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

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Abstract:

As an essential molecule for Treg cell function, TCR plays a critical role in various immune responses including cell development, immune tolerance, and self-antigen recognition. Current research has identified Treg cells in both lymphoid and non-lymphoid tissues as important for maintaining immune homeostasis and preventing autoimmune reactions. However, the TCR pairing types and CDR3 characteristics of these Treg cells are largely unknown. Through innovative analysis of scRNA+TCR-seq data, we discovered that 10.1% to 21.4% of dual TCR Treg cells exist in mouse lymphoid and non-lymphoid tissues. Dual TCR Treg cells in different tissues exhibit differential phenotypic and TCR repertoire characteristics. These findings provide key insights into the complex biological functions of Treg cell V(D)J recombination, tolerance selection, development, and migration.

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]. Due to technical limitations, 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 has been challenging 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][10][11].

First, in the Treg study conducted by Oliver T et al.[12], 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.[13], 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 1D); The proportion of dual TCR Treg cells in mouse non-lymphoid tissues: blood=11.9%; kidney=11.2%; liver=13.6%, LPL=10.1%; pancreas=13.2%, The proportion of dual TCR T cells in mouse skin tissue: 18.6% (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). There are significant differences in TCR pairing types between different tissues, with A1+A2+B1+B2 significantly increased in skin and kidney, and B+A1+A2 significantly higher in spleen and liver than in other groups (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.

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. 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 2E), 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 significantly increased; Kidney: *Fos* and *Cenpa* related to immune

cell proliferation and differentiation and *Ifit3* involved in the body's antiviral response were significantly increased; Liver: *Cenpa* related to immune cell proliferation and *Birc5* involved in the body's anti-infection immune response were significantly increased; LPL: *Flnb* related to cell adhesion and migration regulation and *Atp1a1* related to cell proliferation, differentiation, and apoptosis regulation were significantly increased; Pancreas: *Ccl5, Ccr2, Cxcr6* related to immune cell migration and *Lbr* and *Id2* related to immune cell proliferation and differentiation, as well as *Fkbp4* 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 significantly increased; mLN: *Glcci1* involved in inflammation and immune cell migration was significantly increased; skin: *Areg* involved in immune cell proliferation, differentiation, migration, and immune suppression functions and *Ccr10* involved in T cell migration and positioning were significantly increased; spleen: *Casc3* related to T cell activation and proliferation was significantly 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]. This study, for the first time 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, 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. 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[14]. 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.

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[15]. 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[16], 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.

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)Individual Treg cells (including CD3+ T cells in skin tissues) were screened for sequencing of both the TCR alpha chain (A) and TCR beta chain (B); (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.

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, 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 scRNA-seq results of the two datasets were quality-controlled to exclude data with nFeature >5,000, nCount <3,000 and >20,000, and percent.MT >5 (skin, spleen, iLN and mLN tissue datasets) or 10 (blood, kidney, liver, LPL and pancreas tissues). Subsequently, the “DoubletFinder” R package was used to remove doublets from four tissue sources (including skin), and

doublets/negatives were eliminated from five tissue sources (including LPL) based on HTO labels. T cells were then clustered and analyzed from different tissue sources after quality control. The scTCR-seq results were analyzed in conjunction with the 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.Cytokine, cytokine receptor, and transcription factor expression analysis: Comparative analysis of the homogeneity of Treg cell signature molecules expressed in single TCR Treg and dual TCR Treg; Comparative analysis of the heterogeneity of the expression of the top 10 mRNA molecules of dual TCR Treg in different tissue origins; 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.

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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.

Conflict of interest:

The authors declare no conflict of interests.

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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%

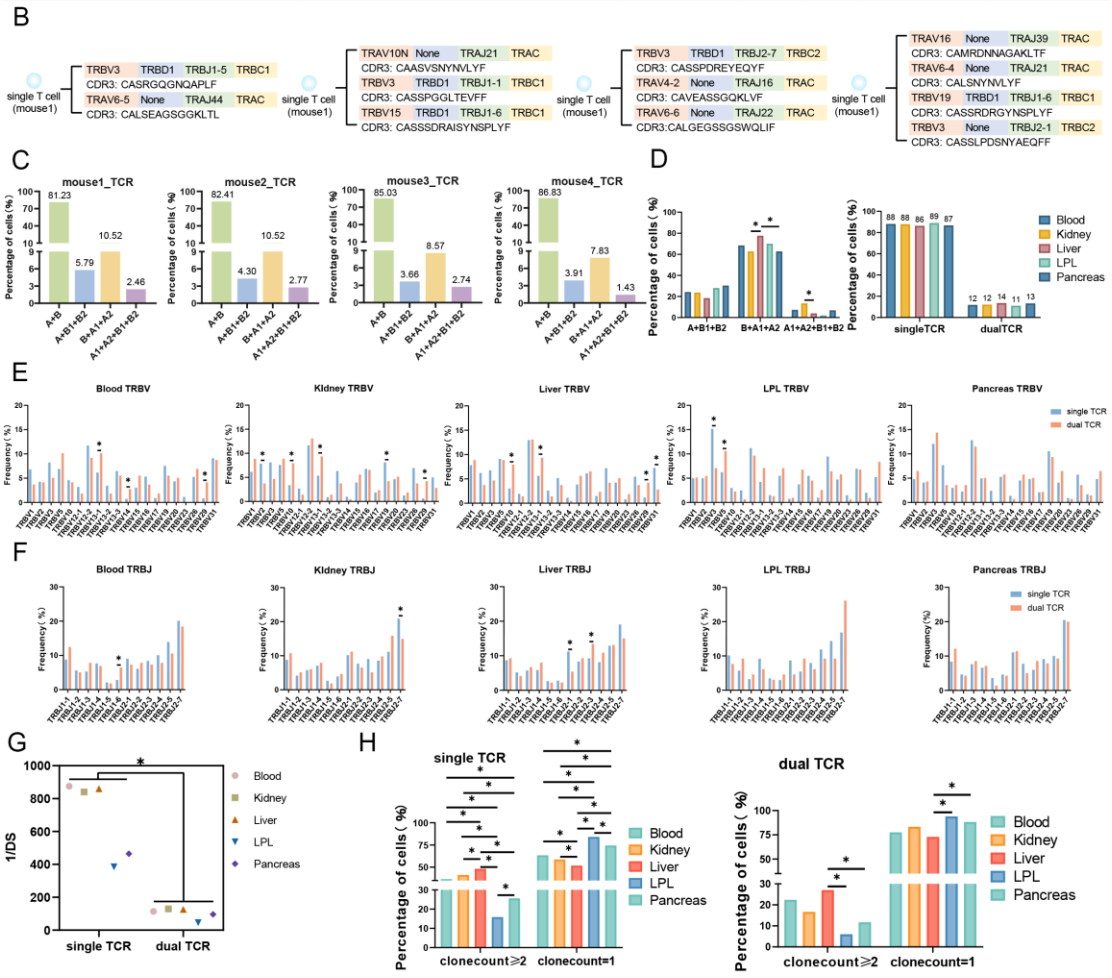

Figure 1. Characterizes single Treg TCR and dual TCR Treg in the Blood, Kldney, Liver, LPL, and Pancreas of mice.**A,**Mouse source names, GEO accession number, total number of paired TCR Treg cells, and number and proportion of TCR paired types.**B,**Four different TCR pairing types, VDJ gene family 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.

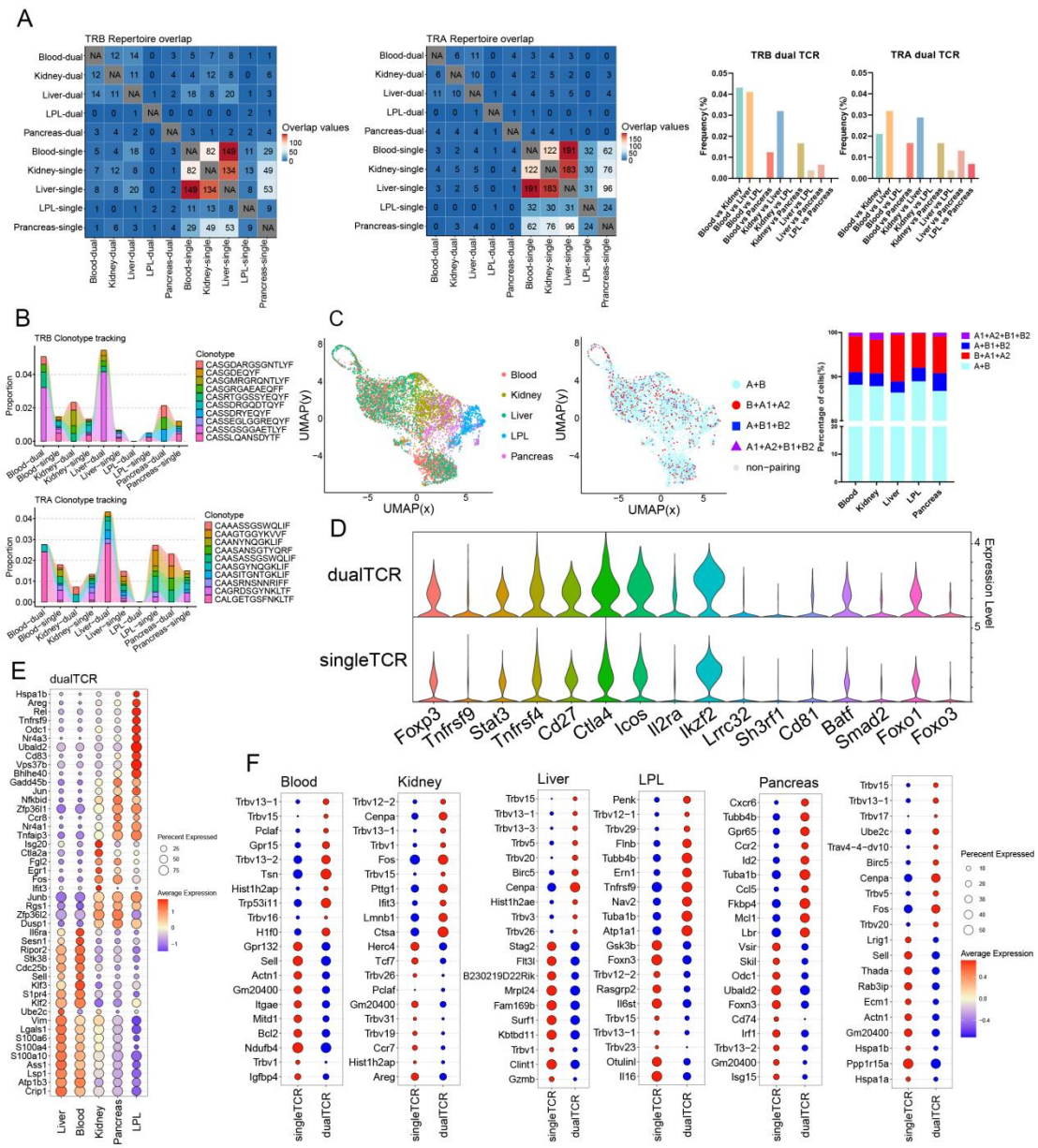

Figure 2. Comparative analysis of single Treg TCR and dual TCR Treg CDR3 overlap and gene mRNA expression in mice Blood, Kidney, Liver, LPL, and Pancreas.**A,**Clonal overlap analysis and jarcard index of TRA/B-chain CDR3 region in single /dual TCR Treg cells.**B,**Tracking results of the first 10 high-frequency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

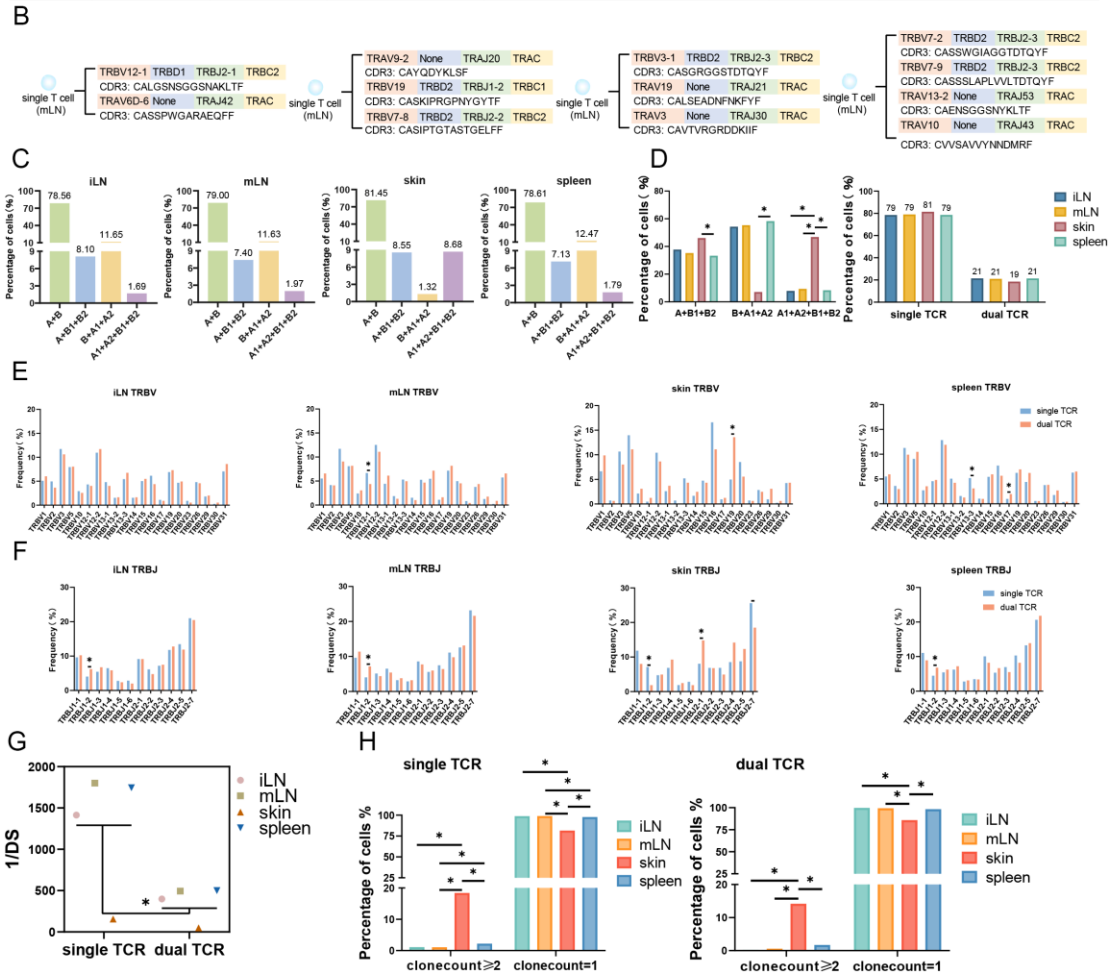

Figure 3. Characterizes single Treg TCR and dual TCR Treg in the iLN, mLN, skin, spleen of mice.**A,**Tissue source names, GEO accession number, total number of paired TCR T cells, and number and proportion of TCR paired types.**B,**Four different TCR pairing types, VDJ gene family names and CDR3 AA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

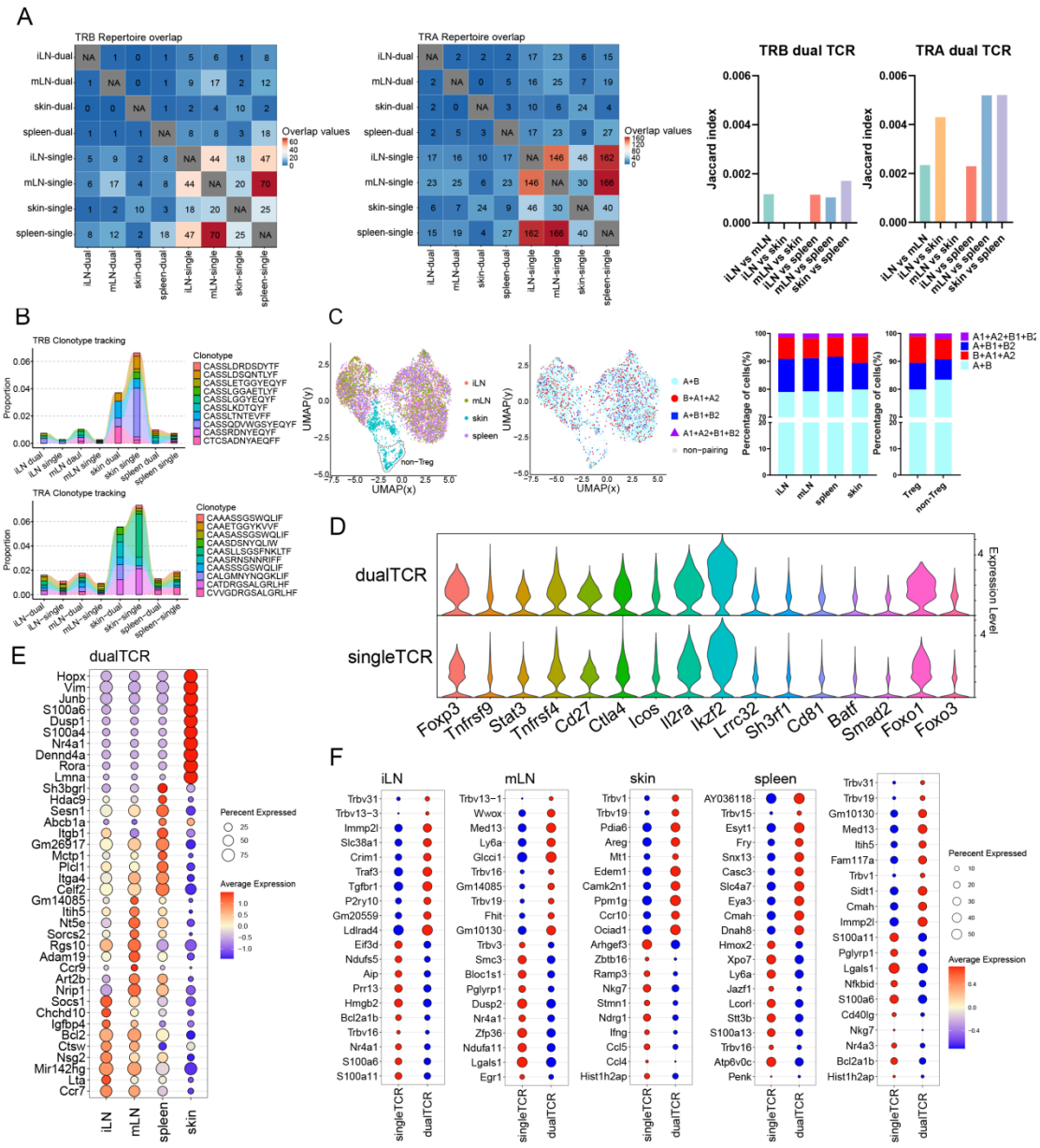

Figure 4. Comparative analysis of single Treg TCR and dual TCR Treg CDR3 overlap and gene mRNA expression in mice iLN,mLN,skin,spleen.**A,**Clonal overlap analysis and jarcard index of TRA/B-chain CDR3 region in single /dual TCR T cells.**B,**Tracking results of the first 10 high-frequency overlapping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

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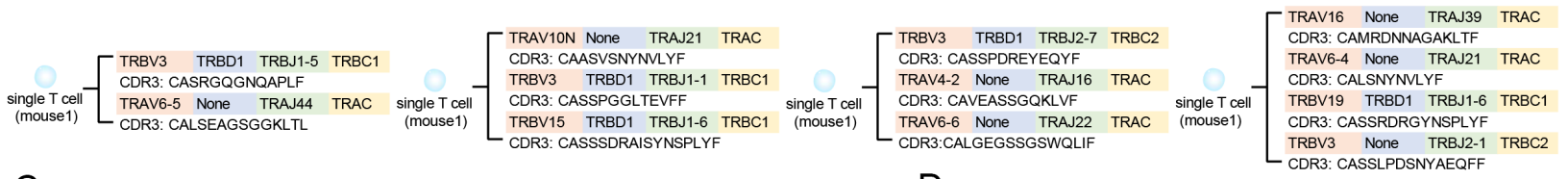
sample	GEO accession		Total cells/paired TCR cells	Single TCR T cells(A+B)/proportion%	Dual TCR T cells/proportion%			
	scRNA-seq	scTCR-seq			Total	A+B1+B2	B+A1+A2	A1+A2+B1+B2
mouse1	GSM8240877	GSM8240878	2598/2072	1683/81.23%	389/18.77%	120/5.79%	218/10.52%	51/2.46%
mouse2	GSM8240880	GSM8240881	3198/2672	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



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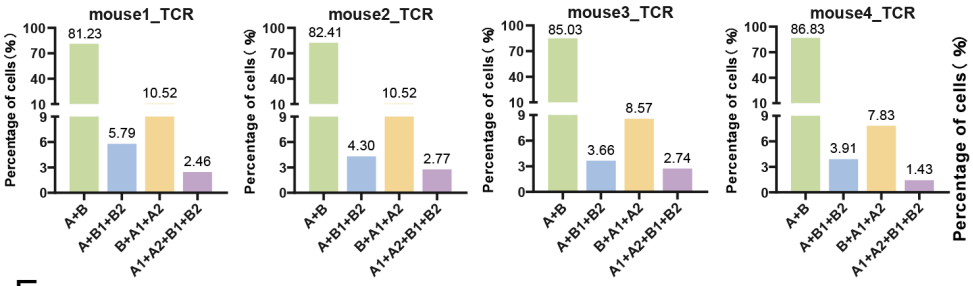

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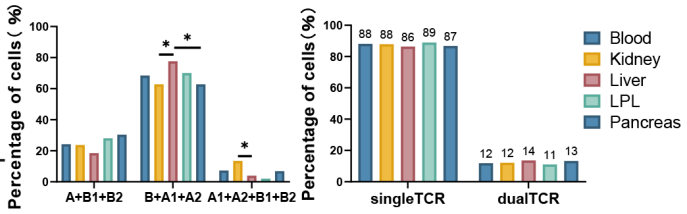

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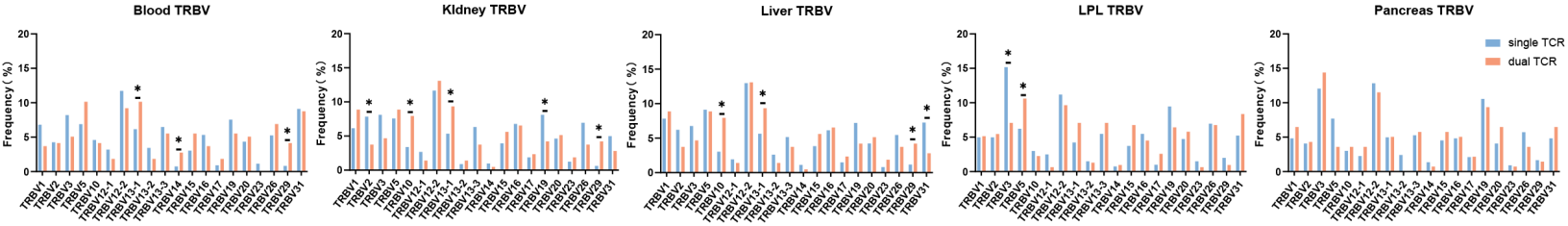

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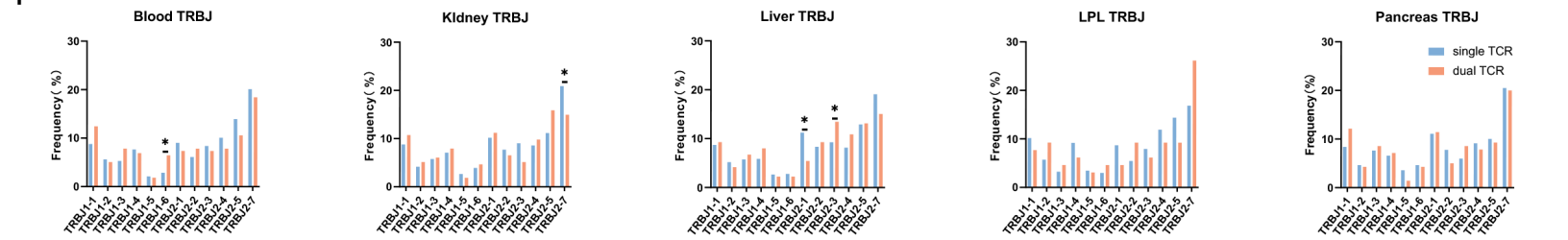

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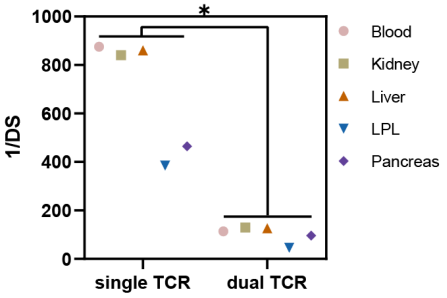

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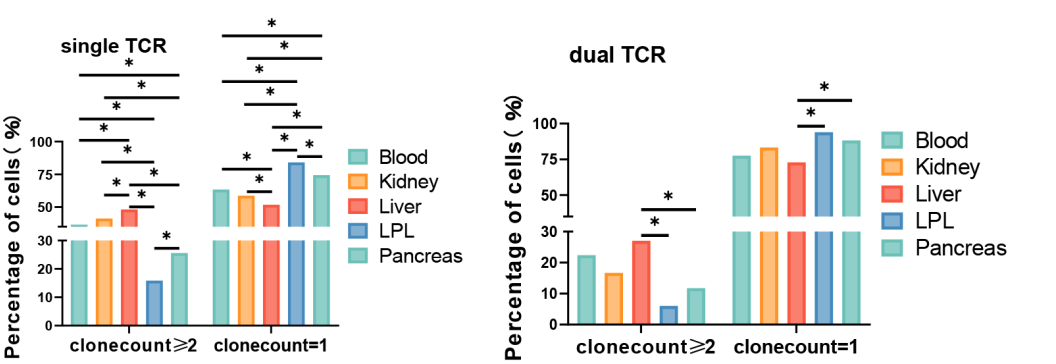
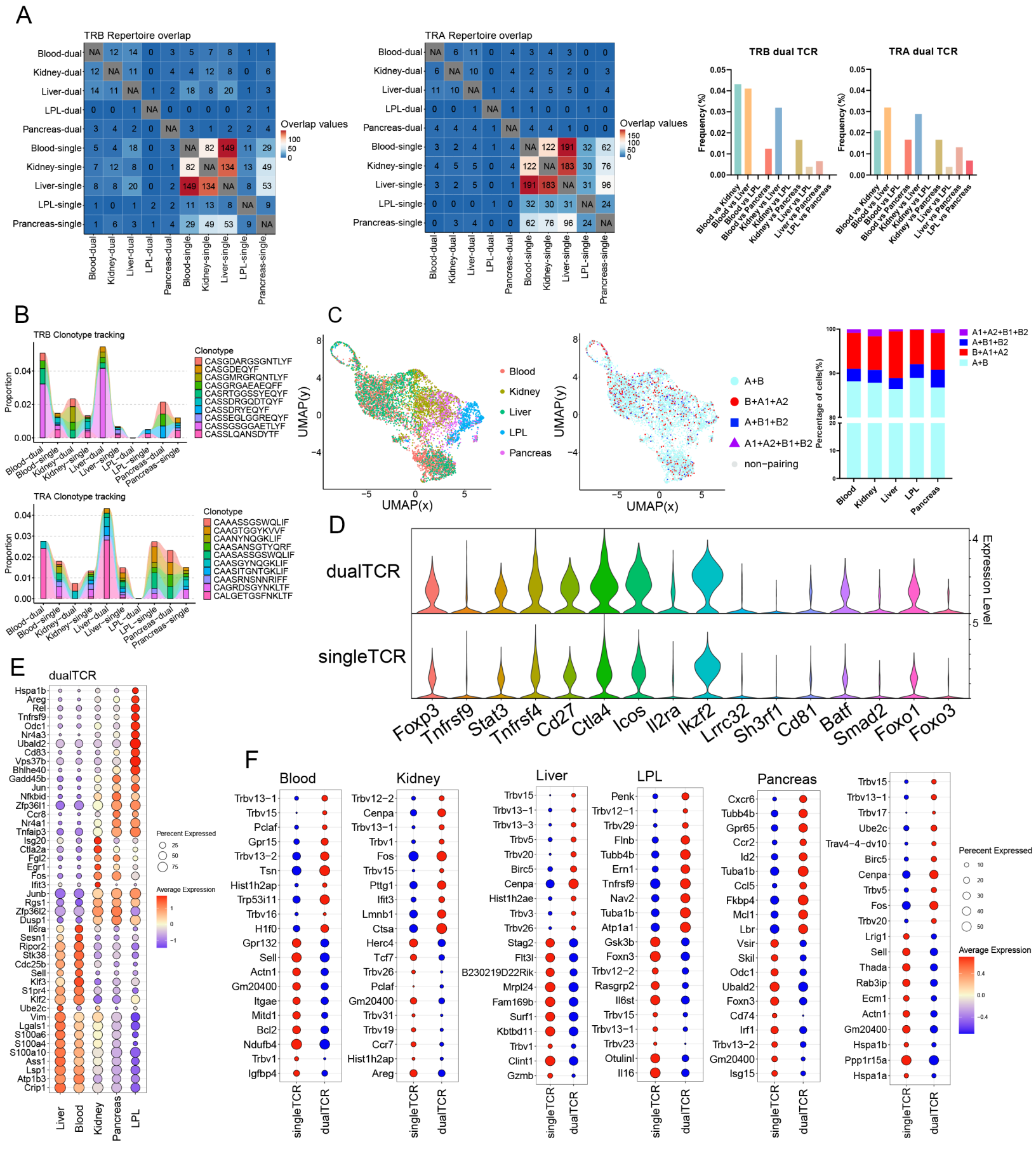
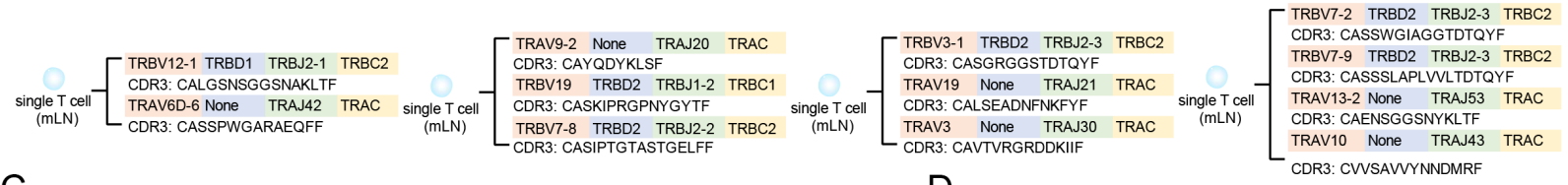



A
TRB Repertoire overlap
Blood-dual NA 12 14 0 3 5 7 8 1 1
Kidney-dual 12 NA 11 0 4 4 12 8 0 6
Liver-dual 14 11 NA 1 2 18 8 20 1 3
LPL-dual 0 0 1 NA 0 0 0 0 2 1
Pancreas-dual 3 4 2 0 NA 3 1 2 2 4
Blood-single 5 4 18 0 3 NA 82 149 11 29
Kidney-single 7 12 8 0 1 82 NA 134 13 49
Liver-single 8 8 20 0 2 149 134 NA 8 53
LPL-single 1 0 1 2 2 11 13 8 NA 9
Prancreas-single 1 6 3 1 4 29 49 53 9 NA
Blood-dual Kidney-dual Liver-dual LPL-dual Pancreas-dual Blood-single Kidney-single Liver-single LPL-single Prancreas-single
Overlap values
100
50
0
TRA Repertoire overlap
Blood-dual NA 6 11 0 4 3 4 3 0 0
Kidney-dual 6 NA 10 0 4 2 5 2 0 3
Liver-dual 11 10 NA 1 4 4 5 5 0 4
LPL-dual 0 0 1 NA 1 1 0 0 1 0
Pancreas-dual 4 4 4 1 NA 4 4 1 0 0
Blood-single 3 2 4 1 4 NA 122 191 32 62
Kidney-single 4 5 5 0 4 122 NA 183 30 76
Liver-single 3 2 5 0 1 191 183 NA 31 96
LPL-single 0 0 0 1 0 32 30 31 NA 24
Prancreas-single 0 3 4 0 0 62 76 96 24 NA
Blood-dual Kidney-dual Liver-dual LPL-dual Pancreas-dual Blood-single Kidney-single Liver-single LPL-single Prancreas-single
Overlap values
150
100
50
0
TRB dual TCR
TRA dual TCR
Frequency (%)
0.05
0.04
0.03
0.02
0.01
0.00
Blood vs Kidney
Blood vs Liver
Blood vs LPL
Blood vs Pancreas
Kidney vs Liver
Kidney vs LPL
Kidney vs Pancreas
Liver vs LPL
Liver vs Pancreas
LPL vs Pancreas
B
TRB Clonotype tracking
Proportion
0.04
0.02
0.00
Clonotype
CASGDARGSGNTLYF
CASGDEQYF
CASGMRGRQNTLYF
CASGRGAEAEQFF
CASRTGGSSYEQYF
CASSDRGQDTQYF
CASSDRYEQYF
CASSEGLGGREQYF
CASSGSGGAETLYF
CASSLQANSDYTF
Blood-dual
Blood-single
Kidney-dual
Kidney-single
Liver-dual
Liver-single
LPL-dual
LPL-single
Pancreas-dual
Pancreas-single
TRA Clonotype tracking
Proportion
0.04
0.03
0.02
0.01
0.00
Clonotype
CAAASSGSWQLIF
CAAGTGGYKVVF
CAANYNQGKLIF
CAASANSGTYQRF
CAASASSGSWQLIF
CAASGYNQGKLIF
CAASITGNTGKLIF
CAASRNSNNRIFF
CAGRDSGYNKLTF
CALGETGSFNKLTF
Blood-dual
Blood-single
Kidney-dual
Kidney-single
Liver-dual
Liver-single
LPL-dual
LPL-single
Pancreas-dual
Pancreas-single
C
UMAP(y)
UMAP(x)
8
4
0
−4
−5
0
5
Blood
Kidney
Liver
LPL
Pancreas
A+B
B+A1+A2
A+B1+B2
A1+A2+B1+B2
non−pairing
Percentage of cells(%)
100
90
80
20
10
0
A1+A2+B1+B2
A+B1+B2
B+A1+A2
A+B
Blood
Kidney
Liver
LPL
Pancreas
D
dualTCR
singleTCR
Expression Level
4
5
Foxp3
Tnfrsf9
Stat3
Tnfrsf4
Cd27
Ctla4
Icos
Il2ra
Ikzf2
Lrrc32
Sh3rf1
Cd81
Batf
Smad2
Foxo1
Foxo3
E
dualTCR
Hspa1b
Areg
Rel
Tnfrsf9
Odc1
Nr4a3
Ubald2
Cd83
Vps37b
Bhlhe40
Gadd45b
Jun
Nfkbid
Zfp36l1
Ccr8
Nr4a1
Tnfaip3
Isg20
Ctla2a
Fgl2
Egr1
Fos
Ifit3
Junb
Rgs1
Zfp36l2
Dusp1
Il6ra
Sesn1
Ripor2
Stk38
Cdc25b
Sell
Klf3
S1pr4
Klf2
Ube2c
Vim
Lgals1
S100a6
S100a4
S100a10
Ass1
Lsp1
Atp1b3
Crip1
Liver
Blood
Kidney
Pancreas
LPL
Perecent Expressed
25
50
75
Average Expression
1
0
−1
F
Blood
Trbv13−1
Trbv15
Pclaf
Gpr15
Trbv13−2
Tsn
Hist1h2ap
Trp53i11
Trbv16
H1f0
Gpr132
Sell
Actn1
Gm20400
Itgae
Mitd1
Bcl2
Ndufb4
Trbv1
Igfbp4
Kidney
Trbv12−2
Cenpa
Trbv13−1
Trbv1
Fos
Trbv15
Pttg1
Ifit3
Lmnb1
Ctsa
Herc4
Tcf7
Trbv26
Pclaf
Gm20400
Trbv31
Trbv19
Ccr7
Hist1h2ap
Areg
Liver
Trbv15
Trbv13−1
Trbv13−3
Trbv5
Trbv20
Birc5
Cenpa
Hist1h2ae
Trbv3
Trbv26
Stag2
Flt3l
B230219D22Rik
Mrpl24
Fam169b
Surf1
Kbtbd11
Trbv1
Clint1
Gzmb
LPL
Penk
Trbv12−1
Trbv29
Flnb
Tubb4b
Ern1
Tnfrsf9
Nav2
Tuba1b
Atp1a1
Gsk3b
Foxn3
Trbv12−2
Rasgrp2
Il6st
Trbv15
Trbv13−1
Trbv23
Otulinl
Il16
Pancreas
Cxcr6
Tubb4b
Gpr65
Ccr2
Id2
Tuba1b
Ccl5
Fkbp4
Mcl1
Lbr
Vsir
Skil
Odc1
Ubald2
Foxn3
Cd74
Irf1
Trbv13−2
Gm20400
Isg15
Trbv15
Trbv13−1
Trbv17
Ube2c
Trav4−4−dv10
Birc5
Cenpa
Trbv5
Fos
Trbv20
Lrig1
Sell
Thada
Rab3ip
Ecm1
Actn1
Gm20400
Hspa1b
Ppp1r15a
Hspa1a
singleTCR
dualTCR
Perecent Expressed
10
20
30
40
50
Average Expression
0.4
0.0
−0.4

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



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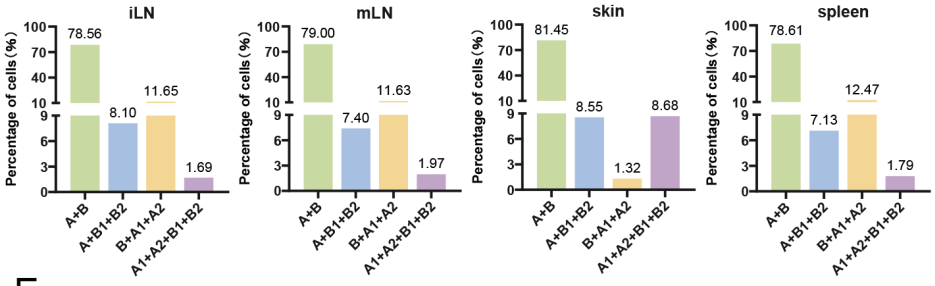

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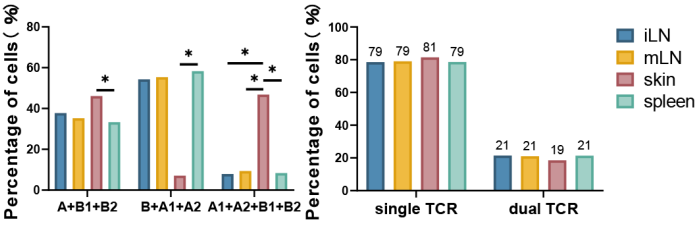
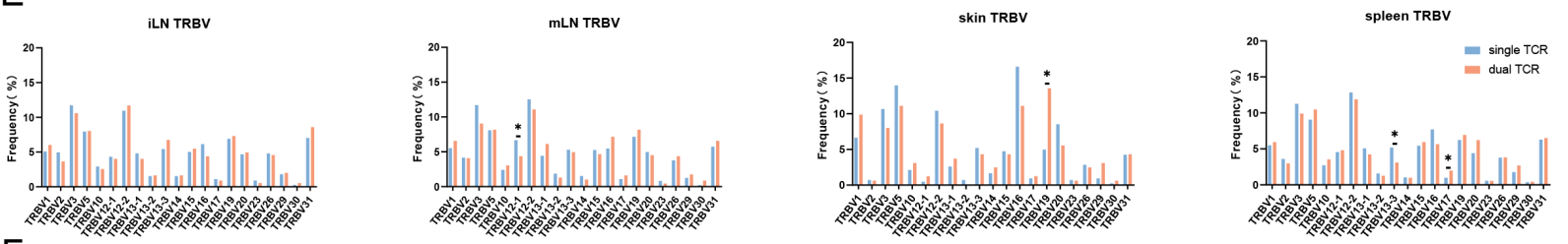


E



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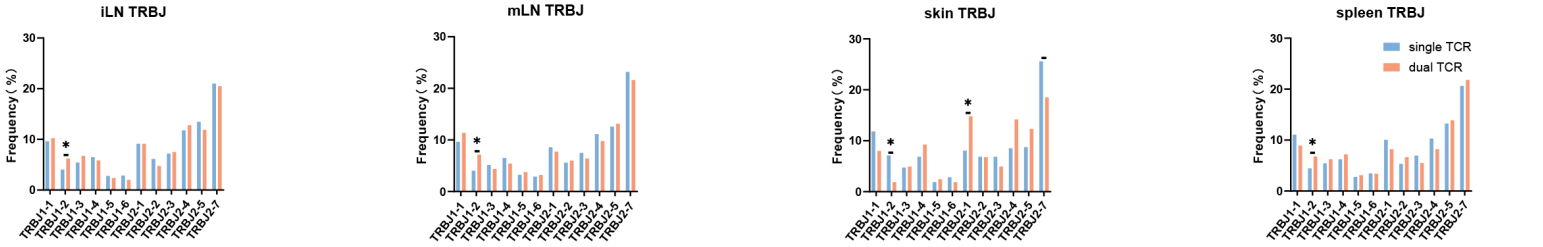

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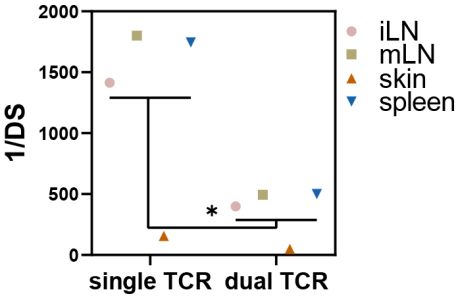

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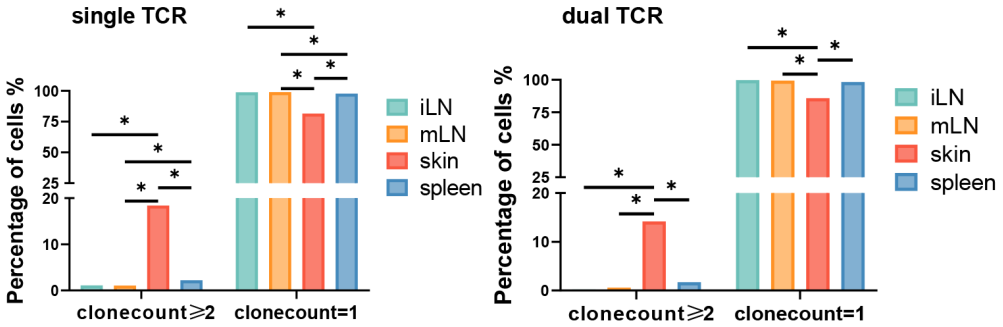

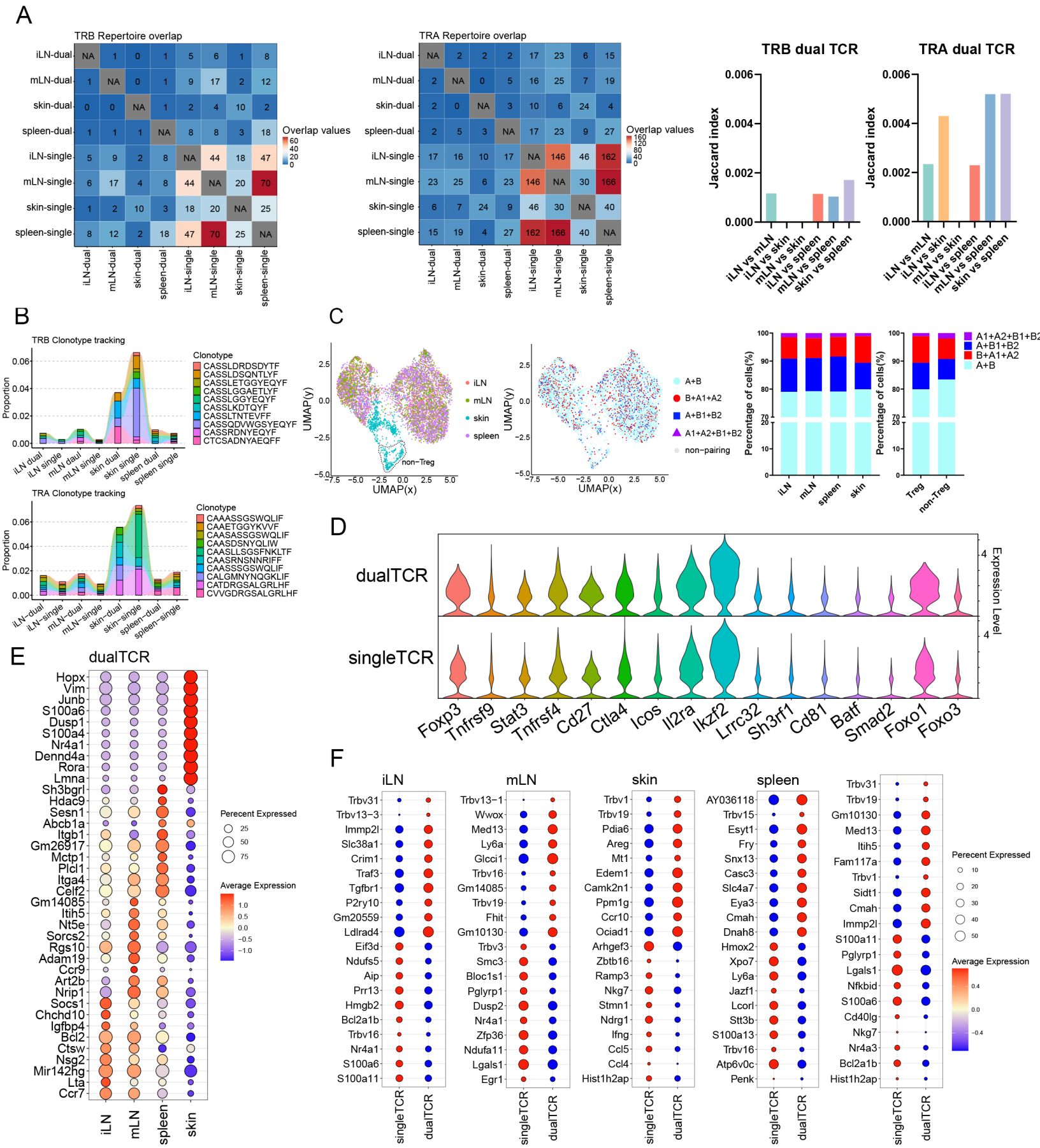
A
TRB Repertoire overlap
iLN-dual
mLN-dual
skin-dual
spleen-dual
iLN-single
mLN-single
skin-single
spleen-single
Overlap values
TRA Repertoire overlap
TRB dual TCR
TRA dual TCR
Jaccard index
iLN vs mLN
iLN vs skin
mLN vs skin
iLN vs spleen
mLN vs spleen
skin vs spleen
B
TRB Clonotype tracking
Proportion
Clonotype
CASSLDRDSDYTF
CASSLDSQNTLYF
CASSLETGGYEQYF
CASSLGGAETLYF
CASSLGGYEQYF
CASSLKDTQYF
CASSLTNTEVFF
CASSQDVWGSYEQYF
CASSRDNYEQYF
CTCSADNYAEQFF
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
C
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
D
dualTCR
singleTCR
Expression Level
Foxp3
Tnfrsf9
Stat3
Tnfrsf4
Cd27
Ctla4
Icos
Il2ra
Ikzf2
Lrrc32
Sh3rf1
Cd81
Batf
Smad2
Foxo1
Foxo3
E
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
F
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