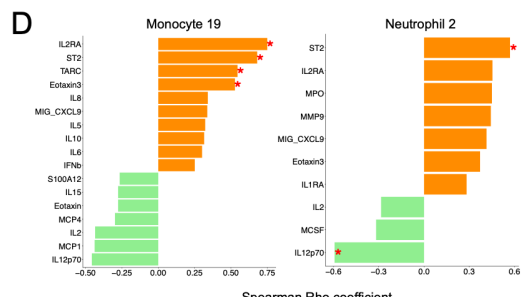
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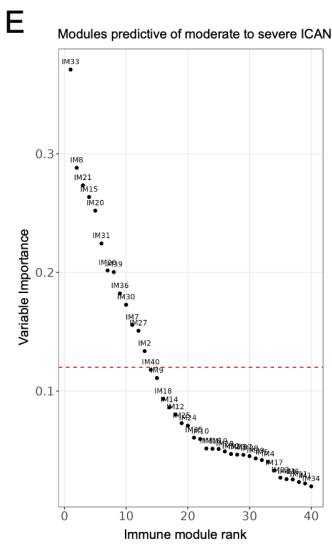
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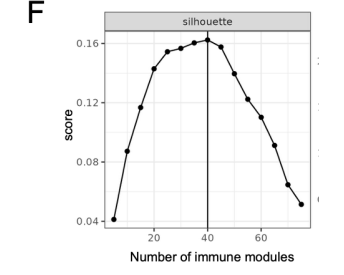
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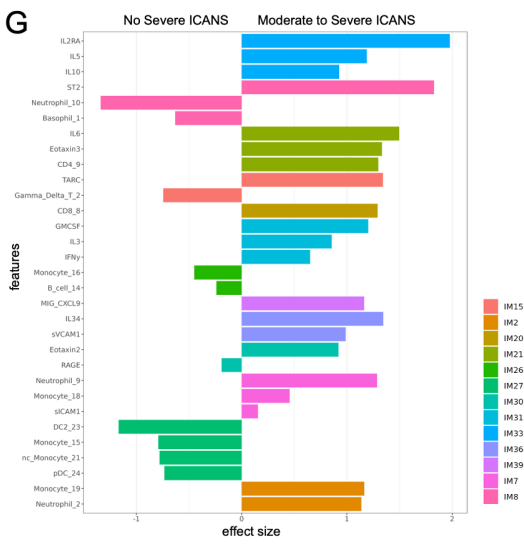


Figure 5. Elevated CD163⁺ monocyte and CD10⁺ CD101⁺ neutrophil subsets correlate with peripheral ST2 and IL2RA at peak ICANS onset

- A. Correlation between clusters, cytokines, and ICANS severity at peak ICANS onset as determined based on Spearman correlation
- B. Monocyte 19 and Neutrophil 2 proportions quantified within patients at peak ICANS onset.
- C. Cytokine correlations with Mononuclear 19 and Neutrophil 11 clusters at the peak ICANS as measured by Spearman correlation. $\rho < -.25$ and $\rho > .25$ depicted.
- D. Linear regression of cytokine concentrations measured against Monocyte 19 and Neutrophil 2 cluster abundance at peak ICANS onset.
- E. Scree plot highlighting individual immune model predictive power in moderate to severe ICANS development
- F. Analysis to determine the number of immune modules maximizing silhouette score
- G. Effect size of features comprising top immune modules predictive of moderate to severe ICANS

3.5 Supplemental Figures

Supplemental Table 1.

Grade	CRS	ICANS
0	3	9
1	12	8
2	15	6
3	2	8
4	1	2
5	0	0

Supplemental Table 2.

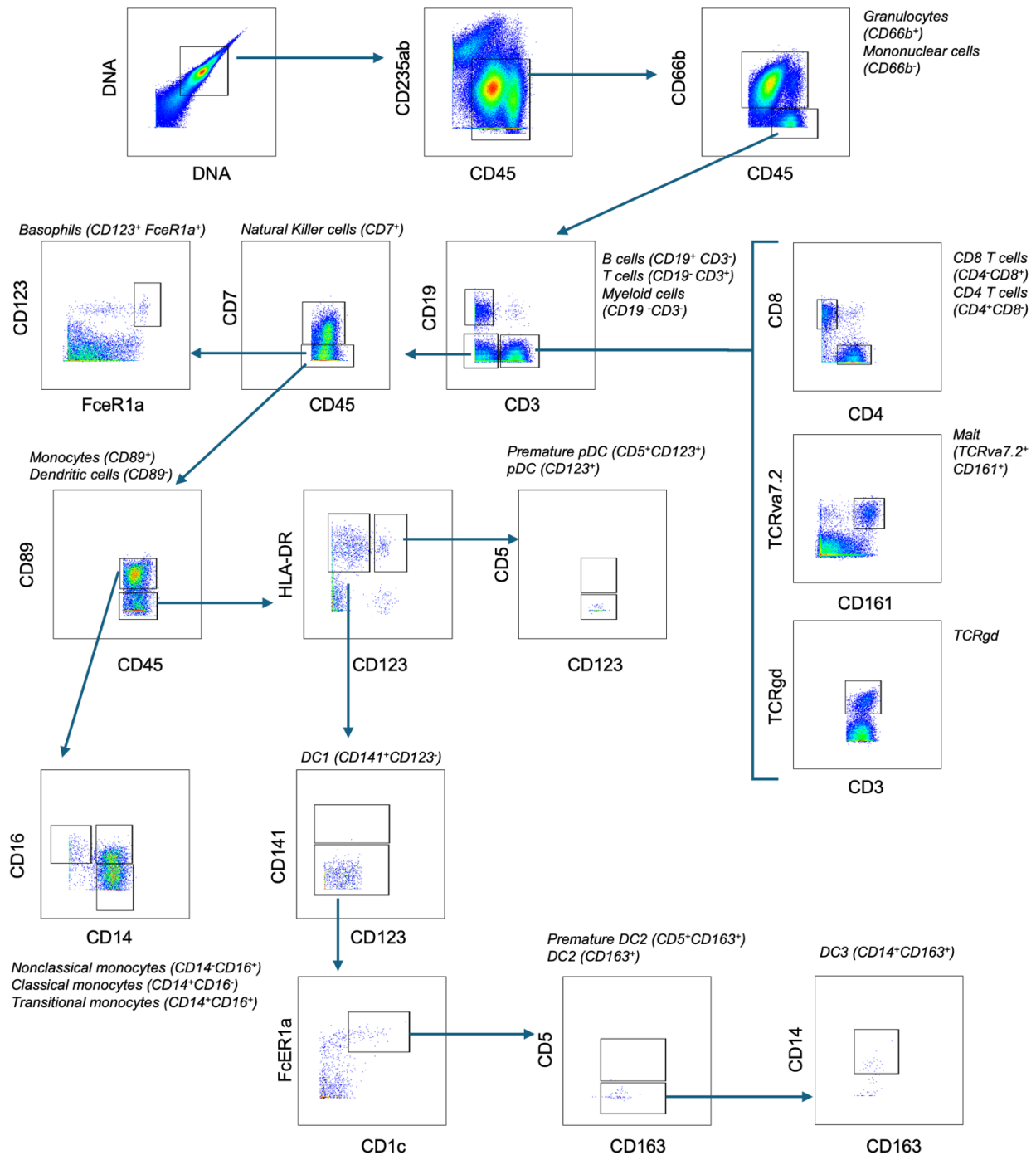
Target	Clone	Metal Isotope	Source	Part number
CD45	HI3O	89 Y	Standard Biotoools	3089003
CD4	RPA-T4	110 Cd	BioLegend	300502
CD8a	RPA-T8	111 Cd	BioLegend	301002
CD7	CD7-6B7	112 Cd	BioLegend	343102
CD63	H5C6	113 Cd	BD Pharmingen	556019
BDCA3/CD141	1A4	114 Cd	BD Pharmingen	230282
CD66b	G10F5	116 Cd	BioLegend	305102
CD10	HI10a	141 Pr	BioLegend	312202
CD101	BB27	142 Nd	ThermoFisher	14-1019-82
CD123	6H6	143 Nd	Standard Biotoools	3143014B
TCRgdAnti-FITC	5A6.E9/FIT-22	144 Nd	ThermoFisher/Standard Biotoools	MHGD01/3144006B
CD163	GHI/61	145 Nd	Standard Biotoools	3145010B
CD69	FN50	146 Nd	BioLegend	310902
CD16	3G8	148 Nd	Standard Biotoools	3148004B
CD24	ML5	149 Sm	BioLegend	311102
FcεR1α	AER-37	150 Sm	Standard Biotoools	3150027B
CD226	DNAM-1	151 Eu	BioLegend	338302
CD62L	DREG-56	152 Sm	BioLegend	304802
CD38	HIT2	154 Sm	BioLegend	303502
CD95	DX2	155 Gd	BioLegend	305602
TCRva7.2	3C10	156 Gd	BioLegend	351702
CD15	W6D3	157 Gd	BioLegend	323002
CD235AB	HIR2	160 Dy	BioLegend	306602
CD161	HP-3G10	161 Dy	BioLegend	339902
CD71	OKT-9	162 Dy	ThermoFisher	14-0719-82
CD1c	L161	163 Dy	BioLegend	331502
CD64	10.1	165 Ho	BioLegend	305002
CD19	HIB19	168 Er	BioLegend	302202
CD45RA	HI100	169 Tm	Standard Biotoools	3169008B
PDL1/CD274	29E.2A3	170 Er	BioLegend	329702
CD56	HCD56	171 Yb	BioLegend	318345
CD193	5E8	172 Yb	BioLegend	310702
HLA-DR	L243	173 Yb	Standard Biotoools	3173005B
CD89	A59	174 Yb	Standard Biotoools	3174012B
CD3	UCHT1	194 Pt	BioLegend	300402
CD5	UCHT2	195 Pt	BioLegend	300602
CD14	HCD14	198 Pt	BioLegend	325602
CD11b	ICRF44	209 Bi	Standard Biotoools	3209003B
CD11c	3.9	106 Cd	BioLegend	301602
pSTAT1	58D6	153 Eu	Standard Biotoools	3153003A
pSTAT3	4/P-STAT3	158 Gd	Standard Biotoools	3158005A
pSTAT5	47	147 Sm	Standard Biotoools	3147012A
pCREB	87G3	176 Lu	Standard Biotoools	3176005A
prpS6	N7-548	175 Lu	Standard Biotoools	3175009A
pMAPKAPK2	27B7	159 Tb	Standard Biotoools	3159010A
pNFkB	K10x	166 Er	Standard Biotoools	3166006A
pERK1/2	D1314.4E	167 Er	Standard Biotoools	3167005A
Ikb	L35A5	164 Dy	Standard Biotoools	3164004A

Supplemental Table 3. Meso-scale Discovery Panels

Assay	Vendor	CATALOG #	Format	Analyte	Sample dilution
A	MSD	K15067M-2	UPLEX Custom Biomarker	GM-CSF, IL-10, MIP1a, IL-2, IL-22, IL-12p70, IL-5, MCP4, IL-4, IL-13	1
B	MSD	K15067M-2	UPLEX Custom Biomarker	IFNy, IL-18, IL-1RA, IL-15, IL-6, IL-8, IP-10, MCP1, sIL-2Ra, MIG	4
C	MSD	K151198D-2	V-PLEX Vascular Injury	CRP, SAA, ICAM-1, VCAM-1	10,000
D	MSD	K151P3S-2	SPLEX	IFN-a2a	1
E	MSD	K151ADQS-2	SPLEX	IL-3	1
F	MSD	K151ADRS-2	SPLEX	IFNb	2
G	R&D	DY119	ELISA	IL-18BP	40 or 200
H	MSD	K151AEM-2	UPLEX Custom Immuno-oncology	MMP9(total), S100A12	100
I	MSD	K1514ER-2	RPLEX	MPO	20

3.5 Supplemental Figures

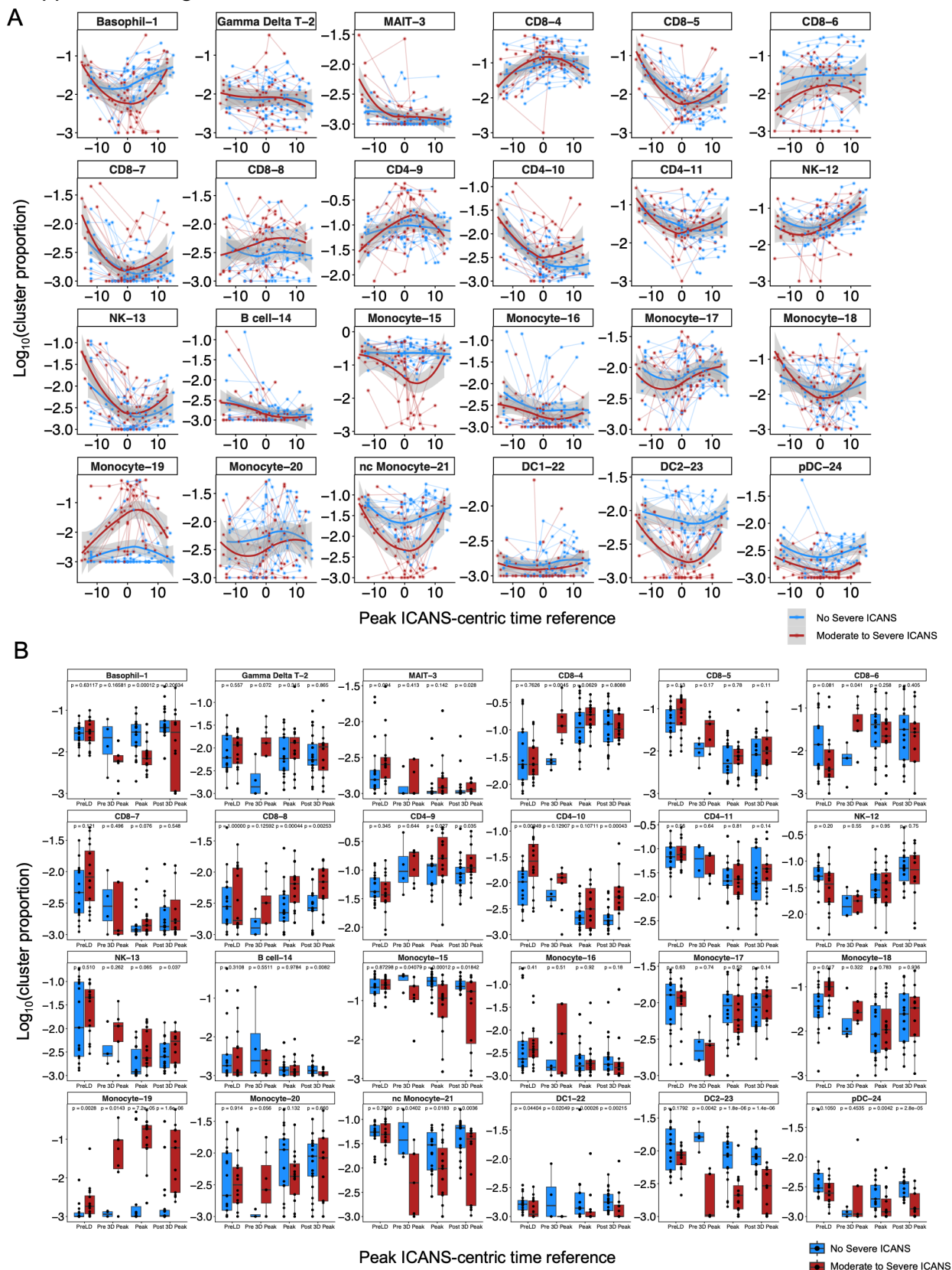
Supplemental Figure 1.



Supplemental Figure 1. Mass cytometry gating scheme

Schematic of manual gating strategy to resolve immune populations from whole blood with mass cytometry

Supplemental Figure 2

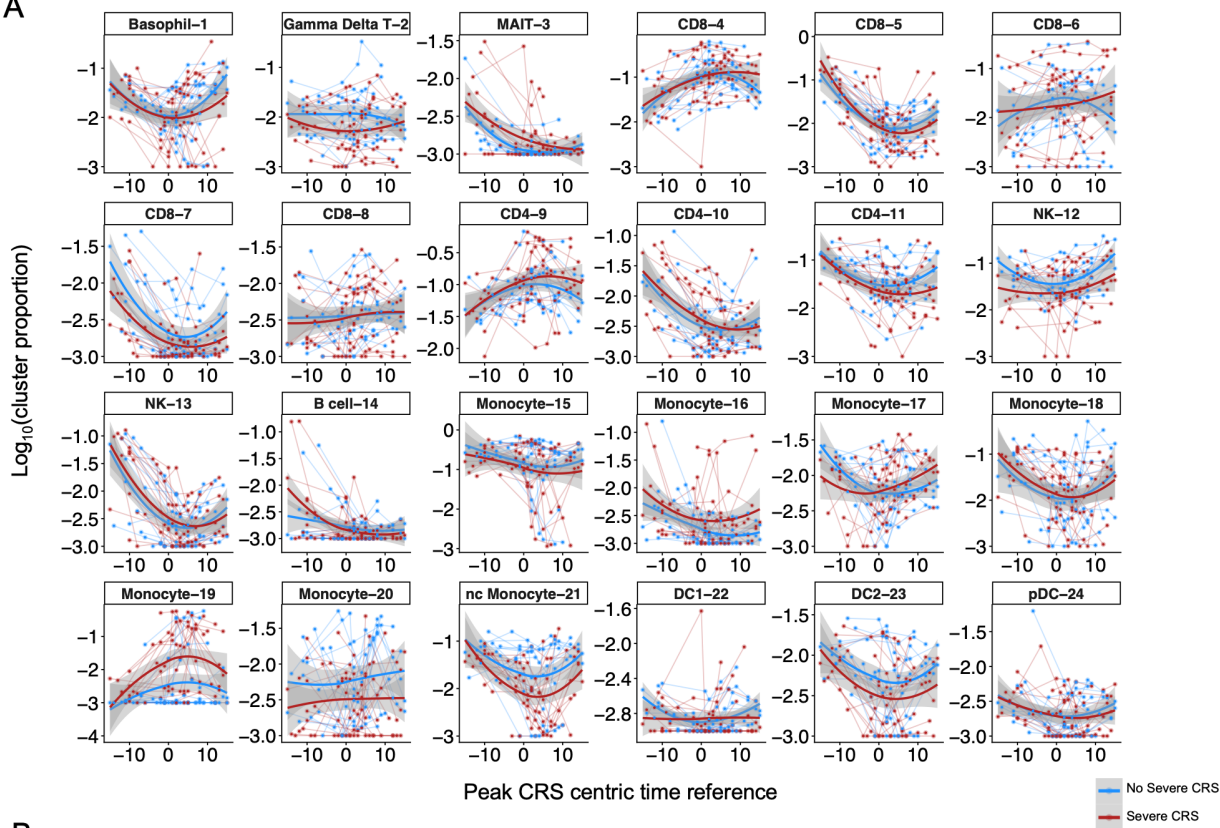


Supplemental Figure 2. Longitudinal profiling of CD66b⁺ clusters relative to peak ICANS

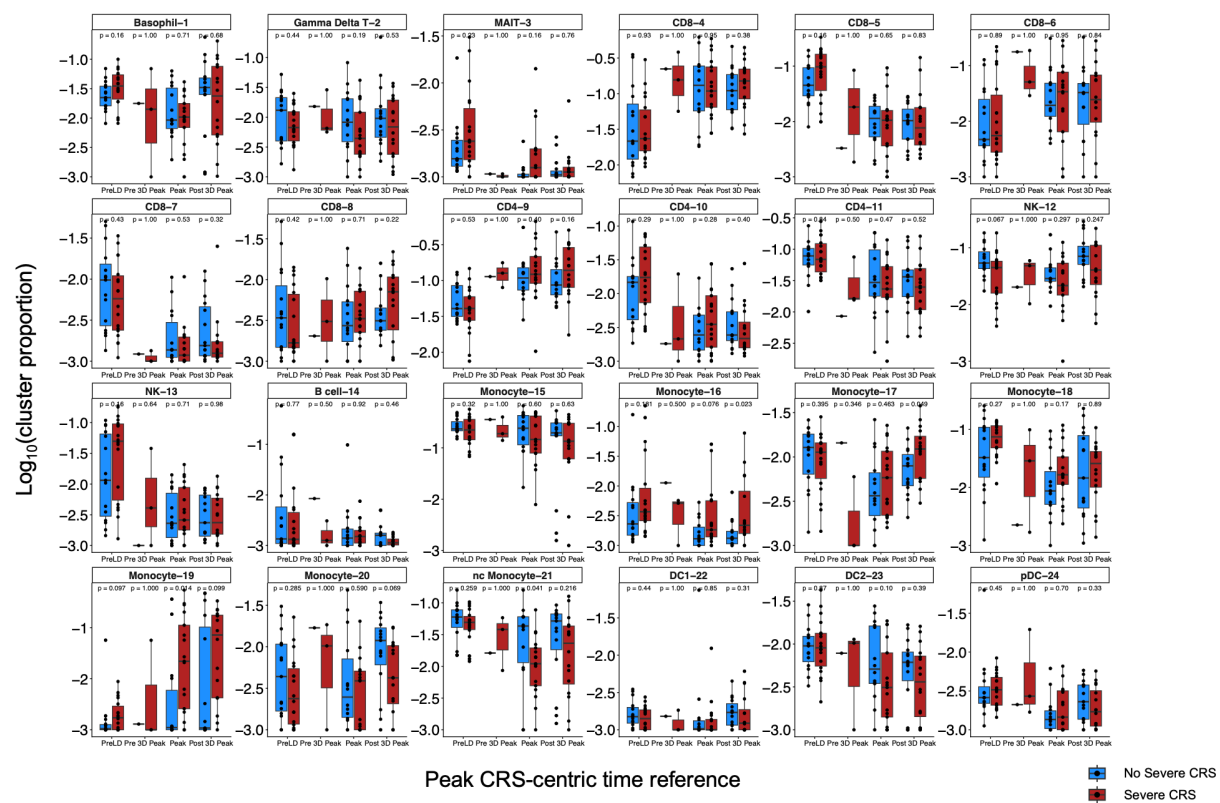
- A. Mononuclear cluster abundances relative to peak ICANS onset in non-severe ICANS (blue) and moderate to severe ICANS patients (red)
- B. Mononuclear cluster abundances relative to peak ICANS centric bins in non-severe ICANS (blue) and moderate to severe ICANS patients (red)

Supplemental Figure 3

A



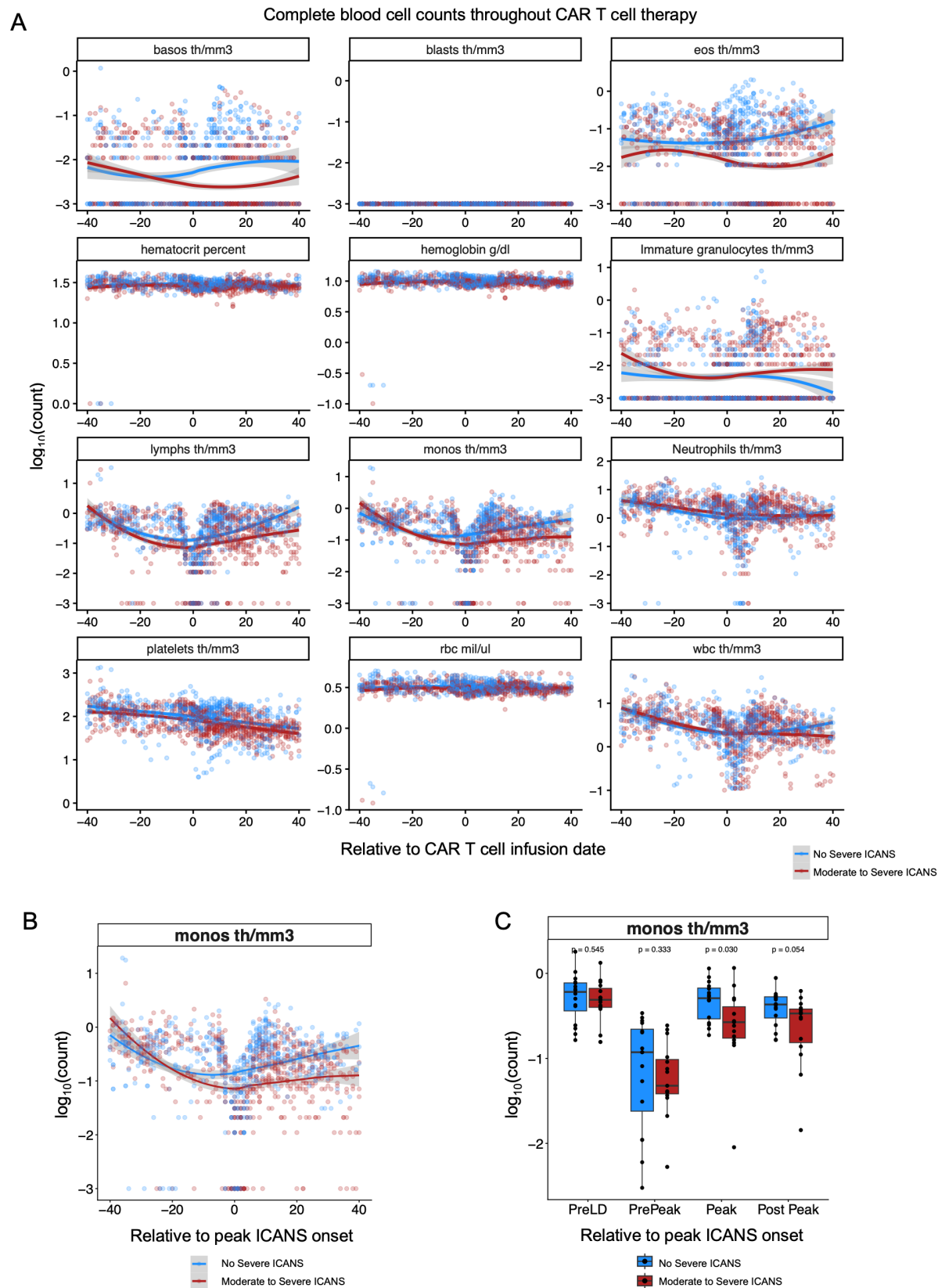
B



Supplemental Figure 3. Longitudinal profiling of CD66b⁺ clusters relative to peak CRS

- A. Mononuclear cluster abundances relative to peak CRS onset in non-severe CRS (blue) and severe CRS patients (red)
- B. Mononuclear cluster abundances relative to peak CRS centric bins in non-severe CRS (blue) and severe CRS patients (red)

Supplemental Figure 4

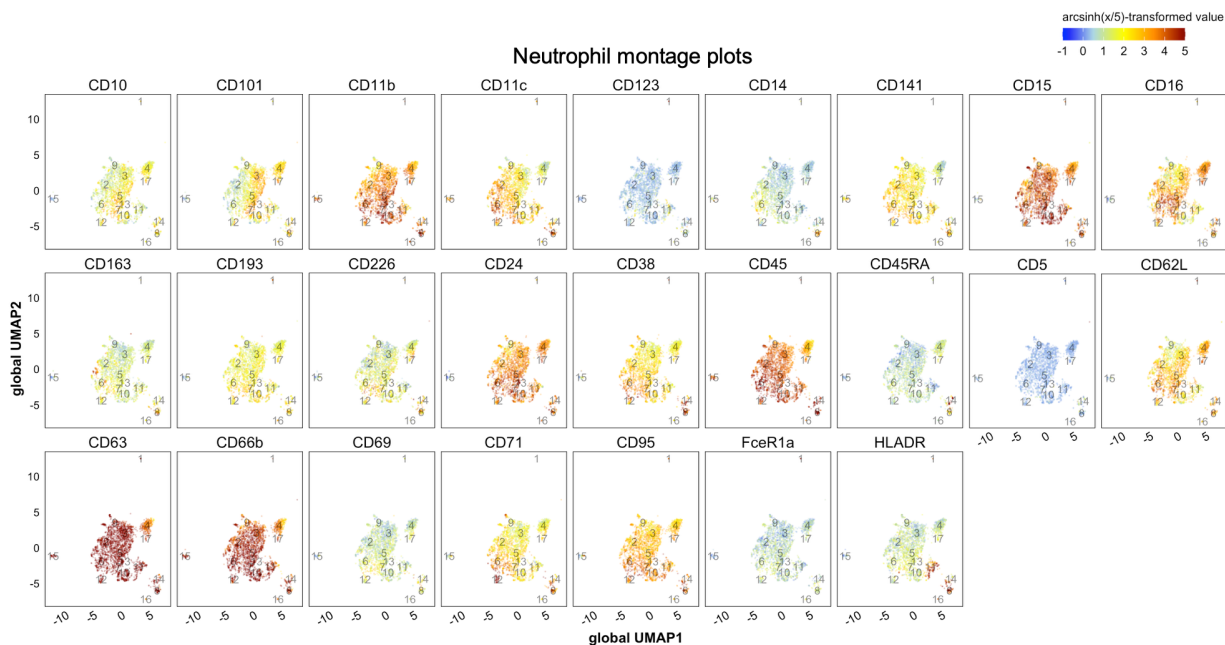


Supplemental Figure 4. Complete blood count analysis

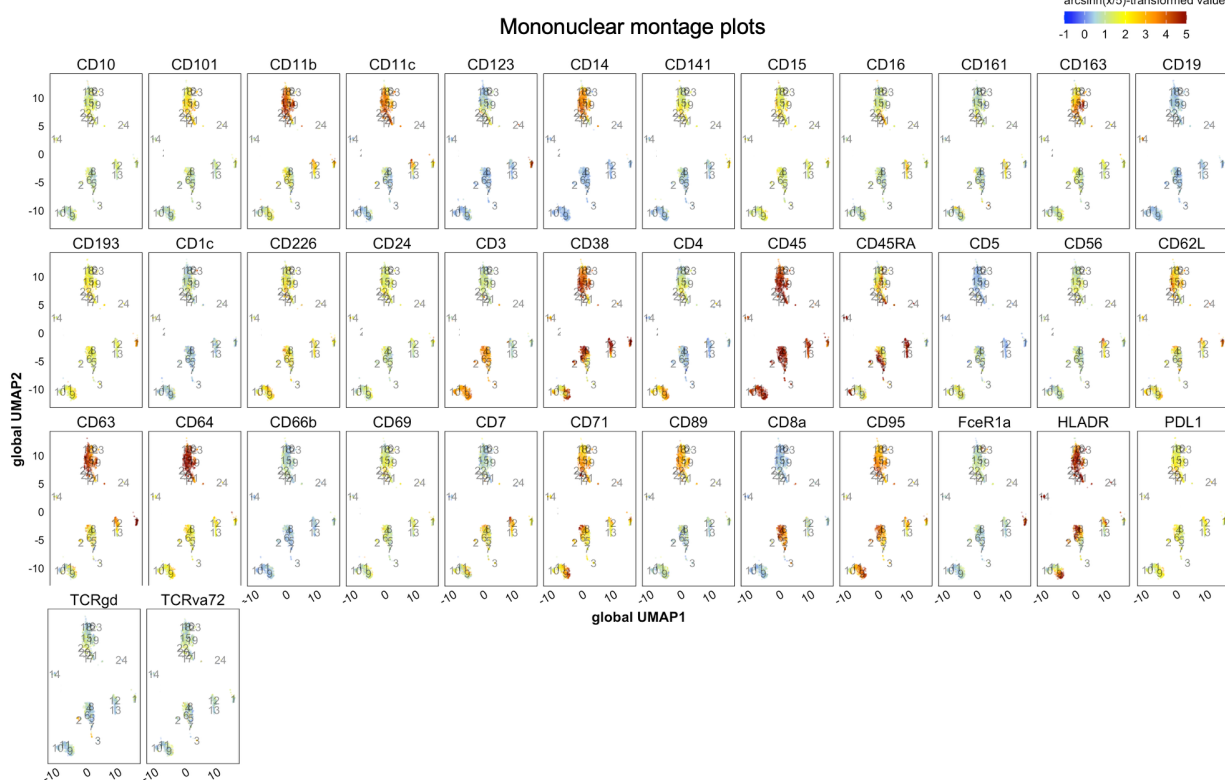
- A. Clinical measurement counts from complete blood at different timepoints relative to the day of CAR T cell infusion
- B. Monocyte counts relative to the day of peak ICANS onset.
- C. Monocyte counts relative to peak ICAN centric bin

Supplemental Figure 5

A



B

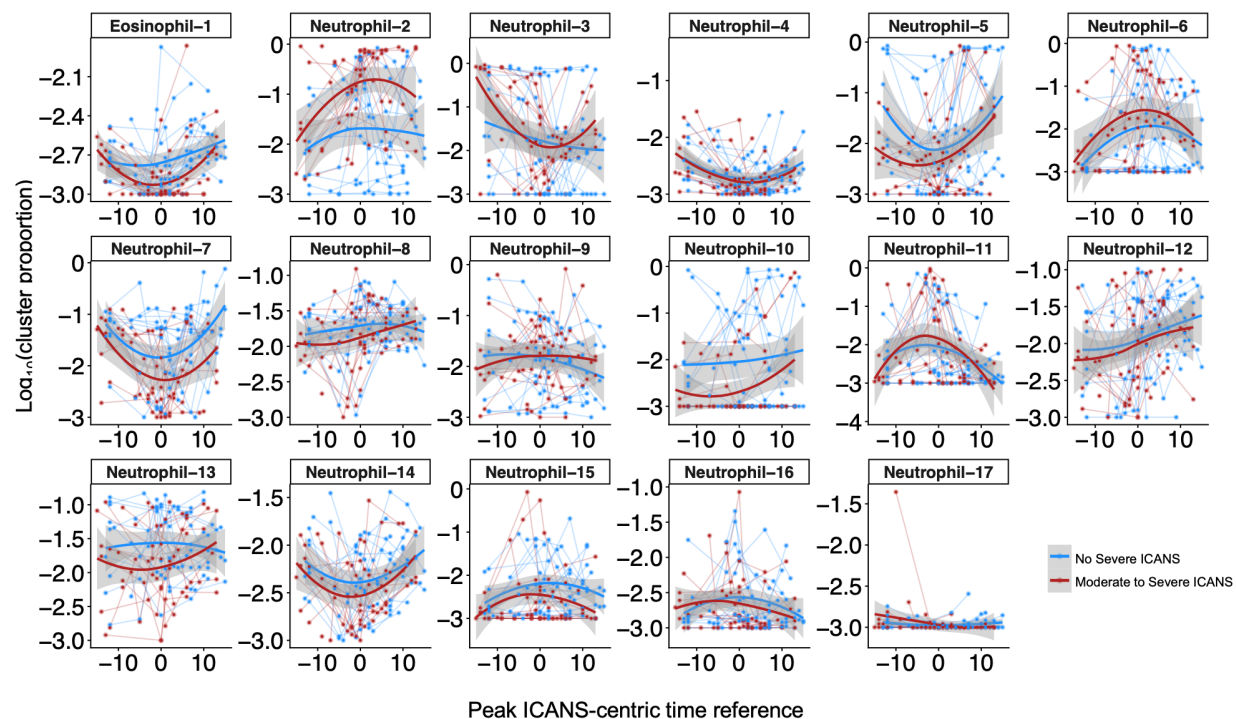


Supplemental Figure 5. Mass cytometry marker-based delineation of CD66b⁺ and CD66b⁻ clusters

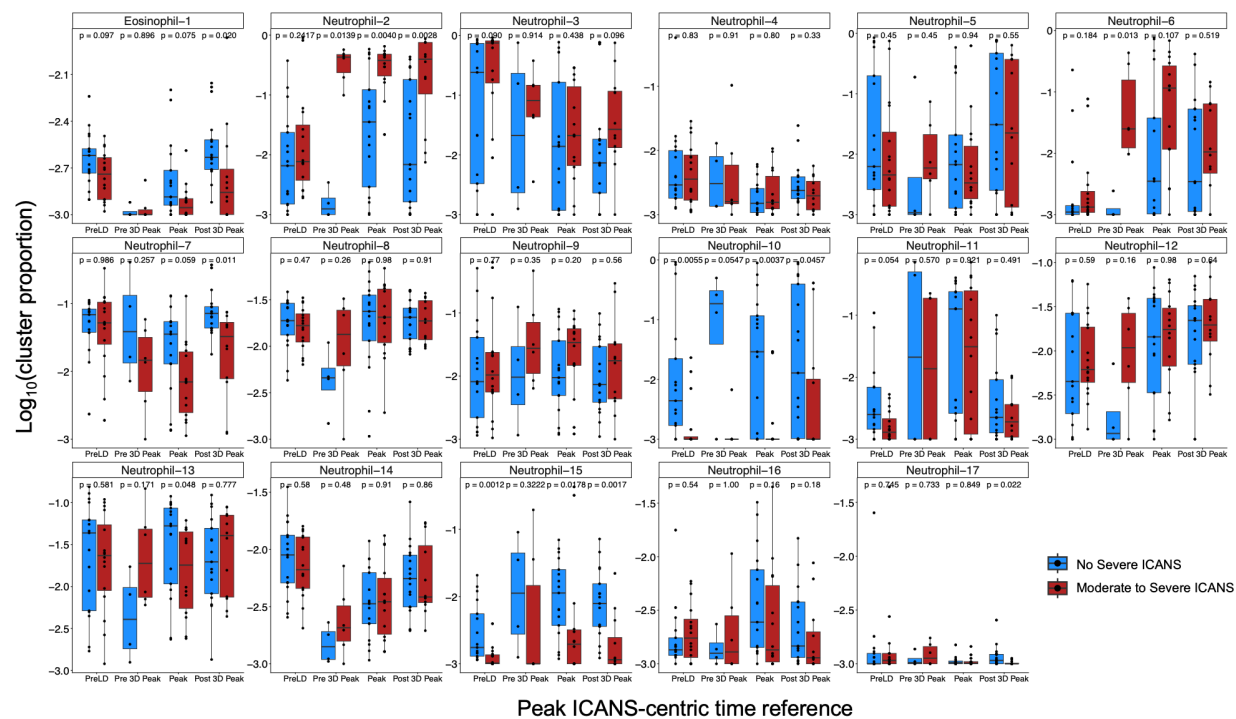
Montage plot of markers from mass cytometry panels overlayed onto neutrophil and mononuclear clusters.

Supplemental Figure 6

A



B

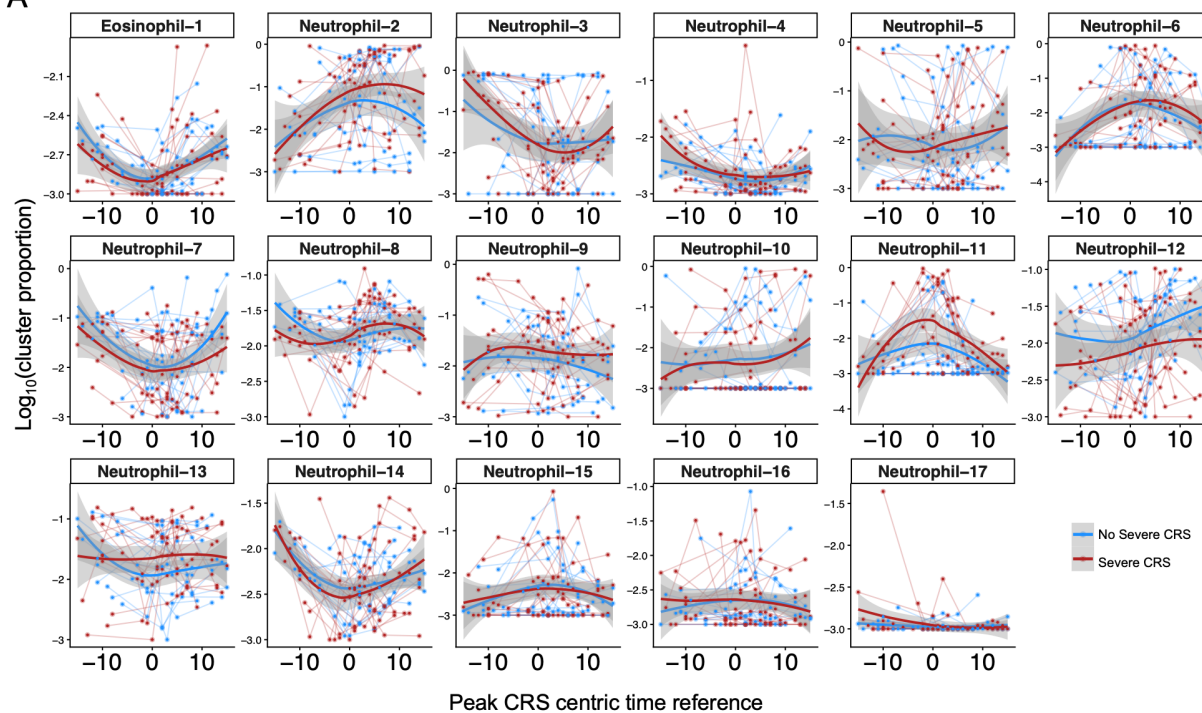


Supplemental Figure 6. Longitudinal profiling of CD66b⁺ clusters relative to peak ICANS

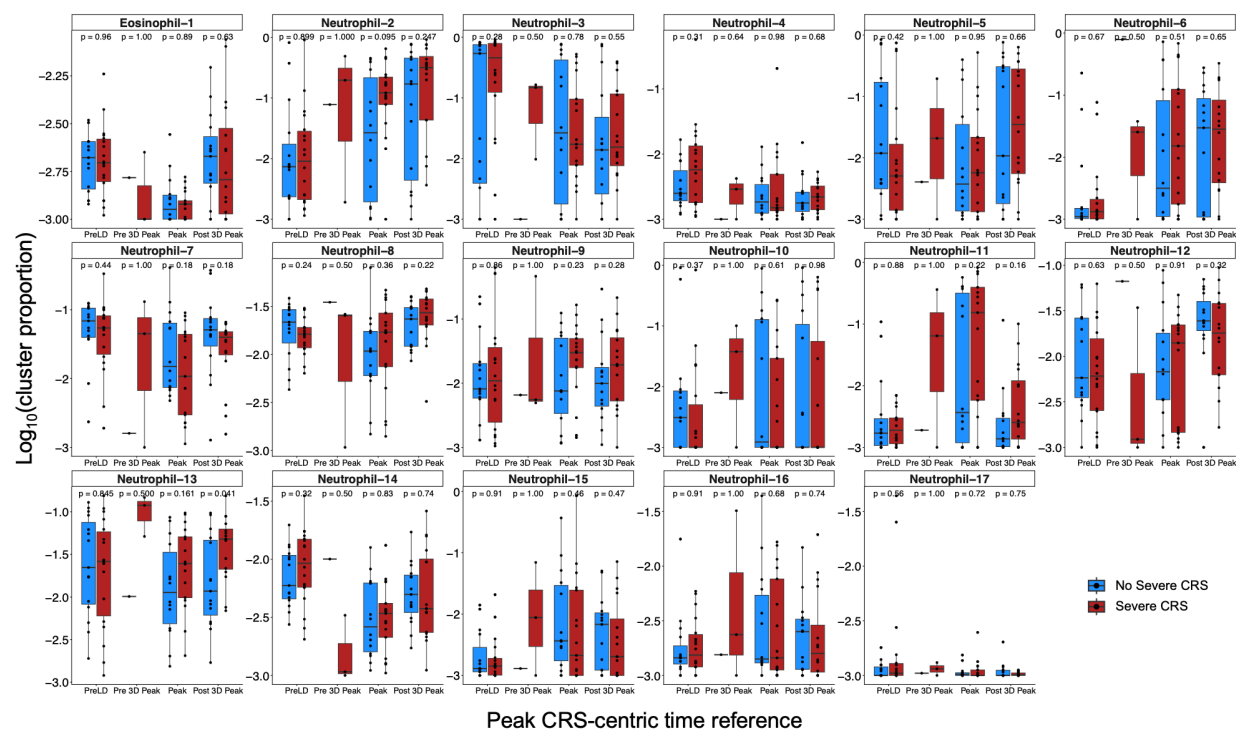
- A. Neutrophil cluster abundances relative to peak ICANS onset in non-severe ICANS (blue) and moderate to severe ICANS patients (red)
- B. Neutrophil cluster abundances relative to peak ICANS centric bins in non-severe ICANS (blue) and moderate to severe ICANS patients (red)

Supplemental Figure 7

A



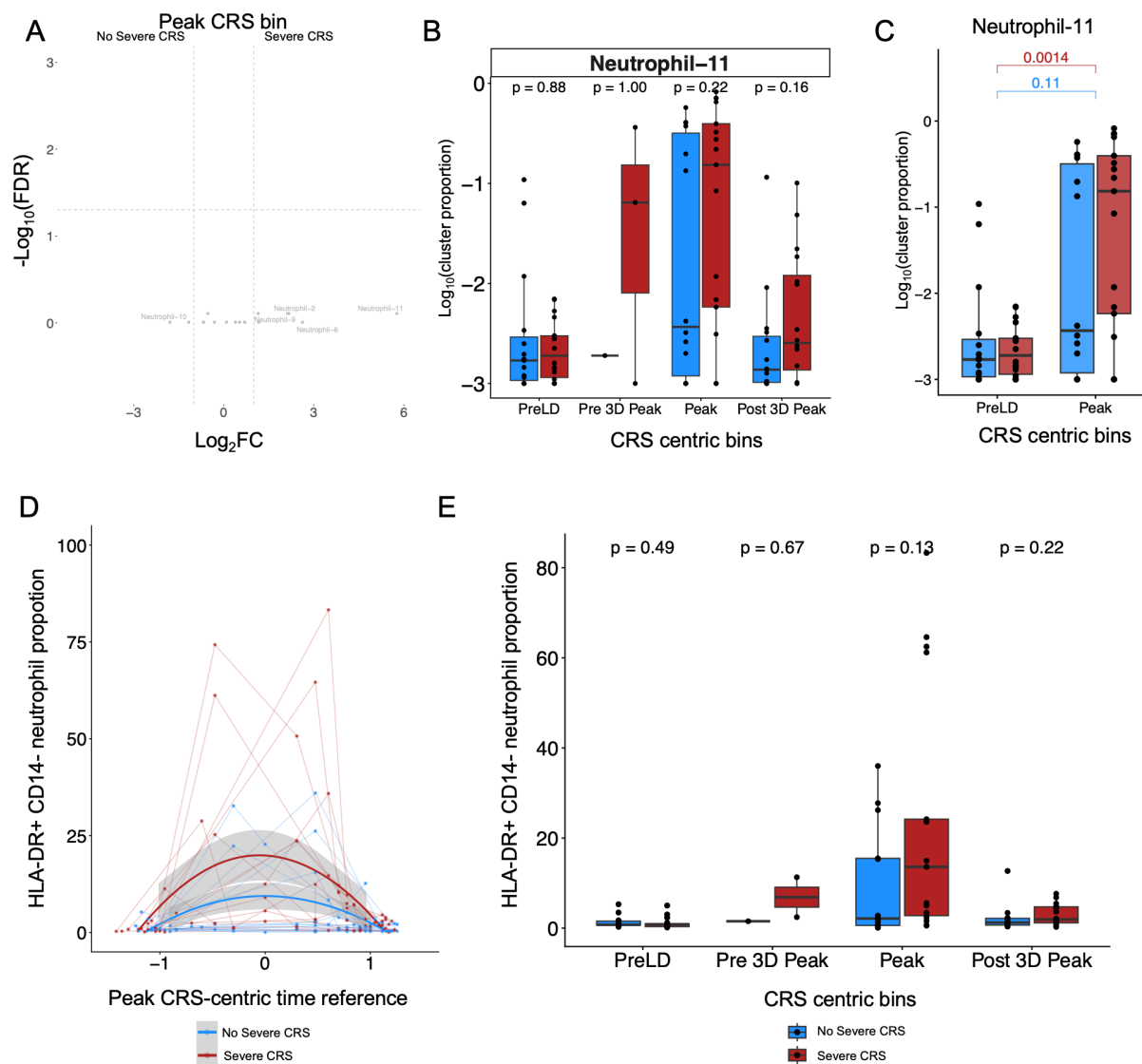
B



Supplemental Figure 7. Longitudinal profiling of CD66b⁺ clusters relative to peak CRS

- A. Neutrophil cluster abundances relative to peak CRS onset in non-severe CRS (blue) and severe CRS patients (red)
- B. Neutrophil cluster abundances relative to peak CRS centric bins in non-severe CRS (blue) and severe CRS patients (red)

Supplemental Figure 8

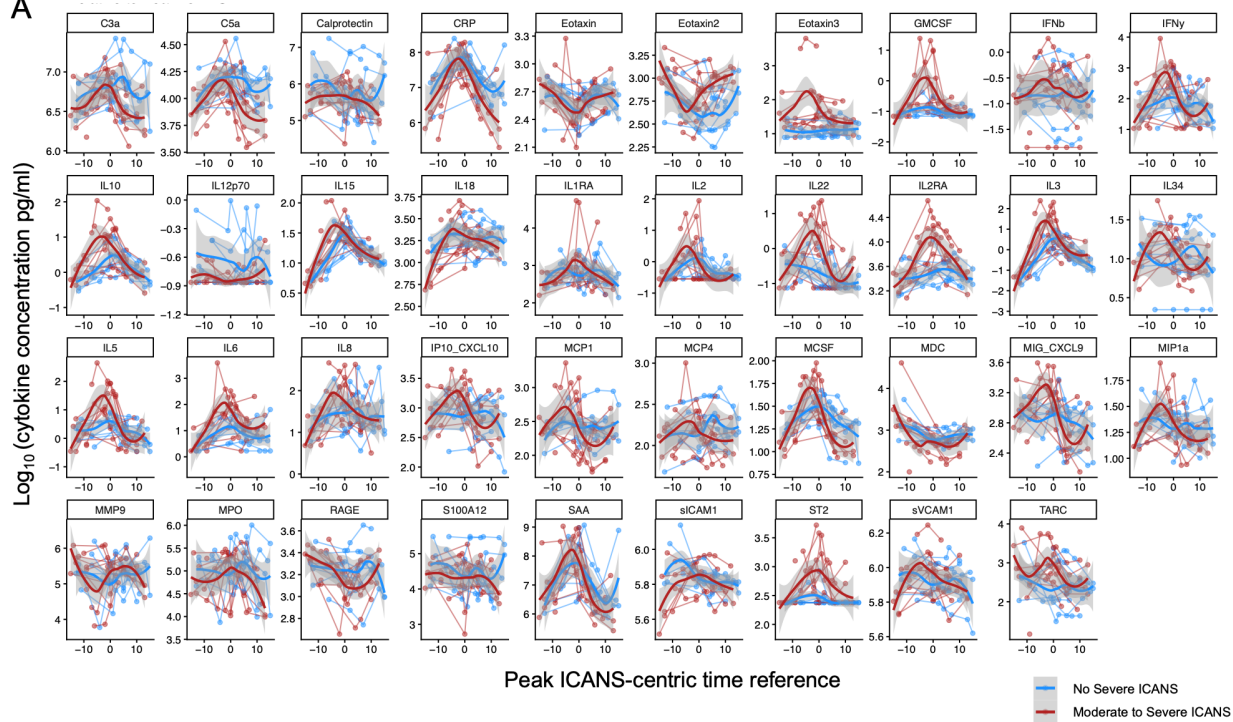


Supplement Figure 8. Longitudinal analysis of HLA-DR⁺ neutrophils across CRS onset

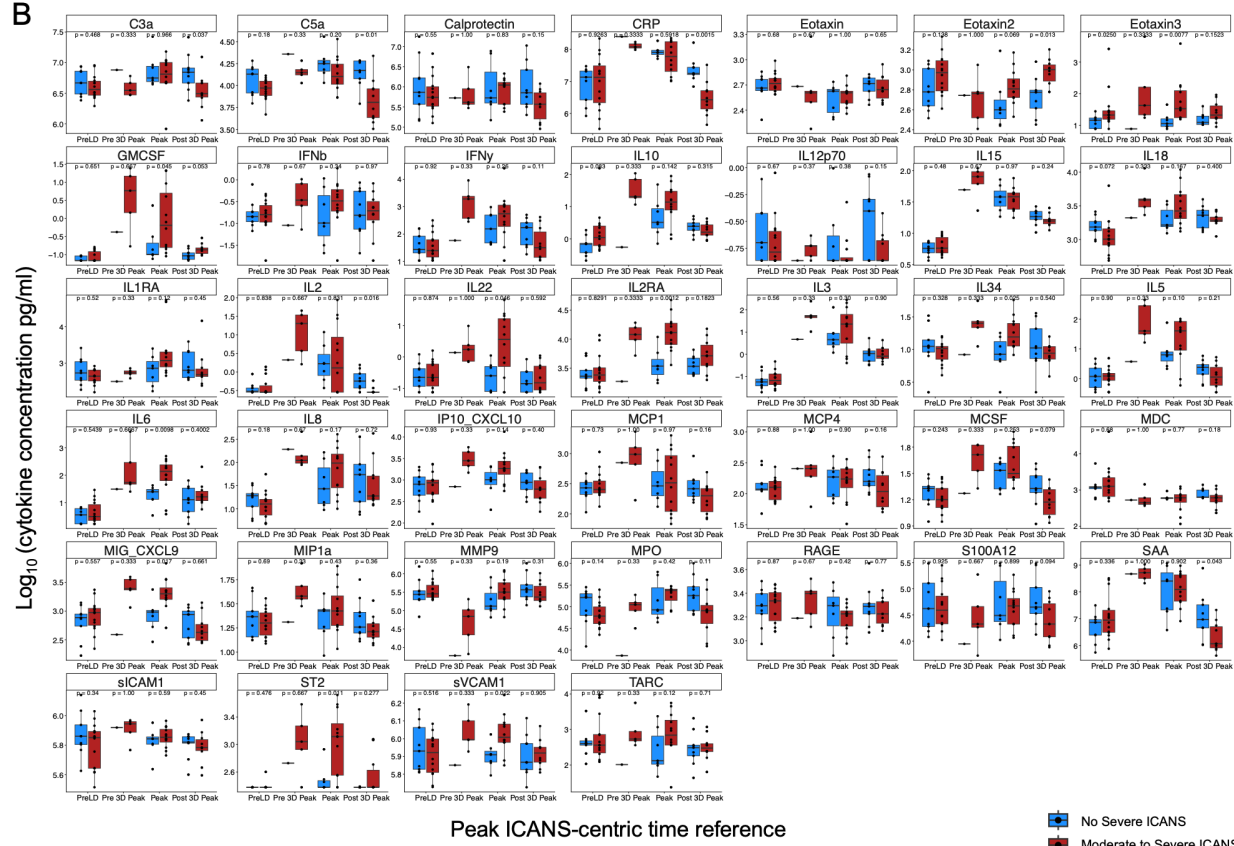
- A. Differentially abundant neutrophil clusters between non-severe CRS and severe CRS patients at peak CRS onset as determined by Wilcoxon signed rank test (FDR < .05).
- B. Box and whisper plot depicting Neutrophil 11 abundances in non-severe CRS or severe CRS patients at peak CRS centric timebins
- C. Neutrophil 11 abundance shift from baseline PreLD to peak CRS onset in non-severe CRS and severe CRS patients
- D. HLA-DR⁺ CD14⁺ monocyte proportion in non-severe CRS and severe CRS patients relative to peak CRS onset as quantified by manual gating
- E. HLA-DR⁺ CD14⁺ monocyte proportion in non-severe CRS and severe CRS patients over CRS centric bins as quantified by manual gating

Supplemental Figure 9

A



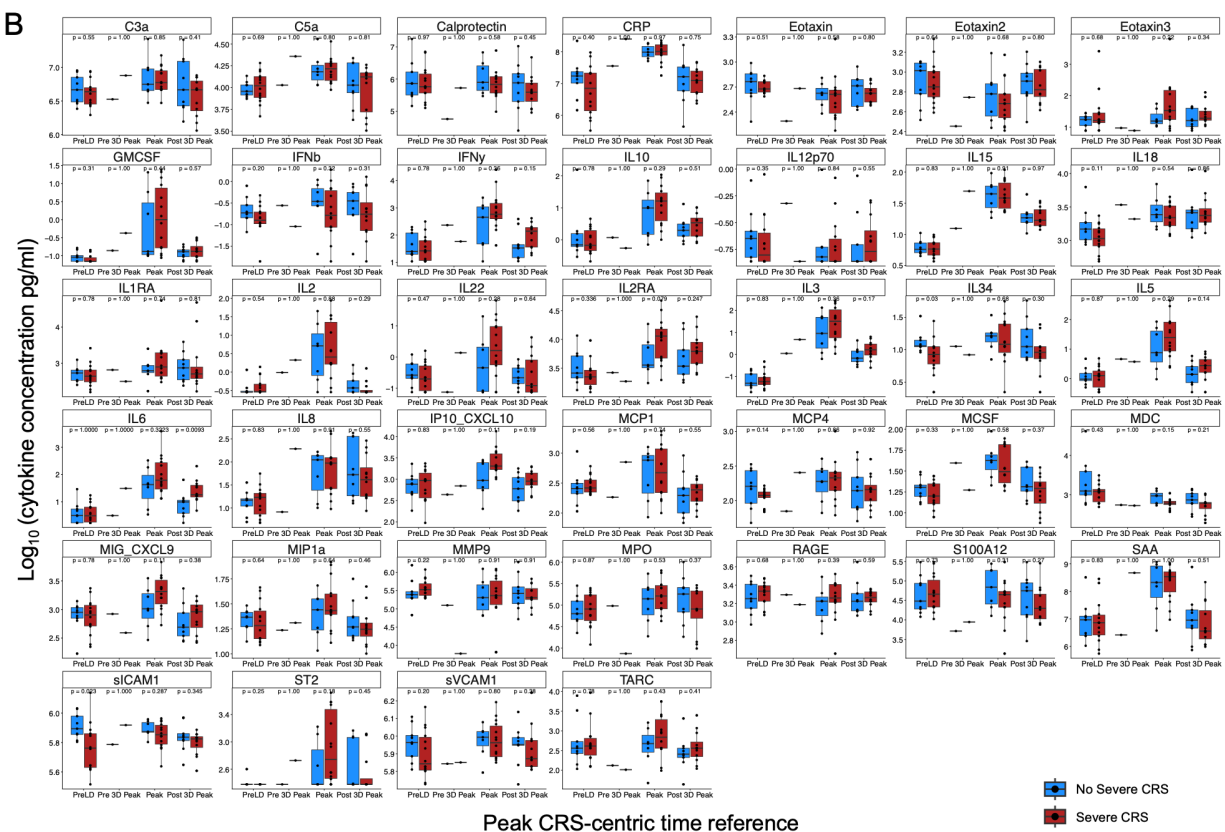
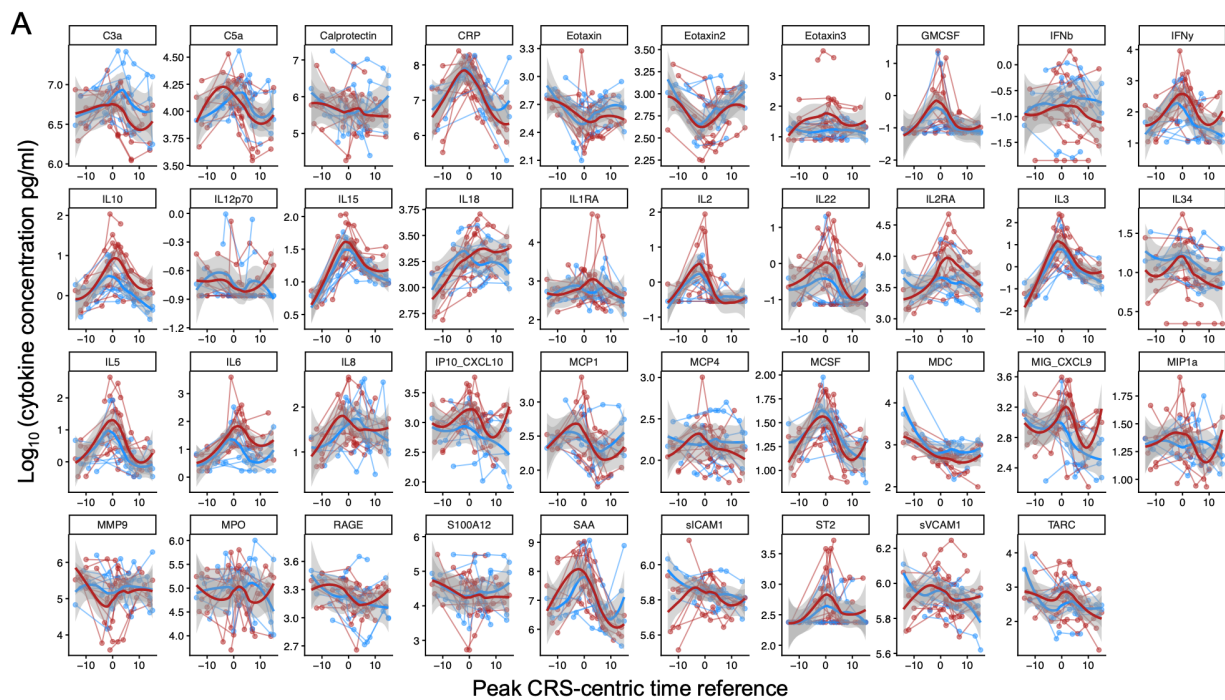
B



Supplemental Figure 9. Longitudinal quantification of serum cytokines relative to peak ICANS onset

- A. Peripheral cytokine concentrations relative to peak ICANS onset in non-severe ICANS (blue) and moderate to severe ICANS patients (red)
- B. Peripheral cytokine concentrations relative to peak ICANS centric bins in non-severe ICANS (blue) and moderate to severe ICANS patients (red)

Supplemental Figure 10

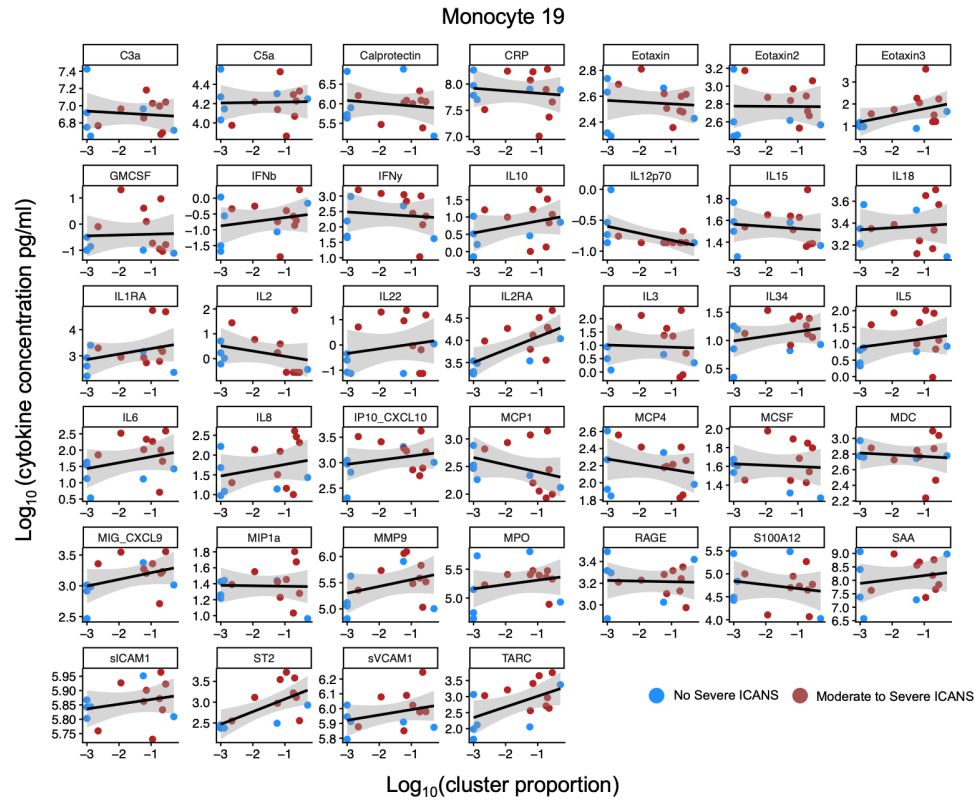


Supplemental Figure 10. Longitudinal quantification of serum cytokines relative to peak CRS onset

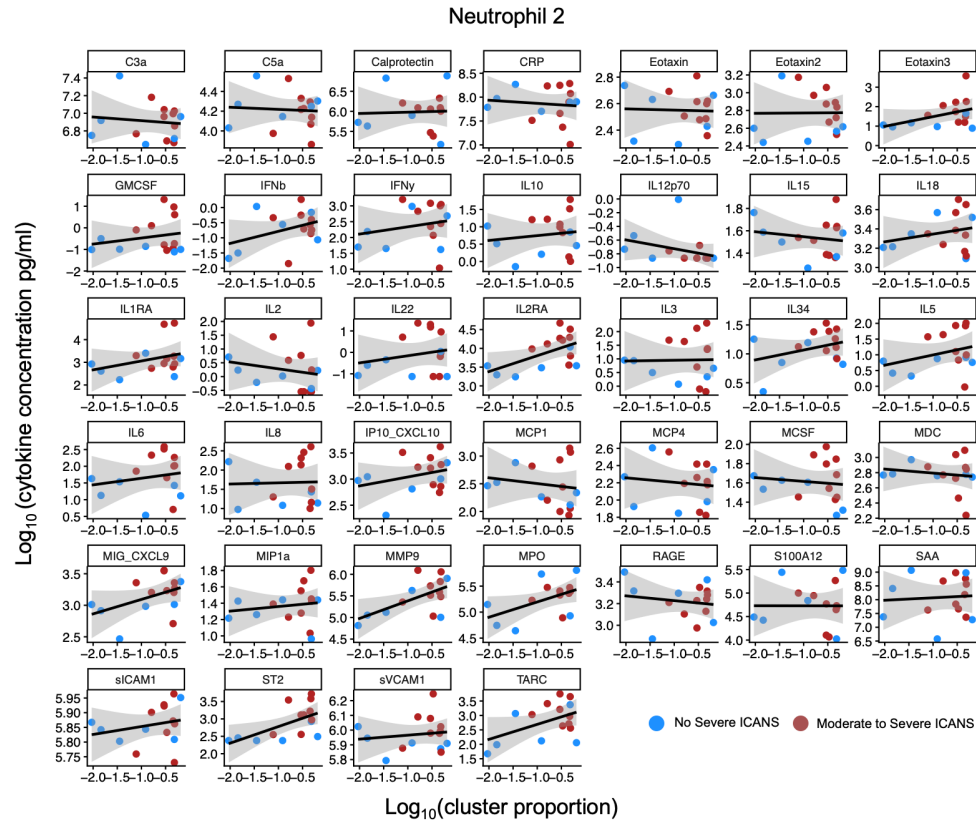
- A. Peripheral cytokine concentrations relative to peak CRS onset in non-severe CRS (blue) and severe CRS patients (red)
- B. Peripheral cytokine concentrations relative to peak CRS centric bins in non-severe CRS (blue) and severe CRS patients (red)

Supplemental Figure 11

A



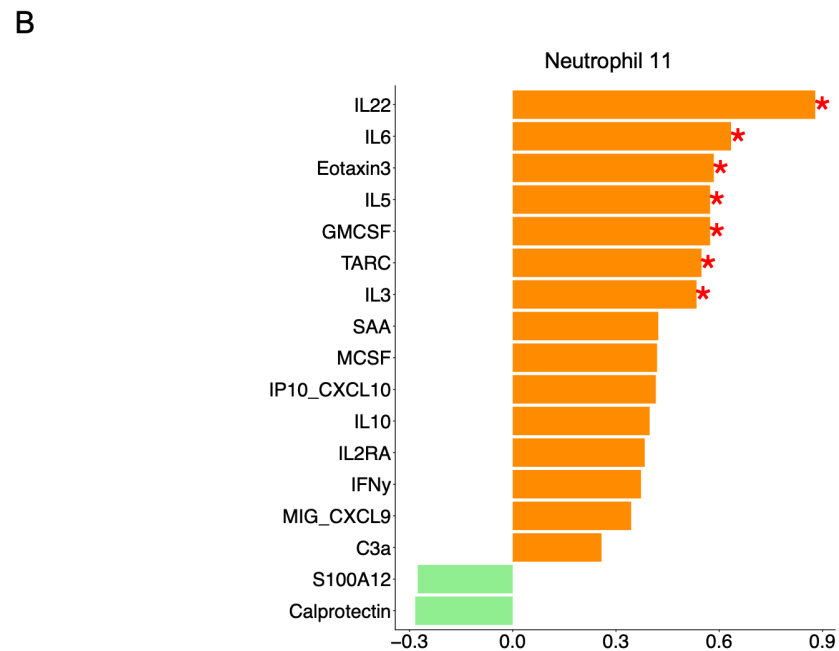
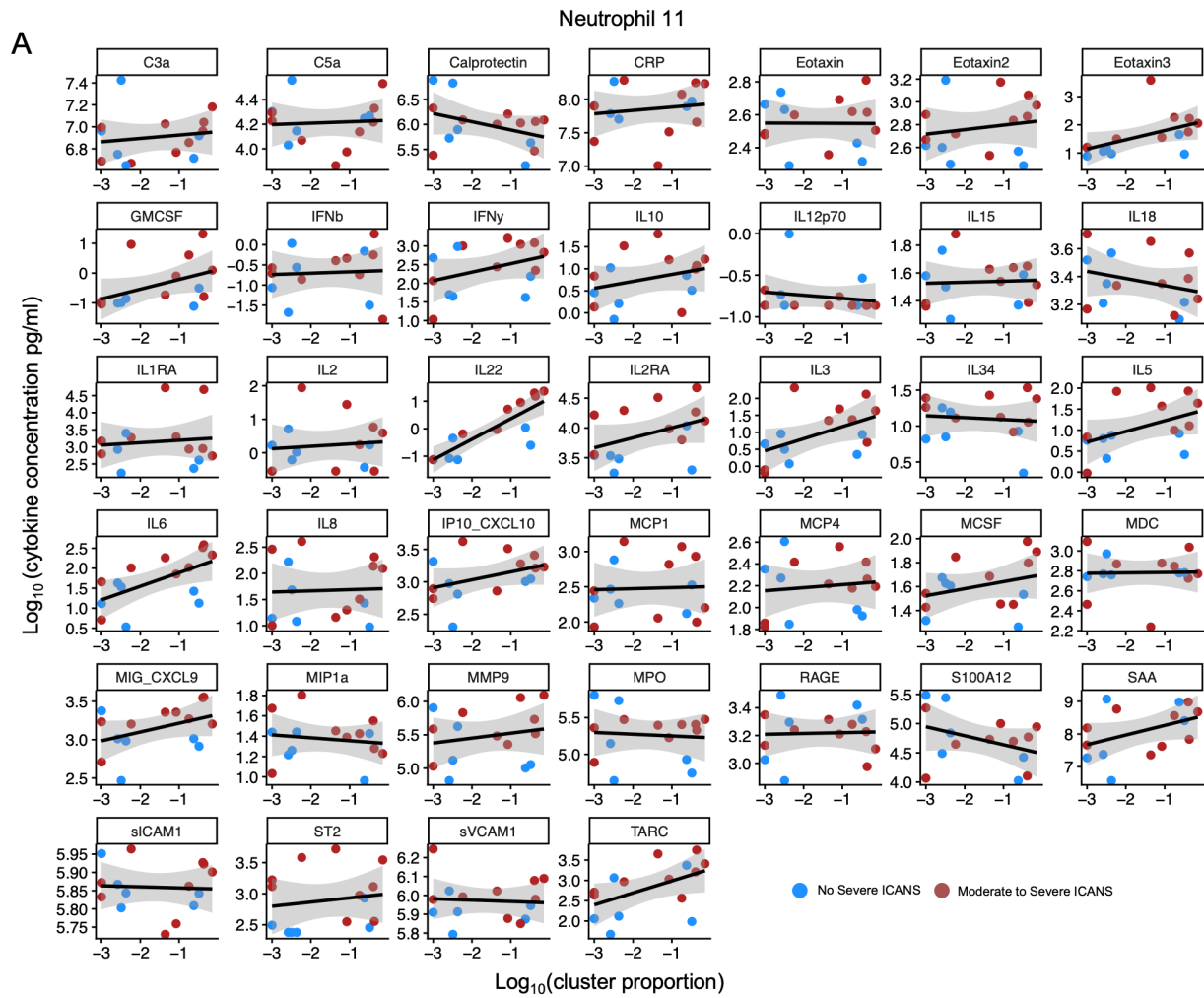
B



Supplemental Figure 11. Monocyte 19 and Neutrophil 2 cytokine correlations at peak ICANS onset

- A. Linear regression of cytokine concentrations measured against Monocyte 19 cluster abundance at peak ICANS onset
- B. Linear regression of cytokine concentrations measured against Neutrophil 2 cluster abundance at peak ICANS onset

Supplemental Figure 12



Supplemental Figure 12. Neutrophil 11 cytokine correlations at peak ICANS onset

- A. Linear regression of cytokine concentrations measured against Neutrophil 11 cluster abundance at peak ICANS onset
- B. Strongest cytokine associations with Neutrophil 11 abundance at peak ICANS onset

3.6 Methods

Study Design and Participants

Thirty three patients enrolled in CD19 CAR T cell therapy with relapsed and/or refractory B cell malignancies were enlisted for our study. The patients received commercial Yescarta (n=18), Tecartus (n=3), Kymriah (n=6), or Brexanzi (n=6) infusions. Of the 33 patients, 18 achieved complete remission by day 28 post CAR T cell infusion, while 15 experienced only partial response or disease progression.

Whole blood fixation and cellular staining

Immediately after blood draw, whole blood was stored in EDTA blood collection tubes for 1-12 hours. Whole blood was mixed with SMARTube Proteomic Stabilizer buffer in 1:1.4 ratio and left at room temperature for 10 minutes prior to storage in -80°C in 2 ml aliquots. On the day of cellular staining, the whole blood and fixation aliquots were thawed on ice for 15 minutes before addition of 2 ml SMARTube Thaw-Lyse buffer and incubated for an additional 10 minutes to deplete red blood cells. 3 rounds of SMARTube Thaw-Lyse washes were performed afterwards prior to transferring cells to a 96 well plate and incubation in a 9:1 Fc Receptor Blocking solution and 200 U Heparin mixture at 4°C for 20 minutes. Cells were washed and resuspended in cytometry buffer (PBS + 0.02% sodium azide + 5% fetal calf serum). Following a wash with cytometry buffer, cells were incubated with PE-conjugated antibody against TCR $\gamma\delta$ on ice for 15 minutes. Afterward, cells were washed with cytometry buffer and stained with a cocktail of metal-conjugated surface marker antibodies at titrated concentrations and incubated on ice for 30 minutes. Following surface marker staining, cells were washed in cytometry buffer twice prior to permeabilization with methanol on ice for 10 minutes. Following permeabilization and two washes with cytometry buffer, cells were incubated with a cocktail of metal-conjugated antibodies against intracellular transcription factors at room temperature for 30 minutes. An additional two rounds of wash with cytometry buffer followed before incubating with 2% paraformaldehyde and DNA intercalator (1:4000) and storing at 4°C until the day of CyTOF acquisition.

CyTOF acquisition

Over the span of several months, all samples were run on the CyTOF in 18 batches, typically composed of the repeated measures from 1-2 patients, as well as a repeated healthy donor control.

Each batch, cells were washed 3× with cytometry buffer and resuspended in 10% EQ beads (Catalog #201078) in MaxPar Cell Acquisition Solution prior to CyTOF processing.

Following CyTOF processing, the signal of each parameter was normalized based on EQ beads with Matlab software (<https://github.com/nolanlab/bead-normalization/releases/tag/v0.3>).

Based on CD66b expression, cellular populations were gated and exported for clustering analysis using customized R scripts.

Clustering analysis

Repeated clustering at the single cell level – typically necessary to identify and factor out technical noise, or putative batch effect -- is computationally intractable at the scale of this study. Hence, to arrive at globally defined clusters across all batches, we first ‘over’-clustered within each batch by clustering then subclustering each cluster⁷¹. Next, we computed the centroids of all clusters in each batch, then (meta)clustered these centroids (or “metacells” or “supercells”). At each stage we used the Rphenograph algorithm, with Leiden clustering of shared nearest-neighbor graphs⁷².

To address moderate drift across batches in some parameters, we aligned the repeated control median values across batches for each parameter and applied the corresponding batch- and parameter-specific shifts to the samples before computing metacells for global metaclustering⁷³. We used Uniform Manifold Approximate Projection (UMAP) for dimension reduction and data visualization⁷⁴.

Generalized Estimating Equation

To determine whether clinical grouping by CRS and/or ICANS severity held marginal associations over post-infusion timepoints, for any specific clusters or serum proteins, we applied Generalized Estimating Equations using the R package geepack^{104,105}. We used a first-order autoregressive correlation structure (corstr == “ar1”), with non-uniform patient sample

timepoints centered according to each patient's peak ICANS date (set to 0). Multiple hypothesis correction was employed with a Benjamini-Hochberg FDR < .05 for statistical significance.

Meso-Scale Discovery Assay

To minimize freeze–thaw cycles, serum samples were thawed once, aliquoted into 96-well plates at the required volume for each assay, and then re-frozen until the day of the assay. Given the large number of samples, each assay was run in two batches. Pooled healthy donor serum spiked with the top standard (STD1) was included on all plates as the inter-plate control, since many pro-inflammatory analytes are undetectable in healthy serum. All assays used in the analysis had an inter-plate control coefficient of variation (CV) between 3–16%.

Samples were run in technical duplicate, and the average concentration (pg/ml) for each analyte was interpolated using a weighted four-parameter logistic (4-PL) fit curve defined by 10 non-zero standards using the Meso Scale Discovery software. Assays were performed on either the Meso Scale Discovery (MSD) platform or by traditional ELISA, according to the manufacturer's instructions, with sample dilutions listed in Table 1.

The lower limit of detection (LLOD) was calculated as the average blank (or raw signal in the sample dilution matrix) plus 2.5 standard deviations. Values above the blank but below the LLOD were interpolated by MSD software and left unchanged. Similarly, values above the curve fit of the top standard (upper limit of detection, ULOD) were interpolated by MSD software and left unchanged. Most cytokines fell within detectable ranges, if an analyte had < 20% of samples in detectable ranges those analytes were removed from further analysis (**Supplemental Table 3**). For transparency, both the LLOD and ULOD are indicated on graphs where values fall below or above these ranges.

Missing cytokine values due to signals below the curve fit or below the average blank were replaced with the re-scaled LLOD (based on sample dilution) to avoid zeros in the dataset and enable log transformation.

Immune Module Analysis

Effect sizes for each feature of interest were calculated. Subsequently, dimensionality reduction was performed via PCA to group our features of interest into immune modules. We used random forest to categorically classify each immune module as a predictor of severe ICANS and rank immune modules based on variable importance, selecting the model with the lowest out-of-bag error as a representative model.

Statistical Analysis

We designated time bins according to expected timeframes of biological responses, relative to each patient's peak ICANS diagnosis time, for those with ICANS, or 3 days after infusion for patients without ICANS. When patients had multiple blood and serum samples collected in a single time bin, the maximum cytokine concentration and the median cluster abundance were used. For each time bin, rank-sum Wilcoxon tests were applied to identify statistically significant differences in median cluster abundances between severe and non-severe ICANS patient groups. Benjamini-Hochberg multiple test correction was done using $FDR < .05$ for statistical significance. Firth logistic regression was used to determine statistically significant cytokines associated with severe ICANS within the peak ICANS window. For the cytokine analyses, an FDR threshold of <0.1 was applied to account for the smaller sample size ($n=15$) relative to the mass cytometry dataset ($n=31$). For correlation analysis between cytokine concentrations and cluster abundances, Spearman rank correlation was used.

Chapter 4: Discussion and Future Directions

Discussion

Advances in CAR T cell therapy have dramatically improved outcomes for patients with relapsed or refractory B-cell malignancies. Despite these advances in efficacy, safety responses remain highly variable across patients. Clinical outcomes are shaped by biological variability that begins prior to CAR T cell infusion and evolves dynamically following infusion. The work presented in this dissertation demonstrates that cellular and molecular signatures throughout the course of CAR T cell therapy emerge that correlate with the onset of severe toxicities.

Chapters 2 and 3 describe distinct but interconnected segments of this continuum. In Chapter 2, we characterize heterogeneity in leukapheresis T cell populations and CAR T infusion products across pediatric and adult cohorts, emphasizing that variability introduced at the earliest stages of manufacturing influences the cellular composition and phenotypic differences represented in the final infusion product. In Chapter 3, we define longitudinal immune dynamics following CD19 CAR T cell infusion in adult patients with DLBCL, identifying cellular and cytokine correlates associated with severe ICANS near the time of peak neurotoxicity. Together, these chapters define a unified model in which severe neurotoxicity emerges based on 1) phenotypic disposition of CAR T cell infusion products and 2) recruited neutrophil and myeloid lineages post CAR T cell infusion.

Findings in Chapter 2 emphasize that variance within leukapheresis T cells and infusion products differ across cohorts and are shaped by patient factors and manufacturing context. Infusion products retain variable representation of memory, effector, and activation states that plausibly influence post-infusion expansion kinetics and cytokine production capacity.

Chapter 3 complements this perspective by demonstrating that, following infusion, the peripheral immune landscape becomes highly dynamic, and that severe neurotoxicity is associated with coordinated changes in circulating myeloid subsets and inflammatory mediators within a tightly defined peak window. A major observation from Chapter 3 is that patients with severe ICANS exhibit increased proportions of CD163⁺ monocytes and enrichment of immature neutrophil subsets marked by reduced CD10 (and reduced CD101 across key clusters) at the time of peak toxicity. In parallel, serum profiling identifies elevated concentrations of soluble mediators

including ST2 and IL2RA, with additional increases in canonical inflammatory cytokines and chemokines (including IL-6 and Eotaxin-3) during the same period. Importantly, integration of cellular and cytokine analysis reveals that CD163⁺ monocytes and immature neutrophils co-vary across patients, and these populations associate with soluble mediators such as ST2 and IL2RA, suggesting a complex regulatory network regulates severe ICANS post CAR T cell infusion.

Limitations to our study include a relatively small sample size and collection availability as we could not obtain as many serum samples per patient in comparison to whole blood/cellular samples, hindering our statistical power and ability to integrate multi-omic datasets.

Additionally, confounding factors such as filgrastim and prior chemotherapeutics administered may also skew cellular abundances and cytokine concentrations. Tumor burden at the time of CAR T cell administration may also affect extent of CAR T cell activation, downstream signal strength and resulting ICANS severity. Furthermore, our sample collection timepoints and analysis are based on dates relative to peak ICANS and not set dates post CAR T cell infusion. These limitations could be addressed with a validation cohort with sample collection at set timepoints: PreLD, Day of Infusion, Days 3, 5, 7, 10, 14 post infusion.

Future Directions

Additional experiments to identify transcriptomic signatures and biomarkers correlating severe ICANS onset include GEM-X FLEX analysis in which we profile fixed whole blood samples to identify shifts in neutrophil and mononuclear transcriptomic signatures post CAR T cell infusion to unravel mechanistic insights into ICANS onset in CD19 CAR T cell therapy. We also intend to identify phosphorylated transcription factors to provide insight into activation pathways. Additional work involves murine models to recapitulate neutrophil, leukocyte, and endothelial cell interactions/adhesion contributing towards ICANS onset post CAR T cell infusion.

While we focused our analysis primarily on ICANS onset, we plan to extend our analysis to encompass other adverse events that arise through CAR T cell therapy, namely CRS, or less common adverse events such as immune effector cell-associated hematotoxicity (ICAHT) and immune effector cell-associated hemophagocytic lymphohistiocytosis-like syndrome (IEC-HS)¹⁰⁶. Furthermore, with a larger cohort we can develop predicted probability models of adverse event onset based on phenotypic or soluble mediator abundances.

In summary our work provides a foundation, integrating cellular dynamics and cytokine shifts that represent severe ICANS pathogenesis. Future work can build upon our research to unravel mechanisms that drive adverse events in CAR T cell therapy to develop safer therapeutic practices.

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