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Early expression of CD94 and loss of CD96 on CD8+ T cells after allogeneic stem cell tranplantation is predictive of subsequent relapse and survival

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Abstract

Allogeneic stem cell transplantation is used widely in the treatment of hematopoietic malignancy. However, relapse of malignant disease is the primary cause of treatment failure and reflects loss of immunological graft-*versus*-leukemia effect. We studied the transcriptional and phenotypic profile of CD8+ T cells in the first month following transplantation and related this to risk of subsequent relapse. Single cell transcriptional profiling identified five discrete CD8+ T-cell clusters. High levels of T-cell activation and acquisition of a regulatory transcriptome were apparent in patients who went on to suffer disease relapse. A relapse-associated gene signature of 47 genes was then assessed in a confirmation cohort of 34 patients. High expression of the inhibitory receptor CD94/NKG2A on CD8+ T cells within the first month was associated with 4.8 fold increased risk of relapse and 2.7 fold reduction in survival. Furthermore, reduced expression of the activatory molecule CD96 was associated with 2.2 fold increased risk of relapse and 1.9 fold reduction in survival. This work identifies CD94 and CD96 as potential targets for CD8-directed immunotherapy in the very early phase following allogeneic transplantation with the potential to reduce long term relapse rates and improve patient survival.

Introduction

Allogeneic hematopoietic stem cell transplantation (SCT) is an effective treatment for many patients with hematological malignancy and can provide long term disease control. However, many patients experience disease relapse after transplant and failure to control the primary disease remains a major challenge.[1]

The immunological graft-*versus*-leukemia (GvL) effect plays a major role in control of disease relapse and is derived primarily from the capacity of donor-derived immune cells to eliminate residual cancer cells that remain after transplant conditioning.[2-5] Alloreactive T-cell responses are believed to be primary mediators of GvL and there is considerable interest in understanding factors that determine the efficacy of the alloreactive immune response post transplant.[6,7] A range of studies have identified mechanisms of immune evasion associated with disease relapse including increased expression of inhibitory receptors or human leukocyte antigen (HLA) downregulation on tumor cells.[8-10]

Although most studies of immune reconstitution have been performed at late time points after transplant, often coincident with disease relapse,[8] it is likely that immune setpoints established in the very early post-transplant period will ultimately determine clinical outcome.[11,12] Indeed, use of cyclophosphamide as early as day 3 post transplant has a profound influence on subsequent development of alloreactive immune responses[13] and the degree of iatrogenic immune suppression within the first 2 weeks is correlated with subsequent clinical outcome.[14,15] A challenge for undertaking such work has been the intense degree of lymphopenia in the early post-transplant period. Peripheral T-cell reconstitution becomes detectable in most patients by 14 days after transplant[16,7] but at the current time no studies have assessed how the T-cell profile at this stage may be correlated with subsequent risk of disease relapse.

Transcriptional analysis can provide a global assessment of T-cell function and the development of single cell technology allows assessment of lymphoid heterogeneity. Here we use single cell RNA sequencing (scRNA-seq) to define the transcriptional profile of peripheral CD8+ T-cell subsets at 2 weeks after stem cell transplantation and identify five discrete cellular subclusters. Furthermore, using this discovery analysis we identified a potential gene signature associated with disease relapse and applied this to a larger confirmatory patient cohort. This identified that the relative expression of CD94 and CD96 on CD8+ T cells both act as strong determinants of disease relapse. These features provide novel insights into T-cell differentiation in the early post transplant and identify new opportunities for immunotherapy.

Methods

Patient cohort

Patients gave written informed consent using the BOOST ethics approved by the regional ethics committee (05/Q2707/175). scRNA-seq was performed in three patients on peripheral blood T cells taken 2 weeks after transplantation. Patients underwent myeloablative conditioning with fludarabine and busulphan. 5 mg/kg total rabbit ATG was given for T-cell depletion together with ciclosporin and methotrexate as graft-*versus*-host disease (GvHD) prophylaxis. All patients were in morphological complete remission but had minimal residual disease by genetic analysis. The validation cohort comprised 34 patients with blood collected at 14-28 days post-transplant (*Online Supplementary Table S1*). No patients had GvHD prior to sampling.

Relapse, survival, grades 2–4 acute GvHD and chronic GvHD were recorded according to consensus conference criteria and NIH criteria respectively.[24-25] Relapse and survival data were analyzed by Kaplan-Meier (KM) curve with log-rank test to compare groups and with CoxPH models, using the Survival R package. Multivariate CoxPH models were fitted with clinical covariates chosen *a priori*. Statistical significance was concluded by a *P* value <0.05.

Smartseq2 single cell RNA sequencing

Single T cells were sorted using CD3, CD4 and CD8 into a 96-well plate and sequencing was performed by Oxford Genomics Center (Oxford University, UK). T-cell phenotyping on validation cohort utilized antibodies against CD3, CD4, CD8, CD94, CD96, CD53, KLRG1, CD14 and CD19.

scRNA-seq data reads were genome-aligned (hg38) using BWA-MEM and feature counts determined using htseq-count from HTSeq[17] with parameters: -r pos -m union -s no. Raw feature counts were imported into R version 3.6.1. A single cell experiment object was constructed in R to store counts and metadata and features with zero count across all cells were removed. Quality control metrics were calculated using scater[18] and cells filtered to remove outliers. Cell-specific bias was normalized using the R package scran.[19]

Marker genes for CD4+ and CD8+ T cells were identified using findMarkers and regarded as differentially expressed after *P* value adjustment for multiple testing with false discovery rate <0.1. Uniform manifold approximation and projection (UMAP) embeddings were calculated with scater function runUMAP. Marker genes for CD4+ and CD8+ T-cell clusters were identified using findMarkers with the block parameter set to the patient label.

Functional enrichments within marker gene sets were identified using the R package gProfiler2 (20). Enrichments of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Biological Process GO term and transcription factor targets were calculated by hypergeometric test.

The activity of gene sets from the Molecular Signatures database was estimated using AUCell (v1.2.4) (21). MSigDb Accessions for Effector and Naïve CD8+ T-cell signatures used: GS9650 GSE23321, KAECH, GOLDRATH and GSE40666. T-regulatory signatures that were compared include GSE25087, GSE7852, GSE22045 and a published signature of regulatory T cells (Tregs).[22] Cells are assigned as being active for a given gene set if the area under the curve (AUC) score was above the “Global_k1” threshold in the AUC score distribution. T-cell receptor (TCR) genes were reconstructed using TraCer (version 0.6.0) (42) Assemble and summarize functions for TCR reconstruction and summary of TCR recovery rates.

Results

Single cell RNA sequencing analysis of CD4+ and CD8+ T cells in the very early post-transplant period

Blood samples were taken from three patients at 2 weeks after allogeneic SCT. CD4+ and CD8+ T cells were isolated by fluorescence-activated cell sorting (FACS) and underwent scRNA-seq (Figure 1A). Distributions of read and feature metrics confirmed ~3x10^6 reads and 1-5k features per cell indicating high quality analysis (*Online Supplementary Figure S1*).

Two female patients and one male patient were studied whilst all donors were male. Mean expression of genes from the X chromosome was correlated with expression of genes from the Y chromosome in every cell and compared to randomly selected chromosome pairs. Strongly increased expression of Y chromosome-derived genes was observed indicating that sorted cells were predominantly donor-derived (Figure 1B).

UMAP embedding separated the CD4+ and CD8+ T-cell

lineages (Figure 1C) and hierarchical clustering using the top CD4+ and CD8+ marker genes defined distinct transcriptional profiles (Figure 1D). CD4+ T cells were enriched for expression of genes involved in cytokine signaling (*IL2RA, ARID5B, LTB, VIM, ITGB1, TIMP1*) as well as the *TNFRSF18* (GITR) and *CTLA4* markers of Treg differentiation (*Online Supplementary Figure S2A*). In contrast CD8+ T cells showed a cytotoxic genotype with a high expression of granzymes (*GZMB, GZMA, GZMK, GZMH*), perforin and natural killer cell granule protein 7 (*NKG7*) (Figure 1D; *Online Supplementary Figure S2A*). Marker genes confirmed this pattern and pathways previously reported as enriched in GvHD and allograft rejection were also amplified (*Online Supplementary Figure S2*).

Five CD8+ cell clusters are identifiable at 2 weeks post transplant

Clustering analysis was then applied to the UMAP populations and identified five distinct sub-clusters within the CD8+ population. Gene analysis identified two of these as naïve populations and three as effector subsets (N1, N2; E1, E2, E3). Importantly, these showed similar distribution between the three patients (Figure 2A). Distinct subclusters within CD4+ cells were not clear and further analysis therefore focused on the CD8+ T-cell population. Transcriptional analysis identified that NOTCH2 signaling was enhanced in all effector populations indicating a potential common mechanism for T-cell activation in the early post-transplant period. TCR signaling pathways were also enriched in clusters E1 and E3, indicating likely recent engagement with cognate antigen. Of interest, this was not present in cluster E2 which may have expanded through homeostatic proliferation and was enriched in H3 histone gene expression (Figure 2B and C).

The two naïve sub-clusters were distinguishable from each other primarily through increased expression of *CNN2, HNRNPM* and *SLC25A6* in the N2 population (*Online Supplementary Figure S2*). Naïve cell cluster N1 expressed genes associated with glucose metabolism whilst N2 was active for Hif1a stabilization (Figure 2C). Significant interactions were observed among the effector clusters between checkpoint molecule HLA-E and its ligands CD94, KLRK1 and KLRC4 (*Online Supplementary Figure S2F*). Transcription factor (TF) target gene enrichment analysis identified selective activity of several TF within the E1 and N1 clusters but only one TF for each of the other three clusters (Figure 2D). In particular, GATA-2 and estrogen receptor α (ERα) target genes were upregulated in clusters E2 and E3 respectively whilst AML2 was the dominant TF in the N2 cluster. A further characteristic feature of cluster E1 was transcriptional regulation from several interferon-gene regulatory factors (IRF-1, IRF-2, IRF-3) (Figure 2D) whilst heat shock factors (HSF2 and HSF4), which typically regulate genes encoding molecular chaperones and other stress proteins, were the dominant TF associated with the N1 cluster.

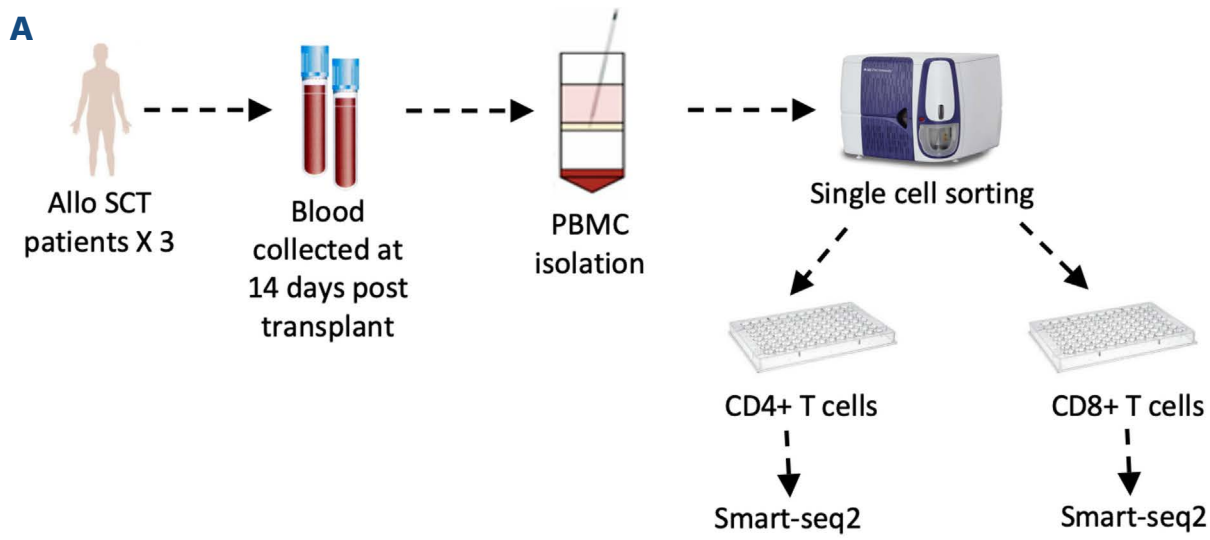
Strong activation of CD8+ T cells early post transplant is associated with subsequent leukemia relapse

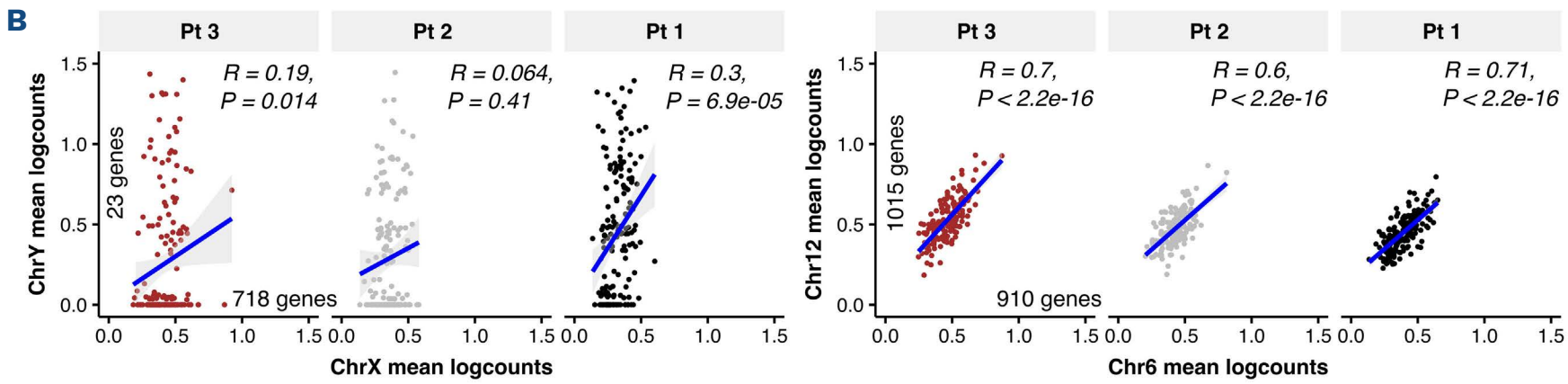
We next assessed the transcriptional profile of CD8+ T cells at day 14 in relation to whether or not the patient subsequently stayed in clinical remission or suffered a relapse of the original leukemia. Two patients were seen to relapse at days 29 and 65 respectively whilst the third remained in long term remission.

A 'relapse gene-signature' of 47 genes that were either upregulated (n=29) or downregulated (n=18) in CD8+ T cells was defined (Figure 3A). High levels of T-cell activation at day 14 were associated with subsequent risk of disease relapse. In particular, strong upregulation of genes involved in the TCR signaling pathway, such as *LCK*, *STAT1* and *STAT4*, was present in the patients who relapsed and KEGG pathway analysis showed increased levels of 'lymphocyte activation' (Figure 3B). The proportion of effector cells was also increased in this group with a particular increase in clusters E1 and E3 (Figure 3C). Although the proportion of naïve cells was decreased in relapse, the N1 cluster was markedly increased and the major change was a marked reduction in the N2 pool. The amino acid sequence of TCRB CDR3 regions from TCR reconstruction analysis on CD8+ T cells and CD4+ T cells revealed heterogeneity and no evidence of marked clonal expansion was observed at this time point (Figure 3D). Within-cluster comparison of relapse status (*Online Supplementary Figure S3*) revealed genes differentially expressed in all effector clusters, with E3 showing the most transcriptional divergence (27 differentially expressed genes [DEG]). Whilst Naive cluster N2 had no significant hits, 21 DEG were identified within N1. Of note, the cytotoxicity gene perforin (*PFN1*) showed reduced expression particularly in E1 cells of relapsed patients, whilst in N1 cells, the immune checkpoint molecule DUSP2 (PAC1) was significantly increased.

Effector CD8+ T cells display a T-regulatory gene signature in patients with subsequent relapse of disease

Genes that were overexpressed in effector CD8+ T cells from patients with subsequent relapse were highly enriched for target genes regulated by transcription factors that are key drivers of regulatory T-cell function such as NF-kB subunits Rela/p65, relB, NOTCH2 and IRF1 (*Online Supplementary Figure S4*). We therefore assessed the relative expression of genes that are associated with CD4+ T-regulatory cell activity within the CD8+ clusters. These were found to be markedly enriched in effector CD8+ cells from patients who went on to relapse (Figure 3A; *Online Supplementary Figure S5*). Of note, this was most prominent within the E1 and E3 subsets which had





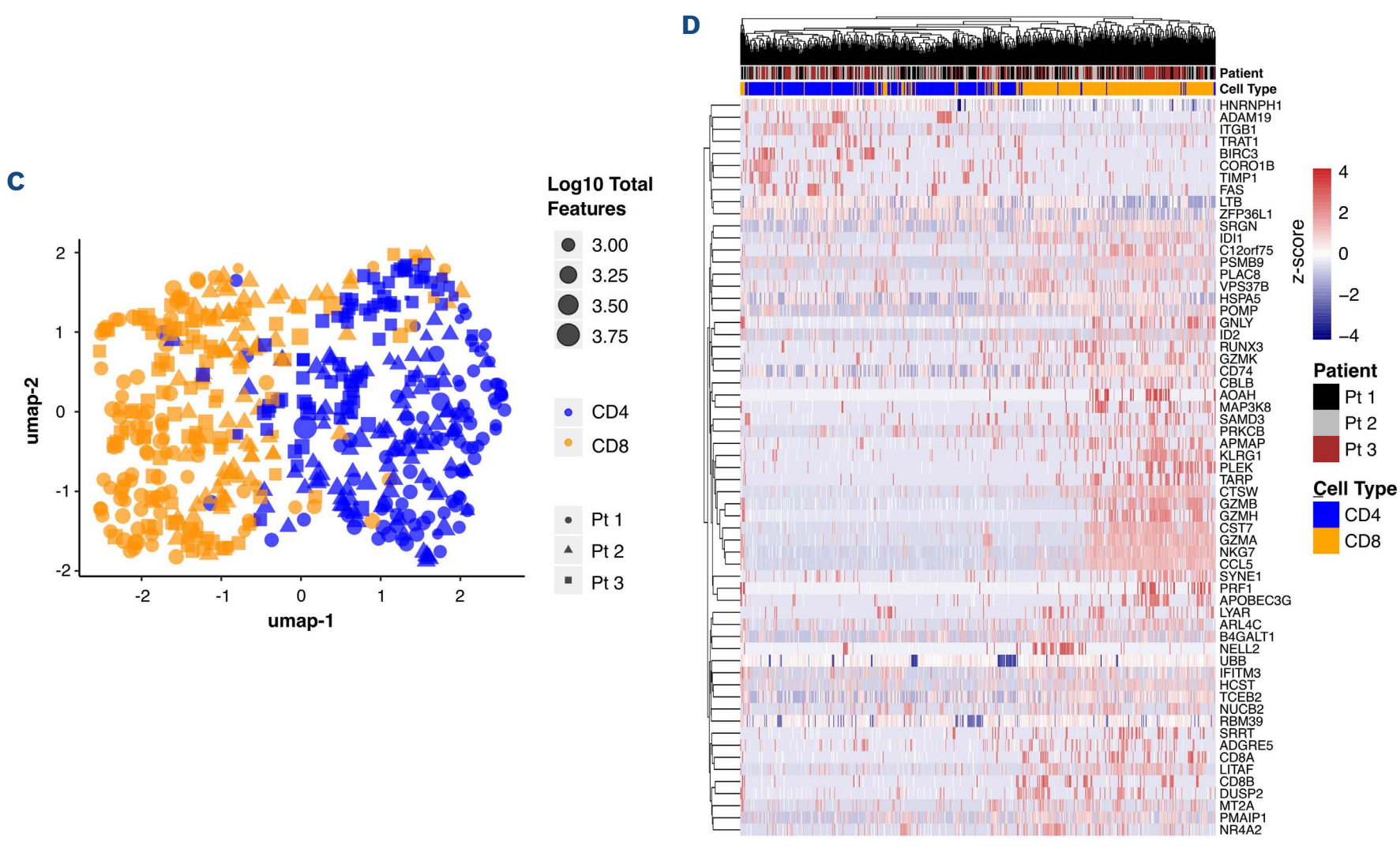


Figure 1. Markers of CD4+ and CD8+ T cells at week 2 post transplant. (A) Sample processing schematic. (B) Y-linked gene expression skew suggesting donor-derived cells. Scatter and Spearman correlation of mean expression of genes expressed on Y chromosome (ChrY) against mean expression of genes on ChrX (left). Right panel show scatter and Spearman correlation of mean gene expression of randomly selected chromosome pairs. (C) Uniform manifold approximation and projection (UMAP) embedding of single cell expression data from CD4+ and CD8+ T cells. (D) Hierarchical clustering of single CD4+ and CD8+ T cells using top marker genes (false discovery rate <0.001; log fold-change >1.5) at day 14. Allo SCT: allogeneic stem cell transplantation.

shown high levels of TCR activation (Figure 3E). 52% of effector cells within these subsets expressed a regulatory transcriptional profile in patients that subsequently relapsed compared to only 24% in the patient who remained in remission (Figure 3E). Of note, this regulatory signature was not observed within the CD4+ T-cell population.

Differential expression of CD94 and CD96 on CD8+ T cells within the first month post transplant is highly predictive of subsequent relapse

Using the 'relapse gene signature' (Figure 3A) that identified genes within CD8+ cells that were differentially expressed in relation to risk of subsequent relapse, we next went on to assess the expression of these genes or their

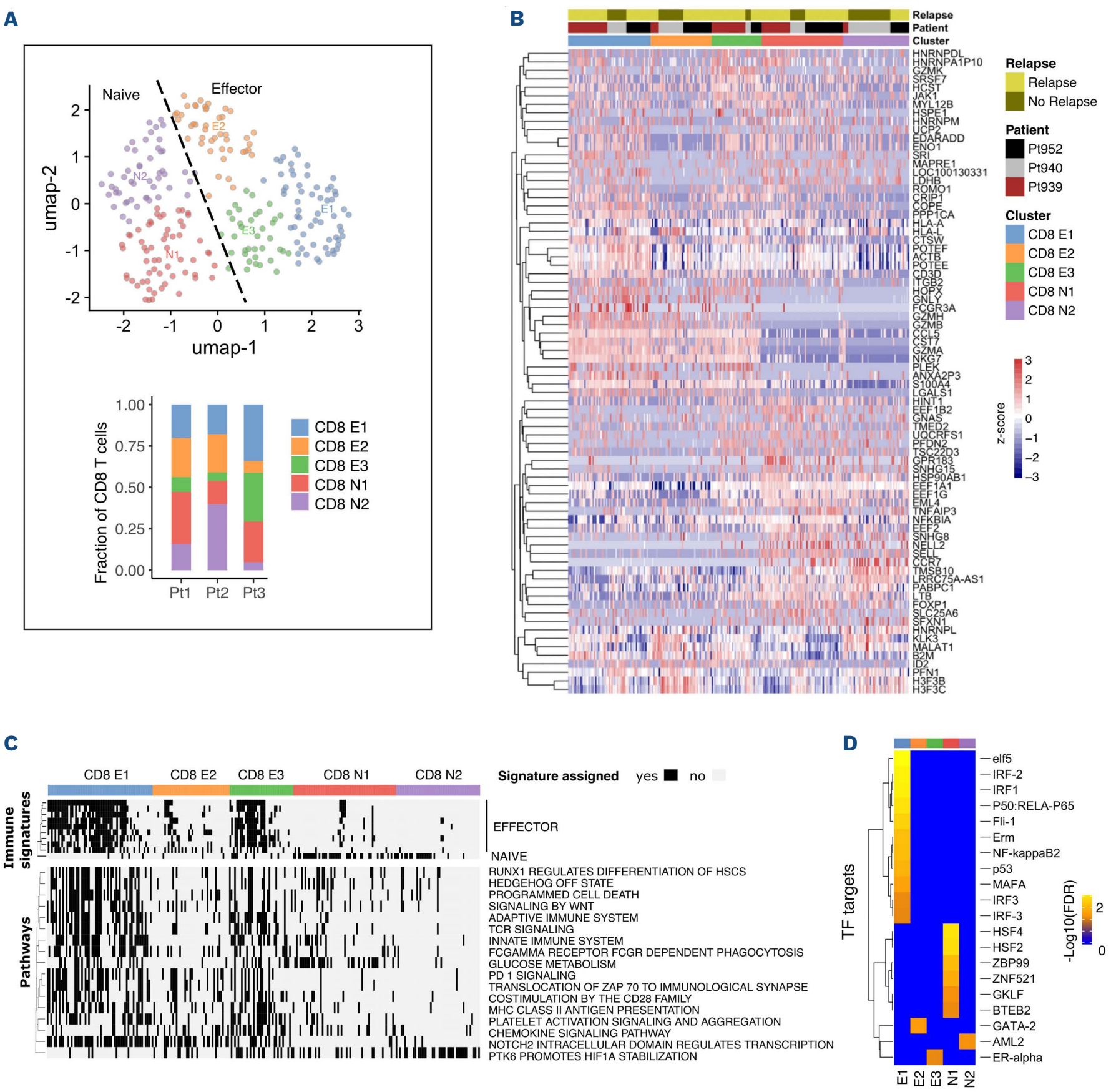


Figure 2. Characterization of week 2 post transplant CD8+ T-cell subsets. (A) Uniform manifold approximation and projection (UMAP) embedding and graph-based clustering of CD8+ T cells identified effector (E) and naïve (N) cell populations. Inset: proportions of cell populations identified in each patient sample. (B) Expression profile of top marker genes for clusters identified in (A). (C) Profile of selected KEGG and REACTOME gene set signature assignments from AUCell. Cells are assigned as being active for a signature if the area under the curve (AUC) signature score is above a threshold value (global k1 threshold). (D) Transcription factor (TF) targets within marker genes overexpressed in week 2 CD8+ T-cell effector (E) and naïve (N) cell populations.

associated proteins in a confirmation cohort of 34 patients (*Online Supplementary Table S1*). As before, patient blood samples were taken within the first month after transplant and flow cytometry was used to determine expression of proteins on CD8+ T cells. Where antibodies were not available we used quantitative polymerase chain reaction (qPCR) to determine relative mRNA level. Protein expression of selected surface markers associated with T-cell function including CD28, CD53, CD63, CD94, CD95, CD96, CD132, CD164, NKG2D, KLRG1 and CCL5 was assessed using flow cytometry. qPCR was used to determine relative expression of the phosphatase *DUSP1* as well as *KLRC4* which encodes the intracellular NKG2F (*Online Supplementary Figure S6*; Figure 4A and D).

The expression of most genes/proteins on CD8+ T cells was not found to be related to subsequent risk of relapse. However, strong and independent correlations were observed between the relative expression of both CD94/NKG2A and CD96 on CD8+ T cells and the subsequent risk or disease relapse. Cell surface expression of CD94 and CD96 is depicted in a representative FACS plot in Figure 4C. Expression of the inhibitory heterodimer CD94/NKG2A was seen on 37% of CD8+ T cells in patients who relapsed (n=16) compared to only 8% of CD8+ T cells in those that remained in remission (n=18) (P<0.005; Figure 4A). Overall median percentage of CD94/NKG2A+ cells was 15% within the CD8+ T-cell population and patients with percentage expression above this level had an 81% probability of disease relapse (n=16; P=0.003, Figure 5A) as well as decreased overall survival (P=0.0047, Figure 5B) (*Online Supplementary Table S1*). Patients with values below the median displayed a 17% chance of relapse. The mean fluorescence index (MFI) of CD94 was also determined in relation to relapse risk and retained statistical significance (*Online*

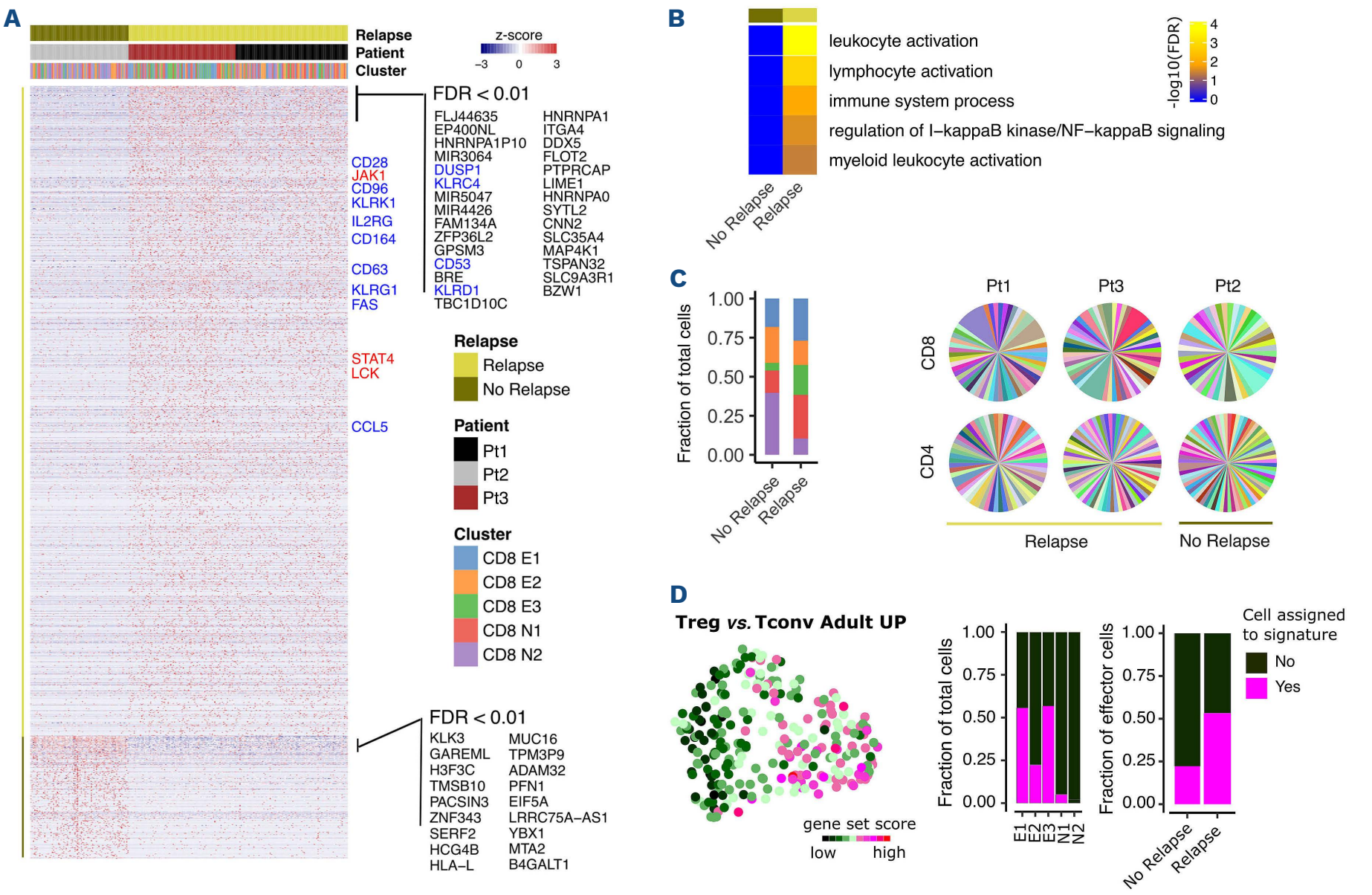


Figure 3. CD8+ T-cell gene signature and T-cell receptor clonality in relation to leukemia relapse at week 2 post transplant. (A) Expression profile of potential relapse marker genes in week 2 (WK2) CD8 T cells (P<0.05). Higher confidence (false discovery rate [FDR] <0.01 and absolute log fold-change >1) markers are labeled. Genes labeled blue were selected for validation by flow cytometry. (B) Proportions of clusters by relapse status. (C) Enrichment of terms, KEGG Pathway analysis of relapse associated genes. (D) T-cell receptor clonality of CD4 and CD8 T cells at WK2 post transplant. Colors represent TRB CD3 amino acid sequences that could be identified from the single cell RNA sequencing data. (E) UMAP embedding overlaid with AUCell signature score for T-regulatory cell (Treg) signature GSE25087 and proportions of cells assigned to this signature stratified by CD8+ T-cell clusters and relapse status.

Supplementary Figure S5). Associations of percentage CD94/NKG2A+ cells with outcome remained significant (Relapse Adj. *P*=0.008 and Survival Adj. *P*=0.008) after adjusting for covariates in multivariate CoxPH models (*Online Supplementary Figure S7*).

Conversely, the expression of CD96 on CD8+ T cells was reduced in patients who suffered disease relapse. In particular, a median of 90% of CD8+ T cells expressed CD96 in this group compared to 97% in those who remained in remission (Figure 4B) and MFI expression was also lower in the relapse cohort (*Online Supplementary Figure S7*). scRNA-seq data showed that expression of CD96 was most pronounced in CD8+ T cell cluster E3 but cells expressing CD96 were also observed in all other clusters (Figure 4C). Expression of CD94 was most apparent in cells of cluster E1 (Figure 4D). Overall median percentage expression was 95% and relapse was observed in 65% of those with expression below-median compared to 29% of patients with higher levels of CD96 (*P*=0.018, Figure 5D). This also translated into lower overall survival for patients in the former group (*P*=0.016, Figure 5E). Following adjustment for covariates, CD96+ associations retained a similar trend (Relapse Adj. *P*=0.10 and Survival Adj. *P*=0.067) (*Online Supplementary Figure S8*). Relapse and survival associations (univariate CoxPH model) of all covariates are listed in the *Online Supplementary Table S2*. There was no significant correlation between GvHD incidence and CD94 or CD96 expression on CD8+ T cells (Figure 5C and F; *Online Supplementary Table S1*).

Discussion

Relapse of primary malignant disease is the major clinical challenge in allogeneic SCT at the current time. Here we undertook detailed assessment of the profile of T-cell reconstitution during the very early post-transplant period in order to determine a relapse risk-associated gene signature and identified CD94/NKG2A and CD96 expression on CD8+ T cells as strong determinants of relapse risk. These observations raise a number of questions relating to the differentiation of T cells in the early post-transplant period, the mechanisms of GvL and potential immunotherapeutic opportunities.

Five different CD8+ subsets were identified within blood at day 14 after allograft. This is the most detailed characterization of T cells at this very early post-transplant time point and is a unique setting in order to assess T-cell differentiation. T cells are exposed to a range of activation stimuli during this period including TCR-mediated recognition of alloantigen and homeostatic proliferation in response to the intense lymphopenia of the transplant conditioning regimen. TCR signaling was a dominant transcriptional program in two of the effector CD8+ clusters and is likely to reflect recent engagement with alloantigen.[26,27] Homeostatic proliferation can also lower the threshold for TCR signaling during this period.[28] The Notch2 signaling pathway was strongly upregulated within effector CD8+ cells and reveals a novel role of this pathway in the early post-transplant period. Importantly, Notch2 expression on CD8+ T cells

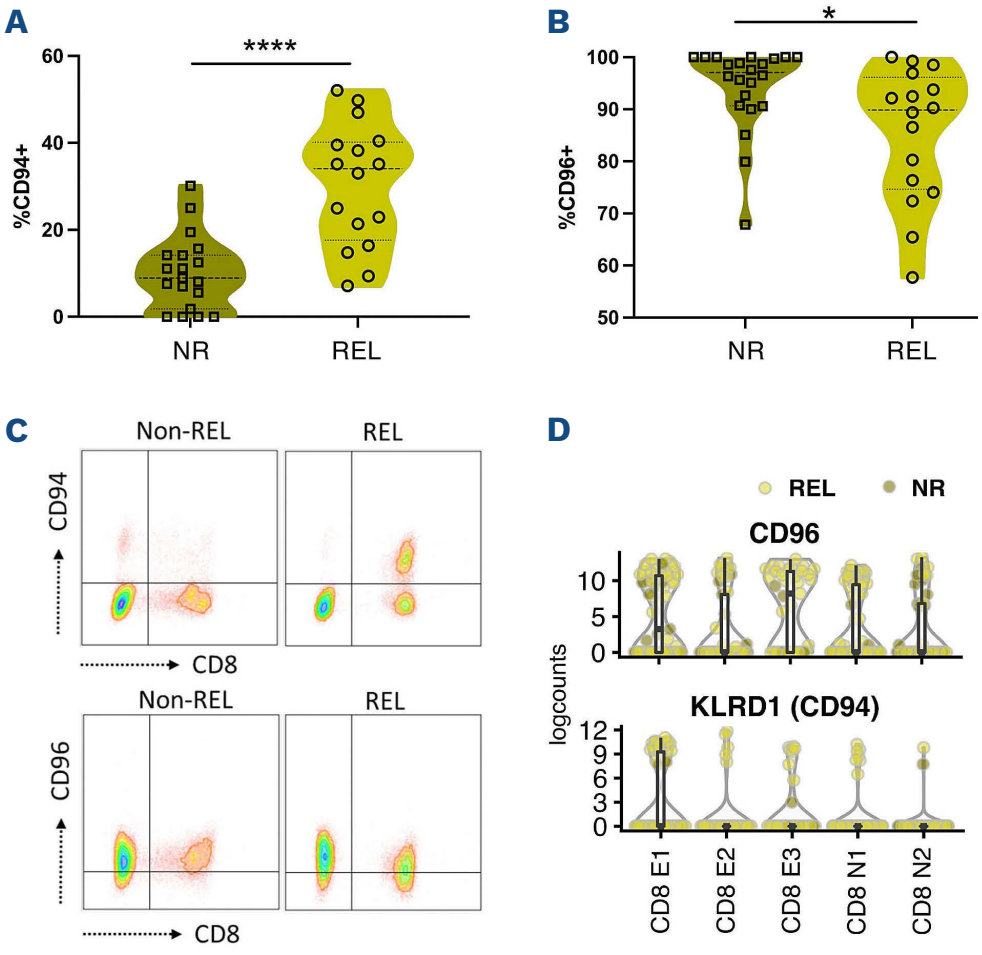


Figure 4. Validation of selected genes by flow cytometry in patients with disease relapse or no relapse post-allogeneic stem cell transplantation. (A and B) Percentage of CD94+ and CD96+ cells within the CD8+ T-cell subset is depicted in disease relapse (REL) patients (n=16) compared to no relapse (NR) patients (n=18) using flow cytometry. (C) Flow cytometry staining for CD94 and CD96 on CD8+ T cells from REL and NR patient is shown in a representative fluorescenec-activated cell sorting (FACS) plot. (D) Cluster specific gene expression of CD94 and CD96 from single cell RNA sequencing. Mann Whitney-U test was performed for statistical analysis, *P<0.05; ****P<0.001.

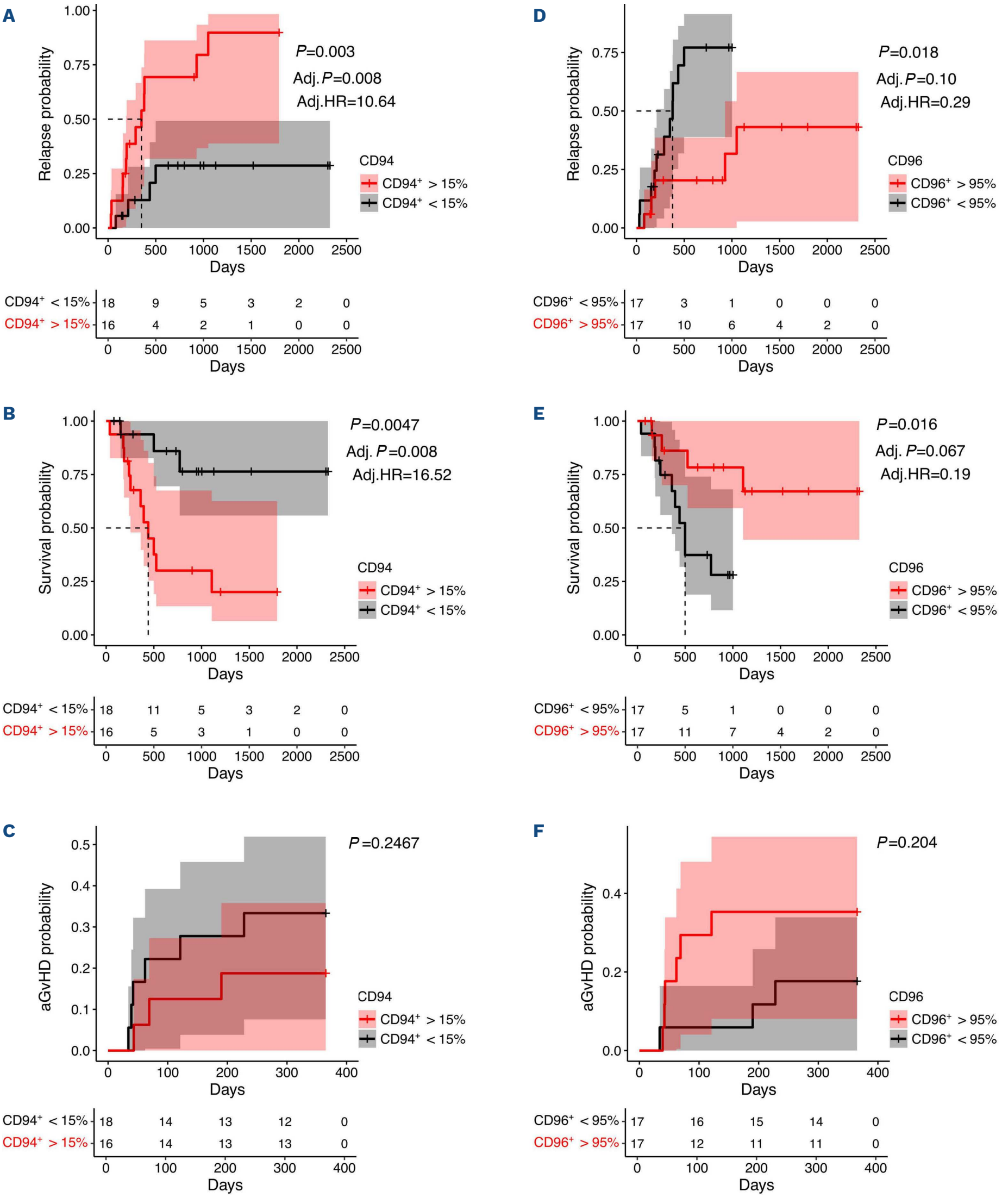


Figure 5. Correlation of CD94 and CD96 expression on CD8+ T cells with clinical outcome. Kaplan Meier curves showing (A) time to relapse, (B) survival and (C) acute graft-*versus*-host disease (aGvHD) incidence post-allogeneic stem cell transplantation in patients with high (>15%) and low (<15%) CD94+ CD8+ T cells. Kaplan Meier curves showing time to (D) relapse, (E) survival and (F) aGvHG disease post-allogeneic stem cell transplantation in patients with high (>95%) and low (<95%) CD96+ CD8+ T cells. Results of log-rank test (p) and Multivariate CoxPH model adjusted *P* (Adj.*P*) and hazard ratio (Adj.HR) are indicated.

plays an important role in the generation of tumor-specific cytotoxic T cells activity and indicates a potential pathway for intervention to optimize effector T-cell development post transplant.[29] A further feature was that interferon regulatory genes were upregulated in the CD8+ cluster and is noteworthy given the central role for type 1 interferon both in generation of CD8+-mediated GvL responses[30,31] and treatment of disease relapse after transplant.[32] Two discrete naïve pools were also apparent with unique features that included glycolytic metabolism and a IL-17 driven differentiation profile. These pathways deserve further investigation given the suboptimal regeneration of the naïve pool after transplantation.

The major ambition of this work was to identify novel pathways that might represent therapeutic targets to decrease the rate of disease relapse after transplantation. As such we used these transcriptional datasets to define a 39-gene signature within CD8+ T cells that was associated with disease relapse. A striking feature was strong upregulation of genes involved in the TCR signaling pathway, including *JAK1*, *LCK*, *STAT1* and *STAT4* in patients who went on to suffer disease relapse. This indicates that intense early activation may potentially lead to exhaustion of the GvL immune response and is supported both by increased expression of the exhaustion-associated granzyme K protein and strong PD-1 signaling within effector clusters.[28] These findings may relate to the clinical importance of reducing tumor burden prior to transplantation. Whilst this is generally assumed to reflect a limitation of the GvL response to clear disease it may actually reflect the induction of T-cell anergy or exhaustion in the setting of a low lymphocyte:tumor cell ratio. Indeed, chimeric antigen receptor T-cell therapy has demonstrated the capacity to mediate clearance of substantial tumor load and uncovers the power of cellular immunity to control malignant disease. The importance of T cell exhaustion at the onset of disease relapse has been clearly demonstrated[33] and had facilitated the assessment of checkpoint blockade as a means to restore disease control.[34-36] Our data suggest that this consequence may already have been determined within the first 2 weeks after transplantation. Why some patients might be particularly prone to intense early T-cell activation is unclear at this time. However, a range of factors could be important such as amount of minimal residual disease, relative expression of alloreactive peptides due to histo-incompatibility, donor T- cell repertoire, early determinants of antigen presentation, and the rate of establishment of donor T-cell chimerism.

Another key finding from scRNA-seq was enrichment of gene targets for the transcription factors IRF1 and NF-kB subunits Rela/p65, relB which are key drivers of the regulatory T-cell response.[37-39] A limitation was that we were only able to gain access to transcriptional datasets from CD4+ T regulatory cells and a comparison with well characterized CD8+ regulatory subsets will be an important future ambition. Furthermore, ATG has been shown to promote development of Tregs cells in some *in vitro* studies. However, 52% of CD8+ T cells acquired a transcriptional profile similar to conventional Tregs in the patients who relapsed and this was particularly evident within the antigen-stimulated effector clusters E1 and E3. We speculate that strong TCR engagement of CD8+ T cells during the unique microenvironmental conditions present during early immune reconstitution, including elevated levels of IL-7 and IL-15, can lead to engagement of a T-regulatory transcriptional program. Considering the active role of Tregs and host antigen-presenting cells (APC) in suppression of alloimmune responses in patients with mixed chimerism following allogeneic HSCT, these findings may potentially contribute to the risk of subsequent disease relapse.[40]

Expression analysis of relapse-associated genes or associated proteins in the confirmatory cohort of 34 patients revealed that the pattern of CD94 and CD96 expression was strongly associated with risk of relapse. CD94/NKG2A is an NK-associated receptor that is present on a subset of memory CD8 cells and expressed following recent TCR engagement. It binds to HLA-E and acts as an inhibitory regulator to downregulate T-cell activation following antigen recognition (Figure 6). The finding that CD94/NKG2A expression is upregulated early, and is predictive of long term relapse rate, is therefore most likely to reflect a negative feedback response to initial strong engagement with antigen. Indeed, HLA-E expression is upregulated on leukemic blasts as a potential mechanism of evading NK cell-mediated immune surveillance and will act to accentuate this inhibition.[41]

The other protein whose expression was highly predictive of subsequent relapse was CD96. CD96 is the member of the PVR/nectin family, expressed on all CD8+ T cells, and binds to CD155 on target cells.[31] CD96 competes with the stimulatory CD244 (DNAM-1) and inhibitory TIGIT for engagement and is a potent costimulatory molecule on CD8+ T cells that can be downregulated following recent T-cell activation. As such, the lower levels of CD96 on CD8+ T cells prior to relapse are again likely to reflect a response to recent antigen engagement (Figure 6).

These findings raise the possibility that immunotherapeutic targeting of CD94 and CD96 may help to reduce the rate of disease relapse after SCT. Indeed, antibodies against CD94/NKG2A are now being assessed as 'checkpoint blockade' in patients with solid tumors and would be readily transferable to this setting. Our data suggest

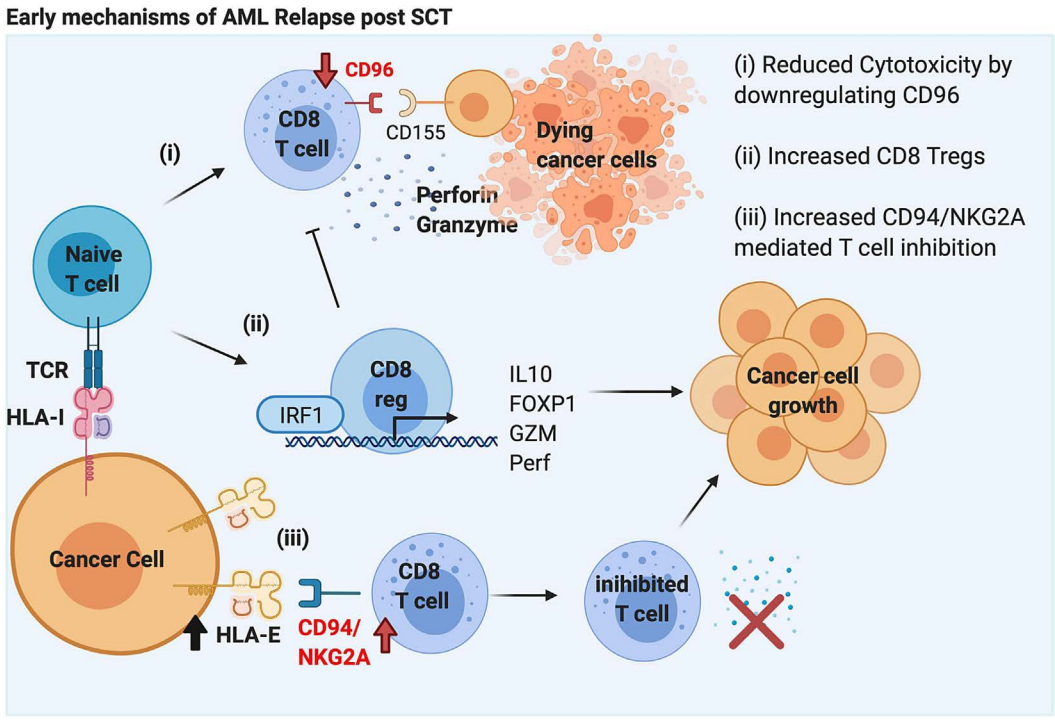


Figure 6. Mechanism of immune escape post-allogeneic stem cell transplantation in patients with acute myeleoid leukemia. Cartoon depicting 3 potential mechanisms by which leukemic cells may escape immune recognition by allogeneic CD8+ T cells within the first few weeks after stem cell transplantation (SCT) leading to disease relapse: (i) CD8 T cells downregulate CD96 on the surface which may result in reduced cytotoxic capacity, (ii) a fraction of CD8 T cells upregulate genes associated with regulatory function which may allow cancer cells to escape, (iii) increased expression of inhibitory receptor complex CD94/NKG2A which interacts with HLA-E expressing leukemic blasts thereby inhibiting a functional T-cell response. TCR: T-cell receptor; Tregs: regulatory T-cells.

that this approach may be valuable when applied very early in the post-transplant setting although must clearly be introduced with caution. In relation to overcoming downregulation of CD96, potential approaches might include blockade of the inhibitory protein TIGIT, with which CD96 competes for binding to CD155, or potentially an agonistic CD96-specific antibody. Again, TIGIT directed checkpoint blockade is currently in trial for patients with malignant disease.

Limitations of our study include the fact that patients received T-cell depletion with ATG and as such the findings are not directly transferable to transplant regimens that utilize T-cell replete stem cell grafts. In addition, the sample size of the discovery cohort was small whilst the validation cohort comprised patients with a variety of underlying malignant disorders.

Disease relapse is a devastating outcome for patients following SCT and an area where novel immunotherapeutic opportunities are limited. Our findings reveal the importance of studying immune reconstitution in the very early post-transplant period and indicate that CD94/NKG2A and CD96 may represent promising therapeutic pathways to enhance protective GvL responses and increase patient survival.

Disclosures

No conflicts of interest to disclose.

Contributions

KV, CS and HP performed the experiments. WC and KV analyzed the data. FAM, RM and JN recruited and consented the patients. JN, CS and KV collected and processed the blood samples. KV, WC, FAM, JZ and PM interpreted the data. KV, WC and PM wrote the paper. PM supervised the study.

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Data-sharing statement

Raw sequencing read of the single cell RNA sequencing experiments have been deposited in Gene Expression Omnibus database under the accession number GSE185498.The authors agree to make original data available to other investigators without unreasonable restrictions.

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