**REVIEWER COMMENTS**  
  
Reviewer #1 (Remarks to the Author):  
  
In the manuscript entitled "Tcf1 and Lef1 orchestrate genomic architecture to supervise mature CD8+ T cell identity” Shan et al. utilize Hi-C together with other high-throughput sequencing techniques to determine the role of Tcf1/Lef1 TFs in supervising mature CD8 T cell identity via organizing genomic architecture and facilitating promoter-enhancer/silencer interactions. First the authors showed that Tcf1/Lef1 impacts genomic structure on multiple scales and maintains an accessible chromatin state and super enhancer activity. By comparing the gene expression profiles in WT and dKO CD8 T cells, the authors further revealed Tcf1/Lef1 TFs are necessary to suppress aberrant expression of non-T lineage and other T cell subset-associated genes. Lastly, the authors perform analyses to document an association between gene expression changes connected to SE activity and the chromatin interactions coupled to Tcf1/Lef1 binding. Collectively these data extend our understanding of the  
mechanisms that maintain naïve CD8 T cells in a quiescent state, however the study falls short in describing the overall contribution of Tcf1 and Lef1 in regulating the chromatin architecture of T cells as they undergo a natural reprogramming during an immune response. The general body of work is very compelling, but there are a few outstanding questions listed below, that will strengthen the claims of the manuscript once they are addressed.  
  
1. The authors possess the tools to describe the overall chromatin architecture changes that occur during T cell effector differentiation. This analysis must be done in order to interpret the chromatin architecture changes that are truly associated with the quiescent state of naïve CD8 T cells. Furthermore, by documenting the overall change that occurs with a naïve CD8 T cell exits its quiesecent the state, the authors will then be able to fully resolve the overall contribution of Tcf1 and Lef1 in the regulating the chromatin architecture of naïve CD8 T cells.  
  
2. In Tcf1/Lef1 TFs-ko CD8 T cells, the expression of effector T cell signature genes are upregulated. What is the physiological relevance of this? For example, do the naïve T cells lacking Tcf1/Lef1 TFs have a stronger tonic TCR signaling, or a lower threshold for activation such that a lower antigen quantity is now required to initiate the effector program compared to WT CD8 T cells?  
  
3. The authors defined Motif+ and Motif- Tcf1 peaks in their CHIP-seq data as direct and indirect binding sites. Is there way to describe the overall contribution of the direct binding of Tcf1 to the effect observed in dKO CD8 T cells? Does the indirect effect also depend on the direct DNA-binding of Tcf1? Does perturbation of DNA-binding motifs results in the same changes in genomic organization or chromatin modeling as the TF ko?  
  
Response: 1. Motif+/Motif- associated signature gene.

2. Open chromatin change between Motif+/Motif-. (figure.xx?)

3. chromatin interaction change between Motif+/Motif-. (figure.xx?)

4. Motif analysis between Motif+/Motif-. (figure.xx?)

Reviewer #2 (Remarks to the Author):  
  
Shan and Li et al. provide an extremely thorough and valuable account of the impact of Tcf1 on post-thymic CD8 T cells, connecting Tcf1 binding with multiple indices of chromatin structure at and around the sites of binding, and at and around the genes affected by loss of Tcf1. Although this in vivo model does not allow identification of the earliest events when the cells lose Tcf1, the authors scrupulously test each effect against the presence or absence of Tcf1 binding at the sites involved, and they also distinguish between sites where Tcf1 appears to be bound directly and sites where the weakness of the motif suggests that it is bound indirectly. The accompanying datasets, especially the tables of differentially regulated genes and signature genes, are filled with valuable results presented in a very useful and lucid way. The context and the interpretations are very well presented in the introduction and discussion. This work will be extremely valuable to anyone interested  
in rigorous cause-effect analyses of transcription factor actions, and because Tcf1 is so important for T cell development and function, it will be valuable to most readers with an interest in T cell molecular biology.  
  
Notable results, in a somewhat different order from the way they are presented, include these: (1) the impact of Tcf1 deletion after thymic egress is much more limited and different from the impact if it is deleted during CD4/CD8 lineage separation; (2) the sets of target genes, positively and negatively regulated by Tcf1, include very strongly affected Myb and Foxp3; (3) whereas Tcf1 binding is often associated with chromatin accessibility increases, many of these sites appear to mediate negative transcriptional regulation; (4) loss of Tcf1 causes not only a shift from primarily “naive CD8” gene expression to “effector CD8” gene expression, as expected, but also striking gains in expression of genes associated with other developmental states. Particularly strong upregulation of genes associated with Treg fate could be due to the massive increase in Foxp3 expression in these cells, although this is very lightly noted in the text. In addition, gene sets associated with  
nonlymphoid (DC, mono, Gr) cells are seen to be upregulated, and there is even slight upregulation of some highly lineage-specific B-cell genes including the Pax5 target Cd19 and the EBF1 target Cd79a.  
(5) Finally, as a technical feature, the authors have generated a new anti-Tcf1 antiserum which appears to make possible Tcf1 ChIP-seq with high sensitivity. If made available to others, this should become a vital reagent for the field.  
  
Some aspects of presentation could be clearer. Addressing these points would help the reader through the logic of the Results section, which is presented in a fairly dry way with only occasional reference to the biology and in a slightly unexpected order.  
  
1. The Results begin with description of the Tcf1 ChIP-seq data, but none is actually presented to the reader until Fig. 4. Not until Fig. 4 can the reader see the quality of the peaks being detected, their excellent signal:noise ratios, and the elimination of these peaks in the KO samples. This seems strange, and it would be very helpful to add an example pair of tracks around some representative loci in Fig. 1.  
  
2. Fig. 3 provides gene expression data only in terms of relative z-scores, but some of these effects involve bare increases of barely detectable trace signals to slightly larger trace signals (e.g. for Cd19) while others, like the effect on Treg “master regulator” Foxp3, are hugely significant, not only in statistical difference but also in the level achieved. Anyone interested in the impact of Tcf1 loss on the biological state of the cells would care about these absolute differences. The data are well presented in the Supplementary Tables, but some of the biologically notable effects should be shown within the figure panels as RNA-seq tracks so that their magnitudes can be appreciated. (It is actually surprising not to see RNA-seq data included.)

Response: 1. RPKM value for expression heatmap. 2. Display RNAseq track (gene?).

3. The text explaining Fig. 3 and Fig. S5 (in lines 253-264) may be a little too brief in explaining what these “signature gene sets” mean when they are up-regulated in the KO.  
(a) Readers may assume that these are highly specific indicators for a unique, alternative developmental path, but it may be more helpful to call them “characteristically enriched in” or “associated with” cells of a given alternative lineage. Some genes in the non-T cell-type signature gene sets appear to be truly lineage-specific, e.g. the B cell genes Cd19 and Cd79a, but not all of them are this clear. For example, the essential T-cell regulatory gene Gata3 is included in the “Pan-NK signature”. In the full ImmGen database, Cish, described as “Treg signature”, is highly upregulated in ILC2 cells as well, and Cx3cr1, described as “effector CD8 signature”, is almost equally high in certain monocytes and much higher, in fact, in microglia.  
(b) Also, although the signature gene set as a whole may be quite biased to a non-T cell type, the particular members of that gene set that are up-regulated in the Tcf1 KO cells are often expressed at one time or another in T lineage cells (spot checks show many of them in thymocytes, etc.). It would be helpful if the authors slightly expanded the description of the signature gene sets in the text to clarify that these particular genes do not mean that the cells are actually transforming into DC or B cells. Otherwise, the reader would look for more detailed flow cytometry to characterize the phenotype of the cells more completely.  
Response: 1. Adopt to definition of signature gene as “characteristically enriched genes” ?

4. Fig. 2 makes a strong distinction between Motif+ and Motif- Tcf1 binding peaks, with the majority of the binding peaks lying in an indeterminate motif score range. After encountering the Tcf1-regulated genes, the question is whether this distinction makes a difference to the effect of the Tcf1 binding on the response of the target genes. Although this is discussed on p. 15, Figure 4a (right panel) shows an indistinct kind of heatmap view where all the Tcf1 binding sites appear to be associated with some kind of Tcf1 motif, but little pattern is detectable. Maybe the Tcf1 motif strength differences are overwhelmed by the divide between Tcf1-bound and Tcf1-unbound sites. If the Tcf1-bound sites are separated out from the unbound sites, can one then see a difference between Tcf/Lef motif strength in C2 and C4, or any relation to the response of the target gene?  
Response: 1. Motif+/Motif- associated signature gene.

2. Tcf1 bound sites motif scores between C2 and C4.

5. On p. 17, the text makes it seem that the effects of Tcf1 KO on positively regulated SEs are just as strong in the opposite direction on negatively regulated SEs. But H3K27ac is clearly lost from specific peaks within the positively regulated SEs in the KO (Ccr7 and Inpp4b loci), while in contrast, the increase in H3K27ac in the negatively regulated ones (around Cish and around Cx3cr1) seems to be just a global increase in background H3K27ac between the peaks. At first glance, the KO samples just appear to have a worse signal-to-noise ratio. Is this the general way that the Tcf1/Lef1 repressed loci all over the genome react? Perhaps the specificity of the effect on the repressed loci would show more clearly if the panels in Fig. 5f were more zoomed-out.

Response: 1. Figure 5f zoomed out.

2. (ratio = islandreads / all reads)

WT: (54,072,465)/(20,720,923+ 16,037468 + 9,764727 + 18,379129 +14,364825+ 11,713709 )

DKO: (36,901,415)/(+25,385,031 + 14,767083+9,514734 + 12,235742 )

|  |  |  |
| --- | --- | --- |
| 54,072,465 | 90,980,781 | 0.594328 |
| 36,901,415 | 61,902,590 | 0.596121 |

3. verify the calculation in Super Enhancer (logFC).

5. Minor:  
(a) in Fig. 6, please clarify where panel b ends and panel c begins. The labeling suggests that the middle hub map is still b, but the red/blue arrow features are only described under the legend for c.  
(b) In Fig. S4, please explain what the small dots are. Are they projections of the large dots onto each 2D plane? (projection onto each 2D plane)  
(c) The biologically interested reader would probably welcome some direct comments about the strong effects of Tcf1 KO on powerful regulatory genes in these CD8 cells, like Myb (greatly down in the KO), Foxp3 (greatly up), Maf (up), and Eomes (down).  
(d) Please clarify whether the new Tcf1 antiserum will be available for others to try using, and where a more complete characterization of its properties relative to commercial antibodies may be found.  
  
  
  
  
Reviewer #3 (Remarks to the Author):  
  
Review of manuscript NCOMMS-21-06081-T "Tcf1 and Lef1 orchestrate genomic architecture to supervise mature CD8+ T cell identity" by Shan, Li et. al..  
  
The authors explore the role of Tcf1 and Lef1 in the maintenance of CD8+ T cell identity with a focus on the integrity of chromatin architecture and chromatin accessibility or gene expression after hCD2-Cre mediated deletion of these two transcription factors in mature peripheral T cells. The authors present a new Tcf1 ChIP and use the determined Tcf1 peaks to systematically asses several global analyses including chromatin topology / Hi–C Seq, chromatin accessibility / DNase seq, superenhancer formation as well as gene expression by discriminating Tcf1+ peak and Tcf1-Motif+ containing from Tcf1- genomic regions and WT/DKO comparisons. Besides providing a profound analysis of the different levels at which Tcf1/Lef1 regulate chromatin accessibility and gene expression or prevent cell-type inappropriate gene expression the most intriguing results are the Tcf1-dependent changes in chromatin topology.  
  
  
Major point 1. The authors study primary CD8+ T cells and have established an elegant system of conditional deletion (plus identification of deleted cells) to study Tcf1 and Lef1 function, but they should also describe or at least mention some of the relevant phenotypes of these DKO CD8+ T cells. If the cells lose their identity, one expects them to show functional impairments for example in killing of target cells, changed cytokine/chemokine production, they should inappropriately adopt certain effector phenotypes, undergo exhaustion, have altered survival and express protein markers of other cell types (CD19?)?  
  
Major point 2. The authors generate new polyclonal anti-TCF1 specific antibodies for ChIP use, but they do not show a validation of specificity.  
  
Major point 3. For Fig. S3a, the authors conclude that Tcf1 peaks are absent from CTCF marked boundaries of TADs. However, Tcf1 peaks appear enriched upstream and downstream of the boundaries (approx. 100kb distance). How is this enrichment explained?  
Moreover, I do not follow how the authors describe the localization of Tcf1 peaks within the displayed TADs for example in Fig. 1i… “a chromatin loop linking Cyct and Prkra gene loci in WT cells with a Tcf1 peak at the anchor proximal to Cyct…”. I rather see the peak positioned at or close to the first exon of Rbm45, and in general (by judging the displayed examples), I would rather see Tcf1 peaks proximal to the genes than at specific anchor points of loops. Please clarify how loop anchors and the presence or absence of Tcf1 peaks within them are defined.  
Response: 1. Using promoter as peaks, draw profile in Fig. S3a.

Major point 4. A criticism for the chromatin topology aspects of this study is that although the authors globally map Tcf1 binding sites by ChIP, define them to be enriched in “transcriptionally more active regions” and in regions with higher TAD scores, they only show diminished TADs in DKO CD8+ T cells for genes that are not prototypic targets of Tcf1/Lef1. Can the authors include examples of a bona fide target (maybe Prdm1 or even CD4 etc.) that have specific Tcf1 binding sites and important functions in T cells and demonstrate that the globally observed effects can also be seen on the well-established and relevant target -- or is the conclusion that the “structural role” of Tcf1 applies rather outside of the set of already established target genes?  
Response: 1. Prdm1 & Cd4 screenshots

Major point 5. The authors generated mouse mutants with ablation of two binding sites in Prdm1 that are associated with unchanged or reduced ChrAcc at these positions in DKO T cells and either reduce or enhance Prdm1 expression. Using these mice they could directly test the suggested interpretation that Tcf1 acts as a pioneer factor via bending of chromatin, increasing accessibility locally and allowing looping and altered chromatin topology, which through interaction with other transcription factors can then have very different impacts on the gene expression.

Response: 1. Address pioneer factor?