# A multi-omic single-cell landscape of cytokine release syndrome in multiple myeloma patients after anti-BCMA CART cell therapy

# Abstract

# Anti-BCMA chimeric antigen receptor T (CART) cell therapy has achieved great success in the treatment of multiple myeloma (MM), yet cytokine release syndrome (CRS) is the most severe and common complication that limits the efficacy and increases the expense of CART cell therapy. However, the comprehensive cellular and molecular mechanisms underlying CRS still remain enigmatic. Here, we conducted a single-cell multi-omic study to profile the transcriptome, immunome and epigenome of CART and bystander immune cells from 61 MM patients at different stages of CRS following CART cell therapy. We also performed multiplex cytokine assays to comprehensively reveal the kinetics of CRS-related cytokines, identifying 3 main cytokines (IL-2, IL-4 and IL-17A) at CRS initiation and 24 biomarkers at the CRS peak stage. Further, scRNA-seq analysis demonstrated systematic and diverse changes across cellular subpopulation secreting particular cytokines and allowed us to define cell-cell interaction networks across the course of CRS. Moreover, integrated analyses of scRNA-seq and scTCR-Seq illustrated the developmental trajectory and clonal diversity of CART and endogenous T cells and detected clonal expansion of CD8 effector memory subsets upon CRS recovery. Meanwhile, combined analyses of scATAC-seq and scRNA-seq deciphered the chromatin accessibility and cis-regulatory network in CART cells at different CRS stages. Finally, we demonstrated that a CD40LG+IL13+CD4+ cluster in pre-infusion CART products is associated with CRS severity and initiated CRS by recognizing tumor cells and activating monocytes and T cells. In summary, our comprehensive dataset provides excellent resources for the understanding of CRS mechanisms, which will bolster the development of therapeutic approaches to ameliorate the clinical burden of CRS and enhance the efficacy of anti-BCMA CART cell therapy.

# Keywords: Chimeric antigen receptor T cells (CART); Cytokine release syndrome (CRS); Multiple myeloma; Multi-omics; Single-cell sequencing;

**Introduction**

Multiple myeloma (MM) is a clonal plasma cell malignancy characterized by hypercalcemia, bone destruction and renal impairment, which constitutes 10% of hematological malignancies (Kumar et al., 2017; Röllig et al., 2015). Although some patients with newly diagnosed MM have more than 10 years of overall survival, which resulted from the advances in new pharmaceuticals including proteasome inhibitors, immunomodulatory agents and anti-CD38 monoclonal antibodies (Kumar et al., 2012; Nijhof et al., 2018), MM remains mostly incurable due to the eventual appearance of resistant clones and consequential relapse. Therefore, there is an imperative need for more effective and durable treatment strategies. B cell maturation antigen (BCMA), a plasma cell surface antigen, is an attractive target for MM immunotherapy due to its high prevalence on malignant plasma cells (Bu et al., 2018). Anti-BCMA **c**himeric antigen receptor **(**CAR**)** T cell therapy has been proven to be safe and effective for relapsed and refractory (R/R) MM in previous work (Raje et al., 2019; Zhang et al., 2021; Zhao et al., 2018). The approval of Abecma (i.e., idecabtagene vicleucel, an anti-BCMA CART cell therapy) for the treatment of R/R MM by Food and Drug Administration further verified the efficacy of BCMA CART cells.

As the most frequent adverse event, 76% ~ 98% of patients experienced cytokine release syndrome (CRS) after CART cell infusion in the above studies, with 6%~45% of patients suffering from severe CRS (CRS grades 3 to 4), that is a life-threatening complication characterized by persistent high fever, hypotension, capillary leakage, coagulopathy, and severe organ dysfunction (Li et al., 2021b; Neelapu et al., 2022). To date, the clinical management of CRS mainly involve in the intervention of signaling pathways (such as IL-1 and IL-6 pathways) (Hu et al., 2016), metabolites (Staedtke et al., 2018) and adapters (Lee et al., 2019). However, CRS limits the clinical benefit of CART therapy and increases the therapeutic cost due to the necessity of close patient monitoring and corticosteroids treatments (Mahadeo et al., 2019; Neelapu et al., 2018). Thus, it is of great importance to delineate the underlying cellular and molecular mechanisms of CRS as a first step toward attenuating toxicity and boosting therapeutic efficacy of CART cell therapies.

Earlier studies on CRS mechanisms have largely focused on binary crosstalk between monocytes and CART cells. For example, Giavridis *et al.* and Norelli *et al.* demonstrated that IL-1 and IL-6 secreted from monocytes and macrophages were primary contributors to CRS in a mouse model (Giavridis et al., 2018; Norelli et al., 2018). Recently, this view has shifted to a model where CRS is the result of a multicellular network, not only a binary interaction of CART cells and monocytes. Liu *et al.* found that tumor cell pyroptosis by the pore-forming protein gasdermin E after anti-CD19 CART infusion triggered CRS through the activation of macrophages in a mouse model (Liu et al., 2020). In addition, Deng *et al.* performed single-cell RNA sequencing of anti-CD19 CART cell products in 24 patients with large B cell lymphomas and found that a rare cell population with monocyte-like transcriptional features in CART products was associated with high-grade immune effector cell-associated neurotoxicity syndrome, and revealed that high grade CRS had a negative association with exhausted CD8 T cells and a positive association with exhausted CD4 T cells (Deng et al., 2020). These studies raise the question of whether a specific cell subpopulation in CART cell products could initiate severe CRS. To date, few studies have focused on the dynamics of various cell subtypes *in vivo*. The specific mechanisms of CRS in the clinic, particularly which components of CART cell products may affect the efficacy, safety and side effects of treatment, still remain unclear. A deep understanding of the CRS mechanisms at play is essential to design the next generation of CART cell products to minimize adverse effects.

Single-cell technologies including single-cell RNA sequencing, single-cell ATAC sequencing and mass spectrometry have become powerful tools for profiling the dynamics of heterogenous cell populations in the context of CART cell potency and immunotoxicity. For example, 10X genomic single-cell RNA sequencing and TCR sequencing were employed to characterize the clonal kinetics and transcriptional profiles of CART cells in non-Hodgkin lymphoma and leukemia, and showed that clonal diversity of CART cells was highest in the CART infusion products and declined following infusion (Sheih *et al.*, 2020). In another study, Chen *et al.* found that chronic IFN signaling regulated by IRF7 was associated with poor CART cell persistence by combining bulk and single-cell ATAC sequencing (Chen et al., 2021; Xue et al., 2017). However, there is a lack of systematic and integrated multi-omic studies on CRS after CART cell therapy.

In this study, we aimed to determine the cellular source of CRS biomarkers and profile the landscape and interactions among CART and endogenous cell subtypes towards a better mechanistic understanding of CRS. We employed multi-omic single-cell profiling to characterize the transcriptome, immunome, and epigenome of peripheral blood mononuclear cells (PBMCs) from MM patients at different stages of CRS following anti-BMCA CART treatment. We identified new CRS biomarkers, uncovered their cellular sources at the initiation and peak phases of CRS, and pinpointed a key cellular subpopulation in pre-infusion CART products is closely related with CRS severity. Our work provides potential new therapeutic targets to ameliorate the clinical burden of CRS and boost the efficacy of anti-BCMA CART cell therapy as well as other CART cell therapies.

**Results**

**Clinical characterization of CRS after anti-BCMA CART cell therapy**

According to the standard of our center (Zhang et al., 2021), 61 patients with R/R MM were enrolled for a clinical trial involving anti-BCMA CART cell therapy. After therapy, we did not find obvious evidence for tumor cell persistence by positron emission tomography computed tomography (PET-CT) and immunohistochemistry (Figure 1A). Further flow cytometry revealed the frequency of tumor cells (BCMA+CD138+) reduced from 31.4% to 0.36% in patient 2 (Figure S1A). Among 61 R/R MM patients, the objective response rate was 98.3% and the complete response (CR) rate was 70.3%, including 41 patients with CR, 5 patients with very good partial response (VGPR), 11 patients with partial response (PR) and 1 patient with stable disease (SD) (Figure 1B). The severity of CRS was graded using the Lee scale (Lee et al., 2014). 98.4% (60/61) patients developed CRS, including 11.5% grade 1 (7 patients), 42.6% grade 2 (26 patients), 37.7% grade 3 (23 patients), 6.6% grade 4 (4 patients) after CART cell therapy (Figure 1C). Patients with grade 3 CRS had a higher proportion (78.3%) of complete remission (CR) when compared with patients with grade 1~2 CRS (64.7%), suggesting that patients with high CRS might have a better outcome (Figure S1B). The clinical presentations of all 61 patients which resulted from CRS were detailed in Figure 1D, which showed that CRS is a systemic disease affecting multiple organ systems, including respiratory system, cardiovascular system, gastrointestinal system, urinary system, and etc. Apart from constitutional symptoms like fever and fatigue, gastrointestinal and cardiovascular manifestations were among the most common ones in our patients, while acute kidney injury was the most severe one, which required continuous renal replacement therapy (Figure 1D). In addition, clinical manifestations in nervous system, lymphatic system, rheumatologic system and other systems were all observed in previous studies (Fajgenbaum and June, 2020). Although CRS was reversible in all patients, corticosteroids and/or tocilizumab (an anti-IL-6 receptor monoclonal antibody) were required in nearly half of the patients (n = 27) (Zhang et al., 2021). However, complications caused by grade 4 CRS may be life-threatening evidenced by two deceased patients: patient 34 died of cerebral hemorrhage 8 days after CART infusion and patient 51 died of infection 14 days after CART fusion, which was consistent with the results of Zhang group (1 patient die 22 days after CART infusion) (Zhao et al., 2018) and Kochenderfer group (1 patient die about 30 days after CART infusion) (Raje et al., 2019). The high incidence, along with the severe and various manifestations, demonstrated that CRS is a pressing issue in CART therapy.

**Flow cytometry and multiplexed cytokine assay revealed the dynamics of CART cell and distinct CRS biomarkers in peripheral blood**

To better demonstrate the mechanisms underlying CRS procedure following CART cell therapy, we obtained the multimodal data pertaining to CRS-related parameters by routine clinical blood results, multiplexed cytokine assay and multi-omic single-cell sequencing (Figure 1E). In stark contrast with the steep decline of tumor cells in peripheral blood (PB), CART cells expanded significantly during the first 2 weeks after infusion. Then, CART cells sustained a relatively stable population in the following 20 days and were still detectable at 2 months after infusion (Figure 1F and S1C). The trends of CAR copy number quantification further supported the dynamic change of CART cells (Figure S1D).

At the CRS peak stage (CRSp), we detected an average increase in 2~4 degrees of body temperature in patients (Figure S1E), and the levels of known CRS biomarkers (CRP, ferritin, IL-6 and IFNγ) changed significantly in the majority of patients with CART cell therapy by the routine clinical blood results (Figure S1F). To identify new CRS biomarkers, we performed a multiplexed cytokine assay on 6 patients’ plasma samples before fludarabine and cytoxan chemotherapy (FCb), before CRS develop (CRSb), at the CRS initiation (CRSi), CRSp and CRS recovery stage (CRSr) (Figure 1E) and revealed a dynamic expression of cytokines across the course of CRS, which was in agreement with the clinical blood test results (Figure 1G and S1F). The 24 cytokines enriched at CRSp included IL-1β, IL-3, IL-6, GM-CSF, CCL20, TNFα, IFNγ and GZMB (Figure 1H). Interestingly, the highly expressed cytokines at CRSi were IL-2, IL-4 and IL-17A, pointing to their potential stage-specific roles in CRS initiation stage. Except for IL-2 known for T cell activation, IL-4 and IL-17A were our newly discovered biomarker at CRSi, consistent with previous studies that IL-4 enables strong and highly selective expansion of functional CD19 CART cells (Ptáčková et al., 2018) and Th17 (secreting IL-17) cells engineered with a CAR regressed large human tumors to a greater extent both *in vitro* and in mouse model (Nelson et al., 2020).

Together, we identified 24 CRS-related biomarkers at the CRSp stage and 3 initiation biomarkers at CRSi stage, which greatly expanded the known pool biomarkers for CRS in the clinic.

**Transcriptome landscape of CART and endogenous cell subsets by scRNA-sequencing**

To further decipher the dynamics of contributing cell types during anti-BCMA CART therapy and subsequent CRS mechanisms, we used scRNA-seq to characterize the cellular landscape using samples from 4 patients (patients 1 and 2 with grade 3 CRS, and patients 3 and 4 with grade 2 CRS) across the course of CRS (at FCb, CRSb, CRSp and CRSr time points). We could not isolate any CART cells by FACS sorting at CRSi because of the limited cell number and safety concerns surrounding in blood collection from patients at this stage (Figure 1D). We used the R package Seurat 3.0 with Harmony to remove batch effects, and uniform manifold approximation and projection (UMAP) visualization of all 28 samples revealed that all samples were well integrated (Figure 2A, 2B and S2A). Cells from all samples, patients, and time points were interspersed across multiple clusters (Figure 2B), and all clusters contained cells from multiple samples, while CART and endogenous T (EndoT) cells were also partially integrated (Figure 2C and 2D). Detailed sample and cell information was summarized in Table S1.

We exploited previously described marker genes and the R package SingleR (Zhao et al., 2020) to define cell types and functional states using the scRNA-seq data. We identified 10 clusters (Figure 2D), including cycling T cells, CD4 T cells, CD8 T cells, natural killer (NK) cells, T regulatory cells (Treg), plasma/tumor cells, CD14 monocytes, CD16 monocytes, dendritic cells (DC) and platelets, all defined by characteristic gene expression profiles (Figure 2E, 2F and S2B). Based on these profiles, we analyzed the expression patterns of specific cell subset marker genes and defined the differentially expressed genes (DEGs) of each cell type (Table S2).

The relative abundances of CART and non-CART cell subsets in PBMCs varied across the time course (Figure 2G and 2I). Among the CART cells, the percentage of cycling T cells significantly decreased from CRSp to CRSr, while the percentage of CD8 T cells significantly increased and the percentage of CD4 T cell remained constant (Figure 2G). CART cells at CRSb were mainly in the active state shown by the enrichment of oxidative phosphorylation, proteasome, cell cycle and carbon metabolism pathways. CART cells at CRSp were largely enriched in the effector state evidenced by the pathways involved in the natural killer (NK) mediated cytotoxicity and chemokine signaling pathways. CART cells at CRSr were predominantly in the dysfunctional state as shown by the enrichment of cellular senescence and apoptosis pathways (Figure 2H). The relative abundance of monocytes declined from CRSb to CRSp, suggesting a potential role for monocytes in the initiation of CRS with significant monocyte decrease at later stages of CRS (Figure 2I and S2C). Moreover, we found that the percentages of endogenous CD8 T cells and NK cells increased by the peak and recovery phases of CRS (Figure 2I), suggesting that these cell types switch from an inactive state to an active killing-competent state following CART cell therapy and across the progression of CRS (Figure S2D and S2E). In addition, in both CART and EndoT cells, the cell cycle and cytotoxic score analysis demonstrated that cellular proliferation was decreased and cytotoxicity was increased from CRSi to CRSr (Figure S2F). We also observed that CART cells were first amplified and maintained in a high proportion, (about day 7~day 21) and then decreased (day 21~day 55), while EndoT cells showed an opposite trend (Figure 2J).

Altogether, we show that across the course of CRS, CART cells experienced a transition from active to effector or dysfunctional state. Meanwhile, CART treatment activated the endogenous immune system including EndoT, NK and myeloid cells.

**Transcriptome uncovered the diverse source of CRS biomarkers and cell-cell interaction network by scRNA-sequencing**

Taking into account our multiplexed cytokine data as well as previous literature, we next screened a list of 80 inflammatory genes (Table S3) and detected 34 important cytokines (Figure 3A). Looking across our CRS time course in the patients, we carefully analyzed the cells that secreted individual cytokines at different CRS stages. At CRSb, we detected significant production of 8 cytokines by CART products, including IL-17A, IL-13, IL-2 and TNFα, which partially explained the specific biomarkers of CRSi stage (Figure 1E). CART cells highly expressed CSF2, IFNG and IL-13 at CRSp, indicative of CART mediated activation of monocytes and endogenous T cells, which were concordant with previous studies that IFNγ and IL-13 were critical for CAR T cell-mediated myeloid activation and induction of endogenous immunity (Alizadeh et al., 2021). We also found significant production of 17 of these cytokines by monocytes at CRSp, indicative of the important role of monocytes in CRS. At CRSr, endogenous NK and EndoT cells released high levels of GZMB/CCL4 and IL2/IL2RA/PDCD1/FLT3LG, respectively (Figure 3A).

To elucidate the interaction network among different cell types, we predicted ligand-receptor (L-R) pairs and molecular interactions among different cell clusters with the R package CellPhoneDB (Vento-Tormo *et al.*, 2018) and analyzed the specific signaling pathways in PBMCs at different times after CART product infusion with the R package CellChat (Jin *et al.*, 2021). Many ligands for which cognate receptors were expressed among different clusters, especially monocytes and dendritic cells, demonstrated extensive strong interactions in different cell types (Figure 3B and Figure S3A). The differential interaction strength between CRSp and CRSb suggested that CART cells play crucial roles in activating EndoT cells, monocytes, and NK cells (Figure 3B), while that of CRSr and CRSp suggested that the functions of EndoT cells, NK cells and monocytes were improved (Figure 3C).

A total of 149 significantly differential L-R pairs were identified (Table S7). Among these LR pairs, the most frequent ligands and receptors were involved in the TNF pathway (30 pairs) and CCL pathway (12 pairs). Furthermore, gene ontology (GO) and KEGG enrichment analyses showed enrichment in lymphocyte activation, cell adhesion, cytokine-cytokine receptor interaction and natural killer cell-mediated cytotoxicity. Owing to the important role of CART cells and monocytes in CRS, we found that their specific L-R pairs largely overlap (such as TNF, CCL, CD40 and IFNG pairs), supporting the notion that CART might activate monocytes (Figure 3D, 3E and 3F). The strength of these specific L-R pairs at CRSb, CRSp and CRSr is also shown for CART cells (Figure S3B and S3C), EndoT cells (Figure S3D and S3E), NK cells (Figure S3F and S3G), and plasma/tumor (Figure S3H and S3I). Among them, CD40LG-(ITGA5/ITGB1), IL2-(IL2RB/IL2RG), IL1B-IL1R2 and IL6-(IL6R/IL6ST) generally play similar roles in CRSb, CRSp and CRSr, while TNF-TNFRSF1A/1B is differentiated and complex (Figure 3E and Figure 3F). Furthermore, these differentially expressed LRs were mainly enriched in the CD40, IFN-II, FLT3, CCL, IL-1, IL-2, IL-6 and GRN pathways (Figure 3G and Figure S3J). Among them, CART and EndoT cells appeared to be largely responsible for the production of IL-2, IFN-II, CD40, FLT3 and CCL, while IL-1, IL-6, GRN, IFN-I, CSF and BAFF (TNFSF13B) were the significant pathways involved in monocyte activation (Figure 3G and Figure S3J). The VEGI (TNFSF15) and CD30 (TNFRSF8) pathways were specifically involved at the CRS peak (Figure S3K). Importantly, the TNF signaling pathway is closely associated with the initiation and development of CRS after CART cell infusion. However, the TNF pathway was mainly enriched in CART cells at CRSb, while it was enriched in monocytes at CRSp and CRSr (Figure 3H).

In summary, these data suggested that expression of CRS-related cytokines was dynamic across CRS stages, reflecting the varied immune microenvironment and cell-cell interaction network as CRS progresses and recovery.

**Transcriptome and immunome illustrated the trajectory and clonal diversity of CART across the CRS course by scRNA-seq and scTCR-seq**

The trajectory and clonal diversity of CART and EndoT cells are significantly related to their efficacy (Sadelain et al., 2017) and persistence (Melenhorst et al., 2022) *in vivo*. To examine on T cell development in our patient cohort, CART and EndoT cells were separated from the peripheral blood cells based on CD45 and CD3E expression. All the T cells from the same 4 patients at different time points (total 28 samples) were integrated well without any apparent batch effects (Figure 4A-D and Figure S4A). CART and EndoT cells also clustered with each other (Figure 4C) and could be classified into 8 clusters: CD4 naive T cells (Tn), CD4 central memory T cells (Tcm), CD4+GZMB+ effector T cells (Teff), CD4+PRF1 effector T cells (Teff), T regulatory cells (Treg), PRF1+GZMB+ gdT cells, CD8 central memory T cells (Tcm), and CD8 effector memory T cells (Tem), all of which were defined by characteristic gene expression profiles (Figure 4D-E, S4B-C and Table S4).

To explore the developmental trajectory of CART and EndoT cells across the stages of CRS, we employed the R package monocle2. The trend of pseudotime and real time was consistent, indicating a transition from the naive/memory state to the effector memory state both in CART and EndoT cells, including both CD4 and CD8 subsets of each (Figure 4F and 4G). For CART cells, the percentage of CD4 Tn cells significantly decreased after CART cell infusion, while that of CD8 Tem cells significantly increased (Figure 4F). There was a similar but less dramatic pattern for endogenous CD4 and CD8 cells, as well as a mild increase in the proportion of endogenous Treg and gdT cells across CRS (Figure 4G). We confirmed the trends in T cell dynamics that we observed from the transcriptome data by multicolor flow cytometry to quantify relative abundances of naive T cells (CCR7+CD45RA+ T cells), central memory T cells (CCR7+CD45RA- T cells, Tcm), effector T cells (CCR7-CD45RA+ T cells), and effector memory T cells (CCR7-CD45RA- T cells, Tem) in both CD4 and CD8 T cell subsets (Figure 4H). All of these cellular dynamics were generally consistent with our scRNA-seq results. Intriguingly, expression of T cell exhaustion markers (LAG3 and PD1) were gradually increased in CART and EndoT cells at later stages of CRS, concordant with the elevated percentage of Tem and Teff cells (Figure 4I).

To determine how the CART and EndoT cell populations change *in vivo* after CART cell infusion, we next examined the T cell receptor (TCR) repertoire over the course of CRS. We used high-throughput single-cell sequencing of the TCR CDR3 region in CART and EndoT cells at FCb, pre-infusion CART products, CRSb, CRSp, and CRSr to profile the clonal diversity over time and understand the trajectory of high-abundance clones (Figure 1D). We found that the clonal diversity of CART cells was highest at CRSp and declined throughout the course of CRS (the percentage of the top 3 clones at CRSr was shown in Figure 4J). The clonal diversity of EndoT cells showed a similar tendency as CART cells, with a lower TCR diversity in EndoT cells compared to CART cells at each stage of CRS (Figure 4J). We noted obvious oligoclonal expansion of CART cells in three out of four patients (patients 2, 3, and 4). Patient 1 did not exhibit oligoclonal expansion, but notably their CART cell persistence was low as well (Figure S4D). These results suggested that decreased clonal diversity after infusion might be partially due to expansion of oligoclonal CART cell populations and that the proportion of oligoclonal expanded CART cells was positively correlated with the persistence of CART cells *in vivo.* A comparison of the top CART cell clones’ transcriptome profiles with the UMAP landscape from our earlier analysis revealed that these expanding CART clones were predominantly CD8 effector memory cells, with high expression of FOS, PRF1 and GZMB (Figure 4K and S4E), and mainly enriched in pathways related to cell adhesion molecules, antigen processing and presentation, T cell receptor signaling pathway and phagosome (Figure 4L).

Overall, integrated analysis of single-cell RNA-seq and TCR-seq revealed that naive or memory CART cell differentiated into effector or memory CART cells after infusion, and the expanding CART clones at CRSr were mainly CD8 effector memory cells, not CD4 T cells, which was in line with previous observations that TCR engagement might negatively affect CD8 but not CD4 CAR T cell expansion and leukemic clearance (Yang et al., 2017).

**Epigenome deciphered the cis-regulatory network in CART cells across the course of CRS by scATAC-seq**

Given the major changes we observed in gene expression and cell surface markers in CART cells across the progression of CRS, we next turned our attention to their cis-regulatory networks. Using single-cell ATAC-seq (scATAC-seq), we explored the dynamic changes in chromatin accessibility in CART cells during CRS progression and identified differentially accessible regions (DARs) of CART cells at CRSb, CRSp and CRSr (Figure 1D). All 6 samples from 2 patients (patient 1 and patient 2, both with grade 3 CRS) were integrated well in UMAP that passed quality control (Figure S5A-S5E). Cells from all samples and different time points were interspersed across multiple clusters (Figure 5A), and all clusters contained cells from multiple samples (Figure 5B) (Table S5). Next, we clustered the cells into 5 groups based on the similarity of their scATAC-seq profiles (Figure 5B and 5C): CD4 naive T cells (CD4 Tn), CD4 central memory T cells (CD4 Tcm), CD4+PRF1+ effector T cells (CD4 Teff), CD8 central memory T cells (CD8 Tcm), and CD8 effector memory cells (CD8 Tem). Out of the total CD4 cell population, the percentage of CD4 Tn cells was dramatically decreased, while the percentage of CD4 Tcm cells increased as CRS progressed. The relative abundance of CD8 Tcm cells decreased after CART cell infusion, and the percentage of CD8 Tem cells was significantly elevated at CRSp and CRSr compared to the earlier time point (Figure 5C), agreeing with our above results from the scTCR-seq and scRNA-seq analyses (Figure 2G and 4F). We performed integration analysis of scRNA-seq and scATAC-seq data (Figure S5F and S5G), showing that the expression patterns of characteristic genes from each cell subset largely agreed with the changes in abundance of their respective cell types; for example, GZMB and IFNG increased over the progression of CRS, consistent with the increase of CD8 T cells, which are major producers of these proteins (Figure 2E, 5D and 5E).

We detected dramatic changes in the chromatin accessibility landscape of CART cells across CRS progression. CART cell products were enriched in chromatin accessibility at these loci related to Th1, Th2 and Th17 cell differentiation, cytokine-cytokine receptor interaction and the JAK-STAT signaling pathway (Figure 5F). The apparent potential of CART products for multidirectional differentiation to Th1, Th2 or Th17 of CART products suggested that CART products were heterogeneous populations. This could imply that particular CART cell subsets might participate in the initiation of CRS. For example, the high expression of TNFα and IL-2 in CART products may be secreted from Th1 cells, while IL-13 in CART products may be released by Th2 cells and IL-17 in CART products may be produced by Th17 cells (Figure 3A). In CART cells at CRSp, we detected DARs related to the activation of the chemokine signaling pathway, T cell receptor signaling pathway and MAPK signaling pathway. In contrast, CRS recovery-stage CART cells were characterized by chromatin accessibility changes in regions associated with cell cycle and cellular senescence (Figure 5F) (Table S6). To provide more clues into how these chromatin accessibility changes may influence the transcriptional program, we identified transcription factor binding motifs overrepresented in the DAR sets for CART cells at each stage of CRS (Figure 5G). We found that motifs for T cell activation-related transcription factors (TFs) (e.g., NFKB1) and JUN and were enriched in DARs of CART cell products prior to infusion, while the motifs enriched in DARs of CART cells at CRSp were related to T cell differentiation and effector functions, including EOMES, IRF1 and RUNX1. At CRSr, we noted an enrichment of DARs at T cell memory genes, such as KLF2 and SMAD2 (Figure 5H). In accordance with these findings, the loci of NFKB1, EOMES, IRF1 and RUNX1, and KLF2 were markedly accessible at CRSb, CRSp and CRSr, respectively (Figure 5I). Apart from changes across the stages of CRS, we also examined the DARs between CD4 Tn and CD8 Tem clusters, identifying IL1R1/IL6R and GZMB/PRF1/RUNX3 loci, respectively (Figure S5H). Furthermore, KEGG analysis of the DARs in CD4 Tcm cells showed enrichment in both Th1 and Th2 cell differentiation, while CD8 Tem clusters displayed increased chromatin accessibility in the chemokine signaling pathway, T cell receptor signaling pathway, and apoptosis (Figure S5I). In addition, the loci of proinflammatory genes (e.g., TNF) were accessible in subpopulations annotated as naive T cells, which mainly belonged to CART products prior to infusion (Figure S5J). In addition, cytotoxic gene loci (e.g., PRF1 and IFNG) were enriched in the highly accessible DARs of subpopulations annotated as effector T cells, which mainly belong to CART cells at CRSp and CRSr (Figure S5J).

To sum up, these combined analyses of scATAC-seq and scRNA-seq data imply that CART cells exhibited distinct cis-regulatory network and chromatin accessibility at different CRS stages.

**CD4+CD40LG+ CART cells in pre-infusion products were associated with the severity of CRS**

It is of utmost importance to identify the initiating factors of CRS, which might help predict CRS risk, detect CRS early and minimize the adverse events of CRS. To determine the contribution of each cell subset in inducing CRS, we next sought to compute a CRS score by calculating the mean expression level of 34 CRS biomarker-related genes (Figure 3A) in each cell type. We found that the CRS scores of monocytes and dendritic cells were the highest among all clusters, consistent with previous work showing their crucial contributions to CRS (Figure 6A). Interestingly, some outlier cells (CRS score >1.5\*(upper quartile- lower quartile) + upper quartile) in CART cells were observed (Figure 6A), among which most cells (1386/1410) were from pre-infusion CART products. Their high CRS scores suggested that they might play pivotal roles in CRS, and we will refer to them as CRS-associated clusters (CRSCs) going forward. We indicated the location of CRSCs in the UMAP plot o in CART cell products using data from 4 patients (Figure 6B and Figure 6C). Interestingly, CRSCs was mainly CD4 T cells (Figure 6B and 6D) and appeared to be more abundant in patients with high-grade CRS (patient 1 and 2, grade 3 CRS) when compared with patients with low-grade CRS (patient 3 and patient4, grade 2 CRS) (Figure 6C). The most notably up-regulated genes in CRSCs compared with other CART cells included CD4, CD40LG, IL-13 and TNF (Figure 6D and 6E), encompassing pathways like cytokine–cytokine receptor interaction, JAK-STAT signaling pathway, T cell receptor signaling pathway, T helper cell differentiation and TNF signaling pathway (Figure 6F). At the epigenetic level, CD4, CD40LG and IL-13 loci exhibited higher chromatin accessibility in CART cell products prior to infusion compared with CART cells at CRSp and CRSr (Figure 6G). These data suggested that CRSCs represent a highly activated and pro-inflammatory CD4 T cell subset of pre-infusion CART products that may be associated with severe CRS (Figure 6F and 6G).

To examine which features in our multi-omics dataset were associated with CRS severity, we first compared the clinical and cellular features between patients with high-grade vs. low-grade CRS. We found that higher tumor burden and higher percentage of CD4 CART cell products were both associated with increased CRS severity (Figure 6H). Furthermore, the percentage of CD40LG+CD4+ CART cells in high-grade CRS patients (approximately 52% of total CART cells) was significantly higher than that in low-grade CRS patients (approximately 34%) (Figure 6I). We conducted transcriptomic analysis of this cell subset, revealing that these CD4+CD40LG+ CART cells have up-regulated T cell activation pathways (such as JAK-STAT and PI3K-Akt pathways) and inflammation pathways (such as TNF, NF-κβ and IL-17 pathway) (Figure S6A, S6B and S6C). Epigenetic analysis using scATAC-seq revealed enrichments for open chromatin at loci encoding these same pathways, as well as enrichment for ARID3A motifs (Figure S6D and S6E). All of these findings point to a potential role for these CD40LG+CD4+ CART products in activating monocytes through interactions between CD40LG and either CD40 or a5b1/a2Bb3 complex, based on our interaction analysis (Figure 3E and S3B), consistent with previous study (Giavridis et al., 2018). The relative abundance of IL-13+CD4+ CART cells showed similar trends as the CD40LG+CD4+ subset, with enrichment in high-grade CRS patients (Figure 6J). This was concordant with high expression of IL-13 and a recent finding that human IL13Rα2-CAR T cells can activate patient-derived endogenous T cells and monocytes/macrophages (Alizadeh et al., 2021). These data suggested the above specific CD40LG+IL-13+CD4+ subpopulation of CART cells, which we refer to as CRSCs, may activate endogenous monocytes and T cells to initiate CRS process.

To functionally investigate the role of CD4+CD40LG+ subsets in CRS and explore the interactions among CD4+CD40LG+ CART products, tumor cells and monocytes, we used a BCMA+ H929 cell line to model MM tumor cells and performed cytokine arrays in samples collected from co-cultured immune cell subsets. We used the THP1 monocyte-like cell lineto mimic monocytes, and FACS-sorted fresh CART cell products prior to infusion from other new patients into the following subsets: CD4+CD40LG+ CART, control CART (total CART products except for CD4+CD40LG+ CART) and EndoT cells. For many of the monocultures and binary co-cultures we investigated, we detected little to no production of CRS-related cytokines in the supernatant after 24 hours. However, CD4+CD40LG+ CART released some CRS related factors (such as TNFα, GM-CSF, IL-2, CCL4, IFNγ and GZMB) after co-culture with MM tumor cells, which also increased with time. We further showed that the supernatant of CD4+CD40LG+ CART + MM (24h) activated monocytes and caused them to release a large number of CRS related cytokines, including IL-1β，IL-6, CXCL1/2, and PDGF-AA (*P* < 0.05) (Figure 6K). These findings held true when we performed similar experiments using CART products derived from two additional patients (Figure S6F and Figure S6G), suggesting that certain CRS-related cytokines (TNFα, CCL20/MIP-3α, IL-3, IL-4 and IL-17A) were released in large amounts as a result of CD4+CD40LG+ CART cells making contact with the tumor cells, and those factors in turn activated monocytes to produce IL-1β, IL-6, CXCL1/GROα and PDGF-AA (Figure 6L).

Taken together, the presence and abundance of the CD40LG+IL13+CD4+ cluster in pre-infusion CART products was associated with higher CRS grade, and our cellular assays show that this CART subset may recognize tumor cells and then activate endogenous monocytes and T cells to initiate CRS.

**Discussion**

CRS remains a challenging and detrimental side effect that must be overcome in order to achieve the full potential of CART therapies. In our present study, we identified a key subpopulation of cells within pre-infusion CART products (CD40LG+IL-13+CD4+ clusters) closely related to CRS severity as well as 24 CRS-related biomarkers at the CRS peak stage. Based on our multi-omic analyses and verification in clinical samples, we propose a three-stage model of the CRS process after CART cell therapy (Figure 7): 1) CRS initiation (CRSi) stage: the above key subpopulation recognizes tumor cells and releases specific cytokines (such as IL-2, IL-4 and IL-17A) to activate monocytes and endogenous T cells; 2) CRS peak (CRSp) stage: the above factors continuously activate CART, monocytes, endogenous T cells and NK cells, which produce a large number and amount of cytokines (such as CCL20, CXCL1, IL-1β and IL-6) and trigger CRS; 3) CRS recovery (CRSr) stage: after killing the tumor cells, the number of CART cells decrease while endogenous T and NK cells increase, and both CART and EndoT cells undergo clonal expansion.

Previous work had identified the classical cytokines IL-1, IL-6 and ferritin as CRS biomarkers, and we have greatly enlarged this list to include IL-3, CCL20 (MIP-3α), CXCL1 (GROα) and other factors. Moreover, we have determined their dynamic and individual cellular sources (Figure 3A). Our findings suggest that, in addition to CART cells and monocytes that have been reported to participate in CRS, activated EndoT cells and NK cells both contribute to the cytokine storm at the peak of CRS during anti-BCMA CART cell therapy. These results were in agreement with recent work showing that anti-CD19 CART cell therapy in large B cell lymphoma patients activated bystander immune cells (including EndoT cells and monocytes), which subsequently constituted the exclusive source of IL-6 (Chen et al., 2020). Meanwhile, we also identified biomarkers at early stages of CRS (CRSi), including IL-2, IL-4 and IL-17A, which may become potential intervention targets for CRS management and may help stratify patients with limited or failed CART cell expansion.

In addition to the above cytokines, the results of crosstalk further showed that the IL-1, IL-6, IFN-I, BAFF, CSF and GRN pathways were significantly involved in monocyte cells, while the IL-2, IFN-II, FLT3 and CCL pathways were involved in CART cells and the FGF and GAS pathways were involved in tumor cells. To date, anti-IL-1R antagonist anakinra, anti-IL-6R tocilizumab, anti-IL-6 siltuximab, anti-TNFα infliximab and anti-IFNγ emapalumab have been used in the management of CRS (Fajgenbaum and June, 2020). In addition, BAFFR has also been used as a CAR target for the treatment of acute lymphoblastic leukemia (Wang et al., 2022) and lymphoma (Qin et al., 2019). Therefore, our list of CRS-implicated pathways agrees with and extends past work in the area of CRS management and provides new potential therapeutically targetable pathways to add to CRS treatment or prevention options. Interestingly, we found that TNF was mainly produced by CART cells at CRSb, whereas it was produced by monocytes at later stages of CRS, raising the possibility that the TNF pathway could be manipulated to help facilitate CART activation in patients with limited or failed CART therapy.

Previous studies mainly focused on the overall characteristics of CRS. However, the dynamics of immune cell populations over time to elucidate initiating, effector, and recovery factors remain largely unknown. Our studies showed that CART cells and EndoT cells were in a highly cytotoxic state at the peak phase of CRS, in agreement with our previous work demonstrating that CART cells at the peak phase gradually converted to a highly cytotoxic state from a highly proliferative state along their developmental trajectory (Li et al., 2021a). Furthermore, our functional pathway analysis suggested a switch from proliferation to cytokine production to apoptosis for CART cells across the phases of CRS (Figure 2H). By tracking the clonal diversity of CART and EndoT cells across these phases, we showed that TCR diversity increased up until the CRS peak and then decreased (Figure 4J). We noted clonal amplification of oligoclonal CART cells that was positively correlated with CART persistence (Figure 4H). These results spark that a specific amplified seeds in CART cell products prior to infusion were exiting, which was in consistent with a recent work that CART cells at later time points (days 26~30 after infusion) showed clonal amplifications (Sheih *et al.*, 2020). However, the clonal expansion of EndoT cells may be caused by recognition of released antigens upon tumor cell killing. This hypothesis could explain the observation that the proportion of clonally amplified EndoT cells was higher than that of CART cells, although the first top 3 clones were less abundant than those of CART cells. At the recovery stage, CD4 CART and EndoT cells were mainly central memory cells, while CD8 cells reached a balance between effector memory and central cells (Figure 4G). Altogether, our results suggest that TCR engagement might negatively affect CD8 but not CD4 CAR T cell expansion, which would be consistent with previous observations *in vitro* and mouse model (Yang et al., 2017).

An important contribution of this study is our identification of a CD4+IL-13+CD40LG+ subpopulation of the CART cell product that is associated with the severity of CRS, which we named the CRS-associated cluster (CRSC). Our *in vitro* co-culture model clearly demonstrated the ability of these cells to highly express several cytokines in response to tumor cells, and to subsequently drive monocyte stimulation. This conclusion is consistent with the previous finding that CD40L-overexpressing murine CART cells prompted more severe CRS symptoms, sustained weight loss, and markedly increased mortality in a mouse model of CRS (Giavridis et al., 2018).

Overall,we provide a comprehensive multi-omics resource identifing key factors, critical cell subsets, the TCR repertoire and the cis-regulatory network in MM patients receiving anti-BCMA CART cell therapy. These data not only facilitate a more in-depth biological understanding of the cellular and molecular mechanisms at distinct stages of CRS after CART therapy, but also provide the possibility for clinical assessment CRS risk, detection and intervention at early time points of CRS, and enhancing the efficacy of CART therapy for MM and potentially other types of cancer.

**Materials and methods**

**Patient enrollment and clinical design**

Patients with relapsed/refractory multiple myeloma were enrolled in the clinical trial (Chictr.org number, ChiCTR1800017404). The study was conducted in accordance with the principles of the Declaration of Helsinki as well as with the approval of the First Affiliated Hospital of Zhejiang University. All patients in this study signed informed consent forms. The manufacturing process of anti-BCMA CART cells, patient demographics and clinical characteristics was provided in our previous studies (Zhang et al., 2021). Multiple myeloma response assessment was conducted according to the International Uniform Response Criteria for Multiple Myelomas (Rajkumar et al., 2014). Cytokine release syndrome (CRS) was graded as previously described (Lee et al., 2014). The clinical trial is completed and the related clinical data was published in clinical cancer research in 2021, with the title of “Risk Factors Associated with Durable Progression-Free Survival in Patients with Relapsed or Refractory Multiple Myeloma Treated with Anti-BCMA CAR T-cell Therapy” (Zhang et al., 2021). Meanwhile, to explore the dynamic changes of various cells in peripheral blood and the mechanism of side effect CRS during anti-BCMA CART cell therapy, the multi-omic analysis was pre-specified for in the clinical trial protocol.

**Sample collection, detection, and preparation**

We collected CART cell products as well as 4 peripheral blood samples at 4 time points as follows: FCb, CRSb, CRSp (based on the cytokine levels and clinical manifestations) and CRSr (based on the MRD test). CART cells (CD3+CAR+) and non-CART cells (CAR-) were isolated from fresh peripheral blood by fluorescence-activated cell sorting (FACS). In addition, dynamic changes in cell subpopulations (such as CART, EndoT and tumor cells) in PBMCs and related cytokines (such as IL-1, IL-6, IFNγ and TNFα) in plasma were detected every day by flow cytometry as described in more detail below.

**Flow cytometry, multicolor flow cytometry and fluorescence-activated cell sorting (FACS)**

Antibodies against CD3, CD4, CD8, CD45RO, CD62L, PD-1, TIM-3 and LAG-3 were used to identify the different subtypes of CART and EndoT cells. All antibodies and isotype controls were purchased from Biolegend. The subsets of CART cells were defined as naive T (CD45RO−/CD62L+), central memory T (CD45RO+/CD62L+), effector memory T (CD45RO+/CD62L−) and effector T (CD45RO−/CD62L−) cells. The tumor/plasma cells were defined by BCMA and CD138. The exhaustion markers of CART cells mainly included PD-1, TIM-3, and LAG-3. All samples were tested by the CytoFLEX Lx Flow Cytometer (Beckman Coulter) and data were analyzed using FlowJo X 10.0.7r2 (FlowJo LLC). All antibodies and detection reagents used in this study are listed in the key resources table (Table S8).

**10X single-cell RNA and TCR sequencing and data analysis**

Cells for single-cell sequencing were isolated from cryopreserved aliquots of CART cells or peripheral blood mononuclear cells (PBMCs). 10X Chromium Single Cell V(D)J Reagent Kits, including 5’ library & gel bead kit(PN-1000006), library construction kit (PN-1000020), V(D)J enrichment kit (PN-1000005), chip kit (PN-120236) and i7 multiplex kit (PN-120262), were purchased from 10X Genomics.

In short, cells at 1\*106 cells/mL were resuspended in DPBS with 0.04% BSA at first. Second, gel beads-in-emulsion (GEMs) were generated, and a reverse transcription reaction was performed to acquire cDNA in the PCR instrument. Then, a scRNA-seq library and TCR library were generated from cDNA according to the manufacturer’s protocol. Libraries were sequenced on the Illumina HiSeq platform by Hangzhou Repugene Technology Co., Ltd. All detection reagents used in this study are listed in the key resources table. The raw sequence reads of scRNA-seq and scTCR-seq generated in this study have been deposited in the Genome Sequence Archive (GSA) of the China National Center for Bioinformation (CNCB) under accession number (subPRO013723).

All raw sample data were separately demultiplexed and aligned to the human genome GRCH38, and the gene (unique molecular identifier, UMI) count of each cell was estimated using the Cellranger toolkit (Version 3.0.0, 10X Genomics) with default parameters. Cells with fewer than 200 detected genes, greater than 20,000 detected transcripts, and a total percentage of mitochondrial gene expression exceeding 20% were excluded from the analysis. Genes expressed in fewer than 5 cells were also removed. Then, 28 sample matrices from 4 time points and 4 patients were integrated by using the Seurat (Version 3.0.0) and Harmony R packages. The above data were normalized using the normalizeData function in Seurat (LogNormalize method with a scale factor of 10000). Anchors were identified using FindIntegrationAnchors with 1-50 dimensions and 2000 anchor features. An expression matrix of approximately 12000 cells with an average of 2000 genes per cell was obtained for further analysis. Downstream analysis after integration included data feature scaling (ScaleData), principal component analysis (PCA, RunPCA) and SNN graph building (FindNeighbors). The first 50 dimensions were used for UMAP reduction with the RunUMAP function. Cells were clustered using the FindClusters function in Seurat with resolution = 0.6. A total of 10 clusters were defined by common markers, with marker genes identified using the FindMarkers function. The name of the cell subgroup was identified by single R and classical cluster markers. All code, including the analysis code at the levels of RNA, TCR and ATAC was provided together on GitHub (https://github.com/lixia2017/CRS-after-BCMA-CART-for-MM).

**Communication networks identified by the R package CellChat and CellPhoneDB**

Analyses were performed with the use of R (v4.0.0) software. The R package “CellChat” (v0.0.2) and software “CellPhoneDB” (v2.0.0) were applied for cell–cell interaction analysis among different cell clusters. Cell–cell interactions were calculated based on the expression of ligand–receptor pairs stored in the database by CellPhoneDB with default parameters. The total number of interactions between cell types was plotted with the “heatmap plot” function. Cell–cell interactions among different cell clusters were also calculated by CellChat and visualized using the “netVisual\_circle” function. The "weight.scale" was set, and the "groupSize" was used as the weight of the vertex. The numbers of inferred communication nodes between different CRS stages were compared and visualized with circle plots. Specific significant signaling pathways in each cell type were shown using a heatmap generated by CellChat. Individual ligand–receptor pairs with inferred communication probability and *P value*s were extracted from CellChat objects. Specific significant signaling pathways in each cell type between each CRS stage were presented using a bubble plot generated by ggplot2. Ligands and receptors that were significantly enriched in each cell type (*P value* <= 0.05) were used to perform KEGG/GO enrichment analysis with the R package “clusterProfiler” (v4.0.5). The cutoff of the *P value* was set at 0.05, and the top 20 categories were displayed in dotplots.

**scATAC sequencing and data analysis**

CART cells, which were used to build single-cell ATAC-seq libraries, were collected as described in the scRNA-seq section. CART cell nuclear extraction, Tn5 transposition and library construction were performed according to the manufacturer’s instructions for 10x Genomics Chromium Next GEM Single Cell ATAC Reagent Kits v1.1. The libraries were sequenced on a NovaSeq6000 platform (Illumina). The raw data of scATAC-seq used in this study have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0002442.

scATAC-seq data analysis: Cellranger-ATAC (v1.1.0) was used to perform scATAC-seq data processing, including alignment, deduplication, peak calling and generation of a cell × peak raw count matrix composed of all samples. Quality control, dimensional reduction and clustering were performed with the R package Signac (v1.1.0), which is an extension of the R package Seurat for the analysis of single-cell chromatin datasets.

The peaks of signals detected in more than 10 cells were retained. The cells were retained with peak region fragments > 5000, peak region fragments < 50000, blacklist ratio < 0.01, nucleosome signal < 10, percent reads in peaks > 20, mitochondrial ratio < 20 and TSS enrichment score > 2. The peaks on the Y chromosome were removed to eliminate the influence of sex. Then, the filtered matrix was used for linear dimension reduction by latent semantic indexing (LSI). Briefly, we performed term frequency-inverse document frequency (TF-IDF) normalization, and then the peaks present in >10 cells were used to run singular value decomposition (SVD) on the TD-IDF matrix by function RunSVD. The R package Harmony was used to remove the batch effects between samples with the default settings on the first 50 LSI components. Because the second component result in Harmony was closely associated with cell read depth, we used 1 and 3:20 components to perform cell clustering and nonlinear dimensionality reduction (uniform manifold approximation and projection, UMAP) by Signac function FindNeighbors, FindClusters (resolution = 0.8) and RunUMAP.

Gene activity scores were calculated with the GeneActivity function and log normalized with the NormalizeData function. We also used the RunChromVAR function to compute per-cell motif activity scores using human TF motif position weight matrices (PWMs) provided in the R package chromVARmotifs ("human\_pwms\_v2")[4]. Then, we used the gene activity score and TF activity score to define the clusters identified in the above steps according to the genes or TFs specifically activated in the T cell subtypes. Chromatin accessibility of specific regions was visualized with the CoveragePlot function. The Seurat function FindAllMarkers was used to calculate differential chromatin accessibility regions and gene activity. The motifs enriched in a specific peak set were calculated with FindMotifs. Peaks were annotated to the nearest genes with the R package ChIPseeker[5] function annotatePeak (promoter region defined as TSS ±3 kb).

scRNA-seq and scATAC-seq integration analysis was conducted to visualize all the cells together. First, the anchors between the scATAC-seq dataset and the scRNA-seq dataset were identified with FindTransferAnchors (dimension reduction method was CCA). Then, the normalized expression matrix of the top variable genes in the scRNA-seq data was used as reference data to make an imputed scRNA-seq matrix for each of the ATAC cells by TransferData (dimensional reduction used for the weighting anchors was 1 and 3:20 components resulted in Harmony in scATAC-seq data). Then, scATAC-seq data were combined with scRNA-seq data by an imputed matrix. Finally, we ran PCA and UMAP on this combined object to visualize the scRNA-seq and scATAC-seq cells in the same low-dimensional space. We calculated the average gene expression of each cell subtype in the scRNA-seq data and the average gene activity of each cell subtype in the scATAC-seq data. The correlations between the cell subtypes in these two datasets were calculated by Pearson correlation analysis.

**Functional enrichment analysis**

Functional enrichment analysis, including Gene Ontology (GO) term enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, was performed for dimensionality reduction of DEGs by the R package clusterProfiler. GO term analysis consisted of 3 subontologies: biological process, cellular component, and molecular function. The significance of the KEGG and GO enrichment was set at *P* value <0.05.

***In vitro* co-culture experiments and cytokine array**

To further verify the specific initiation function of CD4+CD40LG+ subsets in CART products and explore the sequence of interactions among CD4+CD40LG+ CART products, tumor cells and monocytes, BCMA+ H929 cell line was used as MM tumor cells and THP1 was used as monocyte-like cells in vitro, while CART products were divided into CD4+CD40LG+ CART, control CART (the all CART products except for CD4+CD40LG+ CART) and EndoT cells by FACS. The 50ml CART co-culture media was consisted of 47.5 ml X-VIVO media, 2.5ml patient plasma and 300U/Ml IL-2, while the media for H929 and THP1 cell lines was RPMI 1640 media and 10% fetal bovine serum. The proportion of co-cultured different cell types was 1:1 with 1 million cells each in a 6-well plate. According to the each cell type pair, specific group, the co-culture time is divided into 12 hours (only CD4+CD40LG+ + MM group) and 24 hours. The group CD4+CD40LG + CART + MM (24h) + THP1 (24h) (group 5) represented that using the 2mL supernatant from the co-culture of CD4+CD40LG+ CART + MM (24h) further activate THP1 cells (1 million cells). The detection of cytokines in the supernatant of co-culture and the cytokine array of patient plasma after anti-BCMA CART therapy were completed by the Shanghai Tissuebank Biotechnology Co., Ltd.

**Statistical analysis**

Each experiment was repeated independently at least three times. All the data are represented as mean ± standard deviation. Student’s t-test was used for statistical analysis of two groups, and one-way ANOVA followed by Tukey’s post-hoc tests was used for comparisons among multiple groups. The P value was calculated using GraphPad Prism 8 (GraphPad Software). Significant differences are indicated as \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001.

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**Author contributions**

L.X., H.Y., P.Q and H.H. designed and performed experiments, analyzed data and wrote the manuscript. L.X., P.J., Y.X. and J.H. performed bioinformatics analysis of single cell RNA-seq/TCR/ATAC sequencing and published data. Z.C., X.Z. and H.L. assisted to bioinformatics analysis. L.X., X.S. and D.K. constructed in vitro model. S.H., T.Y., T.G., M.S., J.C., H.X., Y.H., X.Y., F.N., W.L., L.Z., H.Z. R.H., H.Z., Y.Z. and Y.L. provided technical assistance. Y.L., M.Z., G.W., W.W., J.Z., H.L. and J.H assisted to manuscript discussion. P.Q. and H.H. supervised the overall project and co-wrote the manuscript. All authors contributed to reading and editing the manuscript.

**Competing financial interests**

The authors declare no competing financial interests.

**Figures and tables:** 7 main figures, 6 supplemental figures, 7 supplemental tables.

**Figure Legends**

**Figure 1. Clinical characterization and kinetics of CRS-related biomarkers.**

(A) Positron emission tomography-computed tomography and immunohistochemistry showed that the tumor disappeared after CART cell therapy. (B) Percentage of prognosis after anti-BCMA CART cell therapy. (C) Percentage of CRS grade after anti-BCMA CART cell therapy. (D) Manifestations of CRS in multiple organ systems, including respiratory system, gastrointestinal system, cardiovascular system and urinary system. (E) Flowchart depicting the overall experimental design of the present study. Sixty-one multiple myeloma (MM) patients were enrolled for anti-BCMA CART cell therapy. Twenty-nine samples from 6 MM patients were selected for the multiplexed cytokine assay. Twenty-eight samples from 4 MM patients were selected for single-cell RNA (scRNA-seq) and single-cell TCR sequencing (scTCR-seq). Six samples from 2 MM patients were selected for single-cell ATAC sequencing (scATAC-seq). (F) Dynamic change of CART cells in 4 patients’ peripheral blood mononuclear cells (PBMCs) after CART cell infusion. (G) Heatmap of 45 CRS-related proteins at different times (FCb, CRSb, CRSi, CRSp and CRSr) by multiplexed cytokine assay. (H) Histogram of 24 significantly altered CRS-related proteins at CRSp.

**Figure 2. Transcriptome atlases of CART and other cell subsets by scRNA-seq****.**

(A) Uniform manifold approximation and projection (UMAP) visualization of PBMCs that passed QC with the patient number, different time points (B), CART and non-CART cells (C) and different clusters (D). (E) Specific markers (including CD3E, CD4, CD8B, GZMB, NKG7, FOXP3, CD14, CD16, MKI67, PPBP, CD138 and BCMA) of 10 clusters in the feature plot. (F) Heatmap depicting the expression levels of the top 100 specific marker genes expressed in each subpopulation. (G) Proportions of each cell type for CART cells at CRSb, CRSp and CRSr. (H) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of CART cells at CRSb, CRSp and CRSr. (I) Proportions of each cell type for non-CART cells at FCb, CRSb, CRSp and CRSr. (J) Dynamic changes of CART and non-CART cells.

**Figure 3. Cellular resources of CRS-related biomarkers and cell-cell interaction networks across the CRS course.**

(A) The source of CRS-related biomarkers at CRSb, CRSp and CRSr. (B) Capacity for intercellular communication among CART cells, EndoT cells, NK cells, plasma/tumor cells, platelets, monocytes and DCs. Each line color indicates the ligands expressed by the cell population represented in the same color. The lines connect to the cell types that express the cognate receptors. The line thickness is proportional to the number of ligands when cognate receptors are present in the recipient cell type. The loops indicate autocrine circuits. (C) The differential interaction strength of L-R pairs between CRSp and CRSb. The line thickness is proportional to the number of ligands when cognate receptors are present in the recipient cell type. (D) The differential interaction strength of L-R pairs between CRSr and CRSp. The line thickness is proportional to the number of ligands when cognate receptors are present in the recipient cell type. (E) Overview of selected L-R interactions of CART cells as ligands and receptors. P values are indicated by circle size, with the scale to the right (permutation test). The means of the average expression levels of interacting molecule 1 in cluster 1 and interacting molecule 2 in cluster 2 are indicated by color. Assays were carried out at the mRNA level but were used to extrapolate protein interactions. (F) Overview of selected L-R interactions of monocytes as ligands and receptors. (G) Significant pathways (including the CD40, IFN-II, FLT3, CCL, IL-1, IL-6 and GRN pathways) involved in the interactions among plasma/tumor cells, CART cells, NK cells, EndoT cells, monocytes, dendritic cells (DCs), and platelets. (H) Detailed view of the TNF signaling pathway at CRSb, CRSp and CRSr. Numbers indicate the quantity of ligand–receptor pairs for each intercellular link.

**Figure 4. Trajectory and clonal diversity of CART and EndoT cells** **during CRS by scRNA-seq and scTCR-seq analyses.**

(A) UMAP visualization of T cells with the patient number, different time points (B), CART and EndoT cells (C) and different clusters (D). (E) Specific markers of 8 clusters in the violin plot. (F) Trajectory analysis of CART cells from pseudotime, time and cluster perspectives (left panel); proportions of each cell type among CART cells at CRSb, CRSp and CRSr (right panel). (G) Trajectory analysis of EndoT from pseudotime, time and cluster perspectives (left panel); proportions of each cell type for EndoT cells at CRSb, CRSp and CRSr (right panel). (H) Multicolor flow cytometry showed the dynamic changes in CART and EndoT cell types (Tn, Tcm, Teff and Tem) during anti-BCMA CART cell therapy in 3 patients (upper); dynamic changes in LAG3 and PD1 in CART and EndoT cells during CRS progression (low); (I) TCR diversity of CART and EndoT cells at FCb, CRSb, CRSp and CRSr. (J) Location and characterization of clonally amplified T cells in the T cell UMAP visualization chart. (K) KEGG enrichment analysis of up- and down-regulated DEGs between clonally amplified T cells and non-clonally amplified T cells.

**Figure 5. Dynamic landscape of chromatin accessibility of CART cells during CRS progression by scATAC-seq.**

(A) UMAP projection showing the distribution of CART cells at CRSb, CRSp and CRSr. (B) The annotated scATAC-seq profile of CART cells in UMAP visualization. (C) The cell proportion of annotated cell subsets (CD4 Tn, CD4 Tcm, CD4+PRF1+ Teff, CD8 Tcm and CD8 Tem) at CRSb, CRSp and CRSr. (D) The gene scores of marker genes in the UMAP projection. (E) Dot plot showing the gene activity scores of marker genes of each cell subtype. Dot size represents the percentage of cells with values detected in each subtype. Color represents the average gene activity score of each cell subtype. Dark red indicates a higher gene activity score, and light yellow indicates a lower gene activity score. (F) Differentially accessible regions of CART cells at CRSb, CRSp and CRSr. The right panel shows the enriched KEGG pathways of genes around differentially accessible regions. (G) Motifs of CART cells enriched in differentially accessible regions at CRSb, CRSp and CRSr. (H) Regulatory network of motifs and their corresponding target genes at CRSb, CRSp and CRSr. (I) Track profiles of NFKB1, EOMES and KLF2 at CRSb, CRSp and CRSr.

**Figure 6．CD4+CD40LG+IL-13+ CART cell products were associated with high CRS severity.**

(A) The CRS score (the average expression values of CRS-related biomarkers) for each cell type. (B) UMAP plot showing the locations of CRS-associated clusters (CRSCs) and other cells in CART cell products. (C) UMAP plot showing the location of the CRS-associated cluster (CRSC) in different patients. (D) The specific genes of CRSC between CRSC and other cells at CRSb by violin chart, including CD4, CD40LG, IL-13 and TNF. (E) Differentially expressed genes (DEGs) of CRSC compared with other cells. (F) Gene ontology (GO) term enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of specific genes in CRSC. (G) Track profiles of CD4, CD40LG and IL-13 in CART cells at CRSb, CRSp and CRSr. (H) Tumor burden and CD4+ CART cell products were associated with CRS severity. (I) Percentage of CD4+CD40LG+ cells in high CRS (CRS grade ≥3) and low CRS (CRS grade <3). (J) Percentage of CD4+IL-13+ CART cells in high CRS (CRS grade ≥3) and low CRS (CRS grade <3). (K) Heatmap of 45 cytokines in the co-culture supernatant from different treatment with one patient CART products. (L) Bar chart of TNFα, CCL20/MIP-3α, IL-3, IL-4, IL-17A, IL-1β, IL-6, CXCL1/GROα and PDGF-AA in 5 groups.

**Figure 7．The three-stage model of CRS after CART cell therapy.**

(A) CRS initiation (CRSi) stage: CRSC (a cluster of naive cells in CART cells) recognizes tumor cells and releases specific cytokines (such as IL-2, IL-4 and IL-17A) to activate monocytes and endogenous T cells. (B) CRS peak (CRSp) stage: the above factors continuously activate CART, monocytes, endogenous T cells and NK cells, which produce a large amount of cytokines and trigger CRS (such as CCL20, CXCL1, IL-1β and IL-6). (C) CRS recovery (CRSr) stage: after killing the tumor cells, number of CART cells decreased while number of endogenous T and NK cells increased, and both CART and EndoT showed the trend of clonal expansion.

**Supplementary Figure Legends**

**Figure S1. Patient inclusion criteria and basic information.**

(A) Dynamics of tumor cells (BCMA+CD138+) in 4 patients’ bone marrow (BM) by flow cytometry during anti-BCMA CART cell therapy. (B) Sankey diagram of the prognosis of MM patients and CRS grade. (C) The dynamic change of CART cells after product infusion by flow cytometry. (D) Dynamics of 4 patients’ CAR copy numbers in PBMCs after CART cell infusion. (E) Dynamics of 4 patients’ temperatures after CART cell infusion. (F) Dynamics of 4 patients’ classical cytokines (CRP, Ferritin, IL-6 and IFNγ) after CART cell infusion.

**Figure S2. Characteristics of CART cells and other cell subsets by scRNA-seq after CART cell therapy.**

(A) Uniform manifold approximation and projection (UMAP) visualization of 28 samples of PBMCs that passed QC. (B) Average relative expression of each cluster-specific gene in the dimplot. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of monocytes at CRSb, CRSp and CRSr. (D) KEGG pathway enrichment of EndoT cells at CRSb, CRSp and CRSr. (E) KEGG pathway enrichment of NK cells at CRSb, CRSp and CRSr. (F) Distribution of the cell cycle (upper) and cytotoxicity (lower) at different stages (FCb, CRSb, CRSp and CRSr) in both CART cells and non-CART cells.

**Figure S3. Cell-cell interaction network and key pathways of various clusters at CRSb, CRSp and CRSr during CART cell therapy.**

(A) Detailed separated view of the ligands expressed by CART, EndoT, NK, plasma/tumor, platelets, monocytes and DCs and the cells expressing the cognate receptors primed to receive the signal. Numbers indicate the quantity of ligand–receptor pairs for each intercellular link. (B-I) Overview of selected L-R interactions among CART cells as ligands (B) and receptors (C), EndoT cells as ligands (D) and receptors (E), NK cells as ligands (F) and receptors (G), plasma/tumor cells as ligands (H) and receptors (I) at CRSp, CRSb and CRSr. (J) Significant pathways (FGF, GAS, IFN-I, CSF and BAFF pathways) involved in the interactions among plasma/tumor, CART, NK, EndoT, monocytes, DCs and platelets. (K) Detailed view of specific pathways (VEGI and CD30 pathway) at CRSp and CRSr.

**Figure S4. Trajectory and clonal diversity of CART and EndoT cells by scRNA-seq and scTCR-seq integration analysis.**

(A) UMAP visualization of CART and EndoT cells at FCb, CRSb, CRSp and CRSr in four patients (total 28 samples). (B) Proportions of each cell type for EndoT and CART cells at FCb, CRSb, CRSp and CRSr. (C) Average relative expression levels of each cluster-specific gene in the dim plot. (D) The top clone percentages of EndoT and CART cells at FCb, CRSb, CRSp and CRSr in 4 patients. (E) The DEG between clonally amplified T cells and non-clonally amplified T cells.

**Figure S5. Characteristics of CART cells during CRS progression.**

(A) Profile plot showing accessibility signals over all TSS sites. **(**B) Violin plot showing the read number, feature peak number, percentage of read number in the peaks and mitochondrial ratio for all cells. (C) The density map shows the distribution of insert fragment length. (D) The nucleosome signal of peak region fragments. (E) Peak distribution of differentially accessible regions among different CRS stages.

(F) UMAP projection showing the integration results of scATAC-seq and scRNA-seq data. (G) The correlations between the cell subtypes annotated in scATAC-seq data and the cell subtypes annotated in scRNA-seq data. (H) Differentially accessible regions of CD4 Tn, CD4 Tcm, CD4+PRF1+ Teff, CD8 Tcm and CD8 Tem clusters. (I) KEGG enrichment analysis of CD4 Tcm and CD8 Tem clusters. (J) Track profiles of PRF1, IFNG, TNF and IL-6 in each cell subtype.

**Figure S6. Characterization of CD4+CD40LG+ CART cell products at the transcriptomic and epigenetic levels.**

(A) DEGs of CD40LG-positive and CD40LG-negative CART cell products at the transcriptomic level. (B) KEGG (upper) and GO (lower) enrichment analysis of CD40LG-positive CART cells at the transcriptome level. (C) UMAP projection showing the distribution of CD40LG, IL-13, FTL, FTH1, IL2RA, IL2RB, JUN, S100A11, TNF, TNFRSF18, CCL5, CCL3, GZMA, FLT3LG, CSF2 and IFNG. (D) KEGG enrichment analysis of CD40LG-positive CART cell products epigenetically. (E) Motifs enriched in differentially accessible regions for CD40LG-positive CART cells. (F-G) Heatmap of 45 cytokines in the co-culture supernatant from different treatment with another 2 patient CART products.

**Supplementary Table Legends**

Table S1. The related information of 28 samples subjected to scRNA-seq and scTCR-seq.

Table S2. List of differentially expressed genes (DEGs) of 10 different cell subsets in PBMCs.

Table S3. List of 80 CRS-related biomarkers.

Table S4. List of differentially expressed genes (DEGs) of 8 different CART and EndoT cell subsets.

Table S5. List of 149 significant differential ligand-receptor pairs.

Table S6. The related information of 6 samples subjected to scATAC-seq.

Table S7. List of DEGs between clonally amplified CART cells and other CART cells.

Table S8 Key resources table

**Reference**

Alizadeh, D., Wong, R.A., Gholamin, S., Maker, M., Aftabizadeh, M., Yang, X., Pecoraro, J.R., Jeppson, J.D., Wang, D., Aguilar, B.*, et al.* (2021). IFNγ Is Critical for CAR T Cell-Mediated Myeloid Activation and Induction of Endogenous Immunity. Cancer discovery *11*, 2248-2265.

Bu, D.X., Singh, R., Choi, E.E., Ruella, M., Nunez-Cruz, S., Mansfield, K.G., Bennett, P., Barton, N., Wu, Q., Zhang, J.*, et al.* (2018). Pre-clinical validation of B cell maturation antigen (BCMA) as a target for T cell immunotherapy of multiple myeloma. Oncotarget *9*, 25764-25780.

Chen, G.M., Chen, C., Das, R.K., Gao, P., Chen, C.H., Bandyopadhyay, S., Ding, Y.Y., Uzun, Y., Yu, W., Zhu, Q.*, et al.* (2021). Integrative Bulk and Single-Cell Profiling of Premanufacture T-cell Populations Reveals Factors Mediating Long-Term Persistence of CAR T-cell Therapy. Cancer discovery *11*, 2186-2199.

Chen, P.H., Lipschitz, M., Weirather, J.L., Jacobson, C., Armand, P., Wright, K., Hodi, F.S., Roberts, Z.J., Sievers, S.A., Rossi, J.*, et al.* (2020). Activation of CAR and non-CAR T cells within the tumor microenvironment following CAR T cell therapy. JCI Insight *5*.

Deng, Q., Han, G., Puebla-Osorio, N., Ma, M.C.J., Strati, P., Chasen, B., Dai, E., Dang, M., Jain, N., Yang, H.*, et al.* (2020). Characteristics of anti-CD19 CAR T cell infusion products associated with efficacy and toxicity in patients with large B cell lymphomas. Nature medicine *26*, 1878-1887.

Fajgenbaum, D.C., and June, C.H. (2020). Cytokine Storm. The New England journal of medicine *383*, 2255-2273.

Giavridis, T., van der Stegen, S.J.C., Eyquem, J., Hamieh, M., Piersigilli, A., and Sadelain, M. (2018). CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. Nature medicine *24*, 731-738.

Hu, Y., Sun, J., Wu, Z., Yu, J., Cui, Q., Pu, C., Liang, B., Luo, Y., Shi, J., Jin, A.*, et al.* (2016). Predominant cerebral cytokine release syndrome in CD19-directed chimeric antigen receptor-modified T cell therapy. Journal of hematology & oncology *9*, 70.

Jin, S., Guerrero-Juarez, C.F., Zhang, L., Chang, I., Ramos, R., Kuan, C.H., Myung, P., Plikus, M.V., and Nie, Q. (2021). Inference and analysis of cell-cell communication using CellChat. Nat Commun *12*, 1088.

Kumar, S.K., Lee, J.H., Lahuerta, J.J., Morgan, G., Richardson, P.G., Crowley, J., Haessler, J., Feather, J., Hoering, A., Moreau, P.*, et al.* (2012). Risk of progression and survival in multiple myeloma relapsing after therapy with IMiDs and bortezomib: a multicenter international myeloma working group study. Leukemia *26*, 149-157.

Kumar, S.K., Rajkumar, V., Kyle, R.A., van Duin, M., Sonneveld, P., Mateos, M.V., Gay, F., and Anderson, K.C. (2017). Multiple myeloma. Nature reviews Disease primers *3*, 17046.

Lee, D.W., Gardner, R., Porter, D.L., Louis, C.U., Ahmed, N., Jensen, M., Grupp, S.A., and Mackall, C.L. (2014). Current concepts in the diagnosis and management of cytokine release syndrome. Blood *124*, 188-195.

Lee, Y.G., Chu, H., Lu, Y., Leamon, C.P., Srinivasarao, M., Putt, K.S., and Low, P.S. (2019). Regulation of CAR T cell-mediated cytokine release syndrome-like toxicity using low molecular weight adapters. Nat Commun *10*, 2681.

Li, X., Guo, X., Zhu, Y., Wei, G., Zhang, Y., Li, X., Xu, H., Cui, J., Wu, W., He, J.*, et al.* (2021a). Single-Cell Transcriptomic Analysis Reveals BCMA CAR-T Cell Dynamics in a Patient with Refractory Primary Plasma Cell Leukemia. Molecular therapy : the journal of the American Society of Gene Therapy *29*, 645-657.

Li, X., Shao, M., Zeng, X., Qian, P., and Huang, H. (2021b). Signaling pathways in the regulation of cytokine release syndrome in human diseases and intervention therapy. Signal transduction and targeted therapy *6*, 367.

Liu, Y., Fang, Y., Chen, X., Wang, Z., Liang, X., Zhang, T., Liu, M., Zhou, N., Lv, J., Tang, K.*, et al.* (2020). Gasdermin E-mediated target cell pyroptosis by CAR T cells triggers cytokine release syndrome. Science immunology *5*.

Mahadeo, K.M., Khazal, S.J., Abdel-Azim, H., Fitzgerald, J.C., Taraseviciute, A., Bollard, C.M., Tewari, P., Duncan, C., Traube, C., McCall, D.*, et al.* (2019). Management guidelines for paediatric patients receiving chimeric antigen receptor T cell therapy. Nature reviews Clinical oncology *16*, 45-63.

Melenhorst, J.J., Chen, G.M., Wang, M., Porter, D.L., Chen, C., Collins, M.A., Gao, P., Bandyopadhyay, S., Sun, H., Zhao, Z.*, et al.* (2022). Decade-long leukaemia remissions with persistence of CD4(+) CAR T cells. Nature *602*, 503-509.

Neelapu, S.S., Dickinson, M., Munoz, J., Ulrickson, M.L., Thieblemont, C., Oluwole, O.O., Herrera, A.F., Ujjani, C.S., Lin, Y., Riedell, P.A.*, et al.* (2022). Axicabtagene ciloleucel as first-line therapy in high-risk large B-cell lymphoma: the phase 2 ZUMA-12 trial. Nature medicine.

Neelapu, S.S., Tummala, S., Kebriaei, P., Wierda, W., Gutierrez, C., Locke, F.L., Komanduri, K.V., Lin, Y., Jain, N., Daver, N.*, et al.* (2018). Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. Nature reviews Clinical oncology *15*, 47-62.

Nelson, M.H., Knochelmann, H.M., Bailey, S.R., Huff, L.W., Bowers, J.S., Majchrzak-Kuligowska, K., Wyatt, M.M., Rubinstein, M.P., Mehrotra, S., Nishimura, M.I.*, et al.* (2020). Identification of human CD4(+) T cell populations with distinct antitumor activity. Science advances *6*, eaba7443.

Nijhof, I.S., van de Donk, N., Zweegman, S., and Lokhorst, H.M. (2018). Current and New Therapeutic Strategies for Relapsed and Refractory Multiple Myeloma: An Update. Drugs *78*, 19-37.

Norelli, M., Camisa, B., Barbiera, G., Falcone, L., Purevdorj, A., Genua, M., Sanvito, F., Ponzoni, M., Doglioni, C., Cristofori, P.*, et al.* (2018). Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. Nature medicine *24*, 739-748.

Ptáčková, P., Musil, J., Štach, M., Lesný, P., Němečková, Š., Král, V., Fábry, M., and Otáhal, P. (2018). A new approach to CAR T-cell gene engineering and cultivation using piggyBac transposon in the presence of IL-4, IL-7 and IL-21. Cytotherapy *20*, 507-520.

Qin, H., Dong, Z., Wang, X., Cheng, W.A., Wen, F., Xue, W., Sun, H., Walter, M., Wei, G., Smith, D.L.*, et al.* (2019). CAR T cells targeting BAFF-R can overcome CD19 antigen loss in B cell malignancies. Science translational medicine *11*.

Raje, N., Berdeja, J., Lin, Y., Siegel, D., Jagannath, S., Madduri, D., Liedtke, M., Rosenblatt, J., Maus, M.V., Turka, A.*, et al.* (2019). Anti-BCMA CAR T-Cell Therapy bb2121 in Relapsed or Refractory Multiple Myeloma. The New England journal of medicine *380*, 1726-1737.

Rajkumar, S.V., Dimopoulos, M.A., Palumbo, A., Blade, J., Merlini, G., Mateos, M.V., Kumar, S., Hillengass, J., Kastritis, E., Richardson, P.*, et al.* (2014). International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. The Lancet Oncology *15*, e538-548.

Röllig, C., Knop, S., and Bornhäuser, M. (2015). Multiple myeloma. Lancet (London, England) *385*, 2197-2208.

Sadelain, M., Rivière, I., and Riddell, S. (2017). Therapeutic T cell engineering. Nature *545*, 423-431.

Sheih, A., Voillet, V., Hanafi, L.A., DeBerg, H.A., Yajima, M., Hawkins, R., Gersuk, V., Riddell, S.R., Maloney, D.G., Wohlfahrt, M.E.*, et al.* (2020). Clonal kinetics and single-cell transcriptional profiling of CAR-T cells in patients undergoing CD19 CAR-T immunotherapy. Nature communications *11*, 219.

Staedtke, V., Bai, R.Y., Kim, K., Darvas, M., Davila, M.L., Riggins, G.J., Rothman, P.B., Papadopoulos, N., Kinzler, K.W., Vogelstein, B.*, et al.* (2018). Disruption of a self-amplifying catecholamine loop reduces cytokine release syndrome. Nature *564*, 273-277.

Vento-Tormo, R., Efremova, M., Botting, R.A., Turco, M.Y., Vento-Tormo, M., Meyer, K.B., Park, J.E., Stephenson, E., Polański, K., Goncalves, A.*, et al.* (2018). Single-cell reconstruction of the early maternal-fetal interface in humans. Nature *563*, 347-353.

Wang, X., Dong, Z., Awuah, D., Chang, W.C., Cheng, W.A., Vyas, V., Cha, S.C., Anderson, A.J., Zhang, T., Wang, Z.*, et al.* (2022). CD19/BAFF-R dual-targeted CAR T cells for the treatment of mixed antigen-negative variants of acute lymphoblastic leukemia. Leukemia.

Xue, Q., Bettini, E., Paczkowski, P., Ng, C., Kaiser, A., McConnell, T., Kodrasi, O., Quigley, M.F., Heath, J., Fan, R.*, et al.* (2017). Single-cell multiplexed cytokine profiling of CD19 CAR-T cells reveals a diverse landscape of polyfunctional antigen-specific response. Journal for immunotherapy of cancer *5*, 85.

Yang, Y., Kohler, M.E., Chien, C.D., Sauter, C.T., Jacoby, E., Yan, C., Hu, Y., Wanhainen, K., Qin, H., and Fry, T.J. (2017). TCR engagement negatively affects CD8 but not CD4 CAR T cell expansion and leukemic clearance. Science translational medicine *9*.

Zhang, M., Zhou, L., Zhao, H., Zhang, Y., Wei, G., Hong, R., Wu, W., Xu, H., Wang, L., Ni, F.*, et al.* (2021). Risk Factors Associated with Durable Progression-Free Survival in Patients with Relapsed or Refractory Multiple Myeloma Treated with Anti-BCMA CAR T-cell Therapy. Clinical cancer research : an official journal of the American Association for Cancer Research *27*, 6384-6392.

Zhao, W.H., Liu, J., Wang, B.Y., Chen, Y.X., Cao, X.M., Yang, Y., Zhang, Y.L., Wang, F.X., Zhang, P.Y., Lei, B.*, et al.* (2018). A phase 1, open-label study of LCAR-B38M, a chimeric antigen receptor T cell therapy directed against B cell maturation antigen, in patients with relapsed or refractory multiple myeloma. Journal of hematology & oncology *11*, 141.

Zhao, X., Wu, S., Fang, N., Sun, X., and Fan, J. (2020). Evaluation of single-cell classifiers for single-cell RNA sequencing data sets. Briefings in bioinformatics *21*, 1581-1595.