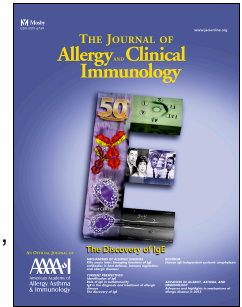


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Deconstructive SCNT reveals novel Treg subsets

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Keywords: Treg; aTreg; nTreg; agonist-induced Treg; naturally occurring Treg; Heterogeneity; Somatic Cell Nuclear Transfer; single-cell transcriptomic

Capsule Summary:

Using single-cell transcriptomic and SCNT, we identify multiple distinct Treg subsets. We show that naturally occurring and agonist-induced Tregs are two of these subsets, which differ amongst in TCR strength, development, differentiation, and Ctla4 expression.

To the Editor:

Regulatory T (T_{reg}) cells have been subject of many debates and controversies. Discrepancies exist in regard to T_{reg} development, their recognition of antigen, specificity of immune regulation, requirement for TCR activation, and regulatory mechanisms^{1,2}. We previously used Somatic Cell Nuclear Transfer (SCNT) to generate a naturally occurring T_{reg} (nT_{reg}) model, T138³. Particularly, we found that the TCR strength of T138 contradicted findings in wildtype (WT) mice, prompting us to hypothesize that T_{reg} cells are heterogeneous with distinctive subsets. With our unbiased SCNT approach, we generated another T_{reg} model (T143) using FoxP3^{GFP}-expressing CD4⁺ T cells from the spleen of NOD-FoxP3^{GFP}-Rag^{+/-} mice as described previously³⁻⁵.

We identified the V(D)J rearrangements underlying T143 as Trav5-4-J40 and Trbv15-J2-3-C2 (Fig. E1A). Thymic development of T143-Rag1^{-/-} revealed a significant increase in CD4 single-positive (CD4SP) cells (~12% WT versus ~39% T143), and a significant decrease in CD4-CD8 double-positive (DP) cells when compared to WT NOD mice (~77% WT versus

~34% T143) (Fig. 1A). Interestingly, CD4 expression levels in DP cells were lower in T143 than WT. Strikingly, we identified thymic FoxP3⁺ T cells in CD4SP of T143-Rag^{-/-} (Fig. 1B, ~6% WT versus 0.3% T143). In the spleen, we found ~10% FoxP3⁺ CD4⁺ T cells in T143-Rag1^{-/-} compared to ~17% in WT (Fig. E1B). Given that we had previously identified a fate-determining role for nT_{reg}-derived TCRβ, we tested whether TCRβ of T143 contributes to lineage choice. We found that two copies of T143 TCRβ skewed T cell development similarly as seen in T143-Rag1^{-/-} (lower frequency of DP, higher frequency of CD4SP, and lower CD4 levels on DP), while a single copy had negligible impact on lineage choice (Fig. E1C).

By determining expression levels of Nur77 and CD5, we previously showed that FoxP3⁺ nT_{reg} and FoxP3⁻ pre-nT_{reg} cells possess comparable TCR strength, thus arguing against agonist-dependent development^{3,6}. We determined TCR strength in T143, and found significantly higher Nur77- and CD5-levels in FoxP3⁺ CD4⁺ T cells than their FoxP3⁻ counterpart, suggesting higher TCR strength (Fig. 1C and 1D). Hence, we refer to T143 as agonist-induced T_{reg} (aT_{reg}).

We previously showed that FoxP3⁻ CD4⁺ pre-nT_{reg} cells carrying the same TCR as nT_{reg} were poised towards FoxP3-expression and away from Th1 differentiation. Thus, we determined whether FoxP3⁻ CD4⁺ T cells carrying the same TCR as aT_{reg} were poised similarly. FoxP3⁻ CD4⁺ T cells from T143 were significantly better in differentiating into FoxP3⁺ T cells than WT (~62% T143 versus 52% WT) (Fig. 1E). However, we observed no significant difference in their ability Th1 differentiation (Fig. 1F, ~48% WT versus ~52% T143). For consistency, we refer to FoxP3⁻ CD4⁺ T cells from aT_{reg} as pre-aT_{reg}.

We previously found that CD8⁺ T cells expressing nT_{reg}-derived TCRβ had reduced capabilities to express FoxP3. We observed similar results for CD8⁺ T cells expressing aTreg-

derived TCR β (Fig. E2A, ~48% WT versus ~8% T143), while we found no difference in their capability to differentiate into IFN- γ secreting cells (~76% WT versus ~79% T143, Fig. E2B).

The development, frequencies, and plasticity of T143 were quite distinct from our previously reported T138, and urged us to compare transcriptional programs of both. We performed RNA-Seq on FoxP3⁺ CD4⁺ T cells from T143, T138, and WT. Genome-wide hierarchical clustering revealed differentially expressed genes in FoxP3⁺ T cells from T138, T143 and the bulk from WT (Fig. 2A). We identified a gene cluster that was specifically activated (E.g. Ccr1), and one that was specifically repressed in aT_{reg} (E.g. Ctla4) (Fig. 2A and 2B). Next we used Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) to determine specific protein-protein interaction network in these differentially expressing gene clusters⁷. Ccr1 forms an interconnected gene network with many aT_{reg}-specific active genes, while Ctla4 forms a distinctive repressive gene network absent in aT_{reg} (Fig. 2C). GO term analysis of T143-specific active gene network showed enrichment in immune functions (Fig. E3). These data demonstrated that aT_{reg} executes transcription programs unlike nT_{reg}.

Our data on T138 and T143 demonstrated that nT_{reg} and aT_{reg} are two distinct thymic-derived T_{reg} subsets³. To determine heterogeneity within the WT T_{reg} pool, we performed single-cell RNA-seq (scRNA-Seq) analysis. Principal component analysis (PCA) was performed and visualized as scatter plot using t-distributed Stochastic Neighbor Embedding (t-SNE). Our analysis revealed four potential T_{reg}-Clusters (TrC1-4) within WT T_{reg} cells (Fig. 2D). To determine whether our nT_{reg} and aT_{reg} models align with these four TrC, we averaged our previous RNA-Seq data from T143 and T138 and compared it to TrC1-4. Strikingly, we found that nT_{reg} T138 clustered closely with TrC1, while aT_{reg} T143 clustered with TrC4, suggesting that each cluster represents a transcriptionally different T_{reg} subset (Fig. 2E).

We used SCNT and scRNA-Seq to demonstrate that nT_{reg} and aT_{reg} are two distinct thymic T_{reg} subsets. Despite the lower relative frequency of thymic T_{reg} in aT_{reg}³ (1.1% in T138, and 0.3% in T143), the absolute numbers of thymic T_{reg} cells were comparable, and the overall frequencies in T138 and T143 were higher than current existing transgenic T_{reg} models. While we currently lack a complete understanding of lymphocyte receptor diversification especially in gnathostomata⁸, our data clearly demonstrate that TCRs have profound impact on T cell development and lineage specification, emphasizing the need to generate novel SCNT models on pure backgrounds.

We demonstrated that aT_{reg} develop in an agonist-dependent manner, while nT_{reg} cells develop in an agonist-independent manner. Thus, we hypothesize that the transcription factor AIRE (autoimmune regulator) might support development of some T_{reg}, such as aT_{reg}, but not of nT_{reg}. This would explain why T_{reg} exist -albeit in reduced numbers- in Aire^{-/-} mice and humans (APECED), yet autoimmune diseases occur.

In line with our hypothesis that WT T_{reg} cells are heterogeneous, our scRNA-Seq analysis revealed potentially four T_{reg} clusters. Strikingly, we identified two clusters, TrC1 and TrC4, that closely resemble our nT_{reg} and aT_{reg} models respectively. So far, several mechanisms had been identified by which T_{reg} might regulate effector cells, such as through the expression of Ctla4. Our RNA-Seq data showed that T143 expressed significantly less Ctla4 than T138, which supports the hypothesis that different T_{reg} subsets might possess different T_{reg} function. This suggests that blocking antibodies against CTLA4 used in cancer immunotherapy, might only target certain T_{reg} subsets. Clearly, more SCNT-derived T_{reg} models are needed to determine common features of T_{reg} cells within a subset, and to unambiguously distinguish T_{reg} subsets.

Using SCNT we can deconstruct the T_{reg} pool and generate accurate T_{reg} models that enable us determine the differences in development, TCR strength, signaling, plasticity, and especially their functions. The existence of different T_{reg} subsets could explain the plentitude of controversial data reported on T_{reg} . Importantly, novel insights into the biology of T_{reg} subsets might provide new opportunities for the treatments of autoimmune diseases and cancer, and to optimize vaccination strategies.

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Figure Legends:

Fig. 1. Development and differentiation of SCNT aT_{reg} T143.

FACS plots and scatter dot plots of thymic development (A) and T_{reg} frequencies (B) in thymus.
TCR strength determined as expression of Nur77 (C) or CD5 (D) on indicated cells.
Differentiation of FoxP3⁻ CD4⁺ T cells from indicated mice into T_{reg} (E) or Th1 (F) *in vitro*.
Error bars = mean ± SEM; ****P* < 0.001; n.s. = not significant.

Fig. 2. Transcriptomes of nT_{reg}, aT_{reg}, and WT show distinct subsets.

Hierarchical clustering of RNA-Seq data from T143, T138 and WT CD4⁺ T_{reg} (A). T143-specific
repressed (Ctla4) or active (Ccr1) gene clusters (B) and protein-protein interaction gene network
(C). PCA and t-SNE analyses revealed potentially four T_{reg} clusters (Trc) in WT T_{reg} (D).
Hierarchical clustering shows T138 clusters with TrC1, and T143 with TrC4 (E).

References:

1. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 2012;30:531-564.

2. Shevach EM, Thornton AM. tTregs, pTregs, and iTregs: similarities and differences.
Immunol Rev 2014;259:88-102.
3. Ku M, Chang SE, Hernandez J, Abadejos JR, Sabouri-Ghomi M, Muenchmeier NJ, et al.
Nuclear transfer nTreg model reveals fate-determining TCR-beta and novel peripheral
nTreg precursors. Proc Natl Acad Sci U S A 2016;113:E2316-25.
4. Kirak O, Frickel EM, Grotenbreg GM, Suh H, Jaenisch R, Ploegh HL. Transnuclear mice
with pre-defined T cell receptor specificities against *Toxoplasma gondii* obtained via
SCNT. J Vis Exp 2010;e2168.
5. Kirak O, Frickel EM, Grotenbreg GM, Suh H, Jaenisch R, Ploegh HL. Transnuclear mice
with predefined T cell receptor specificities against *Toxoplasma gondii* obtained via SCNT.
Science 2010;328:243-248.
6. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell
receptor signal strength in Treg and iNKT cell development demonstrated by a novel
fluorescent reporter mouse. J Exp Med 2011;208:1279-1289.
7. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al.
STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic
Acids Res 2015;43:D447-52.
8. Pancer Z, Cooper MD. The evolution of adaptive immunity. Annu Rev Immunol
2006;24:497-518.

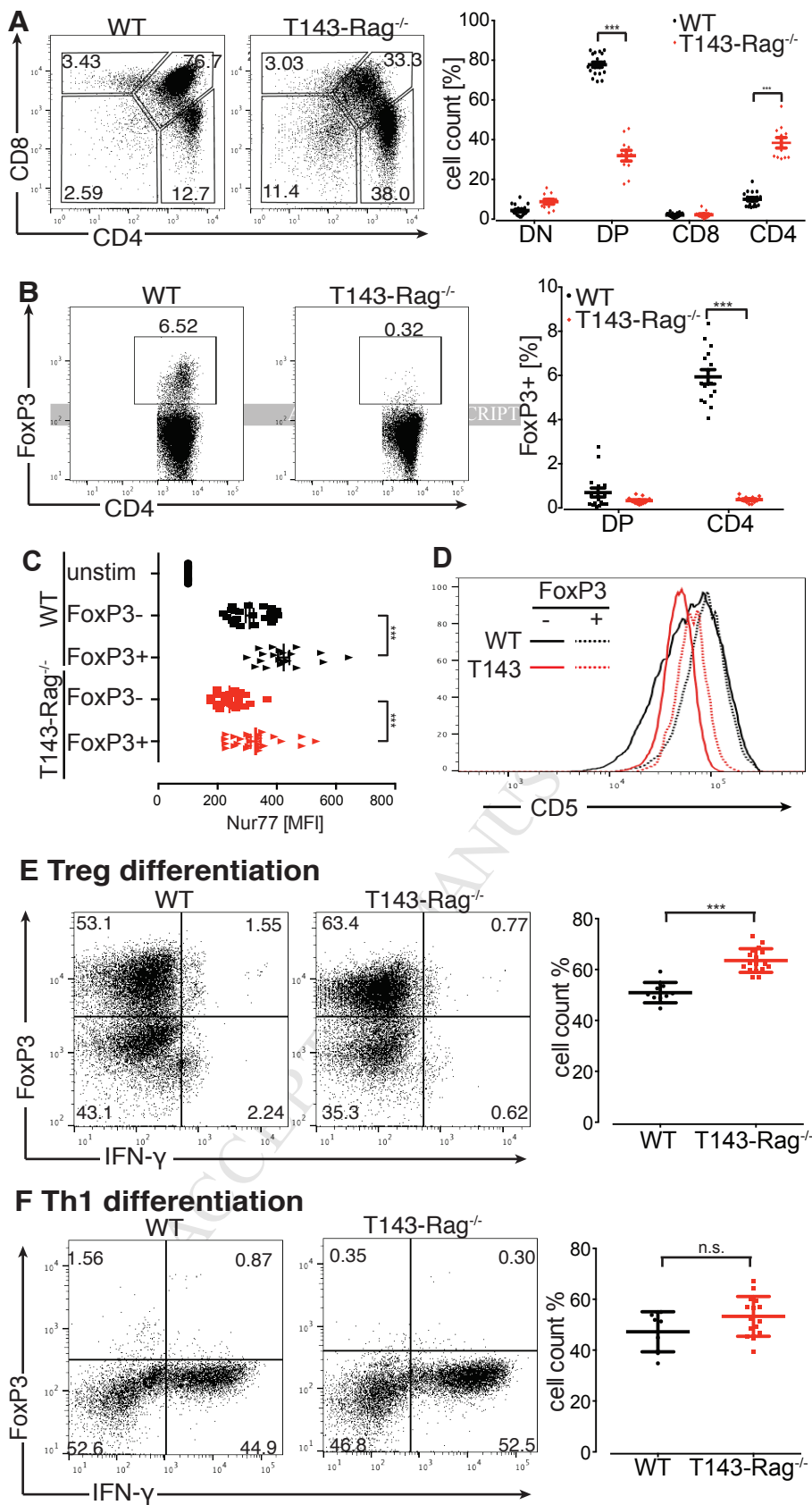


Figure 1

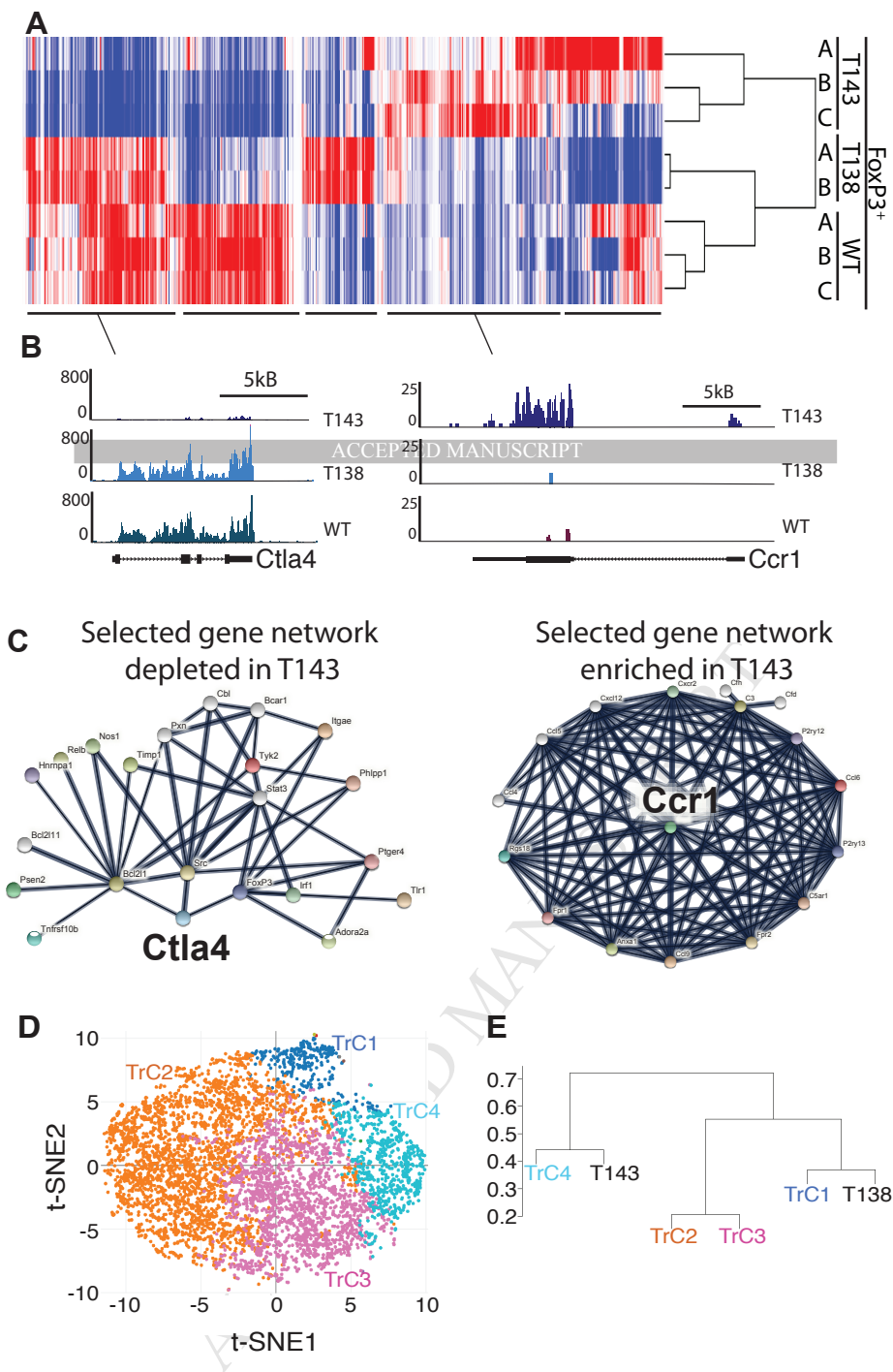


Figure 2

Online Repository:

Materials and Methods:

Mice and Somatic Cell Nuclear Transfer: B6D2F1 females were generated in house by breeding C57BL/6 with DBA/2 mice for the use of oocyte donors. NOD-Rag^{+/-} mice were used to isolate donor T cells for SCNT. The SCNT procedure was performed as previously described^{4,5}. Briefly, oocytes from superovulated B6D2F1 female were enucleated, and donor cells were transferred into enucleated oocytes. ES cells were derived from resulting SCNT blastocysts and used to generate chimeric mice. NOD-T143-Rag^{-/-} mice were obtained through breeding with NOD-Rag^{-/-} mice, and various genotypes were used for analysis as indicated. All mice experiments were approved by the Institutional Animal Care and Use Committee at the Scripps Research Institute.

TCR identification: The TCR of T143 was identified as previously described⁴. Briefly, RNA was isolated from splenocytes of SCNT mice (Macherey Nagel). 5'-RACE was performed, and results were analyzed using the Ensembl Genome Browser (www.ensembl.org) and the international ImMunoGeneTics information system (www.imgt.org).

Fluorescence-activated cell sorting (FACS) and cell sorting: Single-cell suspensions of thymus, spleen or lymph nodes were prepared and red blood cells were lysed. Cells were incubated with various combinations of the following antibodies: CD3, CD4, CD5, CD8, CD19, FoxP3, Nur77, IFN- γ , (eBioscience and Tonbo). Samples were collected using FACS LSRII

(BD) or sorted using FACS Aria (BD), and data were analyzed with FlowJo software (Tree Star). Differences between groups were analyzed using two-tailed *t*-test (Graphpad Prism software).

Nur-77 in-vitro Assay: T Cells were purified from spleen of WT NOD and T143-Rag^{-/-} mice and activated with anti-CD3/28 beads (Dynabeads, Invitrogen). Cells were intra-cellularly stained by Nur77-PE 5-6h after activation (eBioscience). Nur-77 levels were determined using flow cytometry and normalized to unstimulated FoxP3⁻ CD4⁺ T cells from NOD.

Bulk RNA-Seq and data analysis: RNA from indicated populations were isolated from the spleen of either WT NOD or indicated SCNT mice using Nucleospin RNA XS kit and treated with DNase according to manufacturer's protocol (Macherey-Nagel, Bethlehem, PA). RNA was processed into RNA-Seq libraries using Truseq Stranded mRNA-Seq sample prep kit (Illumina, San Diego, CA) following manufacturer's instructions. Briefly, polyA-mRNA was enriched using oligo-dT magnetic beads. Next, RNA was fragmented and reverse-transcribed, and cDNA was end-repaired, adenylated, adapter-ligated and PCR amplified. RNA-Seq libraries were then sequenced on Illumina HiSeq2500 at single-end 50-bp (base pair), resulting 25-30 million reads per library. Sequencing reads were aligned to the mouse genome (mm10, MGSCv38) using STAR (v2.2.0c)⁹. The RNA-Seq data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE97850. Gene expression was quantified using aligned reads to exons of RefSeq transcripts using HOMER¹⁰. Differential gene expression was determined with edgeR¹¹, and StringDB⁷.

Single-cell RNA-Seq and data analysis: Spleenic FoxP3⁺ CD4⁺ T cells from NOD-FoxP3^{GFP} mice were purified using flow cytometry, washed and diluted to 1000 cells per μ L in 0.04%BSA/PBS. Chromium Single-cell 3' RNA-Seq library generation was carried according to 10X Genomics protocols¹² (Pleasanton, CA). Single cell suspension was combined with reverse transcriptase master mixes and loaded onto the Chromium Single Cell 3' Chip together with Single Cell 3' gel beads and partition oil for GEM generation on the Chromium Controller. GEMs were then incubated for 2 hrs at 55C for RT reaction and then cDNA was purified using Silane DynaBeads and SPRISelect beads. cDNA was then amplified for 12 cycles and sheared using Covaris. Fragmented cDNA was then end-repaired, A-tailed and adapter ligated prior to index PCR for 10 cycles. Final Single Cell libraries were sequenced on the Illumina NextSeq500. sc-RNA-Seq data was analyzed using the 10x Genomics Chromium platform. For comparative analysis, expression counts from sc-RNA clusters from the pool of FoxP3⁺ CD4⁺ T cells were averaged per gene and normalized in units of tag per million (TPM) to match normalization of bulk expression counts from nT_{reg} T138 and aT_{reg} T143 models prior to clustering. A representative set of 231 genes for clustering was generated from intersection of differentially expressed genes in four T_{reg} clusters, and differentially expressed genes from our nT_{reg} T138 and aT_{reg} T143 models. To overcome the scarcity of the count matrix from single cell subsets, gene expressions were standardized to mean of zero and standard deviation of 1 across all sets (four clusters TrC1-4 and two models, T138 and T143). Hierarchical clustering of sets was performed using Pearson's correlation as distance measure with average linkage criterion.

T cell differentiation: T cell differentiation experiments were performed as described previously³. Briefly, FoxP3- CD4⁺ T cells were purified and activated using anti-CD3/CD28

beads (Dynabeads, Invitrogen). For Th1 differentiation, 1×10^5 T cells were stimulated in the presence of IL-2 (20 U/mL; eBioscience), IL-12 (20 ng/mL; Tonbo), and anti-IL-4 (10 μ g/mL; Tonbo). For T_{reg} differentiation, cells were stimulated in the presence of IL-2 (100 U/mL; eBioscience), TGF- β (5 ng/mL; Tonbo), and anti-IFN- γ (10 μ g/mL; Tonbo). After 4 d, T cells were stimulated with ionomycin (500 ng/mL) and PMA (50 ng/mL) in the presence of Monensin (eBioscience) for 4 h prior to intracellular staining. FoxP3 staining was performed using FoxP3/transcription factor staining kit (Tonbo).

Supplementary Figure Legends

Fig. E1. TCR of aT_{reg} T143 model, and splenic T_{reg}.

(A) Identification of the V(D)J rearrangements for TCR- α (upper) and TCR- β (lower) chains in aT_{reg} T143. **(B)** Representative FACS plots (left) and scatter plot (right) showing the presence and frequencies of splenic FoxP3⁺ T cells in WT NOD and T143-Rag1^{-/-} mice. **(C)** Representative flow cytometric analysis of the impact of T143-derived TCR β on thymic development in mice possessing a single copy (middle) or two copies of TCR β (right). Error bars are mean $\pm$ SEM; *** P < 0.001.

Fig. E2. Characterization of CD8⁺ T cells plasticity.

(A) CD8⁺ T cells from WT NOD and T143- $\beta\beta$ mice were purified and cultured with anti-CD3 and anti-CD28 coated beads in the presence of IL-2, TGF β , anti-IFN- γ to induce FoxP3 expression. Cells were analyzed after 4 days in culture. Representative flow cytometric analysis (left) and scatter plot (right) are shown. **(B)** CD8⁺ T cells from WT NOD and T143- $\beta\beta$ mice were

purified and cultured with IL-2, IL-12, and anti-IL-4 to induce Th1 differentiation. Cells were analyzed after 4 days in culture. Representative flow cytometric analysis (left) and scatter plot (right) are shown. Error bars are expressed as mean $\pm$ SEM; *** $P < 0.001$; n.s. = not significant.

Fig. E3. GO analysis of T143-specific active gene cluster.

T143-specific active genes are enriched for immune functions.

References in Online Repository Text Page

- E1. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013;29:15-21.
- E2. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* 2010;38:576-589.
- E3. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010;26:139-140.
- E4. Zheng GX, Terry JM, Belgrader P, Ryvkin P, Bent ZW, Wilson R, et al. Massively parallel digital transcriptional profiling of single cells. *Nat Commun* 2017;8:14049.

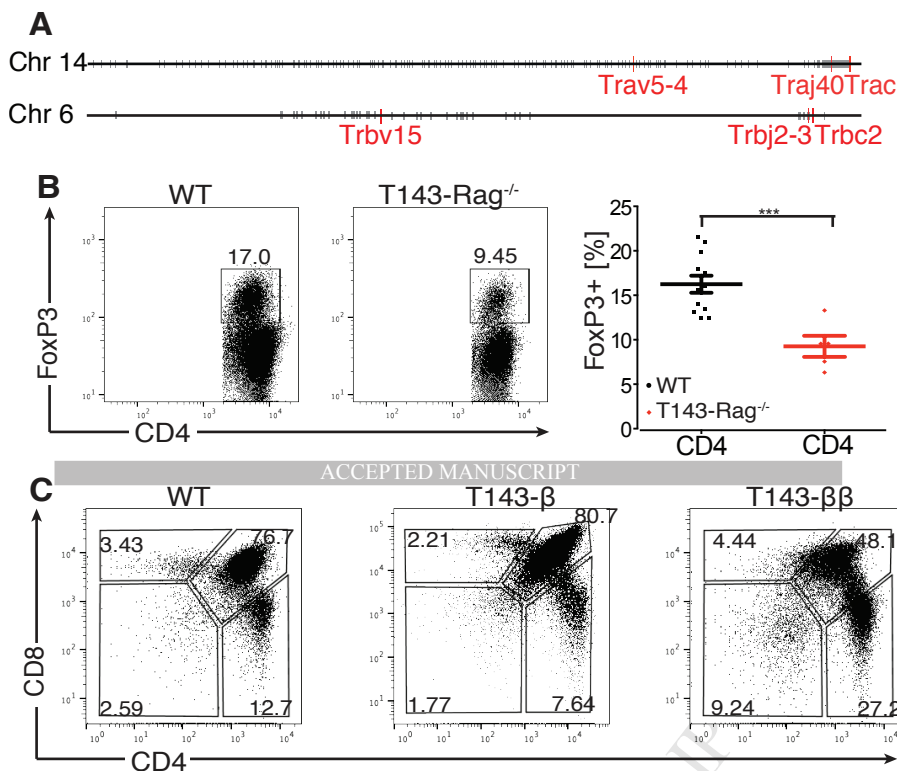


Figure E1

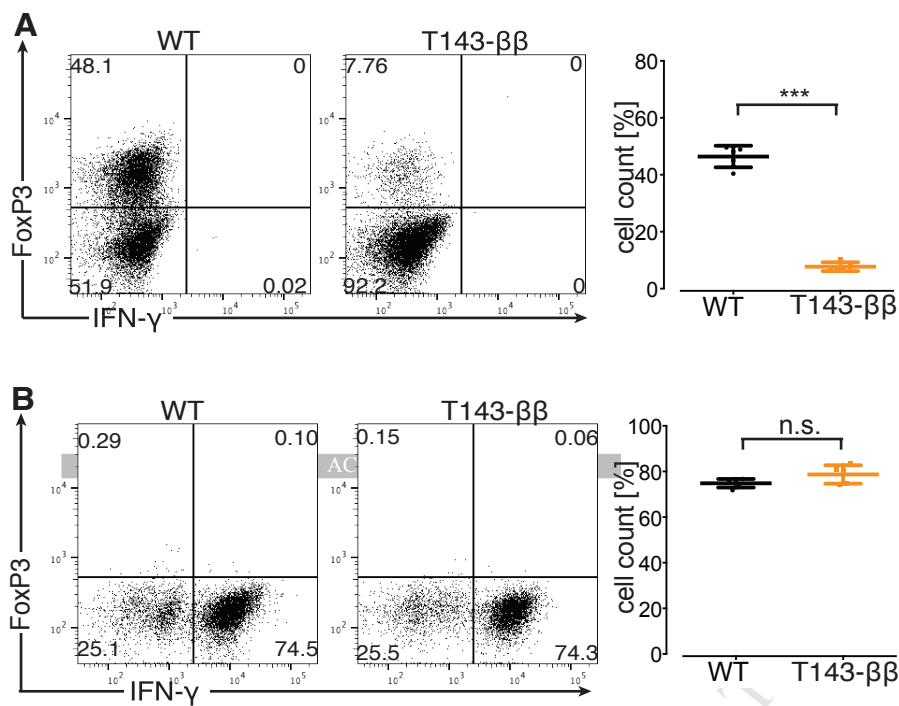


Figure E2

T143-specific active gene cluster GO analysis

Pathway Description	Observed Gene Count	FDR
defense response	73	3.51e-23
response to stress	131	6.94e-23
immune response	64	9.78e-22
inflammatory response	41	2.45e-16
innate immune response	41	3.17e-16

Figure E3