CD19-CAR T cells undergo exhaustion DNA methylation programming in patients with acute lymphoblastic leukemia

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SUMMARY

CD19-CAR T cell therapy has evolved into the standard of care for relapsed/refractory B cell acute lymphoblastic leukemia (ALL); however, limited persistence of the CAR T cells enables tumor relapse for many patients. To gain a deeper understanding of the molecular characteristics associated with CAR T cell differentiation, we performed longitudinal genome-wide DNA methylation profiling of CD8+ CD19-CAR T cells post-infusion in ALL patients. We report that CAR T cells undergo a rapid and broad erasure of repressive DNA methylation reprograms at effector-associated genes. The CAR T cell post-infusion changes are further characterized by repression of genes (e.g., TCF7 and LEF1) associated with memory potential and a DNA methylation signature (e.g., demethylation at CX3CR1, BATF, and TOX) demarcating a transition toward exhaustion-progenitor T cells. Thus, CD19-CAR T cells undergo exhaustion-associated
DNA methylation programming, indicating that efforts to prevent this process may be an attractive approach to improve CAR T cell efficacy.

**Graphical Abstract**

**In brief**
Zebley et al. show that CD8+CD19-CAR T cells undergo genome-wide DNA methylation changes during an antitumor response in patients with B cell acute lymphoblastic leukemia (ALL). Post-infusion CAR T cell differentiation involves acquisition of DNA methylation programs associated with effector function, repression of memory potential, and transition toward exhaustion.

**INTRODUCTION**
Chimeric antigen receptor (CAR) T cell therapy has emerged as a potentially curative treatment modality for patients with relapsed/refractory leukemia (Park et al., 2016, 2018). However, generating a sustained therapeutic response remains a major challenge that necessitates a deeper understanding of the cellular and molecular mechanisms driving CAR T cell expansion, contraction, and persistence in patients. These specific stages of a T cell immune response are governed by discrete gene regulatory programs that are coupled to the differentiation status of the T cell and can be potentially exploited to improve outcomes. Recently, it has become clear that T cell differentiation results in stable epigenetic changes...
in DNA methylation that allow the cell to remain poised to re-elicit acquired properties (Abdelsamed et al., 2017, 2020). This concept of epigenetic imprinting is emerging as a unifying theme to explain immunological memory and provide insight into correlates of durable antitumor responses.

The intimate connection between epigenetic reprogramming and T cell effector and memory potential is well established for endogenous CD8+ T cell immune responses to acute and chronic sources of antigen (Youngblood et al., 2017; Akondy et al., 2017; Gray et al., 2017). During a primary immune response to an acute source of antigen, the differentiation of naive CD8+ T cells into cytotoxic effector T cells is accompanied by broad changes in the epigenetic landscape that enable transcriptional permissivity of effector loci. Following antigen clearance, a subset of effector cells further develops into long-lived quiescent memory T cells that retain a multipotent epigenetic program in addition to the effector-associated epigenetic imprints, which enable recall of effector functions upon antigen reencounter (Kaech et al., 2003; Wherry et al., 2007). If antigen persists, T cells undergo a progressive acquisition of de novo DNA methylation programs that diminishes memory potential and causes exhaustion (Ghoneim et al., 2017). Recent studies exploring the heterogeneity of the pool of exhausted T cells have demonstrated that the process of T cell exhaustion encompasses a transitory state of CX3CR1+ CD8 T cells that maintain effector molecule expression and can contribute to antigen control (Hudson et al., 2019).

While T cell exhaustion has been well described in the prototypical murine model of chronic lymphocytic choriomeningitis virus (LCMV) infection and several human chronic infections, the impact of exhaustion on CAR T cells in a clinical setting remains unclear.

Recent single-cell transcription profiling analyses of CD19-CAR T cell infusion products and post-infusion samples have attempted to define the changes in gene expression associated with a clinical CAR T cell response (Sheih et al., 2020). While clear evidence of a robust effector response was observed from these gene signatures, hallmarks of T cell exhaustion were not detected, raising the question of whether CAR T numerical and functional decline is related to exhaustion. To further investigate whether CAR T cells undergo exhaustion during a clinical response, we examined the epigenetically defined differentiation status of CD8+ CD19-CAR T cells isolated from B cell acute lymphoblastic leukemia (ALL) patients. Here, we report that changes in DNA methylation are coupled to the expansion and eventual contraction of CAR T cells and specifically show that CD8+ CD19-CAR T cells progressively acquire a DNA methylation signature indicative of development into an exhaustion progenitor subset of T cells. This work broadly highlights the DNA methylation programs coupled to a decline in CD19-CAR T cell memory potential and establishment of an exhaustion trajectory, providing a mechanistic path for future efforts to generate long-lived CAR T cell memory post-infusion.

RESULTS

**In vivo CD8+ CD19-CAR T cell expansion is coupled to genome-wide DNA methylation remodeling of gene regulatory elements**

Extensive DNA methylation remodeling is known to occur during T cell effector and memory differentiation (Youngblood et al., 2017; Akondy et al., 2017). These acquired
DNA methylation programs can delineate the differentiation status of CD8\(^+\) T cells and are also known to drive the progressive development of T cell exhaustion (Ghoneim et al., 2017). Despite the known role of epigenetic reprogramming in promoting divergence from a functional memory developmental trajectory, the impact of these DNA methylation programs has not yet been well characterized during the clinical response to human CAR T cell therapy. To determine if CAR T cells undergo DNA methylation reprogramming post-adoptive transfer and if these programs are coupled to clinical outcome, we performed whole-genome DNA methylation profiling on CD8\(^+\) T cells expressing a second-generation CD19-CAR with a 4IBBC\(\zeta\) signaling domain (Talleur et al., 2020) pre- and post-infusion in pediatric patients enrolled on our institutional clinical study for CD19\(^+\) recurrent/refractory B cell ALL (NCT03573700).

Autologous CD19-CAR T cells were manufactured and infused into patients after standard lymphodepleting chemotherapy. The first 15 patients were included in this analysis, and the clinical characteristics and outcome of the first 12 patients have been reported in detail elsewhere (Talleur et al., 2020). To perform DNA methylation analyses, CD8\(^+\) CD19-CAR\(^+\) T cells were sorted pre- and post-infusion, and samples meeting a minimum cell number threshold were subjected to whole-genome bisulfite sequencing (WGBS) (Figures 1A and S1A) to measure variations in DNA methylation programs that are coupled with the stable changes in the developmental status of the cells. Of note, confirmation that our whole-genome DNA methylation analysis was performed on CD8\(^+\) CD19-CAR\(^+\) T cells was verified by identification of the integrated vector sequence in the samples (Figures S1B-S1D). Specifically, we measured the ratio between the average sequencing coverage for the vector and the average coverage for the entire genome and determined that the vector had approximately 2-fold coverage for the CD8\(^+\) CD19-CAR samples (Figure S1D). Moreover, the relative level of integration was consistent between the CAR\(^+\) T cells sorted from the good manufacturing practice (GMP) samples pre-infusion and the post-infusion CAR\(^+\) T cells, confirming our ability to isolate and analyze the CD8\(^+\) CD19\(^+\) CAR T cells after they have expanded in patients. To assess the relationship between DNA methylation states pre- and post-infusion, we performed a principal-component analysis (PCA) of all samples described in the panel inset of Figure S1A based on the CpG methylation status across the genome (Figure S2). Notably, the pre-manufacturing and GMP products were clustered together with a clear separation occurring in the samples up to 1 month (weeks 1–4) after in vivo CAR T cell antigen exposure (Figure 1B). To further characterize the DNA methylation events that delineate pre- versus post-infusion CAR T cells, we examined the loss and gain of methylation in samples isolated at weekly intervals post-CAR T cell infusion relative to the GMP product (Figure 1C). In general, the CAR T cells isolated from patients post-infusion were enriched approximately 10 times more for demethylation events as compared to methylation events. K-means clustering of the DNA methylation changes between the GMP and post-infusion samples identified eight gene-associated subsets (Figure 1D). Representative differentially methylated regions (DMRs) at genes with well-established roles in T cell effector function and differentiation are shown in Figure 1E.

We next proceeded to determine if these changes in methylation occurred at gene regulatory elements. Using established databases that define enhancers and promoters across the genome, we assessed the relative enrichment of DMRs at these regions. A permutation
analysis estimating the statistical chance that a given DMR occurred at an enhancer revealed that the overlap in post-infusion DMRs was significantly enriched at enhancer sites (Figure 1F). This was observed for both the gain of methylation and demethylation events that occur in CAR T cells post-infusion, indicating that these DMRs play a role in gene regulation.

To further characterize the genomic elements undergoing DNA methylation reprogramming, we interrogated the DMRs for enrichment of transcription factor binding site consensus sequences. Logo plots showing the relative abundance of conserved nucleotides for several known transcription factors revealed a highly significant association for transcription factors that are coupled to T cell effector and memory differentiation (Figure 1G). Most notably, some of these—including FOS:JUNB, STAT5, and TCF7—have established roles in regulating CAR T cell anti-tumor responses (Lynn et al., 2019; Zheng et al., 2021; Chen et al., 2021). Taken together, these data document the vast changes in DNA methylation that occur in CAR T cells post-infusion and show that these epigenetic events parallel the modifications that arise during endogenous T cell differentiation.

**CD8^+ CD19-CAR T cell post-infusion-associated DNA methylation programs are coupled to T cell stemness and effector function**

Having determined that post-infusion DNA methylation reprogramming occurs at gene regulatory elements linked to established molecules involved in effector and memory differentiation, we sought to gain further insight into the associated biological processes. Using DMRs conserved among all week 1–3 post-infusion CAR T cell patient samples, we performed independent Gene Ontology (GO) analyses (Figure 2A) on both the loss and gain of methylation events to further resolve biological processes that are broadly associated with epigenetic permissivity versus repression, respectively. The GO analysis for demethylated CpGs enriched for pathways involved in CD8^+ CD19-CAR T cells effector properties such as cell killing, proliferation, and apoptosis. Conversely, the gain of methylation analysis revealed that the CD8^+ CD19-CAR T cells undergo repressive programming that limits stem-associated properties (beta-catenin-TCF complex) and lymphocyte activation.

Given the significant enrichment of DMRs post-infusion in biological pathways that promote T cell effector differentiation, we proceeded to more broadly assess the CAR T cell DNA methylation state relative to the developmental spectrum of endogenous CD8 T cells. Accordingly, we performed a PCA using the post-infusion genome-wide DNA methylation profiles and our established naive and functional memory T cell subset profiles isolated from healthy donors (Figure 2B). The PCA revealed that, consistent with their effector response, the post-infusion CD8^+ CD19-CAR T cells distinctly segregated away from naive CD8^+ T cells. This separation was generally mirrored by the endogenous Tscm and Tcm, along principal component 1 (PC1). However, the CAR T cell population also shows a striking separation from the endogenous functional memory CD8^+ T cell subsets among the PC2 axis. While these data indicate that CD8^+ CD19-CAR T cells undergoing in vivo differentiation generally parallel the developmental trajectory observed with endogenous CD8^+ T cells, they also acquire distinct programs from the canonical memory subsets, suggesting that their developmental trajectory branches off from a functional memory lineage. Collectively, these data show that CD8^+ CD19-CAR T cells undergo broad DNA
methylation reprogramming events at gene regulatory elements that impact biological processes critical for CAR T cell function and survival.

**DNA-methylation-based quantification of CAR T cell developmental potential predicts CAR T cell expansion**

To better define the overall change in differentiation status of post-infusion CD8^+^ CD19-CAR T cells, we applied our previously reported DNA methylation-based multipotency index (MPI). This analysis utilizes CpG methylation of loci with established links to human CD8^+^ T cell memory differentiation and converts the methylation states of these genes into a normalized score for relative developmental potential (Abdelsamed et al., 2020). The MPI calculation was performed on both the pre-infusion product (GMP) and the post-infusion CD8^+^ CD19-CAR T cells isolated weekly from patients. In general, the GMP process yielded a CAR T cell product with a high MPI, consistent with the T cells’ ability to give rise to more differentiated T cell subsets and an overall capacity to mount an effective anti-tumor response. Further, a progressive decline in the MPI was observed from the GMP stage to the weeks 1/2 and 3/4 time points, consistent with CD8^+^ CD19-CAR T cells undergoing *in vivo* differentiation (Figure 3A). The progressive decline in CD8^+^ CD19-CAR T cells’ epigenetically defined developmental potential is further exemplified by tracking the MPI of longitudinal samples isolated from individual patients (Figure 3B). The MPI decreases at each week post-CAR T cell infusion, with the steepest decline occurring immediately after infusion, suggesting that the CD8^+^ CD19-CAR T cells rapidly undergo *in vivo* differentiation programming. Recent efforts to identify determinants of CD19-CAR T cell expansion and efficacy have reported an inverse association between enrichment of exhaustion-associated gene expression programs and the ability of the infusion product to expand within the first week of infusion (Deng et al., 2020). We next sought to determine whether application of our MPI to the GMP product could predict which products will undergo the highest expansion *in vivo*. Indeed, linear regression analysis showed a positive correlation between MPI score of the GMP product and expansion at the 1-week time point (Figure 3C). This analysis suggests that we can apply the MPI to predict CD8^+^ CD19-CAR T cell expansion in patients prior to the initial CAR T cell product infusion. Taken with our prior results showing post-infusion DMRs enriched for genes associated with T cell effector and memory functions, the predicted decline in developmental potential suggests that CD8^+^ CD19-CAR T cells progressively restrict gene regulatory programs that control T cell fate potential as they undergo differentiation programming.

**CAR T cell post-infusion differentiation includes acquisition of exhaustion-associated DNA methylation programs**

Given the progressive epigenetic repression of T cell developmental potential, we sought to determine if the CAR T cell post-infusion DNA methylation programs were enriched at established gene signatures that delineate development of functional memory versus exhausted T cells. We first identified human effector and memory gene expression signatures using publicly available transcriptional profiles for antigen-specific effector and long-lived memory CD8^+^ T cells isolated from individuals vaccinated for yellow fever (Akondy et al., 2017). We then used the weeks 1, 2, and 3 post-infusion DMRs (ranked from unmethylated to methylated) to perform a gene set enrichment analysis (GSEA) assessing for enrichment
of effector and memory gene expression programs. Demethylated regions from weeks 1, 2, and 3 were all enriched for effector-associated genes (Figure 4A); in contrast, when we analyzed the post-infusion methylation programs using the memory-associated gene signature, we observed that samples from each of the time points demonstrated a progressive negative association with unmethylated memory-associated genes (Figure 4B). Specifically, the week 1 samples had a much greater enrichment for unmethylated regions relative to the week 2 and 3 samples. Further, the decline in the memory gene signature among unmethylated loci was mirrored by a corresponding increase for enrichment among the methylated loci. These data document a loss of memory potential among CD8+ CD19-CAR T cells over time in patients.

We next sought to determine whether this loss of memory potential was associated with acquisition of exhaustion-associated gene expression programs. Using a second independently established gene expression signature that has been recently generated to describe a progenitor subset of exhausted CD8+ T cells, we interrogated the post-infusion DMRs. Indeed, samples isolated from all three time points were enriched for an exhaustion progenitor-associated gene signature. Furthermore, the progressive loss in the memory gene signature at weeks 2 and 3 is coupled to a gain in the exhaustion progenitor (Tpex) signature relative to week 1 (Figure 4C). To further interrogate the CAR T cells for evidence of exhaustion, we cross-referenced our current dataset with data obtained from the canonical model of T cell exhaustion, chronic LCMV infection of mice. Importantly, this murine model was recently used to establish the causal relationship between DNA methylation programs and functional exhaustion by comparing effector potential of wildtype (WT) and DNMT3A knockout (KO) (deficient in acquiring new DNA methylation programs) antigen-specific CD8+ T cells exposed to chronic LCMV (Figure S3A). Similar to the human progenitor exhaustion GSEA, the analysis using the DNA methylation-driven exhaustion gene signature shows an enrichment over time for these programs among the CD8+ CD19-CAR T cells.

Building upon our findings from the murine LCMV model system, we recently identified loci targeted by DNMT3A in human CAR T cells undergoing exhaustion in preclinical model systems (Prinzing et al., 2021). Using the DNMT3A targeted genes conserved in both murine and human model systems, we assessed these loci for enrichment of DNA methylation during the post-infusion CAR T cell response. Quite strikingly, many of these loci are progressively methylated, including DNMT3A, BACH2, and LEF1 genes (note that TCF7 is already methylated in the GMP samples and remains methylated) (Figures 4D and S3B). Interestingly, roughly half of the de novo DNA methylation programs identified using the chronic stimulation preclinical model systems remain unmethylated at week 3. Parsing these epigenetic changes into demethylation (Figure 4E) versus methylation (Figure 4F) events, we characterized the biological pathways associated with these gene subsets. Notably, the loci enriched for an unmethylated state were involved in transcriptional regulation and host defense, whereas the methylated loci were enriched for pathways involved in T cell differentiation and, specifically, the beta-catenin TCF complex. Collectively, these data show that the pool of CD8+ CD19-CAR T cells acquires permissive effector-associated DNA methylation programs within the first week after infusion and progressively acquires repressive DNA methylation programs that are associated with a
loss of memory developmental potential. Further, these data indicate that the loss of T cell memory potential is coupled to acquisition of T cell exhaustion DNA methylation signatures.

**CD19-CAR T cells transition toward exhaustion**

Having observed progressive enrichment for human and murine exhaustion-associated programs among the genes targeted for epigenetic modification during a CD8⁺ CD19-CAR T cell response, we proceeded to interrogate the DNA methylation programs that delineate the stages of T cell exhaustion. We first isolated the recently defined progenitor exhaustion (Tpex) subset of T cells from healthy donors and generated whole-genome methylation profiles (Figure S4). Using this newly established Tpex DNA methylation profile, we first performed a PCA to assess the relationship between the CAR T developmental trajectory and an endogenous CD8⁺ T cell exhaustion pathway by applying an unbiased genome-wide approach. This analysis demonstrates a progression of the CAR T cells toward the exhaustion subsets and places the post-infusion CAR T cells in a developmental stage preceding Tpex (Figure 5A).

The cellular heterogeneity existing within the exhaustion developmental trajectory has recently been highlighted by studies showing a clear transitory state of T cells that lose stem-associated gene regulatory programs while acquiring hallmarks of exhaustion (Hudson et al., 2019; Utzschneider et al., 2020). Specifically, this transitory state can be demarcated by upregulated expression of CX3CR1 with commensurate repression of TCF7 and LEF1. Further, expression of CX3CR1 has been coupled to expression of BATF, an AP-1 transcription factor that plays a prominent role in the development of T cell exhaustion (Yao et al., 2019; Utzschneider et al., 2020). To resolve the stage of exhaustion into which CD8⁺ CD19-CAR T cells develop, we interrogated the DNA methylation profiles for the transitory hallmarks. After infusion, the LEF1 locus becomes methylated, while the CX3CR1 and BATF loci become unmethylated, indicating a repression of stem-associated genes in conjunction with the acquisition of exhaustion characteristics (Figures 5B and S4). We next examined loci of the inhibitory receptors PD-1 (PDCD1) and TIGIT, which have historically served as hallmarks of T cell exhaustion. Importantly, these loci retain unmethylated states (Figures 5B and S4), consistent with prior reports showing that these epigenetic programs become reinforced during exhaustion (Youngblood et al., 2011). Lastly, we also examined the TOX locus, given that this molecule has recently been shown to be an exhaustion-associated transcriptional regulator (Alfei et al., 2019; Khan et al., 2019). Indeed, the TOX locus becomes significantly demethylated during the post-infusion CAR T cell response (Figures 5B and S4), which further demonstrates the establishment of exhaustion programs during a therapeutic response.

Having observed that these nuanced hallmarks of transitory exhaustion exist among CD8⁺ CD19-CAR T cells post-infusion, we confirmed our findings by referencing a publicly available longitudinal single-cell RNA sequencing analysis of CD19-CAR T cells isolated from patients (Sheih et al., 2020). Specifically, we analyzed these datasets for the expression level of molecules that delineate the progression of exhaustion. Quite strikingly, these data show that LEF1 is repressed, CX3CR1 expression is most prominently detected at the late
time point (approximately 1 month post-infusion), and \textit{BATF} and \textit{TOX} are expressed at both the late and very late time points (approximately 100 days post-infusion) (Figure 5C). Collectively, our results demonstrate a silencing of stem-associated gene expression with progressive upregulation in exhaustion molecules that support the transition of CD19-CAR T cells down a developmental path toward T cell exhaustion.

\textbf{CD19-CAR T cells exhibit impaired \textit{in vivo} recall potential}

Once acquired, exhaustion epigenetic programming can persist even when antigen levels are reduced (Youngblood et al., 2011, 2013; Ahn et al., 2016; Pauken et al., 2016; Ghoneim et al., 2017). Given the long-lived nature of this acquired program, we sought to resolve whether there was evidence for maintenance of T cell exhaustion among CAR T cells that persisted in treated patients. One such hallmark is a limited ability of the cells to expand in response to antigen (Figure 6A). While CAR T cell therapy initially induces significant tumor control tumor, individuals can undergo endogenous CD19+ B cell recovery and relapse with antigen-positive disease. B cell reconstitution occurred among half the patients in this cohort. Notably, CD8+ CD19-CAR T cell quantity in the peripheral blood was detectable at the time of B cell reemergence but did not undergo expansion in response to the presence of antigen-positive cells. This observation is in striking contrast to the initial proliferative burst that the CD8+ CD19-CAR T cells underwent in these same patients upon initial infusion (Figures 6B and S5). These data indicate that the CD8+ CD19-CAR T cells have a functional impairment that limits their ability to mount a recall response. To further assess the \textit{in vivo} function of persistent CD8+ CD19-CAR T cells, we examined patients with CD19+ leukemia relapse for evidence of CD8+ CD19-CAR T cell expansion (Figure 6C). Among the treated individuals in our study, two individuals relapsed with CD19+ ALL while having detectable CAR T cells in their peripheral blood mononuclear cells (PBMCs). Despite the reemergence of tumor antigen, the CD8+ CD19-CAR T cells remained unexpanded. Moreover, when a second infusion of CD19-CAR T cells was given to these patients, a detectable expansion of this product was observed. These data demonstrate that the CD19-CAR T cells in the second infusion retained a greater capacity to expand relative to the existing CAR T cells derived from the primary infusion. To investigate whether these differences in expression between the primary and secondary infusions were associated with the development of exhaustion, we further analyzed whole-genome methylation data from the CD8+ CD19-CAR T cells at the 2-week time point of the primary infusion (Figure 6C). Quite strikingly, the loci associated with a transitory exhaustion program were enriched for the accompanying exhaustion methylation programs (Figure 6C). Importantly, these methylation data were consistent with the gene expression data in Figure 5C with methylation and the decreased expression of \textit{LEF1} and demethylation and increased expression of \textit{CX3CR1}, \textit{BATF}, and \textit{TOX}. While these results are limited due to cohort size, they are consistent with the prevailing concept that T cell exhaustion epigenetic programs can become reinforced and are coupled to repression of the cell’s recall potential.
DISCUSSION

In this study, we performed a longitudinal DNA methylation assessment of CD8\(^+\) CD19-CAR T cell differentiation from patients with B cell ALL to determine if CAR T cells undergo exhaustion. It is important to note that our analysis not only focuses on CD8\(^+\) CD19-CAR T from the GMP product (after CAR T cell manufacturing), but also encompasses samples isolated at several weeks post-infusion. This rich dataset allows for the opportunity to characterize the DNA methylation programming that occurs during a CD8\(^+\) CD19-CAR T cell response. Our data demonstrate that extensive DNA methylation reprogramming of CD8\(^+\) CD19-CAR T cells occurs post-infusion. In comparing the CD8\(^+\) CD19-CAR T cells from the GMP product to CAR T cells isolated from patients longitudinally, we observed a significant overlap between DMRs and gene regulatory elements. Specifically, we found that DMRs were located within enhancers as well as enriched for transcription factor binding sites that regulate T cell effector and memory differentiation. Furthermore, CD8\(^+\) CD19-CAR T cells exhibit DNA methylation programming consistent with progressive acquisition of effector functions, loss of memory potential, and transformation into a precursor exhausted state. Thus, while preclinical studies have suggested T cell exhaustion can occur in CAR T cells, our findings show that CD8\(^+\) CD19-CAR T cells obtain DNA methylation programs consistent with progression on the exhaustion developmental trajectory post-adoptive transfer in humans.

In order to reverse or prevent CAR T cell dysfunction, there is a need to accurately identify the differentiation status and anticipate the developmental trajectory of these cells during a therapeutic response. With mounting evidence that T cell differentiation can be defined by the DNA methylation state of the cell, the field is moving away from the historical reliance on surface molecule expression, including the use of inhibitory receptors to demarcate T cell exhaustion. The potential discordance between inhibitory receptor expression and T cell exhaustion has recently become clear, as several studies have documented the direct link between the duration of inhibitory receptor expression on T cells and their real-time exposure to antigen. In settings where antigen is cleared after a prolonged exposure to the T cell, inhibitory receptor expression is downregulated. Despite the downregulation of these receptors, the T cells retain an imprint of exhaustion, which includes epigenetically permissive states for inhibitory receptors that ultimately impact on the cell’s recall potential (Youngblood et al., 2011; Utzschneider et al., 2016; Pauken et al., 2016; Philip et al., 2017; Schietinger et al., 2012; Ahn et al., 2016). Conversely, upregulation of inhibitory receptors also occurs during the generation of functional effector T cells and may be falsely interpreted as exhaustion. As a result, the field is moving toward a DNA-methylation-based characterization of T cell differentiation, and here, we apply this definition to CD8\(^+\) CD19-CAR T cells.

While several studies have examined the epigenetic mechanisms that reinforce CAR T cell dysfunction in pre-clinical model systems, our findings document that CD8\(^+\) CD19-CAR T cells undergo exhaustion-associated DNA methylation reprogramming during a clinical response. Recently, CAR T cell exhaustion has been associated with increased chromatin accessibility of AP-1 transcription factor motifs. Engineering CAR T cells to overexpress the canonical AP-1 factor c-Jun preserved the CAR T cell’s ability to expand and kill...
tumors during chronic in vitro stimulation (Lynn et al., 2019). Other strategies to prevent CAR T cell dysfunction include limiting activation of the T cells (Weber et al., 2021; Feucht et al., 2019; Zebley and Youngblood, 2021) or erasing the DNA methylation programs that are causal in establishing exhaustion. For instance, treating T cells with the DNA methyltransferase inhibitor decitabine during the manufacturing of the CAR T cells enhanced their proliferative capacity and antitumor ability in both in vitro and murine models (Wang et al., 2021). A more targeted approach involves deletion of the de novo methyltransferase DNMT3A in CAR T cells, which directly inhibits the acquisition of exhaustion-associated DNA methylation. Indeed, we have shown that DNMT3A KO CAR T cells exhibit enhanced proliferation with sustained antitumor ability as compared to their control counterparts (Prinzing et al., 2021). By characterizing the DNA methylation programs acquired during CD8+ CD19-CAR T cell manufacturing and expansion, our data suggest that the different approaches to mitigate T cell dysfunction may be targeting a core set of gene regulatory programs that limits stemness and enforces exhaustion in CAR T cells.

With an enhanced proliferative capacity and ability to eradicate tumors, multipotent memory CD8+ T cells are believed to be the ideal population to use in T-cell-based immunotherapies such as CAR T cell therapy (Flynn and Gorry, 2014; Gattinoni et al., 2011; Sabatino et al., 2016; Klebanoff et al., 2012). However, most CAR T cell manufacturing protocols use the total pool of T cells extracted from the patient without enriching for T cells with desired properties (Chang et al., 2015). CD19-CAR T cells derived from the less-differentiated central memory T cell subset have been reported to exhibit greater proliferation, contributing to the enhanced survival in pre-clinical murine tumor models (Sommermeyer et al., 2016). In patients receiving CD19-CAR T cell therapy, the pre-manufactured T cell population with higher proportions of the less-differentiated T cell subsets (naive, Tscm and Tcm) was associated with CAR T cell persistence beyond 6 months (Chen et al., 2021). Furthermore, it has recently been shown that CD19-CAR T cell infusion products enriched for gene expression signatures indicative of T cell exhaustion have poor expansion after infusion into patients (Deng et al., 2020). These data are consistent with our linear regression analysis showing a positive correlation between the MPI of the GMP product and in vivo expansion. The ability to predict which CD8+ CD19-CAR T cells will expand during a primary immune response against a patient’s tumor has tremendous therapeutic implications and directs our focus to optimizing CAR T cell products prior to infusion. Moving forward, an additional major hurdle involves generating CAR T cells that can remain functional in the setting of prolonged tumor antigen exposure as well as maintain the ability to mount a recall response in the event of tumor relapse. These data provide a clinical rationale for developing strategies to preserve developmental plasticity and limit CAR T cell exhaustion by targeting DNA methylation mechanisms regulating the genes or the genes’ products directly (Zebley et al., 2020).

**Limitations of the study**

Although this study illustrates the acquisition of exhaustion-associated DNA methylation programs during a CD8+ CD19-CAR T cell response, there are several limitations to address. First, our analysis was performed on patient samples, which precludes a
comprehensive characterization. Many time points at which CD19-CAR T cells were isolated from patients did not yield high enough cell numbers to perform analysis. Further, given that DNA methylation has now been established as reinforcing T cell differentiation, specifically exhaustion (Ghoneim et al., 2017; Prinzing et al., 2021), our efforts were focused on demonstrating the changes in DNA methylation that occur during an in vivo CAR T cell response. By using patient samples, we are able to use clinical response to validate our studies and focused here on both antigen-positive leukemia and endogenous B cell reconstitution as metrics of CD8+ CD19-CAR T cell function.

While our study documents changes in CAR T cell DNA methylation programming during their post-infusion response, our data currently do not allow us to incorporate information regarding the duration and quantity of tumor antigen exposure into the characterization of the CAR T cell differentiation status and survival. Nevertheless, our findings support that CAR T cells undergo DNA methylation reprogramming during in vivo expansion, consistent with progression on a pathway to exhaustion. In summary, our work sheds light on the molecular determinants of CAR T cell effector and memory functions in patients post-infusion and provides the rationale for considering manipulation of epigenetic “readers” and “writers” as a future approach for generating CAR T cell products that can sustain an anti-tumor response.

STAR+METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ben Youngblood (benjamin.youngblood@stjude.org).

Materials availability—All stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability—Whole genome methylation data have been deposited to GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

This study does not report original code

Any additional information required to reanalyze the data reported in the paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human samples—CD19-CAR T cells were generated using peripheral blood mononuclear cells (PBMC) obtained via apheresis from pediatric and young adult patients with relapsed/refractory B cell ALL, treated on our institutional CD19-CAR T cell clinical study (NCT03573700; Hines et al., 2021). The protocol was approved by the Food and Drug Administration and by the institutional review boards at St. Jude Children's Research Hospital. CD19-CAR T cell products were generated within the St Jude Children’s Research
Hospital GMP facility. Briefly, T cells are selected from the PBMC product using CD8 and CD4 electromagnetic bead reagents (CliniMACS system; Miltenyi Biotech). T cells are then activated using CD3/CD28 monoclonal antibodies, transduced with the clinical grade CD19-CAR lentiviral vector, and expanded using IL-7 and IL-15 for 7 – 10 days until desired dose is achieved. The final CAR T cell product the undergoes QA/QC testing prior to release for clinical use. Patients then receive lymphodepleting chemotherapy, followed by CD19-CAR T cell infusion at protocol defined dosing. Characteristics of the manufactured product and post-infusion expansion were performed as described by A. Talleur (unpublished data).

| Patient number | Age at infusion (years) | Gender |
|----------------|-------------------------|--------|
| 1              | 20                      | M      |
| 2              | 18                      | F      |
| 3              | 16                      | M      |
| 4              | 10                      | M      |
| 5              | 15                      | F      |
| 6              | 1                       | F      |
| 7              | 12                      | F      |
| 8              | 15                      | F      |
| 9              | 6                       | F      |
| 10             | 5                       | F      |
| 11             | 21                      | M      |
| 12             | 12                      | F      |
| 13             | 8                       | M      |
| 14             | 9                       | M      |
| 15             | 9                       | M      |

**METHODS DETAILS**

**DMR and PCA**—Differential methylation analysis was done by R package DSS 2.34 (Feng et al., 2014). Basic two group comparisons were ran and p value threshold of 0.01 was used. DMR results were further filtered for 30% mean methylation difference between the groups. Single sample comparisons between GMP and later time points were carried using similar parameters in DSS-single. Principal component analysis was carried on top 3,000 CpG sites with the highest variance. Only sites with higher than 5 reads coverage in all samples were included. The R function princomp was used to compute components.

**GO annotation and TF motif enrichment**—Gene ontology annotation was carried using GREAT (McLean et al., 2010) web server (http://great.stanford.edu/public/html/index.php). Input regions include top 300 methylated and demethylated DMRs. And enrichment results of GO biological pathway were collected for Revigo (Supek et al., 2011) visualization tool (http://revigo.irb.hr/). Transcription factor motif scanning was done by HOMER (Heinz et al., 2010) findMotifsGenome.pl with parameter -size given.
**Permutation test of enhancer overlap**—The FANTOM5 (Andersson et al., 2014) phase 1 and 2 combined enhancer bed file for GRCh37 was used. Overlap with DMR bed file was calculated by bedtools intersect (Quinlan and Hall, 2010), and 10,000 randomly generated regions files based on DMRs were generated by bedtools shuffle.

**Linear regression analysis**—A linear regression was used to quantify the relationship between MPI (predictor variable) and log10 transformed peak expansion (response variable). Log transformation was used to make response variable more “normal.”

**Gene set enrichment analysis**—Pre-Ranked GSEA (Subramanian et al., 2005) analysis was performed using DMR tables. Ranks for all genes were assigned by multiplying sign of log fold change and negative log10 of p values. Only one DMR with largest absolute rank were used to represent a gene. The effector and memory gene signatures were generated from YFV RNA seq (Akondy et al., 2017) with top 300 DEGs each direction taken as signatures. The progenitor exhausted gene signature was derived from Table S4 of (Galletti et al., 2020). The epigenetically driven exhaustion signature was generated using top 300 DEGs from chronic DNMT3A KO microarray expression data (Ghoneim et al., 2017). Heatmaps were generated using core enrichment genes from all time points and z score normalized mean methylation levels from DMRs were plotted.

**Analysis of publicly available single cell RNA seq data**—Raw gene expression matrix was downloaded from Gene Expression Omnibus with accession number GSE125881 (Sheih et al., 2020). Data preprocessing and UMAP dimension reduction were done by Seurat 3.2.0 (Stuart et al., 2019). Quality control of cells and genes was done similarly to the paper. Gene expression over UMAP and violin plot were done using Seurat functions FeaturePlot and VlnPlot.

**Isolation of human progenitor exhausted CD8 T cells from healthy donor blood**—This study was conducted with approval from the Institutional Review Board of St. Jude Children’s Research Hospital. Human peripheral blood mononuclear cells (PBMCs) were collected through the St. Jude Blood Bank, and samples for methylation profiling were collected under IRB protocol XPD15-086. PBMCs were purified from platelet apheresis blood unit by density gradient. Briefly, blood was diluted 1:2.5 using sterile Dulbecco’s phosphate-buffered saline (Life Technologies). The diluted blood was then overlayed above Ficol-Paque PLUS (GE Healthcare) at a final dilution of 1:2.5 (ficoll:diluted blood). The gradient was centrifuged at 400 xg with no brake for 20 minutes at room temperature. The PBMCs interphase layer was collected and washed with 2% fetal bovine serum (FBS)/1mM EDTA PBS buffer and then centrifuged at 400xg for 5 minutes.

Tpex cells were FACS purified with the following antibodies:

- CD45RO - APC (BioLegend, Cat# 304210, Clone: UCHL1)
- CD8 - APC/Cy7 (BioLegend, Cat#344714, Clone: SK1)
- CCR7 - FITC (BioLegend, Cat#353216, Clone: G043H7)
CD95 - PE/Cy7 (BioLegend, Cat#305621, Clone: DX2)
CD28 - PE (BioLegend, Cat# 302907, Clone: CD28.2)
PD-1 BV421 (BioLegend, Cat#329920, Clone: EH12.2H7)
TIGIT - PerCp-eFluor710 (Invitrogen, Cat#46-9500-41, Clone: MBSA43)
LDA (Tonbo, Cat# 13-0870-T100)

**Flow cytometric analysis of human CD8^+ CD19-CAR T cells**—Human CD8^+ CD19-CAR T cells were stained with the following antibodies:

CD45 - FITC (BD Bioscience, Cat# 555482, Clone: HI30)
CD19 CAR - Biotin (Miltenyi, Cat# 130-115-965)
Anti-biotin - PE (Miltenyi, Cat# 130-111-068, Clone: REA746)
CD3 - APC (TONBO Bioscience, Cat# 20-0038-1500, Clone: UCHTI)
CD14 – APC Cy7 (BD Bioscience, Cat# 333945, Clone: MφP9)
CD16 – APC Cy7 (BD Bioscience, Cat# 563919, Clone: 3G8)
CD8 – BV510 (BD Bioscience, Cat# 563919, Clone: SK1)
CD4- BV786 (Biolegend, Cat# 317442, Clone OKT4)

**Genomic methylation analysis**—DNA was extracted from the sorted cells by using a DNA-extraction kit (QIAGEN) and then bisulfite treated using an EZ DNA methylation kit (Zymo Research). The bisulfite-modified DNA-sequencing library was generated using the EpiGnome™ kit (Epicenter) per the manufacturer’s instructions. WGBS was performed as described previously. Briefly, bisulfite-modified DNA sequencing libraries were generated using the EpiGenome kit (Epicenter) according to the manufacturer’s instructions. Bisulfite-modified DNA libraries were sequenced using Illumina HiSeq 4000 and NovaSeq 6000 systems (Abdelsamed et al., 2017). Sequencing data were aligned to the HG19 genome using the BSMAP v. 2.74 software (Xi and Li, 2009). Differential analysis of CpG methylation among the datasets was determined with a Bayesian hierarchical model to detect regional methylation differences with at least three CpG sites (Wu et al., 2015). Data are shown from only samples that yielded enough genomic material (~or > 2,000 cells) for genome-wide sequencing coverage and other quality control parameters. Notably, bisulfite modification for the patient 15 week 2 sample had low conversion (deamination of unmethylated cytosines) and was therefore excluded from methylation analyses.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Statistical details of experiments can be found in the figure legends, including sample sizes and p values. For sample size, n = the number of human samples as specified. For p values,
ns = not significant, p < 0.05; *, p < 0.01; **, p < 0.001; ***, p < 0.0001; ****. Unpaired t-tests were performed to assess differences. A p value of < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CD19-CAR T cells undergo extensive DNA methylation reprogramming during therapy
- DNA methylation status of the CAR T cell product can predict *in vivo* expansion
- CD8* CD19-CAR T cells acquire exhaustion-associated DNA methylation programs
- Antigen-positive tumor relapse does not elicit CAR T cell expansion
Figure 1. Post-infusion CD8+ CD19-CAR T cells undergo genome-wide DNA methylation reprogramming

(A) Schematic of sample analysis for CD19-CAR T cells at the pre-manufacturing and post-infusion time points. Created with BioRender.

(B) Principal-component analysis (PCA) showing PC1 using genome-wide DNA methylation status for all available patient samples. n = 29 total patient samples.

(C) Summary graph showing the number of differentially methylated regions (DMRs) longitudinally gained and lost across all patient samples relative to the associated GMP. n = 11 matched GMP and post-infusion patient samples.

(D) Heatmap of the top methylated and demethylated CpG sites in all patient samples with longitudinal analysis among GMP, week 1, week 2, and week 3 time points. Clusters 1–8 are K-means clustering to identify groups of closely associated genes listed in Table S1.

(E) Representative methylation and demethylation events at LEF1, PRF1, and TBET loci. Longitudinal samples are from a representative patient. Individual CpG sites are represented by vertical lines, with red indicating methylation and blue indicating lack of methylation. DMRs are represented by a green box, and enhancers are shown with a purple line.
(F) Permutation test assessing enrichment of the DMRs conserved for all week 1–3 samples at enhancer sites defined by FANTOM (Andersson et al., 2014). n = 12 post-infusion patient samples.

(G) Transcription factor consensus sequence analysis of both the loss of methylation and gain of methylation DMRs conserved for all CD8\(^+\) CD19-CAR T cell post-infusion samples. n = 12 post-infusion patient samples.
Figure 2. Post-infusion DNA methylation programs of CD8+ CD19-CAR T cells are associated with effector differentiation

(A) GO for the conserved post-infusion DMRs derived for all week 1–3 CD8+ CD19-CAR T cells. n = 12 post-infusion patient samples.

(B) PCA of healthy donor naive (n = 4), stem-memory (Tscm, n = 3), central-memory (Tcm, n = 3), effector-memory (Tem, n = 3), and n = 13 CD8+ CD19-CAR T cells isolated longitudinally from patients post-infusion.
Figure 3. Multipotency-associated DNA methylation programs are coupled to CAR T cell post-infusion expansion

(A) Cross-sectional analysis of methylation-based multipotency index (MPI) measurement for CD8+ CD19-CAR T cells. n = 13 patient samples.

(B) Longitudinal analysis of MPI measurement for CD8+ CD19-CAR T cells. n = 19 patient samples.

(C) Linear regression of GMP MPI versus week 1 post-infusion CD8+ CD19-CAR T cell quantity (ug DNA on a log scale) determined from peripheral blood. n = 12 patient samples.
Figure 4. Post-infusion CAR T cell DNA methylation programs are coupled to a progressive repression of memory potential

(A) GSEA of post-infusion DMRs identified in Figure 1 at weeks 1, 2, and 3 using effector-associated gene signatures derived from yellow-fever-specific effector CD8+ T cells. Effector and memory-associated gene signatures were derived from gene expression profiles of yellow fever tetramer+ CD8+ T cells obtained after vaccination of healthy individuals (Akondy et al., 2017). Dashed lines indicate the demarcation between unmethylated and methylated genes.

(B) GSEA of post-infusion DMRs identified in Figure 1 at weeks 1, 2, and 3 using a memory-associated gene signature derived from yellow-fever-specific memory CD8+ T cells.

(C) GSEA of post-infusion DMRs identified in Figure 1 at weeks 1, 2, and 3 using an exhaustion progenitor-associated gene signature (Galletti et al., 2020; van Beek et al., 2021).
(D) Heatmap of overlapping human and murine DNMT3A target genes undergoing methylation and demethylation events from the GMP product to samples collected at the week 1, 2, and 3 time points.

(E) GO analysis of the DNMT3A target genes undergoing demethylation in the GMP product post-infusion.

(F) GO analysis of the DNMT3A target genes undergoing methylation in the GMP product post-infusion.
Figure 5. CD8+ CD19-CAR T cells transition into an exhaustion developmental path post-infusion

(A) PCA using DNA methylation profiles of naive (n = 2), progenitor-exhausted (n = 2), and fully exhausted HIV tetramer+ (n = 4) CD8+ T cells for comparison with the GMP (n = 13) and weeks 1 (n = 4), 2 (n = 5), and 3 (n = 3) CD8+ CD19-CAR T cell profiles.

(B) Summary pie charts for methylation status of DMRs residing within transitory-exhaustion-associated genes.

(C) UMAP analysis of single-cell RNA sequencing data from an independent dataset (Sheih et al., 2020) obtained from CD19-CAR T cell infusion product and post-infusion early (1–2 weeks), late (~1 month), and very late (~80–100 days) time points. The left panel shows the distribution of cells based on the time point, and the smaller right panels show the expression of CX3CR1, BATF, LEF1, and TOX among the cells.
Figure 6. CD19-CAR T cells demonstrate impaired recall potential in patients with antigen-positive cells

(A) Schematic depiction of a CD19-CAR T cell quantity relative to antigen-positive cells during a primary and secondary immune response.

(B) Top panel: longitudinal graph of CD19-CAR T cell quantity and antigen-positive B cells from representative patient 5. Bottom panel: summary graph of CD19-CAR T cell expansion during a primary response to antigen (initial infusion) versus during endogenous CD19+ B cell recovery. n = 5 patient samples.

(C) Patients #4 and #6 CAR T cell vector copy is calculated at the given time points relative to the overall quantity of DNA from the PBMCs. DNA methylation profiles at LEF1, CX3CR1, BATF, PDCD1, TIGIT, and TOX loci from patient 6 GMP and week 2 CD8+ CD19-CAR T cells as well as a Tpex sample are shown. Individual CpG sites are represented by vertical lines, with red indicating methylation and blue indicating a lack of methylation.
methylation. DMRs are represented by a green box, and enhancers are shown with a purple line.
## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| FITC anti-human CD45 (clone HI30) | BD Biosciences | Cat#555482; RRID: AB_395874 |
| Biotin anti-human CD19 CAR (clone N/A) | Miltenyi | Cat#130-115-965 |
| PE anti-biotin (clone REA746) | Miltenyi | Cat#130-111-068 |
| APC anti-human CD3 (clone UCHT1) | TONBO Bioscience | Cat#20-0038-T500 |
| APCCy7 anti-human CD14 (clone MFP9) | BD Biosciences | Cat#333945 |
| APCCy7 anti-human CD16 (clone 3G8) | BD Biosciences | Cat#557758; RRID: AB_396864 |
| BV510 anti-human CD8 (clone SK1) | BD Biosciences | Cat#563919; RRID: AB_2722546 |
| BV785 anti-human CD4 (clone OKT4) | BioLegend | Cat#317442; PRID: AB_2563242 |
| APC anti-human CD45RO (clone UCHL1) | BioLegend | Cat#304210; RRID: AB_314426 |
| APCCy7 anti-human CD8 (clone SK1) | BioLegend | Cat#344714; RRID: AB_2044006 |
| FITC anti-human CCR7 (clone G043H7) | BioLegend | Cat#353216; RRID: AB_10916386 |
| PeCy7 anti-human CD95 (clone DX2) | BioLegend | Cat#305621; RRID: AB_2100370 |
| PE anti-human CD28 (clone CD28.2) | BioLegend | Cat#302907; RRID: AB_314309 |
| BV421 anti-human PD1 (clone EH12.2H7) | BioLegend | Cat#329920; RRID: AB_10960742 |
| PerCp-eFluor710 anti-human TIGIT (clone MBSA43) | Invitrogen | Cat#46-9500-42; RRID: AB_10853679 |
| **Biological samples** | | |
| Healthy Donor PBMC | St Jude Blood Donor Center | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Ghost Dye Violet 510 viability dye | Tonbo Biosciences | Cat#13-0870 |
| **Critical commercial assays** | | |
| QIAGEN DNeasy kit | QIAGEN | Cat#69506 |
| EZ DNA methylation kit | Zymo Research | Cat#E5002 |
| EZ DNA methylation-Direct kit | Zymo Research | Cat#50-444-323 |
| **Deposited data** | | |
| WGBS data | This paper | GSE188325 |
| **Oligonucleotides** | | |
| pUC/M13 reverse primer | Promega | Cat#5421 |
| **Software and algorithms** | | |
| Prism 6 | GraphPad | [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/) |
| FlowJo 9.9.5 | FlowJo | NA |
| Model-based analysis of bisulfite sequencing | (Wu et al., 2015) | [https://pubmed.ncbi.nlm.nih.gov/26184873](https://pubmed.ncbi.nlm.nih.gov/26184873) |
| Gene Ontology (GO) enrichment analysis | GREAT | [http://great.stanford.edu/public/html/index.php](http://great.stanford.edu/public/html/index.php) |
| **Other** | | |
| Fortessa flow cytometer | Flow cytometry core facility at SJCRH | N/A |
| Illumina NovaSeq | Hartwell Center at SJCRH | N/A |
| Illumina HiSeq 4000 | Hartwell Center at SJCRH | N/A |
| Ficoll-Paque PLUS | GE Healthcare | N/A |