nature portfolio

Peer Review File

Histone marks identify novel transcription factors that parse CAR-T subset-of-origin, clinical potential and expansion

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The work from Fiorenza S et al. entitled "Histone marks identify novel transcription factors that parse CAR-T cell subset-of-origin, clinical potential and expansion" describes a novel epigenomic approach for the assessment of T cell immunotherapeutic quality, which they present as orthogonal, robust and wide-reaching. The authors aim at analyzing transcriptionally repressive and permissive histone methylation marks to improve identification of T cell subsets and CAR-T manufactured from central memory cells from healthy donors and patients. Importantly, they identified factor KLF7 to be associated with CAR-T cell expansion in patients-

Overall, the research article comes across as clear and well-thought, with sharp language employed and sound logical flow. Epigenomic analyses are getting more and more attention in the field, so I could see this method being relevant for a wide range of readers. It could be important to include a functional validation of their results, for example by modulating the identified gene KLF7, associated with CAR-T cell expansion in patients, with KO studies in order to generate a cell product with ameliorated efficacy profile.

MAIN POINTS
- In light of recent literature, it would have been interesting to include Naïve-derived CAR T cells in the analysis (Filosto S et al., Blood Cancer Discov. 2024; Dickinson MJ et al., Cancer Discov 2023; Arcangeli S et al, JCI 2022). I can relate to the fact that the authors chose to compare populations that are more similar, so that fine differences may emerge with their approach. Indeed, in Filosto et al. CM appeared to functionally cluster with EM rather than naive-like. However, in a translational perspective I feel that Naive cells should have been included.
- The authors focus on CD8 cells, considering that CD8 CM cells are selected as source of cells for CAR T cell manufacturing for the ongoing clinical trial included in the research article. Nevertheless, recent literature highlight CD4 crucial contribution to CAR-T cell responses, and hence it could be relevant for validation of the approach.
- Even if the authors try to stratify the response in high or low expanders, it is clear that the expansion levels were nevertheless sufficient to achieve a clinical response. I understand that here their goal is to validate the method in seeing differences between populations as similar as possible, but in this case I'm not sure it has great functional relevance. In this light, it could be relevant to include also patients with partial responses or not responders.
- I am also curious as to the potential contribution to this analysis made by the manufacturing process. Here the authors employ Dynabeads + IL-2; expanding the analysis (e.g. IL-7/IL-15 or short manufacturing processes) could be relevant to validate the strength of the novel proposed approach.

Minor points
- The authors should include in the discussion their thought on the effect of CAR design (e.g. CD28 endocostimulus) to their results.
- Even though patients included in the study are quite similar (all received CAR-T cells manufactured from the same starting CM CD8+ T cell subset, with the same CAR-T dose and lymphodepletion regimen and all achieved complete response to CAR-T immunotherapy), there could be additional factors to evaluate in the results (e.g. age, previous treatments…). A Table describing patients characteristics should be included.
- The authors identify in figure 2 a bimodal pattern of transcript abundance correlated with different Histone mark pattern. It's not clear to me how it's possible to have genes expressed with a repressed chromatin mark, and discussion/clarification in this regard could be expanded.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors evaluate healthy donor and patient T cell subsets, CAR T cell products, and CAR T cells in blood of treated patients using both RNAseq and CUT&RUN analysis of

histone marks. The authors identify greater distinction between T cell subsets using H3K4me2 and H3K27me3 histone marks than compared with transcriptional changes, and these spanned both differentially expressed and non-differentially expressed high and low abundance gene transcripts. The data presented are interesting and widely expand on existing literature related to transcriptomic and epigenomic comparisons in T cell subsets, CAR T cell IP, and CAR T cells in blood of treated patients. Most interesting, likely, is the association with TFs in high and low expanding CAR T cells in patients. It is still unclear why the TFs that are identified with histone marks likely to be highly expressed are not pulled out by RNAseq or in literature using scRNAseq. While this may highlight the benefit of CUT&RUN histone mark analysis over traditional transcriptomics analysis, previous studies have demonstrated that KLF7 is overexpressed in early N/SCM/CM T cell subsets compared with EM/EFF subsets. So it is unclear what the potential utility of such deep interrogation is with respect to CAR T cells. It might be impactful to look at CAR T cells in blood of patients comparing those who respond and those who do not respond to therapy, which was not done in the current study. Some validation of hits identified and their impact on T cells at least in vitro would also be beneficial.

1. In general, more statistical analyses should be performed, especially to show differences in the replicates, for instance in relation to DEx and non-DEx gene histone marks that characterize T cell subsets.
2. Important data presented in Figure S6 is glossed over, and should be detailed further. It is both hard to read and hard to interpret the TF differences between the subsets. Please clarify.
3. Did the authors look at CAR T cells in patients who did not achieve CR? Are they changes biologically significant given these patients all demonstrated CR even given the changes in expansion? The group focused on cell proliferation genes but this is also related to exhaustion genes, so were there histone mark changes in those genes?

Reviewer #3 (Remarks to the Author):

In this study Fiorenza et al. compare the use of two histone marks, H3K27me3, associated with silencing, and H3K4me2, associated with transcriptional activation, with transcriptomics, to identify changes in gene activity. They start from previous observations that transcriptomics cannot capture subtle changes in expression of transcription factor genes, which are masked by the large changes in effector and metabolism genes. They first evaluate the differences in the histone marks and RNAseq in peripheral blood CD8+ T cell subsets of healthy donors, including naïve, central memory and effector memory. They observe differences in histone marks for genes expressed at high levels, for which they find no changes in gene expression. They further extend the analysis to CAR-T cells engineered from peripheral blood CD8+ T cell subsets of healthy donors, and further extend the analysis to CAR-T cells from large B cell lymphoma patients. They focus on transcription factors that have changes in histone marks, but no changes in mRNA. They compare the histone marks between CM-derived CAR-T cells from healthy donors and CM-enriched CAR-T cells from large B cell lymphoma patients and identified changes in histone marks at TFs associated with exhaustion, not detectable by transcriptomics. They next use the histone marks to uncover genes that predict in vivo expansion of CAR-T cells after infusion in LBCL patients. RNAseq did not identify any changes in TFs, however by histone marks, they identified two TFs KLF7 and ZFP57, and additionally seven targets of KLF7 showed changes in histone marks in the high-expanding CAR-T product. Overall this is an important study and reveals the power of using histone marks rather than transcriptomics to detect activities associated with gene activity and correlated with CAR-T quality.

- It is very important that they extended the study to CAR-T cells used for LBCL patients, including the identification of KLF7 as having differential histone marks between low expanding versus high expanding cohorts. What was the status of the TFs associated with exhaustion?
- The data for CAR-T cells from patients should be expanded to other TFs, including exhaustion-associated, and not only in the context of expansion, similarly to the data in CAR-T from CD8+ T cell subsets.
- Identification of KLF7 is interesting, however the data remains just correlative. Thus KLF7 should

be manipulated at least in CAR-T cells derived from healthy donors and the impact on proliferation should be evaluated, as well as the impact on the targets claimed in Fig. 8E-F, on cytolytic activity, as well as markers of differentiation and exhaustion. This is even more important, as the link of KLF7 with proliferation is rather poor, including the claim of its association with proliferation in multiple cell types.
- the claims on EVI1 on the link with infusion products from high versus low expanders are not clear or supported by sufficient data (Fig. 8G).
- from the genes in Fig. 8E-F, claimed to be targets of KLF7, only TMEM45A seem to be associated with proliferation and very poorly. Perhaps the link is not really with proliferation, and KLF7 is linked to another biological phenomena here, which happens to correlate with high-expanding CAR-T IP.
- KLF7 had elevated H3k27me3 in CAR-T cells from CM versus EM CD8+ T cells from HDs (Fig. 6). How does this correlate with the findings from the patient CAR-T cells? More discussion should be included on these fundings.
- there should be more attention to writing. Sentences are very long and with multiple subordinates and sometimes hard to understand. An example is this sub-title: “Identification of a novel transcription factor and epigenomic regulome in CAR-T products that associates with robust in vivo CAR-T accumulation after infusion into LBCL patients and T cell expansion when over expressed”.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author)

Point 1:
The work from Fiorenza S et al. entitled "Histone marks identify novel transcription factors that parse CAR-T cell subset-of-origin, clinical potential and expansion" describes a novel epigenomic approach for the assessment of T cell immunotherapeutic quality, which they present as orthogonal, robust and wide-reaching. The authors aim at analyzing transcriptionally repressive and permissive histone methylation marks to improve identification of T cell subsets and CAR-T manufactured from central memory cells from healthy donors and patients. Importantly, they identified factor KLF7 to be associated with CAR-T cell expansion in patients-

Overall, the research article comes across as clear and well-thought, with sharp language employed and sound logical flow. Epigenomic analyses are getting more and more attention in the field, so I could see this method being relevant for a wide range of readers. It could be important to include a functional validation of their results, for example by modulating the identified gene KLF7, associated with CAR-T cell expansion in patients, with KO studies in order to generate a cell product with ameliorated efficacy profile.

Reply R1.1.

We thank Reviewer #1 for their comprehensive critique of our manuscript and appreciate the comments that the paper was clear and well-thought out with sound logical flow and relevance to a wide readership. We acknowledge the importance of functional validation of an identified factor, KLF7, and have performed additional experiments in response to the reviewer's comments. We transduced CD8+ T cells to express the KLF7 open reading frame and GFP and found KLF7-induced dose-dependent expansion of T cells relative to control modification (GFP without KLF7). We also identified increased proliferation of CAR-T cells transduced to express KLF7 using Cell Trace Violet (CTV) dilution. This effect was not antigen-dependent. KLF7-transduced CAR-T cells exhibited an increased fraction of CCR7+CD45RA- phenotype cells. We found that KLF7 is predicted to bind to the IL2 promoter. Validating the promoter analysis, we showed greater IL-2 production by KLF7-transduced CAR-T cells, indicating a potential mechanism of enhanced expansion through autocrine IL-2 production by CD8+ T cells.

We have modified the Introduction section at lines 100-103 to read:

"We used this platform to identify an association between the transcription factor, KLF7, and CAR-T proliferation in vivo in lymphoma patients; and found in vitro that transgenic KLF7 expression is associated with an increased CCR7+/CD45RA- phenotype and IL-2 production."

We have modified the Results section at lines 359-371 to read:

"To determine whether KLF7 contributes to increased accumulation of CAR-T, we assessed cell counts, proliferation, phenotype, and cytokine secretion in T cells and/or CAR-T that were transduced to express GFP with or without KLF7. We found that KLF7-transduced T cells showed a dose-dependent increase in accumulation in culture compared to T cells transduced to only express GFP (Fig. 8G). We then transduced T cells to express a CD19-directed CAR with or without KLF7 and demonstrated enhanced proliferation of KLF7-transduced CAR-T (Fig. 8H). KLF7-dependent increased accumulation was only observed in the absence of stimulation through the CAR (Fig. S19A). A higher percentage of

CCR7+ CD45RA- CM phenotype cells was identified in KLF7+ CD8+ CAR-T, consistent with our finding of decreased KLF7 repression in CD8+ CM-derived CAR-T (Fig. 8I & 5E). Autocrine IL-2 production has been shown to enhance memory CD8+ T cell expansion[56]. To investigate the mechanism of enhanced proliferation in KLF7+ T cells, we interrogated the IL2 promoter and found a proposed KLF7 binding site (Fig. 8J). Validating this *in silico* finding, we found higher IL-2 production by CD8+ KLF7+ CAR-T that was only observed in the absence of stimulation through the CAR (Fig. 8K & Fig. S19B)."

We have modified the Methods section at lines 472-485 to read:

"**KLF7 transduction and functional assays**

T cells were immunomagnetically selected using the Pan T cell isolation kit (Miltenyi) as per the manufacturer's instructions, activated with Transact (Miltenyi), and transduced with different volumes of an epHIV7 lentiviral vector containing either the KLF7 open reading frame (NM_001270942) with a ribosomal skip sequence and GFP (KLF7_P2A_GFP) or control P2A_GFP. The cell counts of KLF7_P2A_GFP- and P2A_GFP-transduced cells were quantitated with acridine orange/propidium iodine dyes counted on a Cellaca PLX instrument (Revvity). KLF7 and GFP transcripts were quantitated by RT-qPCR relative to the housekeeping gene ACTB, using primers listed in Table S14. Fold expansions of KLF7_P2A_GFP- and P2A_GFP-transduced cells were calculated by normalizing to GFP transcript level. For functional CAR-T assays, T cells were transduced with lentivirus encoding a CD19-directed CAR with or without KLF7_P2A_GFP. Transduced cells were then sorted on co-expression of GFP and a CAR transduction marker (EGFRt) to high purity and then cultured for 10 days. Cell Trace Violet dilution, immunophenotyping and intracellular IL-2 detection were performed as previously described[2, 64]."

We have modified the Discussion in lines 411-417 to read:

"Our data showed epigenomic suppression of KLF7 in EM-derived CAR-T in comparison to CM-derived CAR-T, and in low proliferating products from CM-derived CAR-T. Together with previous studies showing associations of KLF7 with early murine thymocyte differentiation and naïve human T cells[60, 61], this posits a continuum of function of KLF7 in maintaining a less differentiated CD8+ T cell state[62]. Increased IL-2 production in the KLF7+ CAR-T in the absence of CAR stimulation also suggests a mechanism of autocrine-driven expansion that may increase the proliferative potential of CD8+ CAR-T."

Point 2:
In light of recent literature, it would have been interesting to include Naïve-derived CAR T cells in the analysis (Filosto S et al., Blood Cancer Discov. 2024; Dickinson MJ et al., Cancer Discov 2023; Arcangeli S et al, JCI 2022). I can relate to the fact that the authors chose to compare populations that are more similar, so that fine differences may emerge with their approach. Indeed, in Filosto et al. CM appeared to functionally cluster with EM rather than naive-like. However, in a translational perspective I feel that Naive cells should have been included.

Reply R1.2.

Thank you for the comment. The reviewer is correct in their assumption that we highlighted the central memory subset to enable fine differences between similar subsets to emerge. However, we agree with the reviewer that analyses of naïve-derived CAR-T cells are also of importance. In response to the

reviewer's comment, we have performed additional analyses of naïve-derived CAR-T cells and have included the data in Figure 5, along with updated text in the Results section from lines 255-265:

"This is clinically important, because the presence of less differentiated cells in leukaphereses or CAR-T infusion products has been associated with improved outcomes[33, 34, 35]. We manufactured CAR-T from N, CM or EM CD8+ T cells from HD then isolated CAR-T by flow sorting for the transduction marker truncated epidermal growth factor receptor (EGFRt)[2]. PCA analyses of RNA-seq data did not distinguish N-, CM- and EM-derived CAR-T (Fig. 5A), consistent with the finding that few genes were DEx in pairwise comparisons between CAR-T manufactured from each subset (N vs CM; N vs EM; CM vs EM; Fig. 5B). In contrast, numerous DEn genes were identified in pairwise comparisons between CAR-T manufactured from each subset (Fig. 5B; Table S3).

We focused on the differences between CM- and EM-derived CAR-T to enable subtle but potentially clinically important differences between similar subsets to emerge."

Point 3:
The authors focus on CD8 cells, considering that CD8 CM cells are selected as source of cells for CAR T cell manufacturing for the ongoing clinical trial included in the research article. Nevertheless, recent literature highlight CD4 crucial contribution to CAR-T cell responses, and hence it could be relevant for validation of the approach.

Reply R1.3.

Thank you for this comment. We agree that analyses of CD4+ CAR-T cells are pertinent; and are planning these studies for a future manuscript. Unfortunately, space and scope limitations do not allow analyses of CD4 CAR-T cells to be included in the current revised manuscript, which now comprises 8 main text figures, 19 supplemental figures, and 15 supplemental tables. In response to the reviewer's comment, we have modified the Introduction (lines 94-96) to read:

"We focused this study on CD8+ T cells and CAR-T because of the association of CD8+ CAR-T counts with response to CAR-T immunotherapy[2, 15, 16]."

Point 4:
Even if the authors try to stratify the response in high or low expanders, it is clear that the expansion levels were nevertheless sufficient to achieve a clinical response. I understand that here their goal is to validate the method in seeing differences between populations as similar as possible, but in this case I'm not sure it has great functional relevance. In this light, it could be relevant to include also patients with partial responses or not responders.

Reply R1.4.

Thank you for the comment. We agree with the reviewer that it is relevant to include patients who did not achieve complete response (CR) in the analyses. We performed these studies but did not identify KLF7 as associated with response in the revised larger cohort of analysed patients. This is likely due to the impact of the tumor microenvironment on response. Therefore, to mitigate the impact of the tumor microenvironment we identified histone marked genes that associated with robust in vivo CAR-T cell accumulation in the patients that achieved CR.

In response to the reviewer's point, we have now included new data to show that neither histone methylation marks nor RNA-seq identified differences between patients who did or did not achieve CR (new Fig. S17). We modified the Results section as follows (lines 321-327):

“We first embarked on analyses of IP from 30 LBCL patients (Table S10) treated on a phase 1 clinical trial of CD19 CAR-T immunotherapy (NCT01865617). Transcriptomic and epigenomic analyses did not distinguish the IP of patients who had a complete response (CR) from those who did not have a CR (non-CR; Fig. S17A). This was also the case when we compared IP from patients who received a CAR-T product manufactured from the same subset (CM-enriched CD8+ T cells; Fig. S17B), likely due in part to the complexities of the LBCL tumor microenvironment (TME).”

Point 5:
I am also curious as to the potential contribution to this analysis made by the manufacturing process. Here the authors employ Dynabeads + IL-2; expanding the analysis (e.g. IL-7/IL-15 or short manufacturing processes) could be relevant to validate the strength of the novel proposed approach.

The authors should include in the discussion their thought on the effect of CAR design (e.g. CD28 endocostimulus) to their results.

Reply R1.5.

Like the reviewer, we are equally enthused by the opportunity that this new analysis platform creates for further discovery of determinants of CAR-T cell therapy and the possibility of discovery of new genes that can be manipulated for therapeutic advantage, such as KLF7. For this initial study, we used the method of manufacturing employed in our clinical trial (NCT01865617) to enable comparisons between healthy donor and patient-derived CAR-T cells and to validate the utility of epigenomic analyses in a real-world context.

Furthermore, we agree with the reviewer that an analysis of different manufacturing methods and CAR design may indeed impact CAR-T cell epigenomics and lead to a more detailed understanding of these clinical factors. Further study is indeed warranted, and we have modified the Discussion (lines 420-422) to highlight the reviewer’s point:

*“*This approach opens a suite of possibilities to compare differences in CAR designs, CAR-T manufacturing methods, and treatment regimens, all of which may have heritable effects on CAR-T progeny.”

Point 6:
Even though patients included in the study are quite similar (all received CAR-T cells manufactured from the same starting CM CD8+ T cell subset, with the same CAR-T dose and lymphodepletion regimen and all achieved complete response to CAR-T immunotherapy), there could be additional factors to evaluate in the results (e.g. age, previous treatments…). A Table describing patients characteristics should be included.

Reply R1.6.

We agree with the reviewer and have added a new Table (S10) to show characteristics in the analyzed patients. We have revised the Results section in lines 321-322:

“We first embarked on analyses of IP from 30 LBCL patients (Table S10) treated on a phase 1 clinical trial of CD19 CAR-T immunotherapy (NCT01865617).”

Point 7:
The authors identify in figure 2 a bimodal pattern of transcript abundance correlated with different Histone mark pattern. It's not clear to me how it's possible to have genes expressed with a repressed chromatin mark, and discussion/clarification in this regard could be expanded.

Reply R1.7.

Thank you for the comment. Single repressive and permissive histone marks do not uniformly correlate with transcript abundance, likely because a single transcriptional output can be influenced by epigenetic changes, such as histone modifications and methylation at other loci. We have amended our Discussion (lines 385-387) to now read:

"This is likely because the transcriptome is not necessarily a heritable and transient composite output of histone modifications and methylation at any particular point in time[58]."

Reviewer #2 (Remarks to the Author):

Point 1:
In this manuscript, the authors evaluate healthy donor and patient T cell subsets, CAR T cell products, and CAR T cells in blood of treated patients using both RNAseq and CUT&RUN analysis of histone marks. The authors identify greater distinction between T cell subsets using H3K4me2 and H3K27me3 histone marks than compared with transcriptional changes, and these spanned both differentially expressed and non-differentially expressed high and low abundance gene transcripts. The data presented are interesting and widely expand on existing literature related to transcriptomic and epigenomic comparisons in T cell subsets, CAR T cell IP, and CAR T cells in blood of treated patients.

Reply R2.1.

Thank you. We appreciate the reviewer's comments.

Point 2:
Most interesting, likely, is the association with TFs in high and low expanding CAR T cells in patients. It is still unclear why the TFs that are identified with histone marks likely to be highly expressed are not pulled out by RNAseq or in literature using scRNAseq. While this may highlight the benefit of CUT&RUN histone mark analysis over traditional transcriptomics analysis, previous studies have demonstrated that KLF7 is overexpressed in early N/SCM/CM T cell subsets compared with EM/EFF subsets. So it is unclear what the potential utility of such deep interrogation is with respect to CAR T cells.

Reply R2.2.

We thank the reviewer for their comment and agree that identifying genes that predict high and low expansion is particularly important for the creation of next generation CAR-T cells. The reviewer raises two points. First, the reviewer notes a seeming quandary as to why TFs are, in certain circumstances, identified by epigenomic analyses and not by RNA seq. We were motivated to undertake an analysis of the epigenome using CUT&RUN, because the smaller dynamic range and heritable and stable nature of epigenomic marks being less subject to the statistical noise inherent in analyses of genes with high abundance transcripts. Thus, changes in histone marking patterns may be detected without identified changes in transcript; and may precede or follow transient changes in transcript.

As highlighted by the reviewer, previous studies have identified in KLF7 in memory T cells post-vaccination in humans and following murine thymocyte development, but not in CAR-T cells and not in association with T cell or CAR-T cell proliferation. The utility of using histone mark analyses to investigate CAR-T cells in particular was especially interesting to us because of the wholesale activation of T cells during CAR-T manufacturing. Accordingly, the orthogonal datasets generated by histone mark analyses outperformed those generated by RNA-seq, allowing us to identify differences in KLF7 marking in highly challenging cell types: activated CAR-T cell infusion products. The identification of the association between KLF7 and in vivo CAR-T proliferation is an exemplar of this. Demonstration in vitro of the impact of KLF7 on T and CAR-T proliferation and IL-2 production validated the findings and confirm that KLF7 was not identified only as an epiphenomenon of a less mature cell type.

To emphasize the importance of this approach we state in the Discussion in lines 391-394:

"The identification of TFs is particularly demonstrative in this regard. Despite the importance in governing fate and function, transcription factors are classically lowly and transiently expressed, thus complicating identification by RNA-seq [9, 10]. Assessing stable, heritable epigenomic binary marks in the cell circumvents the low dynamic range and ephemeral nature of TF transcripts."

And we present the validation of KLF7 in the Results section at lines 359-371 to read:

"To determine whether KLF7 contributes to increased accumulation of CAR-T, we assessed cell counts, proliferation, phenotype, and cytokine secretion in T cells and/or CAR-T that were transduced to express GFP with or without KLF7. We found that KLF7-transduced T cells showed a dose-dependent increase in accumulation in culture compared to T cells transduced to only express GFP (Fig. 8G). We then transduced T cells to express a CD19-directed CAR with or without KLF7 and demonstrated enhanced proliferation of KLF7-transduced CAR-T (Fig. 8H). KLF7-dependent increased accumulation was only observed in the absence of stimulation through the CAR (Fig. S19A). A higher percentage of CCR7+ CD45RA- CM phenotype cells was identified in KLF7+ CD8+ CAR-T, consistent with our finding of decreased KLF7 repression in CD8+ CM-derived CAR-T (Fig. 8I & 5E). Autocrine IL-2 production has been shown to enhance memory CD8+ T cell expansion[56]. To investigate the mechanism of enhanced proliferation in KLF7+ T cells, we interrogated the IL2 promoter and found a proposed KLF7 binding site (Fig. 8J). Validating this *in silico* finding, we found higher IL-2 production by CD8+ KLF7+ CAR-T that was only observed in the absence of stimulation through the CAR (Fig. 8K & Fig. S19B)."

To account for this new data, we have modified the Methods section at lines 472-485 to read:

"**KLF7 transduction and functional assays**

T cells were immunomagnetically selected using the Pan T cell isolation kit (Miltenyi) as per the manufacturer's instructions, activated with Transact (Miltenyi), and transduced with different volumes of an epHIV7 lentiviral vector containing either the KLF7 open reading frame (NM_001270942) with a ribosomal skip sequence and GFP (KLF7_P2A_GFP) or control P2A_GFP. The cell counts of KLF7_P2A_GFP- and P2A_GFP-transduced cells were quantitated with acridine orange/propidium iodine dyes counted on a Cellaca PLX instrument (Revvity). KLF7 and GFP transcripts were quantitated by RT-qPCR relative to the housekeeping gene ACTB, using primers listed in Table S14. Fold expansions of KLF7_P2A_GFP- and P2A_GFP-transduced cells were calculated by normalizing to GFP transcript level. For functional CAR-T assays, T cells were transduced with lentivirus encoding a CD19-directed CAR with or without KLF7_P2A_GFP. Transduced cells were then sorted on co-expression of GFP and a CAR transduction marker (EGFRt) to high purity and then cultured for 10 days. Cell Trace Violet dilution, immunophenotyping and intracellular IL-2 detection were performed as previously described[2, 64]."

And we have modified the Discussion in lines 409-415 to read:

"Our data showed epigenomic suppression of KLF7 in EM-derived CAR-T in comparison to CM-derived CAR-T, and in low proliferating products from CM-derived CAR-T. Together with previous studies showing associations of KLF7 with early murine thymocyte differentiation and naïve human T cells[60, 61], this posits a continuum of function of KLF7 in maintaining a less differentiated CD8+ T cell state[62]. Increased IL-2 production in the KLF7+ CAR-T in the absence of CAR stimulation also suggests a mechanism of autocrine-driven expansion that may increase the proliferative potential of CD8+ CAR-T."

Point 3:
It might be impactful to look at CAR T cells in blood of patients comparing those who respond and those who do not respond to therapy, which was not done in the current study. Some validation of hits identified and their impact on T cells at least in vitro would also be beneficial.

Reply R2.3.

We thank the Reviewers for the comment and have enhanced our analysis and modified the Results as follows (lines 321-327):

"We first embarked on analyses of IP from 30 LBCL patients (Table S10) treated on a phase 1 clinical trial of CD19 CAR-T immunotherapy (NCT01865617). Transcriptomic and epigenomic analyses did not distinguish the IP of patients who had a complete response (CR) from those who did not have a CR (non-CR; Fig. S17A). This was also the case when we compared IP from patients who received a CAR-T product manufactured from the same subset (CM-enriched CD8+ T cells; Fig. S17B), likely due in part to the complexities of the LBCL tumor microenvironment (TME)."

As outlined in response to Reviewer 2 in R2.2 we further validated our identification of KLF7.

Point 4:
In general, more statistical analyses should be performed, especially to show differences in the replicates, for instance in relation to DEx and non-DEx gene histone marks that characterize T cell subsets.

Reply R2.4.

We thank Reviewer 2 for suggestions regarding the need for additional statistical analyses across replicates, especially to delineate differences in histone marks among the sorted T cell subsets. We have added three new statistical analyses that underscore the robust nature of the data and highlight consistent patterns of gene marking findings between donors, as outlined below and as shown in Fig. S5, Fig. S6 and Fig. S9:

1. ***MA Plot for Individual Donors:*** *We have included an MA plot for each individual donor, which illustrates the distribution of log-fold changes in gene expression relative to the mean signal counts across all donors (Fig. S5). This visualization that enables assessment of the consistency and variability of gene expression changes among different individual donors and shows that across all donors there is a relative even distribution of up and down-regulated genes across all three modalities of analysis (RNA-seq, H3K27me3, H3K4me2) and that, as expected, in highly expressed genes there are few if any outliers that contribute to differentially expressed genes.*
2. ***Log-Fold Change Analysis with respect to the Donor Replicate Variations:*** *We have added the log-fold changes with respect to the average variance across donors for each gene (Fig. S6). This approach shows that the differential signals detected are not inflated by ultra-small variations across donors and are also not highly variable across donors.*
3. ***Volcano Plot Incorporating Adjusted P-values:*** *Additionally, we have generated a volcano plot that contrasts adjusted p-values with log-fold changes (Fig. S9). This plot not only quantifies the effect size magnitude of gene expression changes (i.e., log-fold change) but also accounts for variation among donors and potential repeated test error by presenting the adjusted P value on the y axis. This plot show that the identification of statistically significant differences*

is not merely due to minor fluctuations across samples but reflects a consistent and substantial effect across the donors.

To account for these additional statistical presentations we have modified the text as follows:

Lines 117-119 of the updated Results section:

"Across donors, consistent distributions of up- and down-regulated genes were observed (Fig. S5) with differential signals being unaffected by small variations (Fig. S6), indicating statistical robustness of these datasets".

Lines 143-147 of the Results section:

"When comparing the numbers of genes statistically differentially expressed (DEx) by RNA-seq or differentially enriched (DEn) by histone marks, larger differences were seen between N and EM subsets than between N and CM, and the smallest differences were observed between CM and EM subsets (Fig.1B, Fig. S9 & Table S1), consistent with known relationships between these subsets[1]."

Point 5:
Important data presented in Figure S6 is glossed over, and should be detailed further. It is both hard to read and hard to interpret the TF differences between the subsets. Please clarify.

Reply R2.5.

We apologize for the oversight. The data is a readout from the University of California Southern California Genome Browser. We have increased the size of the font to ease reading in the new Figure S8. We have also updated the text in lines 134-136 of the Results:

"Genomic tracks coding for TFs that have been typically associated with N and CM subsets were marked by low H3K27me3 in N and CM subsets and low H3K4me2 in EM subsets; and vice-versa for TFs that have been associated with effector differentiation (Fig. S8)[18, 19, 20, 21, 22]."

Point 6:
Did the authors look at CAR T cells in patients who did not achieve CR? Are they changes biologically significant given these patients all demonstrated CR even given the changes in expansion?

Reply R2.6.

Thank you for the comment. We agree with the reviewer that it is relevant to include patients who did not achieve complete response (CR) in the analyses. We performed these studies but did not identify KLF7 as associated with response in the revised larger cohort of analysed patients. This is likely due to the impact of the tumor microenvironment on response. Therefore, to mitigate the impact of the tumor microenvironment and enable identification of genes associated with expansion (an important component of CAR-T cell therapy) we identified histone marked genes that associated with robust in vivo CAR-T cell accumulation in the patients that achieved CR.

In response to the reviewer's point, we have now included new data to show that neither histone methylation marks nor RNA-seq identified differences between patients who did or did not achieve CR (new Fig. S17). We modified the Results, as follows (lines 321 to 327):

“We first embarked on analyses of IP from 30 LBCL patients (Table S10) treated on a phase 1 clinical trial of CD19 CAR-T immunotherapy (NCT01865617). Transcriptomic and epigenomic analyses did not distinguish the IP of patients who had a complete response (CR) from those who did not have a CR (non-CR; Fig. S17A). This was also the case when we compared IP from patients who received a CAR-T product manufactured from the same subset (CM-enriched CD8+ T cells; Fig. S17B), likely due in part to the complexities of the LBCL tumor microenvironment (TME).”

Point 7:
The group focused on cell proliferation genes but this is also related to exhaustion genes, so were there histone mark changes in those genes?

Reply R2.7.

We appreciate the advice of the Reviewer. We examined whether genes that were differentially expressed or enriched within highly proliferating products also overlapped with those in known CAR-T and tumor-infiltrating exhaustion genesets from RNA-seq and scRNA-seq studies. To do so, we assessed overlap between our geneset and genesets from published sources (new Table S12). We found that five histone-marked genes overlapped with published RNA-seq datasets, including SLAMF7, which has a known association with T cell exhaustion. We note that these comparisons are made with RNA-seq datasets; as we emphasize in the manuscript, histone mark analysis is complementary and orthogonal to RNA-seq. We are working on a new manuscript describing histone modification changes in T cell exhaustion. Unfortunately, due to size and scope limitations of the current manuscript, we are not able to include data from studies of CAR-T cell exhaustion here.

We have updated the results section (lines 341-346) as follows:

“Five DEn genes overlapped with previously identified genes from RNA-seq studies of CAR-T exhaustion and tumor-infiltrating lymphocytes (Table S12). Only one of these genes, SLAMF7, has a known association with T cell exhaustion; it was H3K4me2 marked in low expanding CAR-T products[52]. These data demonstrate shared but not identical programming in CAR-T cell functions such as expansion and exhaustion identified by histone modification analyses when compared to those found by RNA-seq.”

Reviewer #3 (Remarks to the Author):

Point 1:
In this study Fiorenza et al. compare the use of two histone marks, H3K27me3, associated with silencing, and H3K4me2, associated with transcriptional activation, with transcriptomics, to identify changes in gene activity. They start from previous observations that transcriptomics cannot capture subtle changes in expression of transcription factor genes, which are masked by the large changes in effector and metabolism genes. They first evaluate the differences in the histone marks and RNAseq in peripheral blood CD8+ T cell subsets of healthy donors, including naïve, central memory and effector memory. They observe differences in histone marks for genes expressed at high levels, for which they find no changes in gene expression. They further extend the analysis to CAR-T cells engineered from peripheral blood CD8+ T cell subsets of healthy donors, and further extend the analysis to CAR-T cells from large B cell lymphoma patients. They focus on transcription factors that have changes in histone marks, but no changes in mRNA. They compare the histone marks between CM-derived CAR-T cells from healthy donors and CM-enriched CAR-T cells from large B cell lymphoma patients and identified changes in histone marks at TFs associated with exhaustion, not detectable by transcriptomics. They next use the histone marks to uncover genes that predict in vivo expansion of CAR-T cells after infusion in LBCL patients. RNAseq did not identify any changes in TFs, however by histone marks, they identified two TFs KLF7 and ZFP57, and additionally seven targets of KLF7 showed changes in histone marks in the high-expanding CAR-T product. Overall this is an important study and reveals the power of using histone marks rather than transcriptomics to detect activities associated with gene activity and correlated with CAR-T quality.

Reply R3.1.

We thank Reviewer #3 for their detailed analysis and comment regarding the importance of this study.

Point 2:
It is very important that they extended the study to CAR-T cells used for LBCL patients, including the identification of KLF7 as having differential histone marks between low expanding versus high expanding cohorts. What was the status of the TFs associated with exhaustion?

The data for CAR-T cells from patients should be expanded to other TFs, including exhaustion-associated, and not only in the context of expansion, similarly to the data in CAR-T from CD8+ T cell subsets.

Reply R3.2.

We agree with the reviewer on the importance of the expanding this analysis to large B cell lymphoma patients. To address overlap with exhaustion, we examined whether genes that were differentially expressed or enriched within highly proliferating products also overlapped with those in known CAR-T and tumor-infiltrating exhaustion genesets from RNA-seq and scRNA-seq studies. To do so, we assessed overlap between our geneset and genesets from published sources (new Table S12). We found that five histone-marked genes overlapped with published RNA-seq datasets, including SLAMF7, which has a known association with T cell exhaustion. We note that these comparisons are made with RNA-seq datasets; as we emphasize in the manuscript, histone mark analysis is complementary and orthogonal to RNA-seq. We are working on a new manuscript describing histone modification changes in T cell

exhaustion. Unfortunately, due to size and scope limitations of the current manuscript, we are not able to include data from studies of CAR-T cell exhaustion here.

To reflect this, we have updated the results section (lines 341-346) as follows:

"Five DEn genes overlapped with previously identified genes from RNA-seq studies of CAR-T exhaustion and tumor-infiltrating lymphocytes (Table S12). Only one of these genes, SLAMF7, has a known association with T cell exhaustion; it was H3K4me2 marked in low expanding CAR-T products[52]. These data demonstrate shared but not identical programming in CAR-T cell functions such as expansion and exhaustion identified by histone modification analyses when compared to those found by RNA-seq."

Point 3:
Identification of KLF7 is interesting, however the data remains just correlative. Thus KLF7 should be manipulated at least in CAR-T cells derived from healthy donors and the impact on proliferation should be evaluated, as well as the impact on the targets claimed in Fig. 8E-F, on cytolytic activity, as well as markers of differentiation and exhaustion. This is even more important, as the link of KLF7 with proliferation is rather poor, including the claim of its association with proliferation in multiple cell types.

Reply R3.3.

We acknowledge the importance of functional validation of an identified factor, KLF7, and have performed additional experiments in response to the reviewers' comments. We transduced CD8+ T cells to express the KLF7 open reading frame and GFP and found KLF7-induced dose-dependent expansion of T cells relative to control modification (GFP without KLF7). We also identified increased proliferation of CAR-T cells transduced to express KLF7 using Cell Trace Violet (CTV) dilution. This effect was not antigen-dependent. KLF7-transduced CAR-T cells exhibited an increased fraction of CCR7+CD45RA- phenotype cells. We found that KLF7 is predicted to bind to the IL2 promoter. Validating the promoter analysis, we showed greater IL-2 production by KLF7-transduced CAR-T cells, indicating a potential mechanism of enhanced expansion through autocrine IL-2 production by CD8+ T cells.

We have modified the Introduction section at lines 100-103 to read:

"We used this platform to identify an association between the transcription factor, KLF7, and CAR-T proliferation in vivo in lymphoma patients; and found in vitro that transgenic KLF7 expression is associated with an increased CCR7+/CD45RA- phenotype and IL-2 production."

We have modified the Results section at lines 359-371 to read:

"To determine whether KLF7 contributes to increased accumulation of CAR-T, we assessed cell counts, proliferation, phenotype, and cytokine secretion in T cells and/or CAR-T that were transduced to express GFP with or without KLF7. We found that KLF7-transduced T cells showed a dose-dependent increase in accumulation in culture compared to T cells transduced to only express GFP (Fig. 8G). We then transduced T cells to express a CD19-directed CAR with or without KLF7 and demonstrated enhanced proliferation of KLF7-transduced CAR-T (Fig. 8H). KLF7-dependent increased accumulation was only observed in the absence of stimulation through the CAR (Fig. S19A). A higher percentage of CCR7+ CD45RA- CM phenotype cells was identified in KLF7+ CD8+ CAR-T, consistent with our finding of decreased KLF7 repression in CD8+ CM-derived CAR-T (Fig. 8I & 5E). Autocrine IL-2 production has been shown to enhance memory CD8+ T cell expansion[56]. To investigate the mechanism of enhanced

proliferation in KLF7+ T cells, we interrogated the IL2 promoter and found a proposed KLF7 binding site (Fig. 8J). Validating this *in silico* finding, we found higher IL-2 production by CD8+ KLF7+ CAR-T that was only observed in the absence of stimulation through the CAR (Fig. 8K & Fig. S19B)."

We have modified the Methods section at lines 472-485 to read:

"**KLF7 transduction and functional assays**

T cells were immunomagnetically selected using the Pan T cell isolation kit (Miltenyi) as per the manufacturer's instructions, activated with Transact (Miltenyi), and transduced with different volumes of an epHIV7 lentiviral vector containing either the KLF7 open reading frame (NM_001270942) with a ribosomal skip sequence and GFP (KLF7_P2A_GFP) or control P2A_GFP. The cell counts of KLF7_P2A_GFP- and P2A_GFP-transduced cells were quantitated with acridine orange/propidium iodine dyes counted on a Cellaca PLX instrument (Revvity). KLF7 and GFP transcripts were quantitated by RT-qPCR relative to the housekeeping gene ACTB, using primers listed in Table S14. Fold expansions of KLF7_P2A_GFP- and P2A_GFP-transduced cells were calculated by normalizing to GFP transcript level. For functional CAR-T assays, T cells were transduced with lentivirus encoding a CD19-directed CAR with or without KLF7_P2A_GFP. Transduced cells were then sorted on co-expression of GFP and a CAR transduction marker (EGFRt) to high purity and then cultured for 10 days. Cell Trace Violet dilution, immunophenotyping and intracellular IL-2 detection were performed as previously described[2, 64]."

We have modified the Discussion in lines 411-417 to read:

"Our data showed epigenomic suppression of KLF7 in EM-derived CAR-T in comparison to CM-derived CAR-T, and in low proliferating products from CM-derived CAR-T. Together with previous studies showing associations of KLF7 with early murine thymocyte differentiation and naïve human T cells[60, 61], this posits a continuum of function of KLF7 in maintaining a less differentiated CD8+ T cell state[62]. Increased IL-2 production in the KLF7+ CAR-T in the absence of CAR stimulation also suggests a mechanism of autocrine-driven expansion that may increase the proliferative potential of CD8+ CAR-T."

Point 4:
The claims on EVI1 on the link with infusion products from high versus low expanders are not clear or supported by sufficient data (Fig. 8G).

Reply R3.4.

We thank the reviewer for highlighting the lack of clarity around the analysis. Given the lack of supportive data for EVI1 and space limitations imposed by addition of new functional KLF7 data, we have removed this panel.

Point 5:
From the genes in Fig. 8E-F, claimed to be targets of KLF7, only TMEM45A seem to be associated with proliferation and very poorly. Perhaps the link is not really with proliferation, and KLF7 is linked to another biological phenomena here, which happens to correlate with high-expanding CAR-T IP.

Reply R3.5.

We apologise for the lack of clarity in the text. The reference to TMEM45A is as an example of genes associated with proliferation that are targets of KLF7. To improve clarity, we highlight the new Table S13, which identifies proposed targets of KLF7 and their association with proliferation and provides references from the literature. In fact, all proposed targets of KLF7 have a known association with cell proliferation and, even more strikingly, this was in the same direction as the association seen in our study of high vs low expanding CAR-T cell production. We have modified the Results section (lines 354-356) to read:

"All proposed targets of KLF7 showed an association with proliferation of various cell types from the literature in keeping with the directionality of association with CAR-T expansion in our analysis (Table S13)."

We agree with Reviewer #3 that the association of KLF7 with robust in vivo CAR-T expansion could be linked to a different biological phenomenon. To address this point we conducted new experiments as described in the Reply R3.3, and confirmed increased proliferation in T cells and CAR-T, a higher fraction of central memory CCR7+CD45RA- phenotype cells, and greater IL-2 production in CAR-T engineered to express KLF7.

Point 6:
KLF7 had elevated H3k27me3 in CAR-T cells from CM versus EM CD8+ T cells from HDs (Fig. 6). How does this correlate with the findings from the patient CAR-T cells? More discussion should be included on these fundings.

Reply R3.6.

KLF7 shows higher repressive H3K27me3 marks in EM-derived CAR-T compared to CM-derived CAR-T. We apologize for any lack of clarity.

Repressive KLF7 marking distinguished EM-derived CAR-T cells from CM-derived CAR-T cells. Even within CM-derived CAR-T cells, repressive KLF7 marking associated with low proliferative capacity. This was demonstrated by our finding that in CAR-Ts infused to patients (all of whom received CAR-T cells manufactured from phenotypically CM cells), we identified more KLF7 repressive marking in those who had low in vivo CAR-T accumulation. Together with studies reporting roles for KLF7 in murine thymocytes and naïve human T cells, the data suggests there may be a continuum of increasing function due to increasing KLF7 associated with less differentiated T cells. We have amended our Discussion in lines 411-417 to emphasize:

"Our data showed epigenomic suppression of KLF7 in EM-derived CAR-T in comparison to CM-derived CAR-T, and in low proliferating products from CM-derived CAR-T. Together with previous studies showing associations of KLF7 with early murine thymocyte differentiation and naïve human T cells[60, 61], this posits a continuum of function of KLF7 in maintaining a less differentiated CD8+ T cell state[62]. Increased IL-2 production in the KLF7+ CAR-T in the absence of CAR stimulation also suggests a

mechanism of autocrine-driven expansion that may increase the proliferative potential of CD8+ CAR-T."

Point 7:
There should be more attention to writing. Sentences are very long and with multiple subordinates and sometimes hard to understand. An example is this sub-title: "Identification of a novel transcription factor and epigenomic regulome in CAR-T products that associates with robust in vivo CAR-T accumulation after infusion into LBCL patients and T cell expansion when over expressed".

Reply R3.7.

We apologize for our lack of attention to writing style. We have revised the sub-title (lines 318-319) and other areas of the text to improve clarity.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have dedicated efforts in addressing all raised points, adding data to main figures, 5 new supplementary figures and 2 tables. However:
• Functional Validation of KLF7. The authors addressed this point by overexpressing this transcription factor in activated T cells and CAR-T cells, and highlighting a relative effect on cell proliferation and IL-2 production. It is not clear to me though why this effect is only visible in absence of antigen stimulation through the CAR, and I am concerned about the implications since antigen-independent chronic proliferation can induce exhaustion affecting overall anti-tumoral responses and pose risks of adverse events. The authors ought to expand discussion on this regard, and also offer more indications on how the experiments with antigen stimulation were executed, since it is lacking.
• Inclusion of Naïve-derived CAR-T cells to the analysis. The authors have performed additional analyses and highlighted numerous DEn genes in the multiple comparison. I would have expected more differentially expressed genes from RNA seq analysis, and I think the authors should properly discuss this point. I acknowledge the difficulty in finding in the literature the exact comparison made by the authors, but at the same time functional differences have been already proven in different manuscripts. It’s difficult to think that such differences are not reflected in transcriptomic changes.
• Inclusion of non-responders to the analysis. As requested, the authors included the analysis in figure S17, and found that neither histone methylation marks nor RNA-seq identified differences between patients who did or did not achieve CR. According to available literature, I would have expected some differences to emerge.

Reviewer #2 (Remarks to the Author):

The authors have addressed many of the reviewer's concerns to improve the quality of the manuscript.

Reviewer #3 (Remarks to the Author):

The authors addressed the concerns of this reviewer. This is a very interesting study and I am looking forward to its publication.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author)

Point 1:
Functional Validation of KLF7. The authors addressed this point by overexpressing this transcription factor in activated T cells and CAR-T cells, and highlighting a relative effect on cell proliferation and IL-2 production. It is not clear to me though why this effect is only visible in absence of antigen stimulation through the CAR, and I am concerned about the implications since antigen-independent chronic proliferation can induce exhaustion affecting overall anti-tumoral responses and pose risks of adverse events. The authors ought to expand discussion on this regard, and also offer more indications on how the experiments with antigen stimulation were executed, since it is lacking.

Reply R1.1.

We thank Reviewer 1 for their comprehensive review of our manuscript. Our additional experiments by transducing KLF7 align with our central aim of this work which was to demonstrate the capacity of epigenomic marks to identify existing and novel transcription factors that influence CAR-T cell product quality and characteristics. We further acknowledge, as outlined by Reviewer 1, that our new KLF7 experiments create questions worthy of discussion and future investigation. Our current working hypothesis is that KLF7 enhances proliferation of T cells and that this is why we observed higher CAR-T cell counts in patients treated with infusion products with decreased H3K27me3 marks on KLF7. A potential underlying mechanism supported by our in vitro experiments is the increased autocrine production of IL-2 from KLF binding upstream of the IL-2 gene. As the reviewer correctly notes, the enhancement of IL-2 production was not noted after antigen stimulation through the CAR, likely because antigen-induced stimulation through the CAR provides robust signalling to the T cell, appropriately inducing IL-2 production above that induced by KLF7. We agree with the reviewer that additional experiments in a future manuscript will be required to dissect the kinetics and conditions under which KLF7 provides support to T cells during and after antigen encounter. With regards to a potential risk of T cell exhaustion or adverse effects due to increased expression of KLF7, we acknowledge that, as with the identification of any new CAR-T-augmenting factor, additional preclinical studies will be required. These experiments are currently underway.

We have modified the Methods section at lines 527-532 to further explain how the experiments with antigen stimulation were performed:

To assess the effects of KLF7 transduction on functions of CAR-T cells with and without antigen stimulation, untransduced, control P2A_GFP-transduced, and KLF7_P2A_GFP-transduced CAR-T cells were co-cultured with CD19-transduced K562 cells (K19), control K562 cells, or with media alone. CAR-T cell proliferation was assessed by Cell Trace Violet dilution at 48, 72 and 96 hours after antigen engagement. Intracellular IL-2 was assessed 24 hours after antigen-engagement by flow cytometry, as previously described{Lynn, 2019 #237;Gauthier, 2020 #190}

We have modified the Discussion section at lines 446-455 as follows:

Furthermore, the in silico predicted binding of KLF7 upstream of the IL-2 gene may also enable autocrine-driven expansion, thereby leading to higher CD8+ CAR-T counts in patients. Thus, transduction of KLF7 during manufacturing to generate less-differentiated CAR-T cells with enhanced

proliferative potential is an attractive strategy that could be investigated to improve CAR-T efficacy. Chronic proliferation due to persistent antigen stimulation or tonic signalling through the CAR can induce CAR-T exhaustion; however, the mechanisms by which KLF7 support CAR-T proliferation are likely different from those induced by chronic signaling through the CAR, the latter often being a consequence of CAR design{Selli, 2023 #383}{Qiu, 2024 #394}. More studies are needed to determine if KLF7 augmentation can improve CAR-T proliferation and durable survival without loss of function.

Point 2:
Inclusion of Naïve-derived CAR-T cells to the analysis. The authors have performed additional analyses and highlighted numerous DEn genes in the multiple comparison. I would have expected more differentially expressed genes from RNA seq analysis, and I think the authors should properly discuss this point. I acknowledge the difficulty in finding in the literature the exact comparison made by the authors, but at the same time functional differences have been already proven in different manuscripts. It's difficult to think that such differences are not reflected in transcriptomic changes.

Reply R1.2.

We thank the reviewer for this observation and opportunity to expand upon the discussion regarding these differences. Our Discussion now reads in lines 426-432:

Previous studies have shown decreased in vivo efficacy of EM-derived CAR-T in comparison to N- and CM-derived CD8+ CAR-T cells{Sommermeyer, 2016 #177}. Our RNA-seq data supports this observation by showing increased expression in EM-derived CAR-T cells of genes that prevent quiescence and stemness in T cells (e.g. LEF1-AS1){Zheng, 2017 #384}. Our epigenetic studies both complement and expand upon transcriptomics by further highlighting the programmatic differences between subset-derived CAR-T cells that may not be detectable by RNA-seq due to the widespread transcriptional activity subsequent to activation and expansion during manufacturing.

Point 3:
Inclusion of non-responders to the analysis. As requested, the authors included the analysis in figure S17, and found that neither histone methylation marks nor RNA-seq identified differences between patients who did or did not achieve CR. According to available literature, I would have expected some differences to emerge.

Reply R1.3.

Thank you for this comment. We have now addressed this gap in the literature in our Discussion in lines 433-439:

Finally, we undertook an analysis of patient derived CAR-T cells. Our initial analysis did not show differences by either RNA-seq or CUT&RUN between patients who had a response versus no response. Similar outcomes have been noted after analyses of single cell RNA-seq data, in which only a small subset of genes and no cell clusters were associated with responses to CAR-T cell therapy in LBCL – likely a consequence of factors, such as the tumor microenvironment, that confound analyses{Li, 2023 #395}. To account for the complexities imposed by the tumor microenvironment, we focused on CM-derived CAR-T from patients who had achieved CR after CAR-T cell infusion.

Reviewer #2 (Remarks to the Author):

The authors have addressed many of the reviewer's concerns to improve the quality of the manuscript.

Reviewer #3 (Remarks to the Author):

The authors addressed the concerns of this reviewer. This is a very interesting study and I am looking forward to its publication.

We thank Reviewers 2 and 3 for their critical analysis and suggestions that have improved the quality of the manuscript.