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scRNA+TCR-seq Reveals the Proportion and Characteristics of Dual TCR Treg Cells in Mouse Lymphoid and Non-lymphoid Tissues

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eLife Assessment

This study reanalyzed previously published scRNA-seq and TCR-seq data to examine the proportion and characteristics of dual-TCR-expressing Treg cells in mice, presenting some **useful** insights into TCR diversity and immune regulation. However, the evidence is **incomplete**, particularly with respect to data interpretation, statistical rigor, and the functionality of dual -TCR Treg cells. The study is potentially of interest to immunologists studying T-cell biology.

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Abstract

The rearrangement of TCR germline *V(D)J* genes during T cell development, including allelic exclusion and tolerance selection, ensures the clonal selection theory, which states that “a lymphocyte expresses only one type of antigen receptor” This forms the basis for T cell-specific responses. However, The existence of “dual TCR T cells” has consistently been supported by specific experimental evidence. detailed reports on the origin, proportion, tissue distribution, and CDR3 characteristics of “dual TCR Treg cells” are currently lacking. In this study, we utilized scRNA+TCR-seq technology to achieve in-depth analysis of single TCR T and dual TCR T pairings, along with their mRNA expressions, from over 5,000 T cells in each sample. Through comparative studies with shared databases, we provided a detailed analysis of the proportions and characteristics of dual TCR Tregs in mouse lymphoid and non-lymphoid tissues (such as iLN, mLN, blood, and skin). Our findings revealed a high proportion of dual TCR Tregs across various mouse tissues, with their TCR pairing patterns, *V(D)J* usage, and mRNA expression showing both homogeneity and certain differences compared to single TCR Tregs, as well as heterogeneity across different tissue sites. This research provides new insights and technical approaches for studying the origins, characteristics, effects, and mechanisms of Treg cells in different tissue locations.

Introduction

The discovery of CD4+CD25+ Treg cells in 1995, has since been linked to the occurrence and development of various human diseases. In addition to their strong regulatory role in self-reactivity and excessive inflammation, Treg cells play an important homeostatic role in the maintenance of tissue homeostasis, muscle damage repair, hair follicle regeneration, fat metabolism, among other complex cellular activities[1][2]. The diverse negative regulatory functions of Treg cells suggest unique characteristics for their TCR diversity, specificity, and memory responses. However, the compositional characteristics of Treg cell TCR, their dependency on antigen epitopes, activation, and maintenance mechanisms, remain largely unelucidated. Some studies have garnered widespread attention, including:V(D)J recombination of nTreg cells TCRs, self-tolerance selection, and migratory settlement with or without lineage characterization;differences in antigen induction and activation mechanisms for iTreg versus conventional helper and effector T cells;diversity and plasticity of Treg cells in lymphoid and non-lymphoid tissues, such as TCR CDR3, and their correspondence to regulatory functions.

Since their initial report in 1988[3], dual TCR expressing lymphocytes have been widely supported by experimental evidence and play important roles in physiological and pathological processes such as autoimmune tolerance, transplant rejection reactions, and T cell tumors[4][5][6][7][8][9][10]. Tuovinen et al. [11] utilized flow cytometry to discover that the frequency of dual TCR expression in human CD4+CD25+ Treg cells is higher than in other T cell subgroups, and that dual TCR Treg cells can effectively transmit signals, suggesting that dual TCR expression may promote the development and function of Treg cells. However, this study primarily focused on the dual TCR α chain, leading to certain limitations in its conclusions. Overall, due to technical constraints, the findings are limited in comparing the proportion, characteristics, origin, and mechanisms of single TCR and dual TCR T cells in a single sample of more than a thousand T cells, posing challenges for immunologists. However, scRNA-seq combined with scTCR-seq can mark over 5,000 T cells from a single research sample at once, obtaining the pairing types of each T cell's TCR beta chain and alpha chain, CDR3 sequence composition characteristics, and complete mRNA expression data. This allows for the accurate analysis of TCR CDR3 characteristics in the development, differentiation, maturation, and response/tolerance processes of each T cell, as well as the corresponding expression of regulatory and effector molecules. This provides unprecedented opportunities for in-depth analysis of dual receptor T cells[8][9][12][13].

First, in the Treg study conducted by Oliver T et al.[14], which involved analyzing single-cell RNA sequencing and T cell receptor (scRNA+TCR-seq) data of Foxp3+ Treg cells sorted from mouse blood, kidney, liver, LPL, and pancreas under physiological conditions, we conducted a detailed comparative analysis of the proportions of single TCR Treg and dual TCR Treg; the usage, diversity, clonality, and overlap of CDR3 VJ; and the homogeneity and heterogeneity of characteristic marker mRNA expression.Additionally, to further explore the differences in dual TCR Treg cells between lymphoid and non-lymphoid tissues in mice, we selected the scRNA+TCR-seq data shared by Annekathrin T et al.[15], which includes sequencing results of CD4+CD25+ Treg cells sorted from mouse spleen, inguinal lymph nodes (iLN), and mesenteric lymph nodes (mLN) under physiological conditions, as well as CD3+ T cells (including Treg and non-Treg) sorted from skin tissue, and performed comparative analysis again.

Results

1. A high proportion of dual TCR Treg cells in mouse lymphoid and non-lymphoid tissues

The proportion of dual TCR Treg cells in mouse lymphoid tissues: spleen=21.4%; iLN=21.4%, mLN=21.0% (**Figure 3D**); The proportion of dual TCR Treg cells in mouse non-lymphoid tissues: blood=11.8%; kidney=12.2%; liver=13.6%, LPL=11.0%; pancreas=13.3%, The proportion of dual TCR T cells in mouse skin tissue: 18.6% (**Figure 1D**, **Figure 3D**). Subsequently, a detailed analysis of the pairing of dual TCR T cells in the two sets of data was conducted, revealing that the TCR pairing types of dual TCR Treg include A+B1+B2, B+A1+A2 (the highest proportion), A1+A2+B1+B2 (the lowest proportion). Significant differences in TCR pairing types exist between tissues, with A1+A2+B1+B2 most highly expressed in skin (common in non-Treg cells of skin) and kidney, and B+A1+A2 most highly expressed in spleen and liver compared to other tissues.(**Figure 1D**, **Figure 3D**).

2. Homogeneity and heterogeneity of TCR composition in mouse dual TCR Treg cells

VJ usage(Figure 1E/F, Figure 3E/F)

The overall *VJ* family distribution pattern of single TCR Treg and dual TCR Treg between different tissues is basically consistent. Compared with single TCR Treg, dual TCR Treg displays specific preferences in *V* and *J* usage, mainly including: *TRBV10* and *TRBV13-1* in the kidney and liver,*TRBV5* in LPL,*TRBV19* in the skin;*TRBJ2-3* in the liver, *TRBJ2-1* in the skin,etc. showing a significant advantage in usage; *TRBV13-1* in peripheral blood, kidney, and liver shows consistent advantage in usage; *TRBJ1-2* in the spleen, iLN, and mLN shows consistent advantage in usage, etc.

Diversity of CDR3(Figure 1G, Figure 3G)

The 1/DS of single TCR Treg CDR3 is significantly higher than that of dual TCR Treg; the CDR3 diversity of dual TCR Treg in lymphoid tissues (spleen, iLN, mLN) is higher than that in non-lymphoid tissues; the CDR3 diversity of dual TCR Treg in skin, LPL, and pancreas is very low.

Clonality of CDR3(Figure 1H, Figure 3H)

The proportion of single TCR Treg with clonal proliferation greater than or equal to 2 is higher than that of dual TCR Treg; the proportion of dual TCR Treg with clonal proliferation greater than or equal to 2 in the liver and skin is significantly higher than that in other tissues.

Overlap of CDR3(Figure 2A/B, Figure 4A/B)

A certain proportion of overlap exists between TRB CDR3 AA and TRA CDR3 AA in different tissues, with single TCR Treg significantly higher than dual TCR Treg, and a lower proportion of TRB CDR3 AA compared to TRA CDR3 AA. This suggests that TRA allelic inclusive recombination involves more CDR3 AA types in TCR pairing of Treg cells. Meanwhile, it was found that single and dual TCR Treg in different tissues can also share common CDR3 AA. This suggests that both single and dual TCR Treg can respond to specific antigen epitopes.

A

sample	GEO accession		Total cells/paired TCR cells	Single TCR T cells(A+B)/proportion%	Dual TCR T cells/proportion%			
	scRNA-seq	scTCR-seq			Total	A+B1+B2	B+A1+A2	A1+A2+B1+B2
mouse1	GSM8240877	GSM8240878	2598/2072	1683/81.23%	389/18.77%	120/5.79%	218/10.52%	51/2.46%
mouse2	GSM8240880	GSM8240881	3198/2672	2202/82.41%	470/17.59%	115/4.30%	281/10.52%	74/2.77%
mouse3	GSM8240883	GSM8240884	3313/2626	2233/85.03%	393/14.97%	96/3.66%	225/8.57%	72/2.74%
mouse4	GSM8240886	GSM8240887	2140/1610	1398/86.83%	212/13.17%	63/3.91%	126/7.83%	23/1.43%

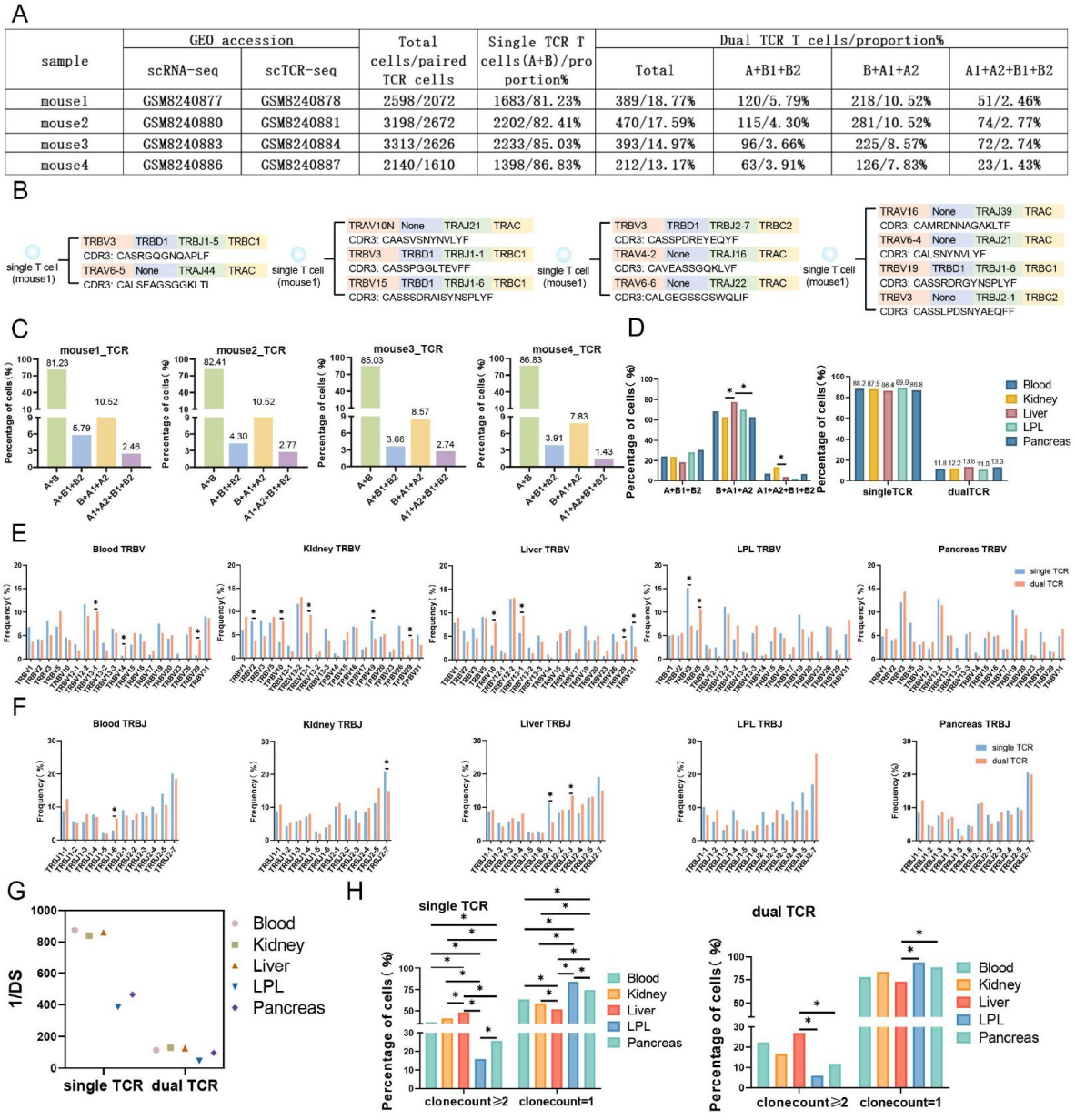

Figure 1.

Characterizes single Treg TCR and dual TCR Treg in the Blood, Kidney, Liver, LPL, and Pancreas of mice.

A, Mouse source names, GEO accession number, total number of paired TCR Treg cells, and number and proportion of TCR paired types. **B**, Four different TCR pairing types, *VDJ* gene family names and CDR3 AA sequences in single T cells. **C**, Proportion of TCR pairing types per mouse. **D**, Statistical analysis of dual TCR Treg cells and the relative proportion of single /dual TCR Treg Cells in Three TCR Pairing Types. **E**, Comparative analysis of single/dual TCR Treg cell *V* gene usage variation. **F**, Comparative analysis of single/dual TCR Treg cell *J* gene usage variation. **G**, Differential analysis of single/dual TCR Treg cell diversity among tissues. **H**, Differential analysis of single/dual TCR Treg cell clone expansion between tissues.

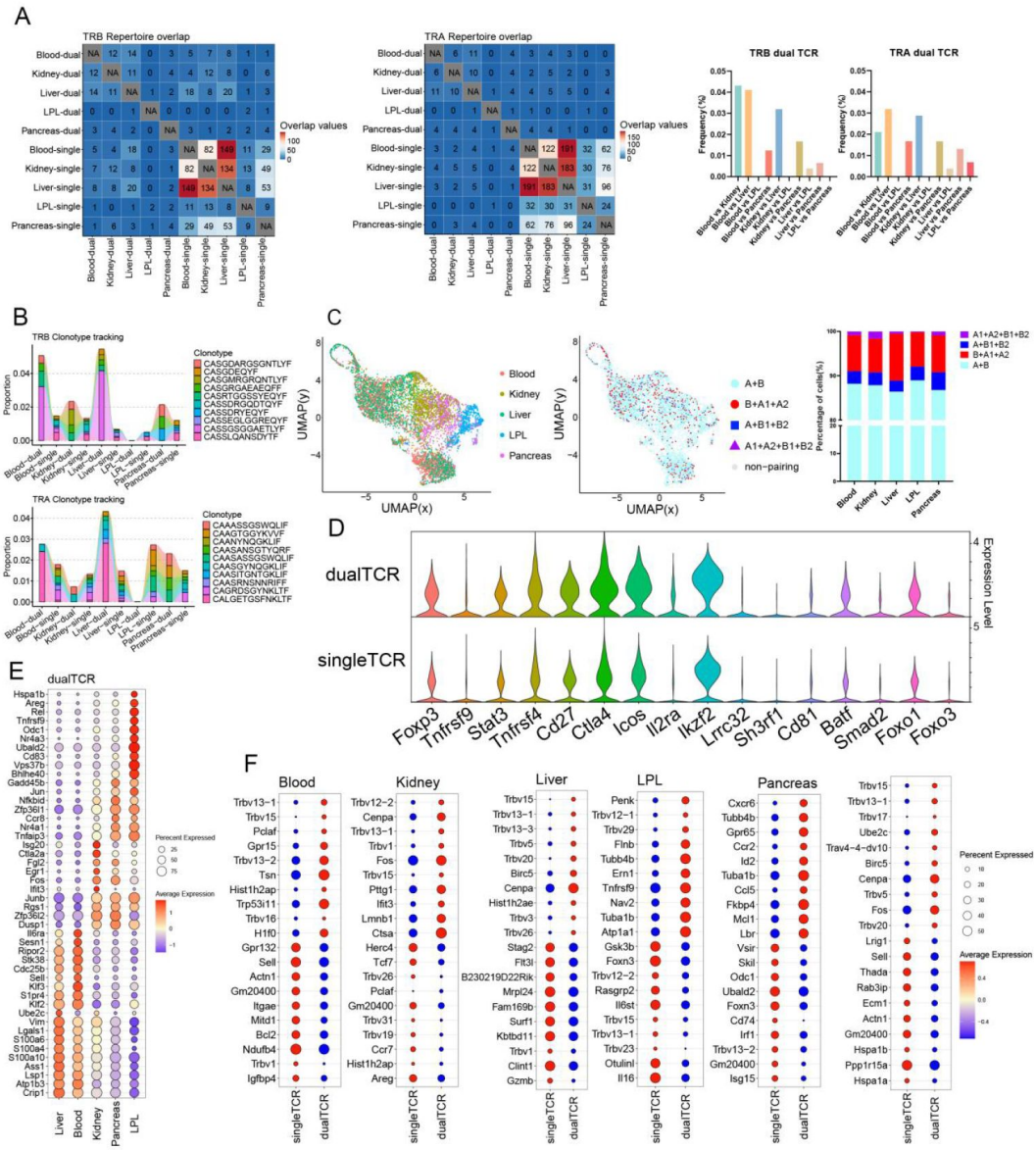

Figure 2.

Comparative analysis of single Treg TCR and dual TCR Treg CDR3 overlap and gene mRNA expression in mice Blood, Kidney, Liver, LPL, and Pancreas.

A, Clonal overlap analysis and jarcard index of TRA/B-chain CDR3 region in single /dual TCR Treg cells. **B**, Tracking results of the first 10 high-frequency overlapping sequences in the CDR3 region of the TRA/B chain of single /dual TCR Treg cells between tissues. **C**, Treg cell clustering results and proportion of Treg cells of the four TCR pairing types among tissues. **D**, Characterized mRNA expression in single/dual TCR Treg cells. **E**, Top 10 mRNA molecules highly expressed by dual TCR Treg cells in each tissue. **F**, Top 10 mRNA molecules highly expressed by single/dual TCR Treg cells in each tissue and overall.

A

sample	GEO accession		Total cells/paired TCR cells	Single TCR T cells(A+B)/proportion%	Dual TCR T cells/proportion%			
	scRNA-seq	scTCR-seq			Total	A+B1+B2	B+A1+A2	A1+A2+B1+B2
iLN	GSM7680409	GSM7680410	2509/1889	1484/78.56%	405/21.44%	153/8.10%	220/11.65%	32/1.69%
mLN	GSM7680409	GSM7680410	3217/2391	1889/79.00%	502/21.00%	177/7.40%	278/11.63%	47/1.97%
skin	GSM7680409	GSM7680410	989/760	619/81.45%	141/18.55%	65/8.55%	10/1.32%	66/8.68%
spleen	GSM7680409	GSM7680410	3412/2510	1973/78.61%	537/21.39%	179/7.13%	313/12.47%	45/1.79%

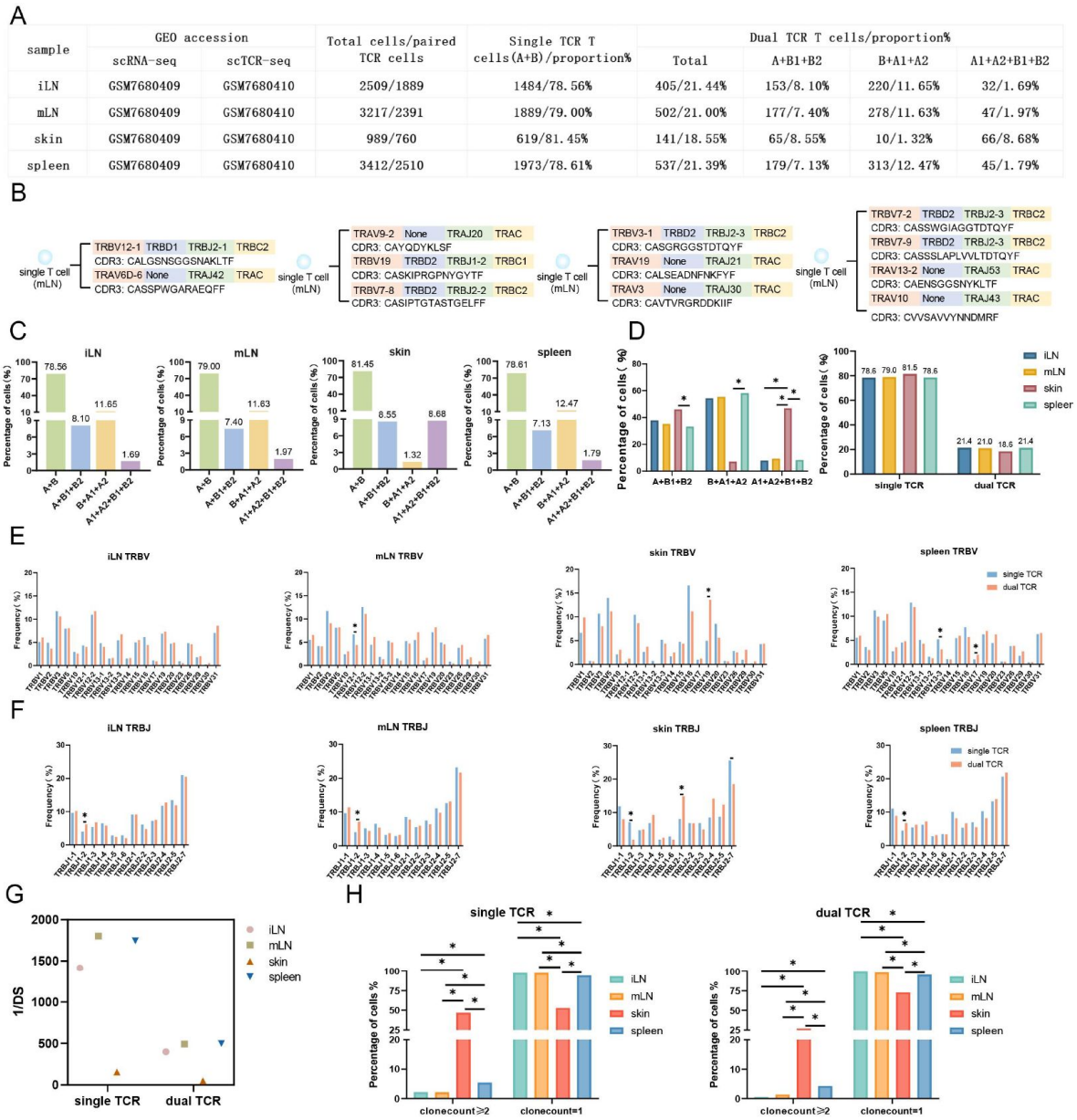

Figure 3.

Characterizes single Treg TCR and dual TCR Treg in the iLN, mLN, skin, spleen of mice.

A, Tissue source names, GEO accession number, total number of paired TCR T cells, and number and proportion of TCR paired types. **B**, Four different TCR pairing types, *VDJ* gene family names and CDR3 AA sequences in single T cells. **C**, Proportion of TCR pairing types per tissue. **D**, Statistical analysis of dual TCR T cells and the relative proportion of single /dual TCR T cells in three TCR pairing types. **E**, Comparative analysis of single/dual TCR T cell *V* gene usage variation. **F**, Comparative analysis of single/dual TCR T cell *J* gene usage variation. **G**, Differential analysis of single/dual TCR T cell diversity among tissues. **H**, Differential analysis of single/dual TCR T cell clone expansion between tissues.

All three tissues, the liver, kidney, and pancreas, show a significantly high proportion of dual TCR Treg CDR3 AA overlap with peripheral blood, suggesting their origin from the influx of peripheral blood (It should be noted that the high overlap phenomenon of the kidneys, liver, pancreas, and blood may not completely eliminate the technical possibility of local Treg migration within the blood), iLN, mLN, and skin all show a significantly high proportion of dual TCR Treg CDR3 AA overlap with the spleen, suggesting their origin from the influx of the spleen; Intriguingly, there is also a high proportion of dual TCR Treg CDR3 AA overlap between the liver & kidney, kidney & pancreas, and iLN & mLN, suggesting that dual TCR Treg cells in these tissue sites may negatively regulate a shared set of antigens. Whereas the mechanism and significance of the high percentage of overlap exhibited between iLN and skin TRA CDR3 AA deserves further analysis.

3. Homogeneity of mouse dual TCR Treg cell phenotypes

Comparative analysis of Treg cells with complete scRNA-seq and scTCR-seq results showed that the TCR pairing types and proportion were consistent with the results of direct analysis of scTCR-seq; the proportion of dual TCR Treg in the skin was higher than that of dual TCR non-Treg (**Figure 2C**, **Figure 4C**); in all tissues,the expression of signature molecules *Tnfrsf9, Stat3, Tnfrsf4, Cd27, Icos, Il2ra, Ikzf2, Lrrc32, Sh3rf1, Cd81, Baff, Smad2*, and *Foxo3* was identical between dual and single TCR Treg in all tissues, with slightly higher *Foxp3, Foxo1, and Ctla4* expression in dual TCR Treg than that in single TCR Treg(**Figure 2D**, **Figure 4D**).

4. Heterogeneity of mouse dual TCR Treg cell phenotypes

Compared with iLN, mLN, and spleen, more characteristic mRNA molecules were highly expressed in dual TCR Tregs in skin (**Figure 4E**), such as *Rora, Dusp1, Junb* involved in immune responses and inflammatory diseases; *S100A6* involved in the proliferation, differentiation, and migration of immune cells; *Hopx* and *Nr4a1* involved in the development of immune cells, etc., suggesting that dual TCR Treg in the skin may be involved in more specific response negative regulations.

Between Blood & Liver; Kidney & Pancreas (**Figure 2E**), iLN & mLN & spleen (**Figure 4E**), the mRNA expression of cytokines, cytokine receptors, and transcription factors in dual TCR Treg shows higher homogeneity, suggesting that dual TCR Treg in these tissue sites may play convergent negative immune response regulations.

Differential mRNA expression analysis between dual TCR Treg and single TCR Treg in each tissue (**Figure 2F**, **Figure 4F**): showed significant high expressions of individual *TRBV* genes in different tissues, consistent with scTCR-seq results, suggesting specific dual TCR Treg lineage origins in different tissues; in addition to *TRBV* usage, each group's dual TCR Treg had significantly different characteristic molecule expressions from single TCR Treg, such as Blood: *H1f0* involved in immune cell proliferation and differentiation and *Hist1h2ap* related to immune cell activation were increased; Kidney: *Fos*($P<0.001$) and *Cenpa*($P=0.003$)related to immune cell proliferation and differentiation and *Ifit3* involved in the body's antiviral response were significantly increased; Liver: *Cenpa*($P=0.022$) related to immune cell proliferation and *Birc5* involved in the body's anti-infection immune response were increased; LPL: *Flnb* related to cell adhesion and migration regulation and *Atp1a1* related to cell proliferation, differentiation, and apoptosis regulation were increased; Pancreas: *Ccl5, Ccr2, Cxcr6* related to immune cell migration and *Lbr*($P=0.009$)and *Id2*($P=0.029$) related to immune cell proliferation and differentiation, as well as *Fkbp4*($P=0.004$) involved in immune suppression signal transduction were significantly increased; iLN: *Tgfbr1* involved in maintaining immune tolerance and regulating inflammatory responses and *Traf3* involved in immune cell activation and cytokine production were increased; mLN: *Glcci*($P<0.001$) involved in inflammation and immune cell migration was increased; skin: *Areg* ($P=0.008$) involved in immune cell proliferation, differentiation, migration, and immune suppression functions and

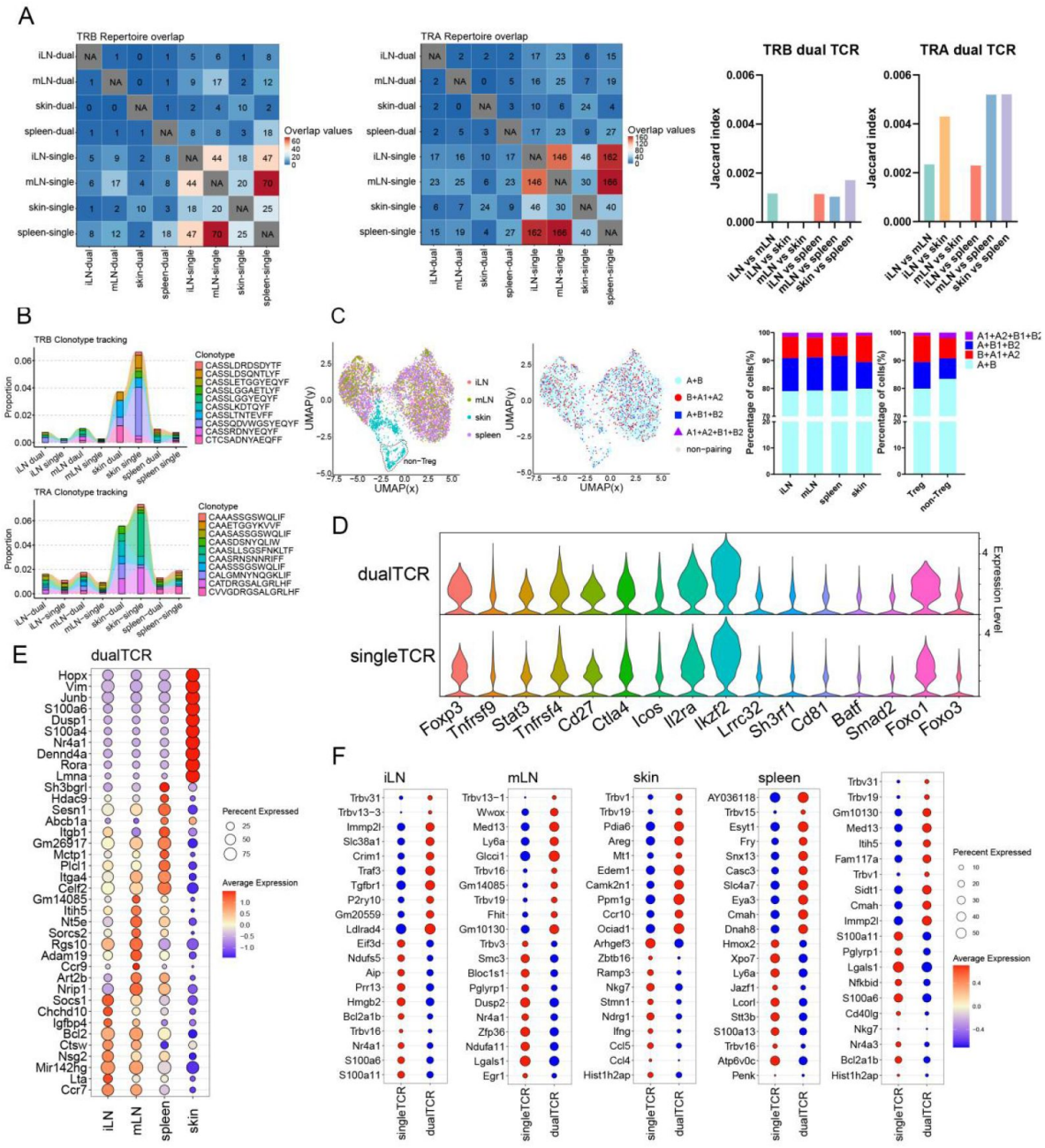

Figure 4.

Comparative analysis of single Treg TCR and dual TCR Treg CDR3 overlap and gene mRNA expression in mice iLN, mLN, skin, spleen.

A, Clonal overlap analysis and jarcard index of TRA/B-chain CDR3 region in single /dual TCR T cells. **B**, Tracking results of the first 10 high-frequency overlapping sequences in the CDR3 region of the TRA/B chain of single /dual TCR T cells between tissues. **C**, Treg cell clustering results and proportion of Treg cells of the four TCR pairing types among tissues. **D**, Characterized mRNA expression in single/dual TCR Treg cells. **E**, Top 10 mRNA molecules highly expressed by dual TCR Treg cells in each tissue. **F**, Top 10 mRNA molecules highly expressed by single/dual TCR Treg cells in each tissue and overall.

Ccr10 involved in T cell migration and positioning were increased; spleen: *Casc3* related to T cell activation and proliferation was increased. This suggests that dual TCR Treg and single TCR Treg in each specific tissue site may be involved in different negative immune response regulations.

Consistent high expression of molecular mRNA between different lymphoid tissues, such as: the presence of the *Nr4a1* gene related to T cell development, activation, and proliferation in both iLN and mLN tissues; partial sharing of molecular mRNA high expression between different non-lymphoid tissues, such as: the expression of *Trbv13−1* and *Trbv5* genes in Blood, Kidney, and Liver tissues, and the common expression of *Trbv1* in skin and Kidney, these results may suggest that dual TCR Treg cells in some tissues have specific lineage origins; and the common expression of the *Cenpa* gene in Kidney and Liver tissues may play a common immune effect; similarly, single TCR Treg also has similar gene expressions, such as the high expression of *Trbv1* in single TCR Treg cells in Blood and Liver tissues, and the high expression of *Hist1h2ap* related to immune cell activation in skin and Kidney.

Discussion

Currently, the proportion of dual TCR T cells is reported to be extremely variable across physiologic and pathologic states (0.1%-30%, etc.) due to differences in assay technology and methodology[4][5][6][7][8][9][10]. This study, in a large number of single Treg cell scRNA+TCR-seq studies, found that mouse lymphoid and non-lymphoid tissues both have a high proportion of dual TCR Treg cells, with lymphoid tissues being higher than non-lymphoid tissues, this is consistent with the findings of Tuovinen et al. [11], which indicate that the proportion of dual TCR Tregs in lymphoid tissues is higher than that of other types of T cells. This will contribute to a better understanding of the distribution characteristics of dual TCR Treg cells in different tissues, and provide a basis for conducting functional experiments on dual TCR Treg cells at various tissue sites based on mRNA expression levels. And the proportion of liver and pancreas dual TCR Treg cells in non-lymphoid tissues is relatively high. In addition, our analysis of dual TCR T cell pairing types revealed significant differences between tissues. These findings not only suggest that lymphoid tissues have a high proportion of dual TCR Treg cells but also provide a comparative baseline and research direction for tissue-specific Treg cells in different locations.

The lower diversity of dual TCR Treg CDR3, the tissue preference in *V/J* gene usage, and the high clonal proliferation frequency in some tissues reflect the limited breadth of the dual TCR Treg subset response. It should be noted that the analysis of CDR3 diversity indicates that the TCR composition of dual TCR Treg cells is diverse, comparable to single TCR Treg cells. Due to differences in the number of single TCR Treg and dual TCR Treg cells in each sample, the diversity indices of single TCR Treg and dual TCR Treg in this study are not suitable for statistical comparative analysis. These cells may play a complementary role in specific physiological or pathological negative regulation, and the tissue-specific preference for *V/J* usage suggests that dual TCR Treg cells may have a characteristic lineage similar to that of γδ T cells. Current research indicates that γδ T cells in different tissues show differences in VJ subfamily usage due to directed migration after specific VJ recombination at different times[16]. Dual TCR Treg cells may have certain lineage characteristics in V(D)J recombination and self-tolerance selection, and migrate to settle in different tissues. The higher clonal proliferation frequency of dual TCR Treg cells in the liver may exert a stronger inhibitory response to certain specific antigenic epitopes. The relatively higher immune tolerance of liver transplantation compared to other organ transplants may be related to the high proportion and clonal proliferation of dual TCR Treg cells in the liver.Based on the diversity and complexity of Treg functions, conducting a comparative analysis of the origins of dual TCR Treg cells and non-T cells with dual TCR will be a highly meaningful direction.

The majority of single/dual TCR Treg cells exhibit consistent expression of characteristic genes, and the high expression of certain genes in dual TCR Treg cells (*Foxp3, Foxo1, Ctla4*) indicates that dual TCR Treg cells can function similarly to or more potently than single TCR Treg cells. In addition, specific or shared *Trbv* genes and mRNA molecule expression in dual TCR Treg cells has been identified in lymphoid or non-lymphoid tissues. Recently, Malte et al. used scATAC-seq technology to reveal organ-specific adaptation and conservation of tissue-resident immune cells such as Treg and Th17, and identify key transcription factors for multiple tissue-resident immune cells[17]. Overall, the homogeneity and heterogeneity of these characteristic molecules within Treg cells suggests their potential induction in response to different antigenic epitopes and corresponding negative regulatory functions.

This study, based on single-cell RNA sequencing and T cell receptor (scRNA+TCR-seq) data of Treg cells in lymphatic and non-lymphatic tissues of mice under physiological conditions, reveals for the first time the phenomenon of a large number of dual TCR Treg cells in mice. Moreover, the CDR3 regions of these dual TCR Treg cells exhibit certain tissue-specific or lineage-specific characteristics in terms of *VJ* usage, diversity, and clonality. Compared to single TCR Treg cells, dual TCR Treg cells highly express immune-inhibitory related molecules, such as *Foxo3, Foxo1, CD27, IL2RA, and Ikzf2*, etc. Dual TCR Treg cells in LPL and skin also have more mRNA differentially expressed characteristic molecules. These results provide a new Treg cell subset (dual TCR Treg) for studying phenotypic characteristics, TCR composition characteristics, etc. This study delves into the complex biological effects and mechanisms of Treg cells from the perspective of TCR mRNA expression, providing a new perspective for Treg cell research and also offering technical solutions for decoding the TCR pairing and characteristics of dual TCR T cells. However, this study still has some shortcomings, such as the lack of protein data for individual dual TCR Treg cells and research on human dual TCR Treg cells. Subsequent studies can use dual receptor transgenic reporter mice[18], combined with FCM, tetramers, and scCITE-seq technology, to provide a foundation for research at the TCR protein expression level. We hope that more laboratories will participate in the study of dual TCR Treg cells, especially to further clarify the diversity and specificity of the immune negative regulatory role of Treg cells in human lymphatic and non-lymphatic tissues. Further experimental studies on their developmental differentiation, origins, functional gene expression, and effects will provide theoretical and technical means for the potential application of tissue-resident dual TCR Tregs.

Materials and Methods

1. Research Subjects and Study Samples

(1) Blood,kidney,liver,LPL and pancreas tissues w ere collected from 4 C57BL/6 mice and CD4+Foxp3+ Thy1.1+ Treg cells were sorted for s cRNA+TCR-seq analysis.(Source document:Oliver T et al.The tissue-resident regulatory T ce ll pool is shaped by transient multi-tissue migration and a conserved residency program.Imm unity. 2024 Jul 9;57(7):1586-1602.e10. Shared Data Link:*https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE266111*)

(2) Spleen,iLN and mLN tissues from 4 mice were collected and sorted into CD4+CD25+T reg cells,while skin tissues from 4 mice were collected and sorted into CD3+ T cells(both Treg and non-Treg),These four tissue-sorted cells were analyzed by scRNA+TCR-seq. (Sourc e document:Nedwed et al.Using combined single-cell gene expression,TCR sequencing and c ell surface protein barcoding to characterize and track CD4+ T cell clones from murin tissu es.Front Immunol. 2023 Oct 12;14:1241283. Shared Data Link:*https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE240041*)

2. The process of sharing raw data analysis

(1)Excel software was utilized for paired analysis of TCR α chain (A) and TCR β chain (B) from single Treg cell scTCR-seq results, including CD3 T cells in skin tissue.; (2) In a single T cell, two types of chains “can pair and assemble into single TCR” T cells named “A+B”; Three or more types of chains “can pair and assemble into dual TCR” T cells named “A+B1+B2;B+A1+A2;A1+A2+B1+B2”, (3) The proportions of total single TCR Treg and dual TCR Treg were calculated for each mouse or each tissue.

3. Examples of single T-cell TCR pairing types

From the two data sets, single T cell was selected to display four different TCR pairing types, *VDJ* gene family names and CDR3 AA sequences:A+ B; A+B1+B2; B+A1+A2; and A1+A2+B1+B2.

4. Analysis of TCR pairing types and V-J usage

Statistically analyze the proportions of five TCR T cell types in each sample, and use SPSS software to perform statistical analysis on the proportions of the three dual TCR T cell types and the single/dual TCR T cell ratios according to tissue origin; classify and calculate the proportions of all corresponding sequences in the ‘β-V’ and ‘β-J’ columns of the single and dual TCR T cell tables, and use SPSS software to conduct differential *VJ* usage analysis between single/dual TCR T cells. It should be noted that the dual TCR Treg and single TCR Treg cells analyzed in our results refer to T cells with functional alpha and beta chains that are paired. Treg cells that possess only a functional alpha chain or beta chain without TCR pairing, or those with non-functional alpha or beta chains participating in TCR pairing, were not included in our analysis.

5. CDR3 diversity and clonality analysis

Use the inverse Simpson’s index to assess TCR Treg cell diversity and perform statistical analysis; define clonal expansion as clone count≥2, and analyze the proportion of clonal expansion of single/dual TCR Treg cells in the two data sets.

6. CDR3 overlap analysis

The TRA CDR3 AA and TRB CDR3 AA of single TCR Treg cells and dual TCR Treg cells from different tissue sources were compared for overlap using the immunarch R package(version 0.9.0, *https://github.com/immunomind/immunarch*), and the Jacard index was used to analyze the frequency of overlap of CDR3 AA between each tissues pair,; the first 10 high-frequency overlapping TRB CDR3 AA and TRA CDR3 AA sequences were traced and analyzed between single TCR Treg cells and dual TCR Treg cells from different tissue sources.

7. Combined scRNA+TCR-seq analysis

The Seurat R package (version 5.0.1, *https://satijalab.org/seurat/*) was used for quality control of scRNA-seq results and preliminary removal of doublets. Data were excluded if nFeature > 5,000, nCount < 20,000 or > 1,000, and percent.MT > 5% (skin, spleen, iLN and mLN tissue datasets) or 10% (blood, kidney, liver, LPL and pancreas tissues). The DoubletFinder R package (version 2.0.4, *https://github.com/chris-mcginnis-ucsf/DoubletFinder*) was further utilized to accurately identify and remove doublets through steps such as simulating doublet generation, data dimensionality reduction, calculating the artificial nearest neighbor ratio (pANN), and setting thresholds. This process enhances the accuracy and reliability of data analysis. Furthermore, in the dataset containing five tissues with LPL, HTO labeling was incorporated (HTO labeling differentiates cells from different tissue sources by assigning unique barcodes to each cell; through the analysis of HTO labeling, double-positive and double-negative cells can be identified) to further exclude doublets and negative cells. T cells were then clustered and analyzed from different tissue sources after quality control. Based on the identical barcode identifiers of each T cell, the AddMetaData

function was used to integrate scTCR-seq and scRNA-seq results, and only Treg cells meeting both criteria were included. Cells containing scTCR-seq and scRNA-seq data were displayed in UMAP plots and analyzed for the proportion of single TCR Treg cells and dual TCR Treg cells from different tissue sources. In this analysis, skin tissue was divided into Treg and non-Treg for comparison.

8. Transcription factor expression analysis

Comparative analysis of the homogeneity of Treg cell signature molecules expressed in single TCR Treg and dual TCR Treg; Differential expression analysis of the top 10 mRNA molecules in single tissue, overall single TCR Treg cells, and dual CR Treg cells was conducted using the DESeq2 R package (version1.38.3 *https://github.com/thelovelab/DESeq2*) : Comparative analysis of the heterogeneity of the expression of single TCR Treg and dual TCR Treg top 10 mRNA molecules in each tissue; Comparative analysis of the heterogeneity of the expression of total single TCR Treg and total dual TCR Treg top 10 mRNA molecules.

9. Statistical Analysis

In this study, statistical analyses were conducted using R language and IBM SPSS Statistics 18 software. Independent sample t-tests were utilized to compare continuous variables between two groups, while chi-square tests were employed for categorical variables, with a p-value < 0.05 considered statistically significant. For the analysis of single-cell RNA sequencing data, the DESeq2 R package was used to perform statistical analysis of differentially expressed genes, and the Wald test was applied to calculate the significance of gene expression differences. A Padj < 0.05 was considered statistically significant.

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Additional information

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Authorship Contributions

Xinsheng Yao completed the experimental design and paper writing, Yuanyuanxu, Qipeng, Xiaoping Lu, completed the data analysis and chart making.

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Note

This reviewed preprint has been updated to correct a typo in the corresponding author's email address, and to correct formatting in figure titles.

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Reviewer #2 (Public review):

Summary:

The manuscript, by Xu and Peng, et al. investigates whether co-expression of 2 T cell receptor (TCR) clonotypes can be detected in FoxP3+ regulatory CD4+ T cells (Tregs) and if it is associated with identifiable phenotypic effects. This paper presents data reanalyzing publicly available single-cell TCR sequencing and transcriptional analysis, convincingly demonstrating that dual TCR co-expression can be detected in Tregs, both in peripheral circulation as well as among Tregs in tissues. They then compare metrics of TCR diversity between single-TCR and dual TCR Tregs, as well as between Tregs in different anatomic compartments, finding the TCR repertoires to be generally similar though with dual TCR Tregs exhibiting a less diverse repertoire and some moderate differences in clonal expansion in different anatomic compartments. Finally, they examine the transcriptional profile of dual TCR Tregs in these datasets, finding some potential differences in expression of key Treg genes such as Foxp3, CTLA4, Foxo3, Foxo1, CD27, IL2RA, and Ikzf2 associated with dual TCR-expressing Tregs, which the authors postulate implies a potential functional benefit for dual TCR expression in Tregs.

Strengths:

This report examines an interesting and potentially biologically significant question, given recent demonstrations that dual TCR co-expression is a much more common phenomenon than previously appreciated (approximately 15-20% of T cells) and that dual TCR co-expression has been associated with significant effects on the thymic development and antigenic reactivity of T cells. This investigation leverages large existing datasets of single-cell TCRseq/RNAseq to address dual TCR expression in Tregs. The identification and characterization of dual TCR Tregs is rigorously demonstrated and presented, providing convincing new evidence of their existence.

Weaknesses:

The existence of dual TCR expression by Tregs has previously been demonstrated in mice and humans, limiting the novelty of the reported findings. The presented results should be considered in the context of these prior important findings. The focus on self-citation of their previous work, using the same approach to measure dual TCR expression in other datasets. limits the discussion of other more relevant and impactful published research in this area. Also, Reference #7 continues to list incorrect authors. The authors do not present a balanced or representative description of the available knowledge about either dual TCR expression by T cells or TCR repertoires of Tregs.

The approach used follows a template used previously by this group for re-analysis of existing datasets generated by other research groups. The descriptions and interpretations of the data as presented are still shallow, lacking innovative or thoughtful approaches that would potentially be innovation or provide new insight.

This demonstration of dual TCR Tregs is notable, though the authors do not compare the frequency of dual TCR co-expression by Tregs with non-Tregs. This limits interpreting the findings in the context of what is known about dual TCR co-expression in T cells. The response to this criticism in a previous review is considered non-responsive and does not improve the data or findings.

Comparison of gene expression by single- and dual TCR Tregs is of interest, but as presented is difficult to interpret. The interpretations of the gene expression analyses are somewhat simplistic, focusing on single-gene expression of some genes known to have function in Tregs. However, the investigators continue to miss an opportunity to examine larger patterns of coordinated gene expression associated with developmental pathways and differential function in Tregs (Yang. 2015. Science. 348:589; Li. 2016. Nat Rev Immunol. Wyss. 2016. 16:220; Nat Immunol. 17:1093; Zenmour. 2018. Nat Immunol. 19:291). No attempt to define clusters is made. No comparison is made of the proportions of dual TCR cells in transcriptionally-defined clusters. The broad assessment of key genes by single- and dual TCR cells is conceptually interesting, but likely to be confounded by the heterogeneity of the Treg populations. This would need to be addressed and considered to make any analyses meaningful.

The study design, re-analysis of existing datasets generated by other scientific groups, precludes confirmation of any findings by orthogonal analyses.

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Reviewer #3 (Public review):

Summary:

This study addressed the TCR pairing types and CDR3 characteristics of Treg cells. By analyzing scRNA and TCR-seq data, it claims that 10-20% of dual TCR Treg cells exist in mouse

lymphoid and non-lymphoid tissues and suggests that dual TCR Treg cells in different tissues may play complex biological functions.

Strengths:

The study addresses an interesting question of how dual-TCR-expressing Treg cells play roles in tissues.

Weaknesses:

This study is inadequate, particularly regarding data interpretation, statistical rigor, and the discussion of the functional significance of Dual TCR Tregs.

Comments on revisions:

Although the authors have provided brief explanations in response to the reviewers' comments, they do not present any additional analyses that would address the fundamental concerns in a convincing manner.
Moreover, the in silico analyses presented in the manuscript alone are insufficient to support the conclusions, and the functional experiments requested by the reviewers have not been conducted.

In the current rebuttal, while some textual additions have been made to the manuscript, the only substantial revision to the figures appears to be the inclusion of statistical significance annotations (e.g., Fig. 1G, Fig. 3G). These changes do not adequately strengthen the overall data or address the core issues raised.

https://doi.org/10.7554/eLife.105504.2.sa1

Author Response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *(1) The use of single-cell RNA and TCR sequencing is appropriate for addressing potential relationships between gene expression and dual TCR.*

Thank you for your detailed review and suggestions. The main advantages of scRNA+TCR-seq are as follows: (1) It enables comparative analysis of features such as the ratio of single TCR paired T cells to dual TCR paired T cells at the level of a large number of individual T cells, through mRNA expression of the α and β chains. In the past, this analysis was limited to a small number of T cells, requiring isolation of single T cells, PCR amplification of the α and β chains, and Sanger sequencing; (2) While analyzing TCR paired T cell characteristics, it also allows examination of mRNA expression levels of transcription factors in corresponding T cells through scRNA-seq.

> *(2) The data confirm the presence of dual TCR Tregs in various tissues, with proportions ranging from 10.1% to 21.4%, aligning with earlier observations in αβ T cells.*

Thank you very much for your detailed review and suggestions. Early studies on dual TCR αβ T cells have been very limited in number, with reported proportions of dual TCR T cells ranging widely from 0.1% to over 30%. In contrast, scRNA+TCR-seq can monitor over 5,000 single and paired TCRs, including dual paired TCRs, in each sample, enabling more precise examination of the overall proportion of dual TCR αβ T cells. It is important to note that our analysis focuses on T cells paired with functional α and β chains, while T cells with non-

functional chain pairings and those with a single functional chain without pairing were excluded from the total cell proportion analysis. Previous studies generally lacked the ability to determine expression levels of specific chains in T cells without dual TCR pairings.

> *(3) Tissue-specific patterns of TCR gene usage are reported, which could be of interest to researchers studying T cell adaptation, although these were more rigorously analyzed in the original works.*

Thank you very much for your detailed review and suggestions. T cell subpopulations exhibit tissue specificity; thus, we conducted a thorough investigation into Treg cells from different tissue sites. This study builds upon the original by innovatively analyzing the differences in VDJ rearrangement and CDR3 characteristics of dual TCR Treg cells across various tissues. This provides new insights and directions for the potential existence of "new Treg cell subpopulations" in different tissue locations. The results of this analysis suggest the necessity of conducting functional experiments on dual TCR Treg cells at both the TCR protein level and the level of effector functional molecules.

> *(4) Lack of Novelty: The primary findings do not substantially advance our understanding of dual TCR expression, as similar results have been reported previously in other contexts.*

Thank you for your detailed review and suggestions. Early research on dual TCR T cells primarily relied on transgenic mouse models and in vitro experiments, using limited TCR alpha chain or TCR beta chain antibody pairings. Flow cytometry was used to analyze a small number of T cells to estimate dual TCR T cell proportion. No studies have yet analyzed dual TCR Treg cell proportion, V(D)J recombination, and CDR3 characteristics at high throughput in physiological conditions. The scRNA+TCR-seq approach offers an opportunity to conduct extensive studies from an mRNA perspective. With high-throughput advantages of single-cell sequencing technology, researchers can analyze transcriptomic and TCR sequence characteristics of all dual TCR Treg cells within a study sample, providing new ideas and technical means for investigating dual TCR T cell proportions, characteristics, and origins under different physiological and pathological states.

> *(5) Incomplete Evidence: The claims about tissue-specific differences lack sufficient controls (e.g., comparison with conventional T cells) and functional validation (e.g., cell surface expression of dual TCRs).*

Thank you for your detailed review and suggestions. This study indeed only analyzed dual TCR Treg cells from different tissue locations based on the original manuscript, without a comparative analysis of other dual TCR T cell subsets corresponding to these tissue locations. The main reason for this is that, in current scRNA+TCR-seq studies of different tissue locations, unless specific T cell subsets are sorted and enriched, the number of T cells obtained from each subset is very low, making a detailed comparative analysis impossible. In the results of the original manuscript, we observed a relatively high proportion of dual TCR Treg cell populations in various tissues, with differences in TCR composition and transcription factor expression. Following the suggestions, we have included additional descriptions in R1, citing the study by Tuovinen et al., which indicates that the proportion of dual TCR Tregs in lymphoid tissues is higher than other T cell types. This will help understand the distribution characteristics of dual TCR Treg cells in different tissues and provide a basis for mRNA expression levels to conduct functional experiments on dual TCR Treg cells in different tissue locations.

> *(6) Methodological Weaknesses: The diversity analysis does not account for sample size differences, and the clonal analysis conflates counts and clonotypes, leading to potential*

> *misinterpretation.*

We thank you for your review and suggestions. In response to your question about whether the diversity analysis considered the sample size issue, we conducted a detailed review and analysis. This study utilized the inverse Simpson index to evaluate TCR diversity of Treg cells. A preliminary analysis compared the richness and evenness of single TCR Treg cell and dual TCR Treg cell repertoires. The two datasets analyzed were from four mouse samples with consistent processing and sequencing conditions. However, when analyzing single TCR Tregs and dual TCR Tregs from various tissues, differences in detected T cell numbers by sequencing cannot be excluded from the diversity analysis. Following recommendations, we provided additional explanations in R1: CDR3 diversity analysis indicates TCR composition of dual TCR Treg cells exhibits diversity, similar to single TCR Treg cells; however, diversity indices of single TCR Tregs and dual TCR Tregs are not suitable for statistical comparison. Regarding the "clonal analysis" you mentioned, we define clonality based on unique TCR sequences; cells with identical TCR sequences are part of the same clone, with ≥2 counts defined as expansion. For example, in Blood, there are 958 clonal types and 1,228 cells, of which 449 are expansion cells. In R1, we systematically verified and revised clonal expansion cells across all tissue samples according to a unified standard.

> *(7) Insufficient Transparency: The sequence analysis pipeline is inadequately described, and the study lacks reproducibility features such as shared code and data.*

Thank you for your review and suggestions. Based on the original manuscript, we have made corresponding detailed additions in R1, providing further elaboration on the analysis process of shared data, screening methods, research codes, and tools. This aims to offer readers a comprehensive understanding of the analytical procedures and results.

> *(8) Weak Gene Expression Analysis: No statistical validation is provided for differential gene expression, and the UMAP plots fail to reveal meaningful clustering patterns.*

Thank you very much for your review and suggestions. Based on your recommendations, we conducted an initial differential expression analysis of the top 10 mRNA molecules in single TCR Treg and dual TCR Treg cells using the DESeq2 R package in R1, with statistical significance determined by Padj < 0.05. Regarding the clustering patterns in the UMAP plots, since the analyzed samples consisted of isolated Treg cell subpopulations that highly express immune suppression-related genes, we did not perform a more detailed analysis of subtypes and expression gene differences. This study primarily aims to explore the proportions of single TCR and dual TCR Treg cells from different tissue sources, as well as the characteristics of CDR3 composition, with a focus on showcasing the clustering patterns of samples from different tissue origins and various TCR pairing types.

> *(9) A quick online search reveals that the same authors have repeated their approach of reanalysing other scientists' publicly available scRNA-VDJ-seq data in six other publications,In other words, the approach used here seems to be focused on quick re-analyses of publicly available data without further validation and/or exploration.*

Thank you for your review and suggestions. Most current studies utilizing scRNA+TCR-seq overlook analysis of TCR pairing types and related research on single TCR and dual TCR T cell characteristics. Through in-depth analysis of shared scRNA+TCR-seq data from multiple laboratories, we discovered a significant presence of dual TCR T cells in high-throughput T cell research results that cannot be ignored. In this study, we highlight the higher proportion of dual TCR Tregs in different tissue locations, which exhibits a certain degree of tissue specificity, suggesting these cells may participate in complex functional regulation of Tregs. This finding provides new ideas and a foundation for further research into dual TCR Treg

functions. However, as reviewers pointed out, findings from scRNA+TCR-seq at the mRNA level require additional functional experiments on dual TCR T cells at the protein level. We have supplemented our discussion in R1 based on these suggestions.

> ***Reviewer #2 (Public review):***
>
> *(1)The existence of dual TCR expression by Tregs has previously been demonstrated in mice and humans (Reference #18 and Tuovinen. 2006. Blood. 108:4063; Schuldt. 2017. J Immunol. 199:33, both omitted from references). The presented results should be considered in the context of these prior important findings.*

Thank you very much for your review and suggestions. Based on the original manuscript, we have supplemented our reading, understanding, and citation of closely related literature (Tuovinen, 2006, Blood, 108:4063 (line 44,line175 in R1); Schuldt, 2017, J Immunol, 199:33 (line 44,line178 in R1)). We once again appreciate the valuable comments from the reviewers, and we will refer to these in our subsequent dual TCR T cell research.

> *(2) This demonstration of dual TCR Tregs is notable, though the authors do not compare the frequency of dual TCR co-expression by Tregs with non-Tregs. This limits interpreting the findings in the context of what is known about dual TCR co-expression in T cells.*

Thank you very much for your review and suggestions. This analysis is primarily based on the scRNA+TCR-seq study of sorted Treg cells, where we found the proportions and distinguishing features of dual TCR Treg cells in different tissue sites. Given the diversity and complexity of Treg function, conducting a comparative analysis of the origins of dual TCR Treg cells and non-T cells with dual TCRs will be a meaningful direction. Currently, peripheral induced Treg cells can originate from the conversion of non-Treg cells; however, little is known about the sources and functions of dual TCR Treg cell subsets in both central and peripheral sites. In R1, we have supplemented the discussion regarding the possible origins and potential applications of the "novel dual TCR Treg" subsets.

> *(3) Comparison of gene expression by single- and dual TCR Tregs is of interest, but as presented is difficult to interpret. Statistical analyses need to be performed to provide statistical confidence that the observed differences are true.*

Thank you very much for your review and suggestions. Based on your recommendations, we performed an initial differential expression analysis of the top 10 mRNA molecules in single TCR Treg and dual TCR Treg cells using the DESeq2 R package in R1, with a statistical significance threshold of Padj<0.05 for comparisons.

> *(4) The interpretations of the gene expression analyses are somewhat simplistic, focusing on the single-gene expression of some genes known to have a function in Tregs. However, the investigators miss an opportunity to examine larger patterns of coordinated gene expression associated with developmental pathways and differential function in Tregs (Yang. 2015. Science. 348:589; Li. 2016. Nat Rev Immunol. Wyss. 2016. 16:220; Nat Immunol. 17:1093; Zenmour. 2018. Nat Immunol. 19:291).*

Thank you for your review and suggestions. This study is based on publicly available scRNA+TCR-seq data from different organ sites generated by the original authors, focusing on sorted and enriched Treg cells within each tissue sample. However, there was no corresponding research on other cell types in each tissue sample, preventing analysis of other cells and factors involved in development and differentiation of single TCR Treg and dual TCR Treg. The literature suggested by the reviewer indicates that development, differentiation, and function of Treg cells have been extensively studied, resulting in significant advances. It

also highlights complexity and diversity of Treg origins and functions. This research aims to investigate "novel dual TCR Treg cell subpopulations" that may exhibit tissuespecific differences found in the original authors' studies of Treg cells across different organ sites. This suggests further experimental research into their development, differentiation, origin, and functional gene expression as an important direction, which we have supplemented in the discussion section of R1.

> ***Reviewer #3 (Public review):***
>
> *(1) Definition of Dual TCR and Validity of Doublet Removal:This study analyzes Treg cells with Dual TCR, but it is not clearly stated how the possibility of doublet cells was eliminated. The authors mention using DoubletFinder for detecting doublets in scRNA-seq data, but is this method alone sufficient?We strongly recommend reporting the details of doublet removal and data quality assessment in the Supplementary Data.*

Thank you very much for your review and suggestions. In the analysis of the shared scRNA+TCR-seq data across multiple laboratories, as you mentioned, this study employed the DoubletFinder R package to exclude suspected doublets. Additionally, we used the nCount values of individual cells (i.e., the total sequencing reads or UMI counts for each cell) as auxiliary parameters to further optimize the assessment of cell quality. Generally, due to the possibility that doublet cells may contain gene expression information from two or more cells, their nCount values are often abnormally high. In this study, all cells included in the analysis had nCount values not exceeding 20,000. Among the five tissue sample datasets, we further utilized hashtag oligonucleotide (HTO) labeling (where HTO labeling provides each cell with a unique barcode to differentiate cells from different tissue sources. By analyzing HTO labels, doublets and negative cells can be accurately identified) to eliminate doublets and negative cells.After the removal of chimeric cells, all samples exhibited T cells that possessed two or more TCR clones. This phenomenon validates the reliability of the methodological approach employed in this study and indicates that the analytical results accurately reflect the proportion of dual TCR T cells. Based on the recommendations of the reviewers, we have supplemented and clarified the methods and discussion sections in the manuscript. It is particularly noteworthy that in our analysis, the discussed dual TCR Treg cells and single TCR Treg cells specifically refer to those T cells that possess both functional α and β chains, which are capable of forming TCR. We have excluded from this analysis any Treg cells that possess only a single functional α or β chain and do not form TCR pairs, as well as those Treg cells in which the α or β chains involved in TCR pairing are non-functional.

> *(2) In Figure 3D, the proportion of Dual TCR T cells (A1+A2+B1+B2) in the skin is reported to be very high compared to other tissues. However, in Figure 4C, the proportion appears lower than in other tissues, which may be due to contamination by non-Tregs. The authors should clarify why it was necessary to include non-Tregs as a target for analysis in this study. Additionally, the sensitivity of scRNA-seq and TCR-seq may vary between tissues and may also be affected by RNA quality and sequencing depth in skin samples, so the impact of measurement bias should be assessed.*

We deeply appreciate your review and constructive comments. Based on the original manuscript, we have further supplemented and elaborated on the uniqueness and relative proportions of double TCR T cell pairs in skin tissue samples in Section R1. Due to the scarcity of T cells in skin samples, we included some non-Treg cells during single-cell RNA sequencing and TCR sequencing to obtain a sufficient number of cells for effective analysis. The presence of non-regulatory T cells may indeed impact the statistical representation of double TCR T cells as well as the related comparative analyses, as noted by the reviewer. T cells with A1+A2+B1+B2 type double TCR pairings are primarily found within the non-regulatory T cell population in the skin. In response to this point, we have provided a detailed explanation of

this analytical result in the revised manuscript R1. Furthermore, concerning the two datasets included in the study, we conducted a comparative analysis in R1, exploring how factors such as sequencing depth at different tissue sites might introduce biases in our findings, which we have thoroughly elaborated upon in the discussion section. We thank you once again for your valuable suggestions.

> *(3) Issue of Cell Contamination:In Figure 2A, the data suggest a high overlap between blood, kidney, and liver samples, likely due to contamination. Can the authors effectively remove this effect? If the dataset allows, distinguishing between blood-derived and tissue-resident Tregs would significantly enhance the reliability of the findings. Otherwise, it would be difficult to separate biological signals from contamination noise, making interpretation challenging.*

We thank you for your review and suggestions. We have carefully verified data sources for tissues such as blood, kidneys, and liver. In the study by Oliver T et al., various techniques were employed to differentiate between leukocytes from blood and those from tissues, ensuring accurate identification of leukocytes from tissue samples. First, anti-CD45 antibody was injected intravenously to label cells in the vasculature, verifying that analyzed cells were indeed resident in the tissue. Second, prior to dissection and cell collection, authors performed perfusion on anesthetized mice to reduce contamination of tissue samples by leukocytes from the vasculature. Additionally, during single-cell sequencing, authors utilized HTO technology to avoid overlap between cells from different tissues.

Analysis of the scRNA+TCR-seq data shared by the original authors revealed highly overlapping TCR sequences in blood, kidney, and liver, despite distinct cell labels associated with each tissue. While these techniques minimize overlap of cells from different sources, they cannot completely rule out the potential impact of this technical issue. As suggested, we have provided additional clarification in R1 of the manuscript regarding this phenomenon of high overlap in the kidney, liver, and blood, indicating that the possibility of Treg migration from blood to kidney and liver cannot be entirely excluded.

> *(4) Inconsistency Between CDR3 Overlap and TCR Diversity:The manuscript states that Single TCR Tregs have a higher CDR3 overlap, but this contradicts the reported data that Dual TCR Tregs exhibit lower TCR diversity (higher 1/DS score). Typically, when TCR diversity is low (i.e., specific clones are concentrated), CDR3 overlap is expected to increase. The authors should carefully address this discrepancy and discuss possible explanations.*

Thank you for your review and suggestions. Regarding the potential relationship between CDR3 overlap and TCR diversity, in samples with consistent sequencing depth, lower diversity indeed corresponds to a higher proportion of CDR3 overlap. In our analysis of scRNA+TCR-seq data, we found that single TCR Tregs exhibit both higher diversity and CDR3 overlap, seemingly presenting contradictory analytical results (i.e., dual TCR Tregs show lower TCR diversity and CDR3 overlap). In R1, we supplemented the analysis of possible reasons: the presence of multiple TCR chains in dual TCR Treg cells may lead to a higher uniqueness of CDR3 due to multiple rearrangements and selections, resulting in lower CDR3 overlap; the lower diversity of dual TCR Tregs may be related to the number of T cells sequenced in each sample. The CDR3 diversity analysis in this study merely suggests that the TCR composition of dual TCR Treg cells is diverse, similar to that of single TCR Tregs. However, the diversity indices of single TCR Tregs and dual TCR Tregs are not suitable for statistical comparative analysis. A more in-depth and specific analysis of the diversity and overlap of the VDJ recombination mechanisms and CDR3 composition in dual TCR Tregs during development will be an important technical means to elucidate the function of dual TCR Treg cells.

> *(5) Functional Evaluation of Dual TCR Tregs:This study indicates gene expression differences among tissue-resident Dual TCR T cells, but there is no experimental validation of their functional significance. Including functional assays, such as suppression assays or cytokine secretion analysis, would greatly enhance the study's impact.*

We sincerely appreciate your review and suggestions: In this analysis of scRNA+TCR-seq data, we innovatively discovered a higher proportion of dual TCR Treg cells in different tissue sites, which exhibited differences in tissue characteristics. Furthermore, we conducted a comparative analysis of the homogeneity and heterogeneity between single TCR Treg and dual TCR Treg cells. This result provides a foundation for further research on the origin and characteristics of dual TCR Treg cells in different tissue sites, offering new insights for understanding the complexity and functional diversity of Treg cells. Based on your suggestions, we have supplemented R1 with the feasibility of further exploring the functions of tissue-resident dual TCR T cells and the necessity for potential application research.

> *(6) Appropriateness of Statistical Analysis:When discussing increases or decreases in gene expression and cell proportions (e.g., Figure 2D), the statistical methods used (e.g., t-test, Wilcoxon, FDR correction) should be explicitly described. They should provide detailed information on the statistical tests applied to each analysis.*

Thank you for your review and suggestions: Based on the original manuscript, we have supplemented the specific statistical methods for the differences in cell proportions and gene expression in R1.

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