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

Yuanyuan Xu 1#, Qipeng 1#, Xiaoping Lu 1, Long Ma 1, Jun Li 1, Xinsheng Yao 1*

1.Department of Immunology, Zunyi Medical University; Guizhou Provincial Research Center for Applied Immunomolecular Engineering; Guizhou Provincial Innovation Base for Graduate Education in Immunology; Guizhou Provincial Key Discipline of Immunology

#Co-first authors; * Corresponding author: immmunology@126.com

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

63 characteristic marker mRNA expression.Additionally, to further explore the differences in dual
64 TCR Treg cells between lymphoid and non-lymphoid tissues in mice, we selected the
65 scRNA+TCR-seq data shared by Annekathrin T et al.[15], which includes sequencing results of
66 CD4+CD25+ Treg cells sorted from mouse spleen, inguinal lymph nodes (iLN), and mesenteric
67 lymph nodes (mLN) under physiological conditions, as well as CD3+ T cells (including Treg and
68 non-Treg) sorted from skin tissue, and performed comparative analysis again.
69

70 **Results**

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

72 The proportion of dual TCR Treg cells in mouse lymphoid tissues: spleen=21.4%; iLN=21.4%,
73 mLN=21.0% (Figure 3D); The proportion of dual TCR Treg cells in mouse non-lymphoid tissues:
74 blood=11.8%; kidney=12.2%; liver=13.6%, LPL=11.0%; pancreas=13.3%, The proportion of dual
75 TCR T cells in mouse skin tissue: 18.6% (Figure 1D, Figure 3D). Subsequently, a detailed analysis
76 of the pairing of dual TCR T cells in the two sets of data was conducted, revealing that the TCR
77 pairing types of dual TCR Treg include A+B1+B2, B+A1+A2 (the highest proportion),
78 A1+A2+B1+B2 (the lowest proportion). Significant differences in TCR pairing types exist between
79 tissues, with A1+A2+B1+B2 most highly expressed in skin (common in non-Treg cells of skin) and
80 kidney, and B+A1+A2 most highly expressed in spleen and liver compared to other tissues.(Figure
81 1D, Figure 3D).
82

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

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

91 **Diversity of CDR3**(Figure 1G, Figure 3G):The 1/DS of single TCR Treg CDR3 is significantly
92 higher than that of dual TCR Treg; the CDR3 diversity of dual TCR Treg in lymphoid tissues

93 (spleen, iLN, mLN) is higher than that in non-lymphoid tissues; the CDR3 diversity of dual TCR
94 Treg in skin, LPL, and pancreas is very low.
95 **Clonality of CDR3**(Figure 1H, Figure 3H):The proportion of single TCR Treg with clonal
96 proliferation greater than or equal to 2 is higher than that of dual TCR Treg; the proportion of dual
97 TCR Treg with clonal proliferation greater than or equal to 2 in the liver and skin is significantly
98 higher than that in other tissues.
99 **Overlap of CDR3**(Figure 2A/B, Figure 4A/B): A certain proportion of overlap exists between TRB
100 CDR3 AA and TRA CDR3 AA in different tissues, with single TCR Treg significantly higher than
101 dual TCR Treg, and a lower proportion of TRB CDR3 AA compared to TRA CDR3 AA. This
102 suggests that TRA allelic inclusive recombination involves more CDR3 AA types in TCR pairing
103 of Treg cells. Meanwhile, it was found that single and dual TCR Treg in different tissues can also
104 share common CDR3 AA. This suggests that both single and dual TCR Treg can respond to specific
105 antigen epitopes.
106 All three tissues, the liver, kidney, and pancreas, show a significantly high proportion of dual
107 TCR Treg CDR3 AA overlap with peripheral blood, suggesting their origin from the influx of
108 peripheral blood (It should be noted that the high overlap phenomenon of the kidneys, liver,
109 pancreas, and blood may not completely eliminate the technical possibility of local Treg migration
110 within the blood), iLN, mLN, and skin all show a significantly high proportion of dual TCR Treg
111 CDR3 AA overlap with the spleen, suggesting their origin from the influx of the spleen;
112 Intriguingly, there is also a high proportion of dual TCR Treg CDR3 AA overlap between the liver
113 & kidney, kidney & pancreas, and iLN & mLN, suggesting that dual TCR Treg cells in these tissue
114 sites may negatively regulate a shared set of antigens. Whereas the mechanism and significance of
115 the high percentage of overlap exhibited between iLN and skin TRA CDR3 AA deserves further
116 analysis.
117
118 **3.Homogeneity of mouse dual TCR Treg cell phenotypes:**
119 Comparative analysis of Treg cells with complete scRNA-seq and scTCR-seq results showed
120 that the TCR pairing types and proportion were consistent with the results of direct analysis of
121 scTCR-seq; the proportion of dual TCR Treg in the skin was higher than that of dual TCR non-Treg
122 (Figure 2C, Figure 4C); in all tissues,the expression of signature molecules *Tnfrsf9, Stat3, Tnfrsf4,*
123 *Cd27, Icos, Il2ra, Ikzf2, Lrrc32, Sh3rf1, Cd81, Baff, Smad2*, and *Foxo3* was identical between dual

124 and single TCR Treg in all tissues, with slightly higher *Foxp3, Foxo1, and Ctla4* expression in dual
125 TCR Treg than that in single TCR Treg(Figure 2D, Figure 4D).
126
127 **4.Heterogeneity of mouse dual TCR Treg cell phenotypes:**
128 Compared with iLN, mLN, and spleen, more characteristic mRNA molecules were highly
129 expressed in dual TCR Tregs in skin (Figure 4E), such as *Rora, Dusp1, Junb* involved in immune
130 responses and inflammatory diseases; *S100A6* involved in the proliferation, differentiation, and
131 migration of immune cells; *Hopx* and *Nr4a1* involved in the development of immune cells, etc.,
132 suggesting that dual TCR Treg in the skin may be involved in more specific response negative
133 regulations.
134 Between Blood & Liver; Kidney & Pancreas (Figure 2E), iLN & mLN & spleen (Figure 4E),
135 the mRNA expression of cytokines, cytokine receptors, and transcription factors in dual TCR Treg
136 shows higher homogeneity, suggesting that dual TCR Treg in these tissue sites may play convergent
137 negative immune response regulations.
138 Differential mRNA expression analysis between dual TCR Treg and single TCR Treg in each
139 tissue (Figure 2F, Figure 4F): showed significant high expressions of individual *TRBV* genes in
140 different tissues, consistent with scTCR-seq results, suggesting specific dual TCR Treg lineage
141 origins in different tissues; in addition to *TRBV* usage, each group's dual TCR Treg had
142 significantly different characteristic molecule expressions from single TCR Treg, such as Blood:
143 *H1f0* involved in immune cell proliferation and differentiation and *Hist1h2ap* related to immune
144 cell activation were increased; Kidney: *Fos*（$P<0.001$） and *Cenpa*（$P=0.003$）related to immune
145 cell proliferation and differentiation and *Ifit3* involved in the body's antiviral response were
146 significantly increased; Liver: *Cenpa*（$P=0.022$） related to immune cell proliferation and *Birc5*
147 involved in the body's anti-infection immune response were increased; LPL: *Flnb* related to cell
148 adhesion and migration regulation and *Atp1a1* related to cell proliferation, differentiation, and
149 apoptosis regulation were increased; Pancreas: *Ccl5, Ccr2, Cxcr6* related to immune cell migration
150 and *Lbr*（$P=0.009$）and *Id2*（$P=0.029$） related to immune cell proliferation and differentiation, as
151 well as *Fkbp4*（$P=0.004$） involved in immune suppression signal transduction were significantly
152 increased; iLN: *Tgfbr1* involved in maintaining immune tolerance and regulating inflammatory
153 responses and *Traf3* involved in immune cell activation and cytokine production were increased;
154 mLN: *Glcci*（$P<0.001$） involved in inflammation and immune cell migration was increased; skin:

155 *Areg*（*P*=0.008） involved in immune cell proliferation, differentiation, migration, and immune
156 suppression functions and *Ccr10* involved in T cell migration and positioning were increased;
157 spleen: *Casc3* related to T cell activation and proliferation was increased. This suggests that dual
158 TCR Treg and single TCR Treg in each specific tissue site may be involved in different negative
159 immune response regulations.

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

170

171 **Discussion**

172 Currently, the proportion of dual TCR T cells is reported to be extremely variable across
173 physiologic and pathologic states (0.1%-30%, etc.) due to differences in assay technology and
174 methodology[4][5][6][7][8][9][10]. This study, in a large number of single Treg cell scRNA+TCR-seq
175 studies, found that mouse lymphoid and non-lymphoid tissues both have a high proportion of dual
176 TCR Treg cells, with lymphoid tissues being higher than non-lymphoid tissues, this is consistent
177 with the findings of Tuovinen et al. [11], which indicate that the proportion of dual TCR Tregs in
178 lymphoid tissues is higher than that of other types of T cells. This will contribute to a better
179 understanding of the distribution characteristics of dual TCR Treg cells in different tissues, and
180 provide a basis for conducting functional experiments on dual TCR Treg cells at various tissue sites
181 based on mRNA expression levels. And the proportion of liver and pancreas dual TCR Treg cells in
182 non-lymphoid tissues is relatively high. In addition, our analysis of dual TCR T cell pairing types
183 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 were collected from 4 C57BL/6 mice and CD4+Foxp3+ Thy1.1+ Treg cells were sorted for scRNA+TCR-seq analysis.(Source document:Oliver T et al.The tissue-resident regulatory T cell pool is shaped by transient multi-tissue migration and a conserved residency program.Immunity. 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+Treg 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. (Source document:Nedwed et al.Using combined single-cell gene expression,TCR sequencing and cell surface protein barcoding to characterize and track CD4+ T cell clones from murin tissues.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 $\geqslant 2$, and analyze the proportion of clonal expansion of single/dual TCR Treg cells in the two data sets.

276

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

283

284 **7.Combined scRNA+TCR-seq analysis:** The Seurat R package (version 5.0.1,
285 https://satijalab.org/seurat/) was used for quality control of scRNA-seq results and preliminary
286 removal of doublets. Data were excluded if nFeature > 5,000, nCount < 20,000 or > 1,000, and
287 percent.MT > 5% (skin, spleen, iLN and mLN tissue datasets) or 10% (blood, kidney, liver, LPL
288 and pancreas tissues). The DoubletFinder R package (version 2.0.4,
289 https://github.com/chris-mcginnis-ucsf/DoubletFinder) was further utilized to accurately identify
290 and remove doublets through steps such as simulating doublet generation, data dimensionality
291 reduction, calculating the artificial nearest neighbor ratio (pANN), and setting thresholds. This
292 process enhances the accuracy and reliability of data analysis. Furthermore, in the dataset
293 containing five tissues with LPL, HTO labeling was incorporated (HTO labeling differentiates cells
294 from different tissue sources by assigning unique barcodes to each cell; through the analysis of
295 HTO labeling, double-positive and double-negative cells can be identified) to further exclude
296 doublets and negative cells. T cells were then clustered and analyzed from different tissue sources
297 after quality control. Based on the identical barcode identifiers of each T cell, the AddMetaData
298 function was used to integrate scTCR-seq and scRNA-seq results, and only Treg cells meeting both
299 criteria were included. Cells containing scTCR-seq and scRNA-seq data were displayed in UMAP
300 plots and analyzed for the proportion of single TCR Treg cells and dual TCR Treg cells from
301 different tissue sources. In this analysis, skin tissue was divided into Treg and non-Treg for
302 comparison.

303

304 **8.Transcription factor expression analysis:** Comparative analysis of the homogeneity of Treg cell
305 signature molecules expressed in single TCR Treg and dual TCR Treg; Differential expression
306 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.

REFERENCES

[1] Sakaguchi S, Mikami N, Wing J B, et al. Regulatory T Cells and Human Disease[J]. Annual Review of Immunology, 2020, 38: 541–566.

[2] Shevyrev D, Tereshchenko V. Treg Heterogeneity, Function, and Homeostasis[J]. Frontiers in Immunology, 2020, 10: 3100.

[3] Malissen M, Trucy J, Letourneur F, et al. A T cell clone expresses two T cell receptor alpha genes but uses one alpha beta heterodimer for allorecognition and self MHC-restricted antigen recognition[J]. Cell, 1988, 55(1): 49–59.

[4] Padovan E, Casorati G, Dellabona P, et al. Expression of Two T Cell Receptor α Chains: Dual Receptor T Cells[J]. Science, 1993;262(5132):422-424

[5] Hinz T, Weidmann E, Kabelitz D. Dual TCR-Expressing T Lymphocytes in Health and Disease[J]. International Archives of Allergy and Immunology, 2001, 125(1): 16–20.

[6] He X, Janeway C A, Levine M, et al. Dual receptor T cells extend the immune repertoire for foreign antigens[J]. Nature Immunology, 2002, 3(2): 127–134.

[7] Schuldt NJ, Binstadt BA, et al. Dual TCR T Cells: Identity Crisis or Multitaskers? J Immunol, 2019. 202(3): 637-644.

[8] Zhu L, Peng Q, Li J, et al. scRNA-seq revealed the special TCR β & α V(D)J allelic inclusion rearrangement and the high proportion dual (or more) TCR-expressing cells[J]. Cell Death & Disease, 2023, 14(7).

[9] Yuanyuanxu, Qipeng, Qingqingma, et al. scRNA + TCR-seq revealed the dual TCR pTh17 and Treg T cells involvement in autoimmune response in ankylosing spondylitis[J]. International Immunopharmacology, 2024, 135: 112279.

[10] Schuldt N J, Auger J L, Spanier J A,et al. Cutting Edge: Dual TCRα Expression Poses an Autoimmune Hazard by Limiting Regulatory T Cell Generation[J/OL]. The Journal of Immunology, 2017, 199(1): 33-38.

[11] Tuovinen H, Salminen J T, Arstila T P. Most human thymic and peripheral-blood CD4+CD25+ regulatory T cells express 2 T-cell receptors[J]. Blood, 2006, 108(13): 4063-4070.
[12] Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity[J]. Nature Reviews. Immunology, 2018, 18(1): 35–45.
[13] Park J-E, Botting R A, Domínguez Conde C, et al. A cell atlas of human thymic development defines T cell repertoire formation[J]. Science, 2020, 367(6480): eaay3224.
[14] Burton O T, Bricard O, Tareen S, et al. The tissue-resident regulatory T cell pool is shaped by transient multi-tissue migration and a conserved residency program[J]. Immunity, 2024, 57(7): 1586-1602.e10.
[15] Nedwed A S, Helbich S S, Braband K L, et al. Using combined single-cell gene expression, TCR sequencing and cell surface protein barcoding to characterize and track CD4+ T cell clones from murine tissues[J]. Frontiers in Immunology, 2023, 14: 1241283.
[16] Papadopoulou M, Sanchez Sanchez G, Vermijlen D. Innate and adaptive γδ T cells: How, when, and why[J]. Immunological Reviews, 2020, 298(1): 99–116.
[17] Simon M, Stüve P, Schmidleithner L, et al. Single-cell chromatin accessibility and transposable element landscapes reveal shared features of tissue-residing immune cells[J]. Immunity, 2024, 57(8): 1975-1993.e10.
[18] Yang L, Jama B, Wang H, et al. TCRα reporter mice reveal contribution of dual TCRα expression to T cell repertoire and function[J]. Proceedings of the National Academy of Sciences of the United States of America, 2020, 117(51): 32574–32583.

Acknowledgements：

We would like to express our gratitude to ***GEO*** and ***IMGT*** databases for providing the availability of the data. Additionally, We thank Oliver T et al. and Nedwed et al. for carrying out the innovative study of Treg in mice and for sharing all original data.

Funding：

This study was supported by the National Natural Science Foundation of China (82471630&82160279) and the Guizhou Provincial Hundred-level Talent Fund [No.(2018)5637].

Authorship Contributions：

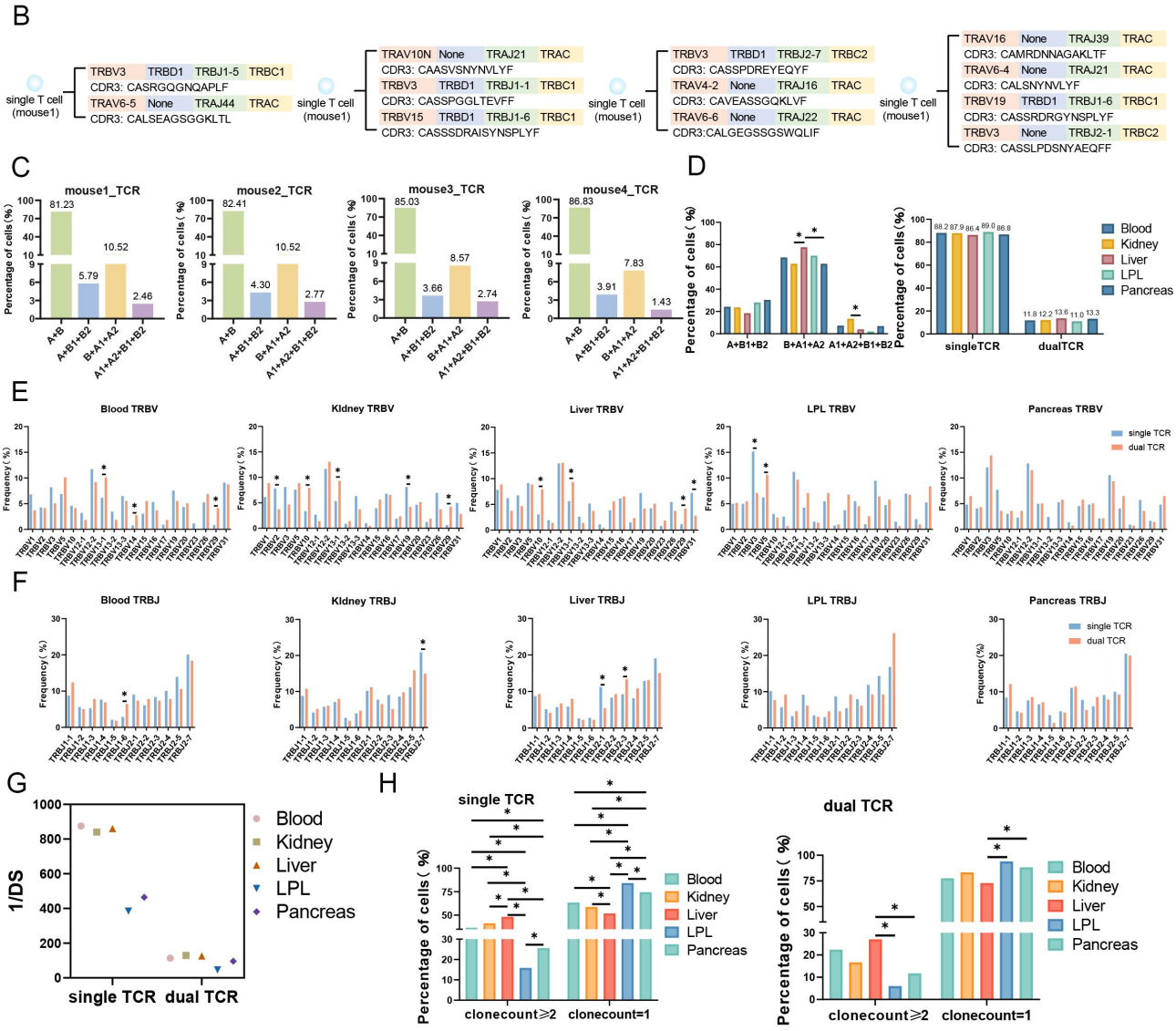
Xinsheng Yao completed the experimental design and paper writing, Yuanyuanxu, Qipeng, Xiaoping Lu, completed the data analysis and chart making.

Conflict of interest:

The authors declare no conflict of interests.

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%



382

383 **Figure 1.**Characterizes single Treg TCR and dual TCR Treg in the Blood, Kldney, Liver, LPL, and
384 Pancreas of mice.**A,**Mouse source names, GEO accession number, total number of paired TCR Treg
385 cells, and number and proportion of TCR paired types.**B,**Four different TCR pairing types, *VDJ*
386 gene family names and CDR3 AA sequences in single T cells.**C,**Proportion of TCR pairing types
387 per mouse.**D,**Statistical analysis of dual TCR Treg cells and the relative proportion of single /dual
388 TCR Treg Cells in Three TCR Pairing Types.**E,**Comparative analysis of single/dual TCR Treg cell
389 *V* gene usage variation.**F,**Comparative analysis of single/dual TCR Treg cell *J* gene usage
390 variation.**G,**Differential analysis of single/dual TCR Treg cell diversity among
391 tissues.**H,**Differential analysis of single/dual TCR Treg cell clone expansion between tissues.

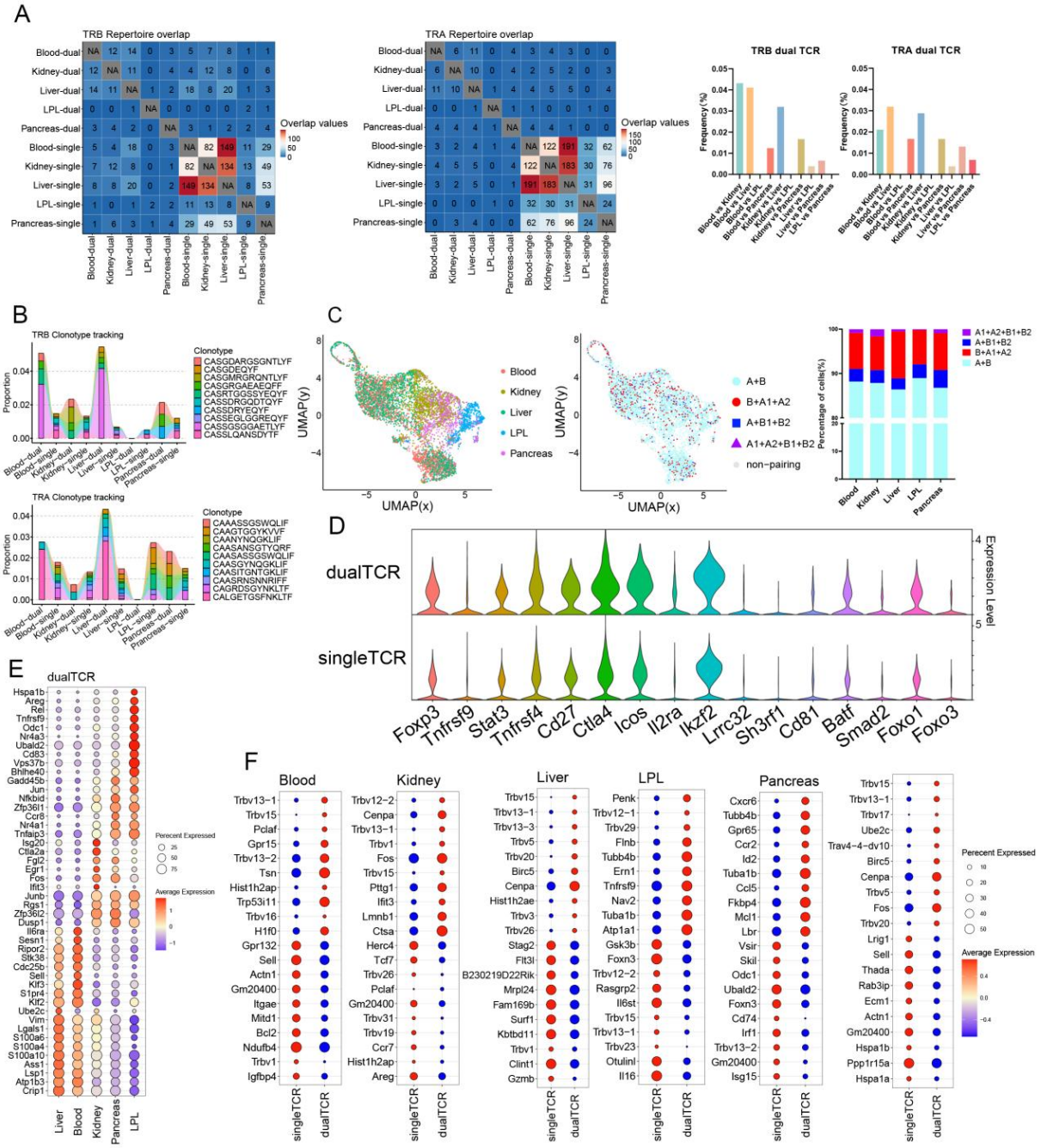


Figure 2. Comparative analysis of single Treg TCR and dual TCR Treg CDR3 overlap and gene mRNA expression in mice Blood, Kidney, Liver, LPL, and Pancreas.**A,**Clonal overlap analysis and jarcard index of TRA/B-chain CDR3 region in single /dual TCR Treg cells.**B,**Tracking results of the first 10 high-frequency 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%

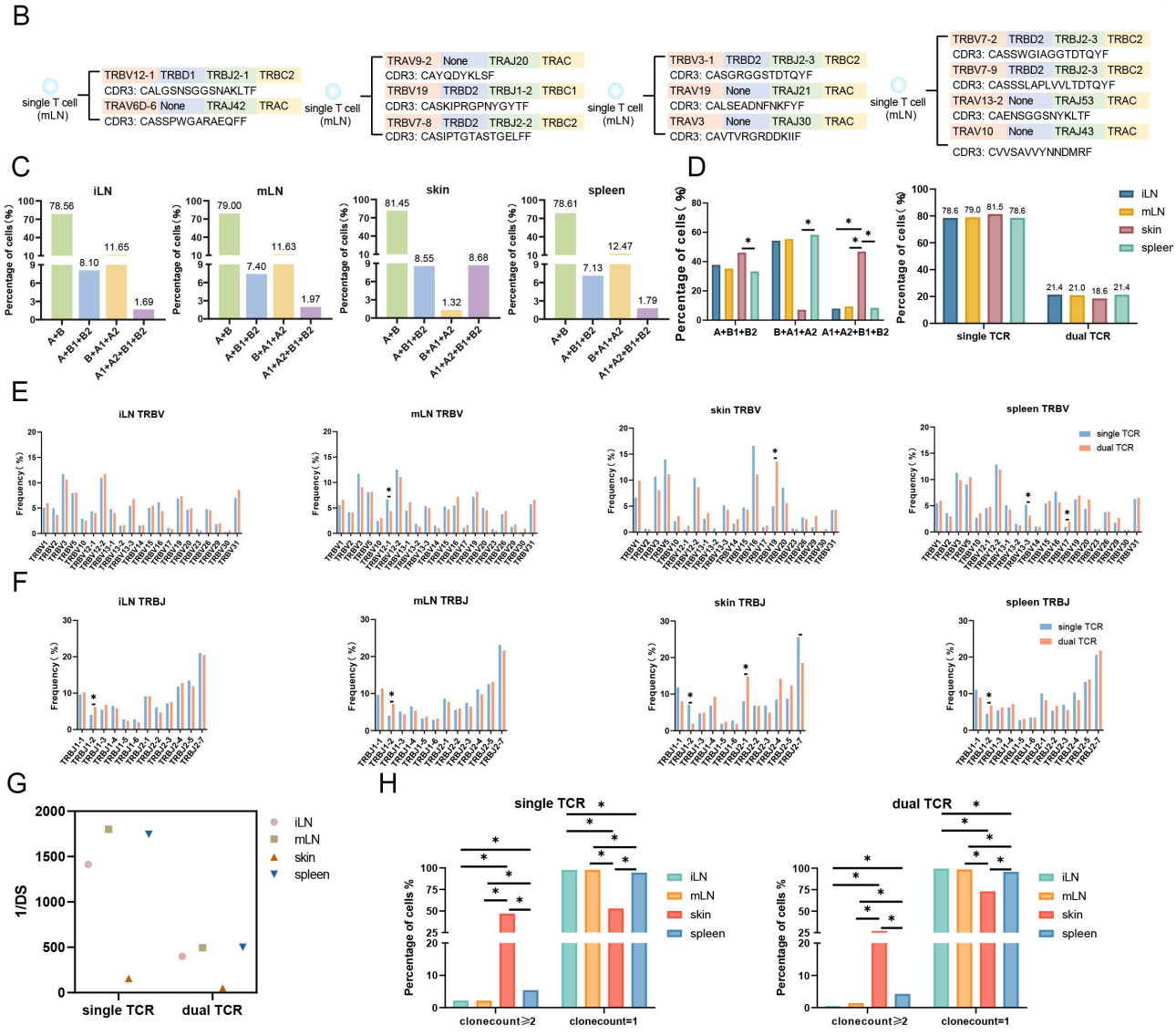


Figure 3. Characterizes single Treg TCR and dual TCR Treg in the iLN, mLN, skin, spleen of mice.**A,**Tissue source names, GEO accession number, total number of paired TCR T cells, and number and proportion of TCR paired types.**B,**Four different TCR pairing types, *VDJ* gene family names and CDR3 AA 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.

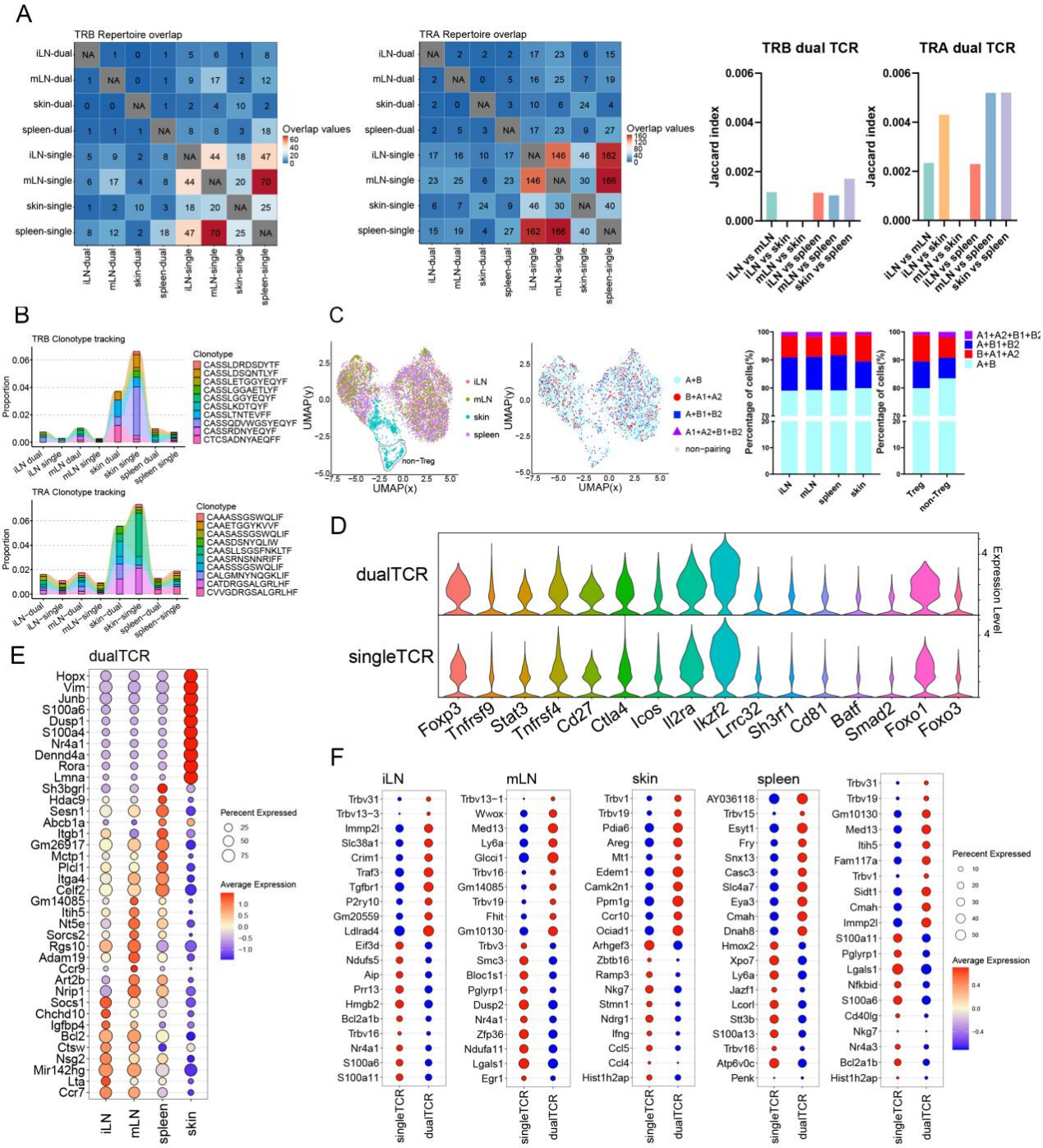
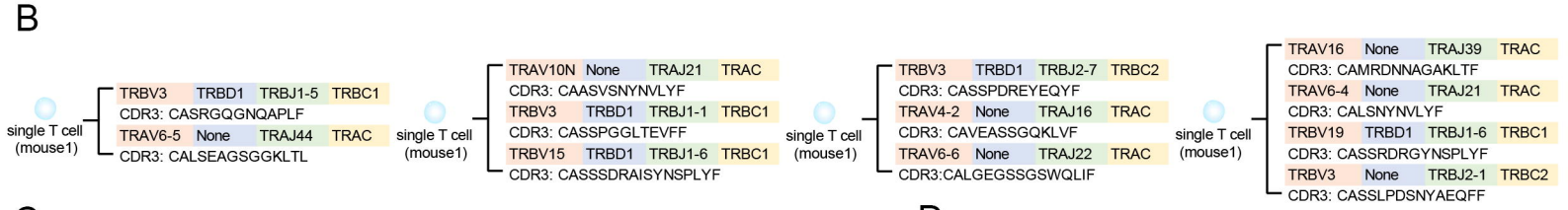


Figure 4. Comparative analysis of single Treg TCR and dual TCR Treg CDR3 overlap and gene mRNA expression in mice iLN,mLN,skin,spleen.**A,**Clonal overlap analysis and jarcard index of TRA/B-chain CDR3 region in single /dual TCR T cells.**B,**Tracking results of the first 10 high-frequency overlapping 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.

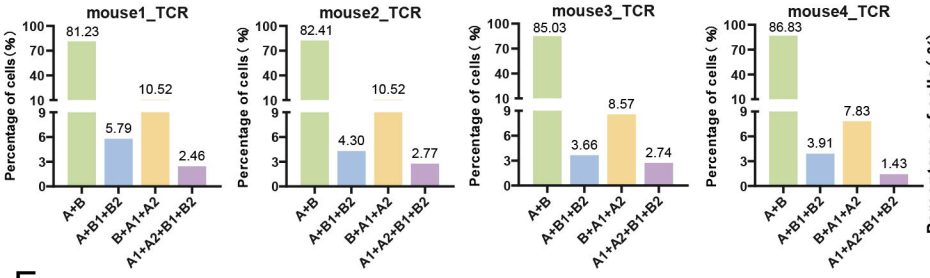
A

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%

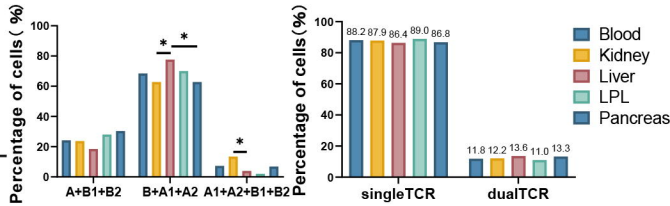
B



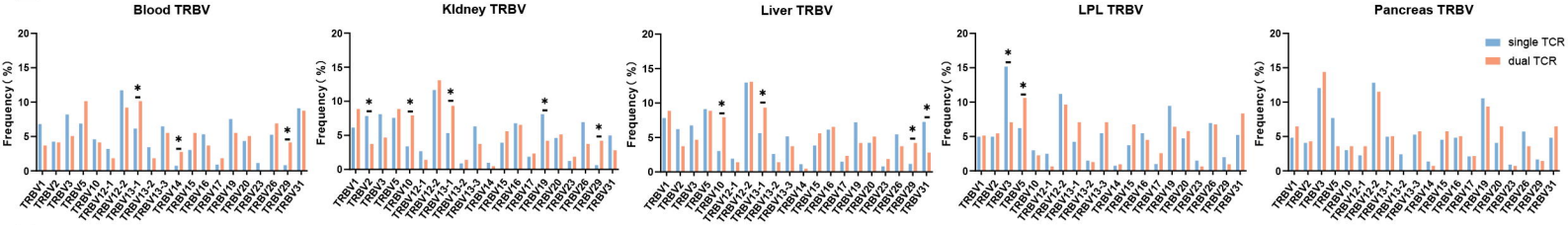
C



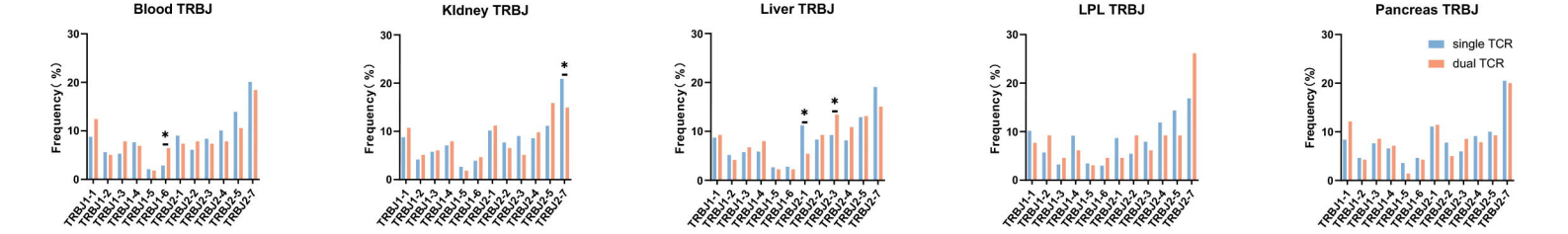
D



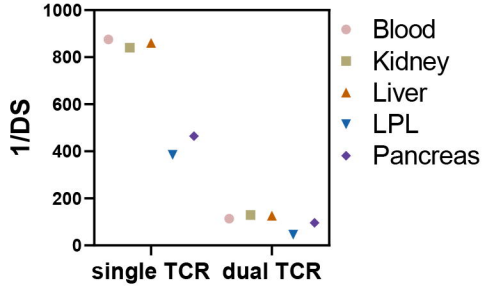
E



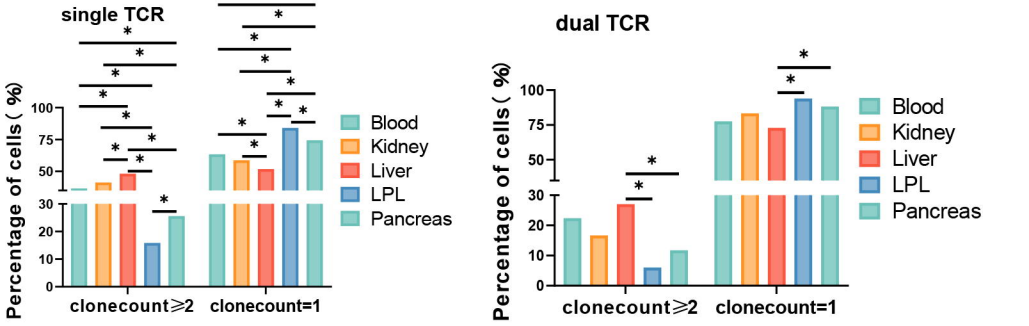
F

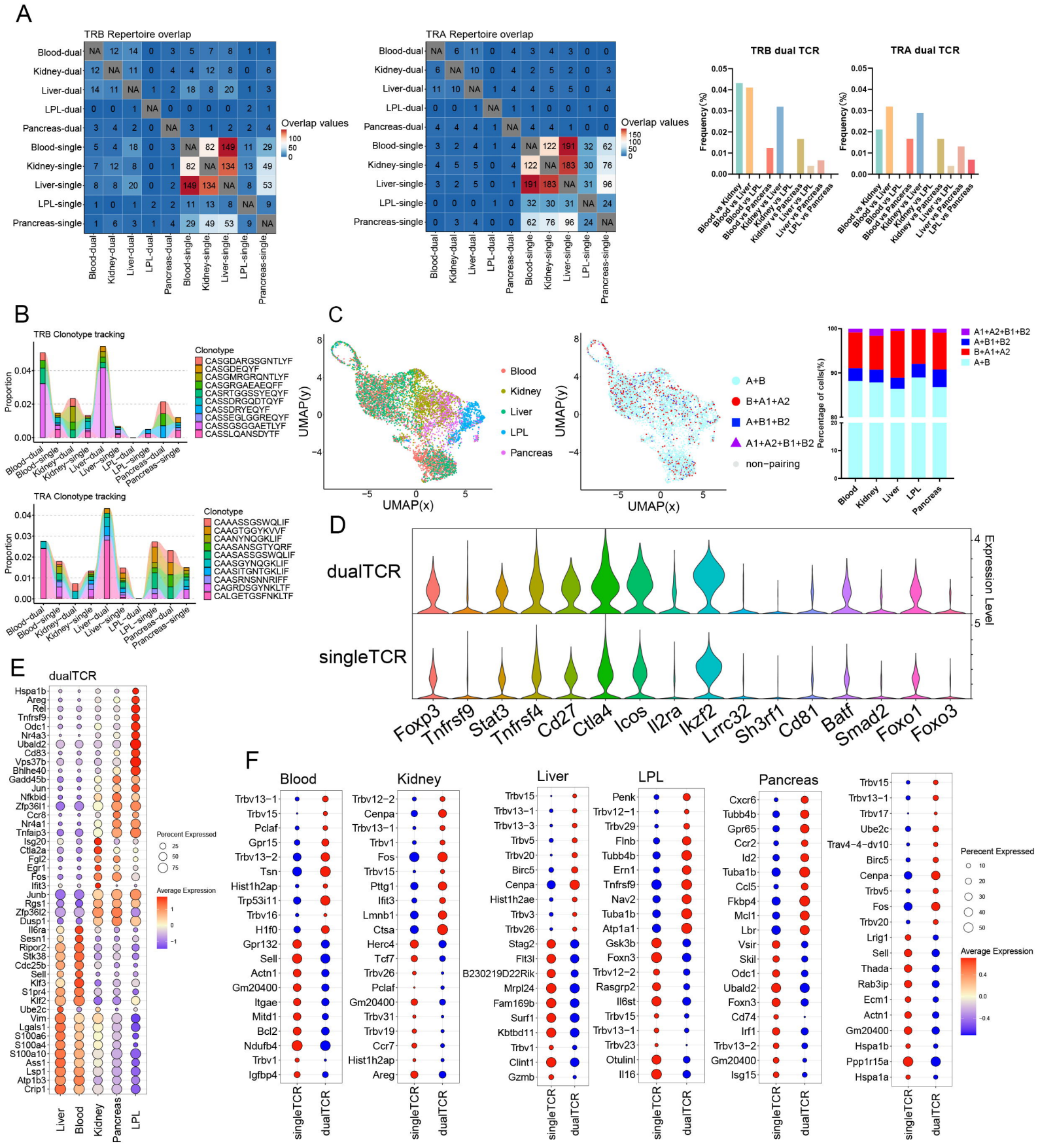


G



H

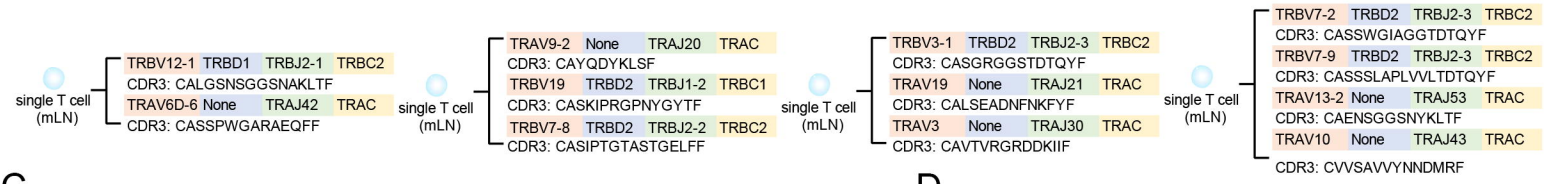



A
TRB Repertoire overlap
TRA Repertoire overlap
Overlap values
TRB dual TCR
TRA dual TCR
Frequency (%)
B
TRB Clonotype tracking
TRA Clonotype tracking
Proportion
Clonotype
C
Blood
Kidney
Liver
LPL
Pancreas
UMAP(x)
UMAP(y)
A+B
B+A1+A2
A+B1+B2
A1+A2+B1+B2
non−pairing
Percentage of cells(%)
D
dualTCR
singleTCR
Expression Level
Foxp3
Tnfrsf9
Stat3
Tnfrsf4
Cd27
Ctla4
Icos
Il2ra
Ikzf2
Lrrc32
Sh3rf1
Cd81
Batf
Smad2
Foxo1
Foxo3
E
dualTCR
Perecent Expressed
Average Expression
Liver
Blood
Kidney
Pancreas
LPL
F
Blood
Kidney
Liver
LPL
Pancreas
singleTCR
dualTCR

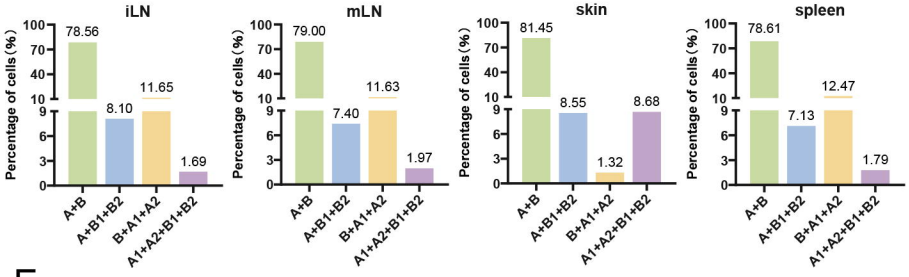
A

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%

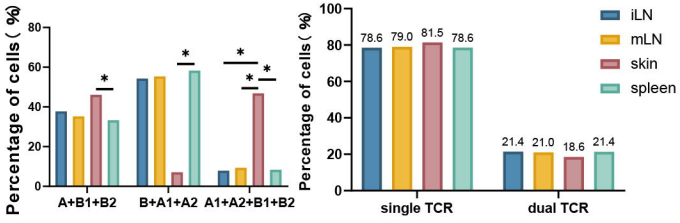
B



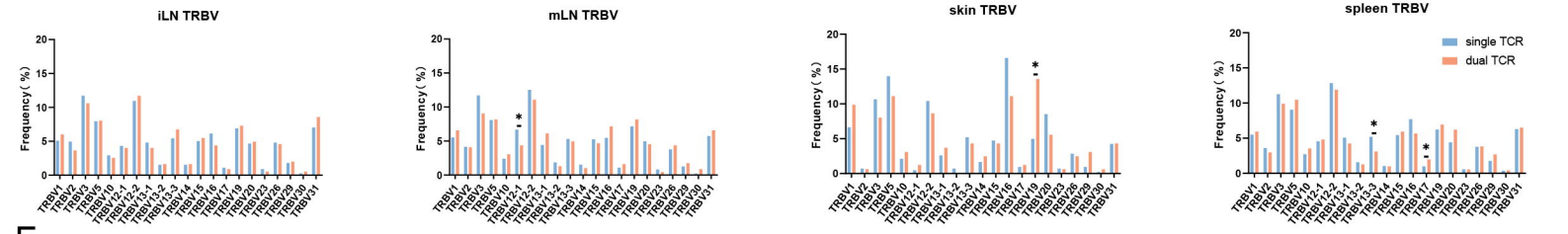
C



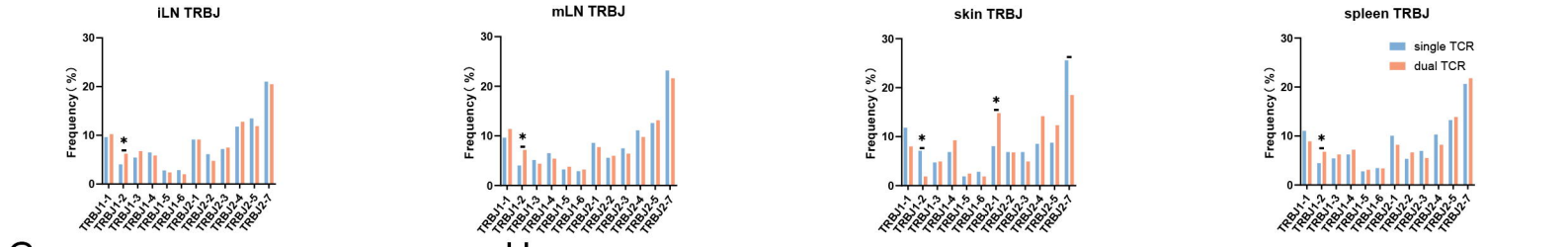
D



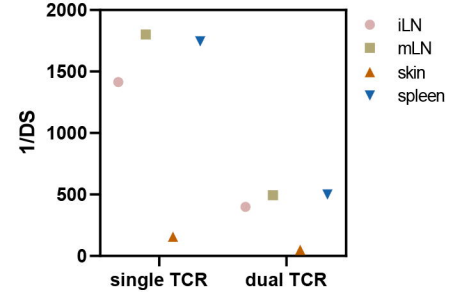
E



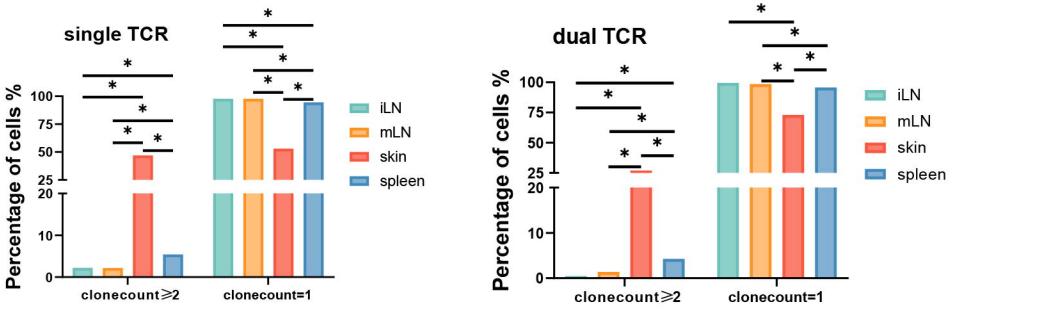
F

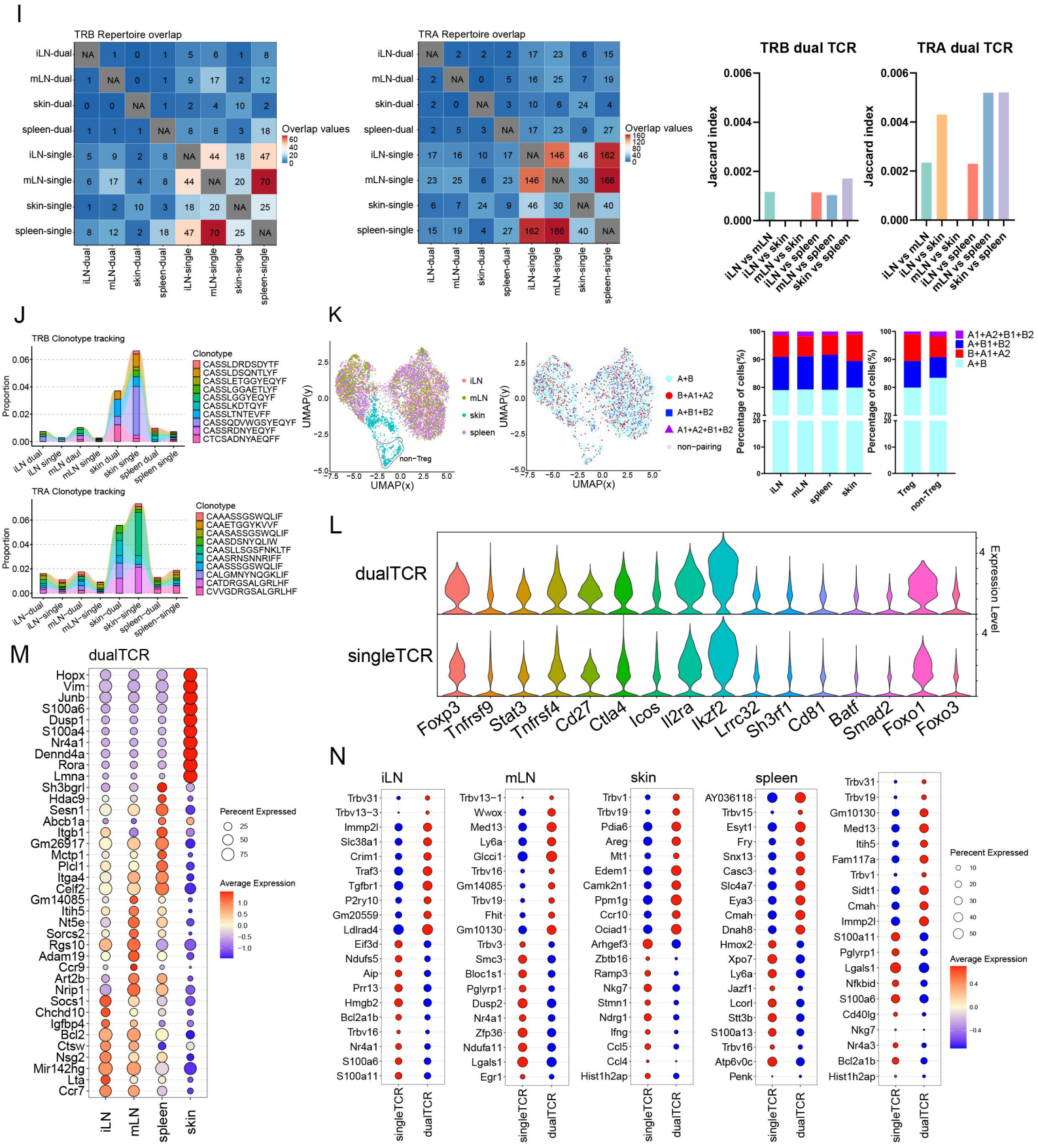


G



H




I
TRB Repertoire overlap
TRA Repertoire overlap
Overlap values
iLN-dual
mLN-dual
skin-dual
spleen-dual
iLN-single
mLN-single
skin-single
spleen-single
TRB dual TCR
TRA dual TCR
Jaccard index
iLN vs mLN
iLN vs skin
mLN vs skin
iLN vs spleen
mLN vs spleen
skin vs spleen
J
TRB Clonotype tracking
Clonotype
CASSLDRDSDYTF
CASSLDSQNTLYF
CASSLETGGYEQYF
CASSLGGAETLYF
CASSLGGYEQYF
CASSLKDTQYF
CASSLTNTEVFF
CASSQDVWGSYEQYF
CASSRDNYEQYF
CTCSADNYAEQFF
Proportion
iLN dual
iLN single
mLN daul
mLN single
skin dual
skin single
spleen dual
spleen single
TRA Clonotype tracking
CAAASSGSWQLIF
CAAETGGYKVVF
CAASASSGSWQLIF
CAASDSNYQLIW
CAASLLSGSFNKLTF
CAASRNSNNRIFF
CAASSSGSWQLIF
CALGMNYNQGKLIF
CATDRGSALGRLHF
CVVGDRGSALGRLHF
iLN-dual
iLN-single
mLN-dual
mLN-single
skin-dual
skin-single
spleen-dual
spleen-single
K
UMAP(x)
UMAP(y)
iLN
mLN
skin
spleen
non−Treg
A+B
B+A1+A2
A+B1+B2
A1+A2+B1+B2
non−pairing
Percentage of cells(%)
Treg
L
dualTCR
singleTCR
Expression Level
Foxp3
Tnfrsf9
Stat3
Tnfrsf4
Cd27
Ctla4
Icos
Il2ra
Ikzf2
Lrrc32
Sh3rf1
Cd81
Batf
Smad2
Foxo1
Foxo3
M
dualTCR
Hopx
Vim
Junb
S100a6
Dusp1
S100a4
Nr4a1
Dennd4a
Rora
Lmna
Sh3bgrl
Hdac9
Sesn1
Abcb1a
Itgb1
Gm26917
Mctp1
Plcl1
Itga4
Celf2
Gm14085
Itih5
Nt5e
Sorcs2
Rgs10
Adam19
Ccr9
Art2b
Nrip1
Socs1
Chchd10
Igfbp4
Bcl2
Ctsw
Nsg2
Mir142hg
Lta
Ccr7
iLN
mLN
spleen
skin
Perecent Expressed
Average Expression
N
iLN
mLN
skin
spleen
Trbv31
Trbv13−3
Immp2l
Slc38a1
Crim1
Traf3
Tgfbr1
P2ry10
Gm20559
Ldlrad4
Eif3d
Ndufs5
Aip
Prr13
Hmgb2
Bcl2a1b
Trbv16
Nr4a1
S100a6
S100a11
Trbv13−1
Wwox
Med13
Ly6a
Glcci1
Trbv16
Gm14085
Trbv19
Fhit
Gm10130
Trbv3
Smc3
Bloc1s1
Pglyrp1
Dusp2
Nr4a1
Zfp36
Ndufa11
Lgals1
Egr1
Trbv1
Trbv19
Pdia6
Areg
Mt1
Edem1
Camk2n1
Ppm1g
Ccr10
Ociad1
Arhgef3
Zbtb16
Ramp3
Nkg7
Stmn1
Ndrg1
Ifng
Ccl5
Ccl4
Hist1h2ap
AY036118
Trbv15
Esyt1
Fry
Snx13
Casc3
Slc4a7
Eya3
Cmah
Dnah8
Hmox2
Xpo7
Ly6a
Jazf1
Lcorl
Stt3b
S100a13
Trbv16
Atp6v0c
Penk
Trbv31
Trbv19
Gm10130
Med13
Itih5
Fam117a
Trbv1
Sidt1
Cmah
Immp2l
S100a11
Pglyrp1
Lgals1
Nfkbid
S100a6
Cd40lg
Nkg7
Nr4a3
Bcl2a1b
Hist1h2ap
singleTCR
dualTCR
Perecent Expressed
Average Expression