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T-cell receptor β-selection is required at the CD4+CD8+ stage of human T-cell development

Edward L. Y. Chen[*,†,§], **Patrick M. Brauer**[†,§], **Elisa C. Martinez**[†], **Xiaotian Huang**[‡], **Ning Yu**[‡], **Michele K. Anderson**[*,†], **Yang Li**[‡,¶], **Juan Carlos Zúñiga-Pflücker**[*,†,¶]

[*]Department of Immunology, University of Toronto, Toronto, Ontario, Canada.

[†]Sunnybrook Research Institute, Toronto, Ontario, Canada.

[‡]Department of Cell Biology, School of Basic Medical Sciences, Peking University Stem Cell Research Center, Peking University, Beijing, 100191, China.

Abstract

T-cell development is predicated on the successful rearrangement of the T-cell receptor (TCR) gene loci, which encode for antigen-specific receptors. Recombination Activating Gene (RAG) 2 is required for TCR gene rearrangements, which occur during specific stages of T-cell development. Here, we differentiated human pluripotent stem cells with a CRISPR/Cas9-directed deletion of the *RAG2* gene (RAG2-KO) to elucidate the requirement for the TCRβ chain in mediating β-selection during human T-cell development. In stark contrast to mice, human RAG2-KO T-lineage progenitors progressed to the $CD4^+CD8^+$ double-positive (DP) stage in the absence of TCRβ rearrangements. Nonetheless, RAG2-KO DPs retrovirally-transduced to express a rearranged TCRβ chain showed increased survival and proliferation as compared to control-transduced RAG2-KO DPs. Furthermore, transcriptomic analysis showed that TCRβ- and control-transduced RAG2-KO DPs differed in gene pathways related to survival and proliferation. Our results provide new insights as to the distinct requirement for the TCRβ chain during human T-cell development.

Keywords

RAG2; pre-TCR signaling; β-selection; human T cell development

Introduction

The V(D)J Recombination Activating Genes (RAG) 1 and 2 are two essential DNA processing enzymes required for the rearrangement of the B- and T-cell receptor (BCR or TCR) gene loci (1, 2). RAG1/2 initiate V(D)J recombination by forming a complex that first recognizes and binds to recombination signal sequences (RSS) found adjacent to each V, D, and J gene segment. Upon formation of a synapse with another RSS, the RAG complex

[¶]Corresponding authors: JC Zúñiga-Pflücker, 2075 Bayview Avenue, M7-619, Toronto, Ontario, M4N3M5, Canada, jczp@sri.utoronto.ca (416) 480-6112; and, Yang Li, 38 Xueyuan Road, Beijing, China, liyang@hsc.pku.edu.cn 86-10-82802164.

[§]Equal contributors

induces a double-strand DNA break, which is repaired by non-homologous end-joining process (3, 4). This ultimately results in the imperfect joining of different V, D, and J gene segments to potentially generate millions of different antigen receptors from a few hundred V(D)J segments.

A dysfunction in either RAG1 or RAG2 results in primary immunodeficiencies (PIDs), characterized by a failure to respond to pathogens or properly identify healthy versus malignant cells (5–7). In gene knock-out (KO) mouse studies, either RAG1- or RAG2-KO mice show a complete lack of mature B- or T-cells. In particular, intrathymic T-cell development is arrested at the $CD4^{-}CD8^{-}$ double-negative (DN)3 stage, when RAG1/2-dependent TCRβ rearrangements are completed (8, 9). Successful rearrangement of the TCRβ chain allows for pairing with the surrogate preTα chain to form the preTCR complex, which is required for survival, proliferation, and differentiation to the $CD4^{+}CD8^{+}$ double-positive (DP) stage (10). Limited access to thymic tissues from individuals with *RAG1* or *RAG2* gene alterations, which are often hypomorph variants rather than null (11), have led to the assumption that developing human RAG1/2-KO T-cell progenitors would also arrest at the $CD7^{+}CD5^{+}CD1a^{+}CD4^{-}CD8^{-}$ stage (DN3-equivalent in humans) (12). However, we previously reported that induced pluripotent stem cells (iPSCs) derived from severe combined immunodeficient (SCID) patients with *RAG1* gene alterations were capable of differentiating well beyond the DN stage and reached the $CD7^{+}CD5^{+}CD1a^{+}CD4^{+}CD8^{+}$ DP stage *in vitro* (13). Recently, similar findings were observed with SCID patient-derived $CD34^{+}$ hematopoietic progenitor cells (14, 15). Nonetheless, this left open the question as to whether preTCR signaling has any function during human T-cell development.

To address the specific roles of the RAG complex and TCRβ chain in human T-cell development, we generated *RAG2*-deficient (RAG2-KO) human pluripotent stem cell (hPSC) lines, which were differentiated towards the hematopoietic lineage and subsequently induced to become T-cells *in vitro*. Control wild-type (WT) and RAG2-KO T-cell progenitors both progressed equivalently to the $CD4^{+}CD8^{+}$ DP stage, but RAG-KO DPs displayed decreased survival and expansion compared to WT DPs. Forced expression of a rearranged TCRβ chain in RAG2-KO DPs resulted in increased survival and expansion, which was further highlighted by the up-regulated expression of survival and proliferation genes. Thus, our results revealed an unexpected stage-specific requirement and function of RAG2-mediated TCRβ rearrangements in developing human T-cells.

Materials and Methods

hESC Maintenance.

Human ESCs (H1; WiCell Research Institute, Madison, WI) were maintained and expanded on plates coated with Matrigel (Corning, NY, USA) in TeSR-E8 medium (STEMCELL Technologies, Vancouver, Canada). Cells were passaged by non-enzymatic dissociation using 0.5 mM EDTA.

Generation of RAG2-KO hESCs.

The pD1321-GFP expression vector, containing cassettes for GFP, Cas9 endonuclease, a CRISPR chimeric cDNA, and the gRNA moiety we designed to target RAG2 [GGTTATGCTTTACATCCAGA], was custom synthesized (DNA2.0). After transfection with Lipofectamine 3000 (Life Technologies, Carlsbad, CA), GFP^+ hESCs were sorted using flow cytometry. Individual clones were picked, expanded, and aliquots were collected for purification of genomic DNA using the Genomic DNA kit (Invitrogen). Mutations (indels) were validated by sequencing products of PCR amplification of the regions flanking the targeting sites. The RAG2 KO clone 1 exhibited a 1bp deletion in one allele and 16bp deletion in the other allele, while clone 4 exhibited a 11bp deletion in one allele and 23bp deletion in the other allele.

Western Blot Analysis.

Briefly, cellular lysates were prepared by incubating the cells in lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA) containing protease inhibitor cocktail (Roche) for 20 min at 4°C, followed by centrifugation at 14000×g for 15 min at 4°C. Proteins were separated by SDS-PAGE, transferred onto PVDF membrane (Millipore, Louis, MO) and probed with anti-RAG2 antibody (Abcam – Ab95955; 1:1000 dilution) overnight at 4°C followed by incubation with secondary antibody. The anti-RAG2 antibody used is a rabbit polyclonal made against a recombinant fragment corresponding to amino acids 271–519 of human RAG2 (Abcam), which are well beyond the gRNA targeting site. Immunoreactive bands were visualized using western blotting Luminol reagent (Thermo). PBMCs from a T-ALL patient were used as positive control (16).

Immunostaining.

Cells were fixed in 4% paraformaldehyde, and permeabilization and blocking were performed in 5% NGS (Abcam, Cambridge, USA) and 1% Triton X-100 (Sigma-Aldrich, Louis, MO) in PBS for 30 min. Cells were stained and analyzed as previously described (17).

Teratoma Formation.

All animal studies were approved by Ethics Committee of Experimental Research of Peking University and were in accordance with the International Animal Care and Use Committee Guidelines. 6–8 week-old non-obese diabetic (NOD)/SCID mice were injected subcutaneously with 1×10^7 hESCs resuspended in DMED-F12 with 50% Matrigel to allow teratoma formation for 8 weeks. Histological analysis was performed as previously described (17).

hPSC Differentiation and $CD34^+$ Isolation.

hPSCs, H1 embryonic stem cells (ESC) were differentiated as previously described (18). After 8 days of differentiation, embryoid bodies were harvested and dissociated into single-cells using Collagenase type IV and trypsin-EDTA as previously described (18), and positively selected using a MACS column (Miltenyi Biotec) with anti-CD34 PE-conjugated

antibody (BD Biosciences) and anti-PE microbeads. The cell yield and purity of the positive selection was assessed pre- and post- MACS by flow cytometry.

OP9-DL4–7FS Co-Culture and Differentiation.

OP9-DL4 cells expressing hIL-7, hFLT3-L, and hSCF (7FS) were generated (manuscript in preparation) and grown in α-MEM containing 10–20% FBS (Gibco), 1% Pen-Strep (Life Technologies), and phospho-ascorbic acid (Sigma-Aldrich) at 37°C, 5% CO_2. For co-cultures, OP9-DL4–7FS cells were plated in 6-well plates the previous day. MACS-purified $CD34^+$ cells were counted and seeded at a density of 5–20 $\times 10^4$ cells per 6-well plate, and differentiated by co-culture with OP9-DL4–7FS cells in OP9 medium at 37°C, 5% CO_2, and cell were passaged and co-cultured with fresh OP9-DL4–7FS cells every ~5 days.

Retroviral Transduction.

PG13 cell lines stably expressing empty vector dTomato, TCRα-dTomato, or TCRβ-dTomato were grown towards 70% confluency, at which point the media was switched to α-MEM with 15% FBS and 1% Pen-Strep to condition the supernatant for 48 h before transducing OP9-DL4–7FS/T-cell cultures. On D8+24 of OP9-DL4–7FS/T-cell cultures, cells were transduced with corresponding PG13 supernatants once a day with 1 μl/ml polybrene and centrifuged at 2000xg for 90 min at RT for 4 d (2–3$\times 10^6$ cells per 2 ml of supe). Cells were rested for 3 d and then sorted as $dTomato^+$ $CD7^+CD5^+CD4^+CD8^+$ DPs. Sorted cells were placed in OP9-DL4–7FS co-cultures for up to 10 days.

Flow Cytometry.

Cells were stained for 30 min on ice with the following mouse anti-human antibodies: CD3-BrilliantViolet421, CD4-AlexaFluor700, CD8β-PE, CD31-FITC, CD34-PE, CD45RA-PE/CF594, TCRgd-FITC (BD Biosciences), CD5-PE/Cy7, CD7-AlexaFluor700, CD45-APC/eFluor780, TCRab-APC (eBiosciences), CD8α-PE/Dazzle, CD38-BrilliantViolet421 (BioLegend). Cells were resuspended in flow cytometry buffer containing DAPI, data was collected using LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo version 9.7.6. For intracellular staining, cells were fixed and permeabilized using Fixation/Permeabilization kit with GolgiPlug™ (BD Biosciences) as per manufacturer's instructions.

RNA-Seq Analysis of Control and RAG2-KO *in vitro* Derived $CD4^+CD8^+$ cells.

Cells were collected at co-culture day 24–28 and stained with fluorochrome-labelled antibodies to CD45, CD7 (eBioscience), CD5, CD4, CD8 (BD Biosciences), DAPI, and sorted into $CD4^+$CD8+ populations using FACSVantage Diva or FACSAria cell sorters (BD Biosciences). Total RNA was extracted from the sorted cell populations using TRIzol. Purified RNA was subjected to RNA sequencing using Illumina Novaseq 6000. Library preparation was done using Illumina TruSeq Strandard Total RNA Sample Preparation kit. Sequencing was done using 100-cycle paired read protocol and multiplexing to obtain ~40 million reads/sample. Samples were aligned to GRCh38 using HISAT2. Read counts were calculated using HTSeq. Differential gene expression analysis was done using edgeR package in the R platform.

Statistical Analysis.

The data and error bars are presented as mean ± standard error of mean. To determine statistical significance, a one-way ANOVA (comparing three means) was performed using Prism version 6. Statistical significance was determined as $*P<0.05$ and $**P<0.01$.

Data Availability.

The data that support the findings of this study are available from the corresponding authors on request. Raw and processed RNA-Seq data are available from the Gene Expression Omnibus under accession number GSE164276 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164276).

Results and Discussion

Generation and characterization of RAG2-KO hPSC lines.

To evaluate the role of RAG2 in human T-cell development, we used CRISPR-Cas9 gene editing to target exon 3 of the *RAG2* gene (Supplemental Fig. 1A). hPSCs were transfected with a plasmid encoding the *RAG2*-targeting guide RNA, the Cas9 enzyme, and green fluorescent protein (GFP). Transfected GFP^+ hPSCs were single-cell sorted and cultured. After expanding individual clones, we identified two clones that contained unique insertion-deletions with bi-allelic mutations (KO-1 and KO-4) (Supplemental Fig. 1A). To evaluate the impact of the *RAG2* mutations on protein expression, we performed Western blot analysis, which confirmed the absence of detectable RAG2 protein in both KO-1 and −4 derived T-lineage cells (Supplemental Fig. 1B).

To assess whether RAG2-KO hPSCs maintained pluripotency, we evaluated the expression of key markers and teratoma formation. Immunofluorescence staining showed that RAG2-KO hPSCs expressed OCT4, NANOG, SOX2, and SSEA-4 (Supplemental Fig. 1C). To functionally test pluripotency, we injected RAG2-KO hPSCs into immunodeficient mice, and histological analysis revealed RAG2-KO teratoma formation with all three germ layers (Supplemental Fig. 1D), indicating that RAG2-KO hPSCs retained key features of pluripotency. To determine the capacity of RAG2-KO hESCs to generate hematopoietic progenitors, we analyzed CD34 expression after 8 days of embryoid-body differentiation cultures (18). Control WT, RAG2-KO-1 and −4 hPSCs gave rise to similar frequencies of hemogenic endothelial $CD34^+$ cells, which could be further enriched by magnetic-assisted cell sorting (MACS) (Supplemental Fig. 1E).

T-cell development from RAG2-KO hPSCs.

$CD34^+$ hemogenic endothelial cells were MACS-enriched and cultured with OP9-DL4 cells, expressing human IL-7, FLT3-ligand and stem cell factor (7FS), to induce T-cell differentiation. After 10 days of culture, Control WT, RAG2-KO-1 and −4 cells proceeded along the T-cell lineage, as marked by expression of both CD7 and CD5 (Fig. 1A). All three groups reached the $CD4^+$ intermediate single positive (ISP) stage by day 15 and displayed intracellular CD3 expression by day 20 (Fig. 1A). After 24 days of culture, the majority of cells from Control WT, RAG2-KO-1 and −4 groups were $CD7^+CD5^+$ (Fig. 1A). Of note, by 29–34 days of culture, Control WT, RAG2-KO-1 and −4 cells all reached the $CD4^+CD8^+$

DP stage (Fig. 1A). However, as expected, only Control WT cells displayed intracellular TCRβ expression, and cell surface CD3/TCRβ expression (Fig. 1A). These results indicate that RAG2-deficient human T-cell progenitors can differentiate up to the $CD4^+CD8^+$ DP stage.

To determine cell survival and expansion, total cellularity was quantified from Control WT, RAG2-KO-1 and −4 developing T-cells. After 20 days of culture, all three samples showed similar cell numbers (Fig. 1B). However, after 40 days of culture, Control WT T-lineage cells further increased their survival and expansion as opposed to RAG2-KO-1 and −4 T-lineage cells, with approximately 10-fold greater cellularity by this time-point (Fig. 1B). This suggests that RAG2-dependent TCRβ rearrangement outcomes are limited to mediating the survival and proliferation of human DP T-cells.

Forced expression of a TCRβ chain in RAG2-KO $CD4^+CD8^+$ DPs.

RAG2-KO hPSC-derived $CD34^+$ cells were cultured on OP9-DL4–7FS cells for 24 days, and DP cells were retrovirally-transduced with an empty vector (dTomato), a rearranged TCRα chain (TRA 1383i), or a rearranged TCRβ chain (TRB 1383i) (Fig. 2A). Transduced RAG-KO cells were sorted for $CD7^+CD5^+CD4^+CD8^+$ DP cells, and placed back on OP9-DL4–7FS cells for an additional 10 days to assess for cell survival and expansion (Fig. 2A). Both dTomato- and TCRα-transduced DPs showed similar cell numbers after 10 days of culture (Fig. 2B). However, TCRβ-transduced DPs displayed significantly higher cell numbers after 10 days of culture compared to dTomato- and TCRα-transduced DPs (Fig. 2B). This suggests that, in contrast to a TCRα chain, expression of a rearranged TCRβ chain promotes cell survival and/or proliferation of developing human T-cells at the DP stage.

Transcriptomic analysis of Control WT, RAG2-KO, and RAG2-KO TCRβ-transduced $CD4^+CD8^+$ DP cells.

Control WT, RAG2-KO control-transduced, and RAG2-KO TCRβ-transduced DP cells were sorted for RNA sequencing (RNA-Seq) analysis. As expected, Control WT DP cells expressed a large set of TCRα, TCRβ, TCRγ, and TCRδ genes that were absent in RAG2-KO control-transduced and RAG-KO TCRβ-transduced DP cells, with the notable exception of some TCR genes, including the 1383i TCRβ used in the transduction and a few TCRα genes, likely the result of germline transcripts induced by the β-selection signals, as seen in mice (19) (Supplemental Table 1, and Supplemental Fig. 2A). Furthermore, we compared the expression of genes from RAG2-KO control- and TCRβ-transduced DP cells with that of umbilical cord-blood (UCB)-hematopoietic stem cell-derived DP cells for a set of DP-associated signature genes (20) (Supplemental Table 1). This analysis revealed that TCRβ-transduced RAG2-KO DP cells gained the expression of a suite of genes that were present in the UCB-derived DP cells, including *RORC*, which is induced following β-selection in mice (21) (Supplemental Fig. 2B).

Differentially expressed gene analysis was performed to determine genes that were up-regulated in Control WT compared to RAG2-KO (KO-1 and KO-4) DP cells, and vice versa. Genes significantly up-regulated in Control WT compared to both RAG2-KO-1 and RAG2-KO-4 include *CCDC152*, *GPR183*, *IL32*, and *MAL* (Supplemental Table 1 and Fig. 3A).

Genes significantly up-regulated ($p<0.05$) in Control WT compared to RAG2-KO-1 or −4 included *ADAMTS17*, *IL1RL1*, *PLXNA2*, and *TEAD1*, or *CTSW*, *IKBKG*, *IL21R*, and *IL4R*, respectively (Supplemental Table 1 and Fig. 3A). Of note, many of these genes (such as *TEAD1*, *IKBKG*, and *IL32*) were also highly expressed in TCRβ-transduced RAG2-KO DP cells similar to Control WT DP cells (Fig. 3A). Interestingly, the expression of a subset of genes (such as *MAL*) were not rescued with forced expression of a TCRβ chain (Fig. 3A). Genes significantly up-regulated in both RAG2-KO-1 and RAG2-KO-4 compared to Control WT include *HBG1*, *HBG2*, *LOC100240735*, and *LOC339975* (Supplemental Table 1 and Fig. 3B). Genes significantly up-regulated in RAG2-KO-1 or −4 compared to Control WT include *CCDC8*, *CPA4*, *EPHA4*, and *ID1*, or *ANKRD1*, *CMTM8*, *MET*, and *RHOU*, respectively (Supplemental Table 1 and Fig. 3B). Of note, many of these genes (such as *ID1*, *RHOU*, and *HBG1*) also showed low expression in TCRβ-transduced RAG2-KO DPs similar to Control WT DP cells (Fig. 3B). Interestingly, the expression of a subset of genes (such as *LOC100240735*) were not reduced with forced expression of a TCRβ chain (Fig. 3B). Phylogenetic tree analysis again showed that TCRβ-transduced RAG2-KO DP cells were more similar to Control WT DP cells than to control-transduced RAG2-KO DP cells (Fig. 3).

Analysis of cell cycle regulators, survival and differentiation genes, which are known to be involved in mouse β-selection (22–24), revealed a set of genes, such as *RORC*, *CD27*, *ERG* and *CCDN3*, that are also regulated following TCRβ expression in RAG2-KO DPs (Supplemental Fig. 3A). A gene ontology analysis to determine biological pathways that involve genes up-regulated in Control WT compared to RAG2-KO DP cells revealed a genetic program involved in “negative regulation of intrinsic apoptotic signaling pathway” (Supplemental Fig. 3B). Furthermore, a gene set pathway pertaining to leukocyte regulation, included genes involved in “positive regulation of lymphocyte activation” and “regulation of lymphocyte proliferation” (Supplemental Fig. 3B). Thus, these data provide additional evidence that RAG2-dependent TCRβ expression in developing human T-cells supports their survival and/or proliferation.

Conclusions.

Here, we generated RAG2-KO hPSCs to assess the role of TCRβ during human T-cell development. In contrast to the effects seen with *Rag1*- or *Rag2*-deficient mice, which show a complete lack of DP thymocytes, developing human T-cells were able to reach the $CD4^+CD8^+$ DP stage in the absence of RAG2 expression. Lack of RAG1/2 in mice results in a definitive block at the $CD44^-CD25^+$ DN3 stage, as it is well documented that RAG1/2-mediated TCRβ rearrangement controls the transition from the DN3 to the $CD44^-CD25^-$ DN4 and DP stages (25, 26). However, the precise developmental block in the absence of RAG1/2 expression or TCRβ rearrangement during human T-cell development was largely unresolved (27, 28).

It was previously suggested that the requirement for TCRβ-induced survival/proliferation, or β-selection, occurs at the $CD4^+$ ISP stage, in which 5% of cells express cell-surface TCRβ protein (29). In another study, it was suggested that β-selection instead happens later, at the $CD4^+CD8\alpha^+CD8\beta^-$ early double positive (EDP) stage in which 25% of cells express

intracellular TCRβ protein (30). Thus, based on these two studies, it was proposed that β-selection begins at the $CD4^+$ ISP stage and continues until the EDP stage. Here, we detected robust development past the ISP and EDP to the DP stage, as CD4, CD8α, and CD8β expression was observed in RAG2-KO developing T-cells. While the requirement for the TCRβ chain in the phenotypic differentiation to the DP stage differs between mice and humans, the TCRβ chain similarly promotes cellular expansion (survival/proliferation) in both mouse and human developing T-cells, as enforced TCRβ expression rescued cellular expansion.

The requirement for TCRβ, and thus the preTCR, during, rather than prior to, the DP stage during human T-cell development may reflect differential expression of cell cycle and survival genes. In mice, expression of a preTCR in DN3 cells leads to expression of Bmi-1, which represses the expression of the cell cycle inhibitor Cdkn2a. Repression of Cdkn2a is required for preTCR-induced cell proliferation and the DN3-DP transition (31). Future studies could investigate whether preTCR-mediated regulation of Bmi-1, Cdkn2a, Cdkn1a (32), as well as other factors involved in cell cycling, are initially acting at the DP stage during human T-cell development. In summary, this study reveals the unexpected timing required for TCRβ-mediated β-selection in developing human T-cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

1. Human RAG2-deficient developing T-cells progress up to the $CD4^+CD8^+$ stage
2. TCRβ-selection of human $CD4^+CD8^+$ cells allows for their survival/proliferation.

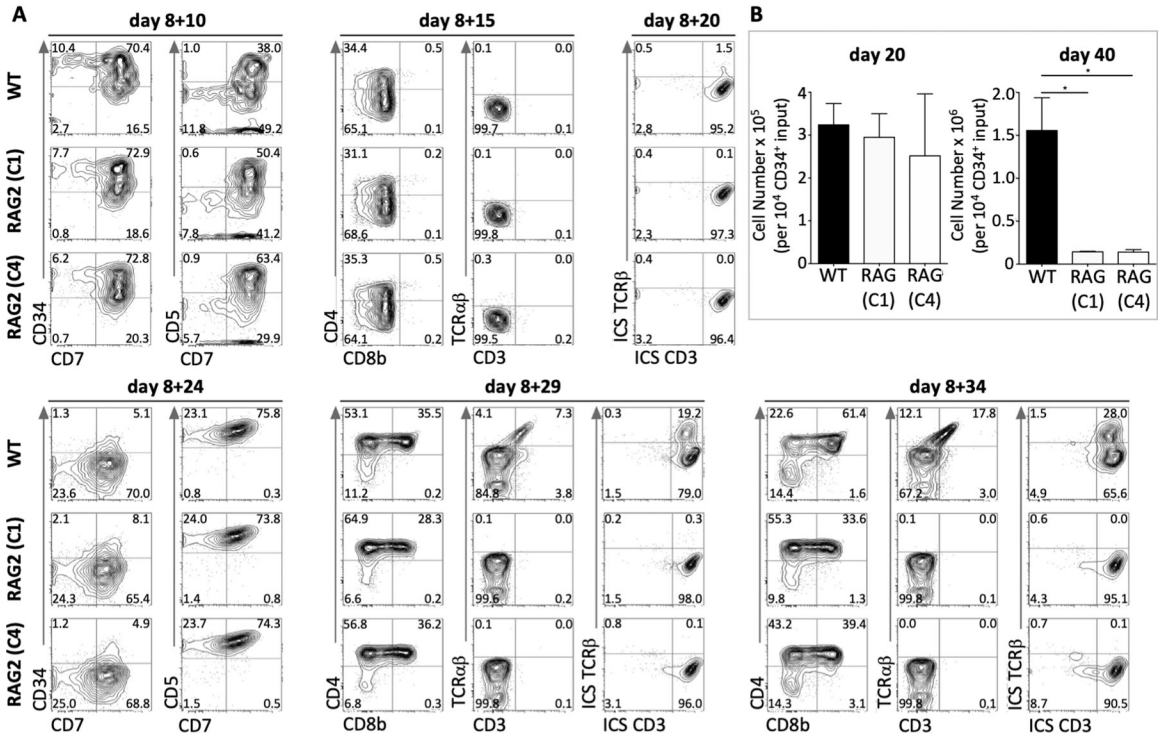

Figure 1. T-cell development from Control WT and RAG2-KO hESC lines.

(A) Representative flow cytometry analysis of Control WT and RAG2-KO (clones 1 and 4) hPSC-derived T-lineage cells from d8 EBs + 10–34d OP9-DL4–7FS co-cultures, as indicated. Cells were pre-gated for $DAPI^{-}CD45^{+}$. (n=5 of five independent experiments). (B) Cell numbers of Control WT and RAG2-KO (clones 1 and 4) hPSC-derived T-lineage cells per 10,000 input of $CD34^{+}$ cells after indicated number of days of culture on OP9-DL4–7FS. (n=3 of three independent experiments).

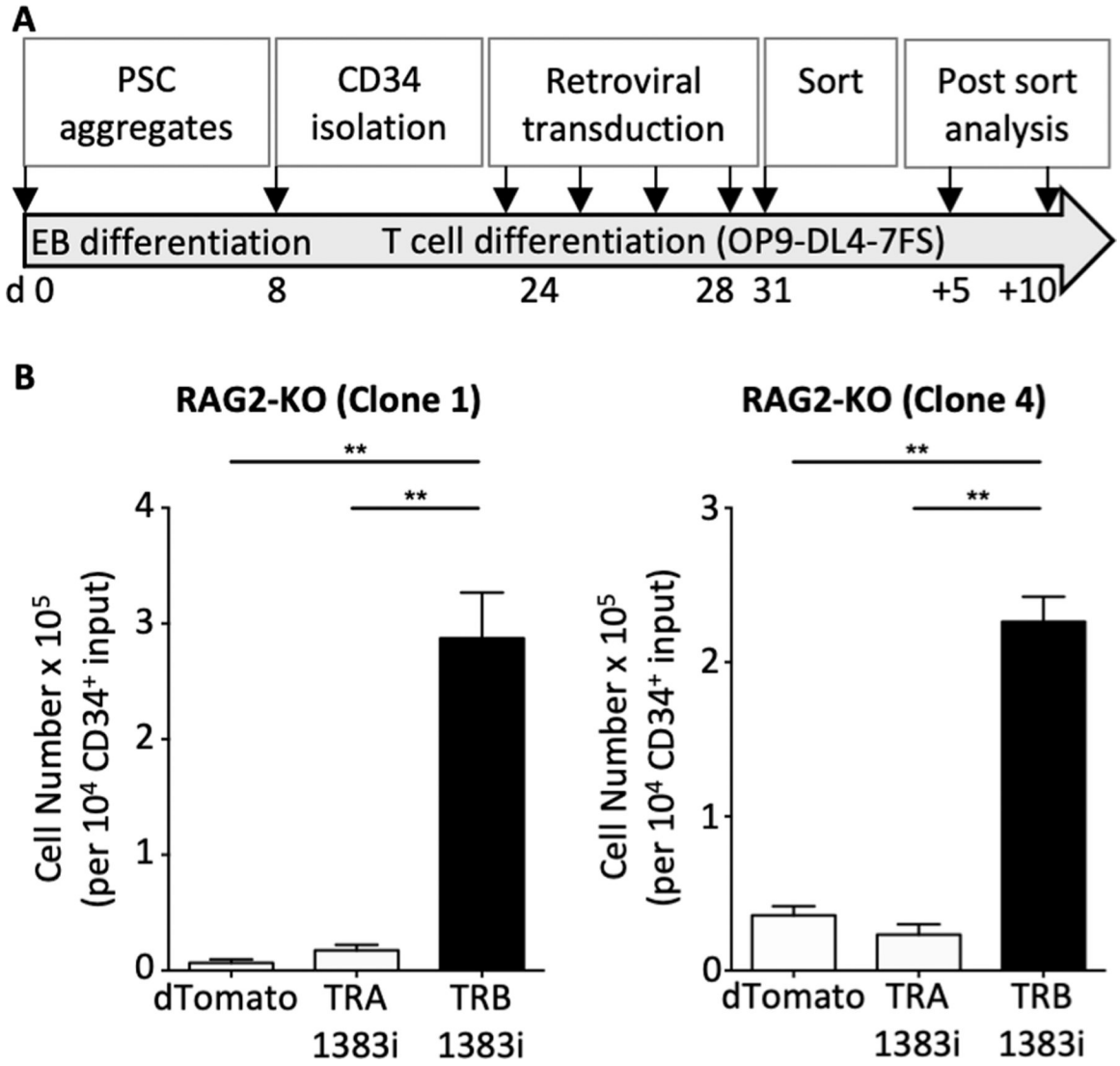

Figure 2. Forced expression of a rearranged TCRβ chain in RAG2-KO CD4$^+$CD8$^+$ DP cells results in cell expansion.

(A) Schematic of the experimental approach. (B) Cell counts of RAG2-KO DPs (clones 1 and 4) retrovirally-transduced with empty vector (dTomato), TCR alpha chain (TRA 1383i), or TCR beta chain (TRB 1383i) per 10,000 input of CD34$^+$ cells. Transduced T-lineage cells (dTomato$^+$) were first sorted for CD7$^+$CD5$^+$CD4$^+$CD8$^+$ double positive cells and then re-cultured on OP9-DL4–7FS for 10 days. (n=3 of three independent experiments).

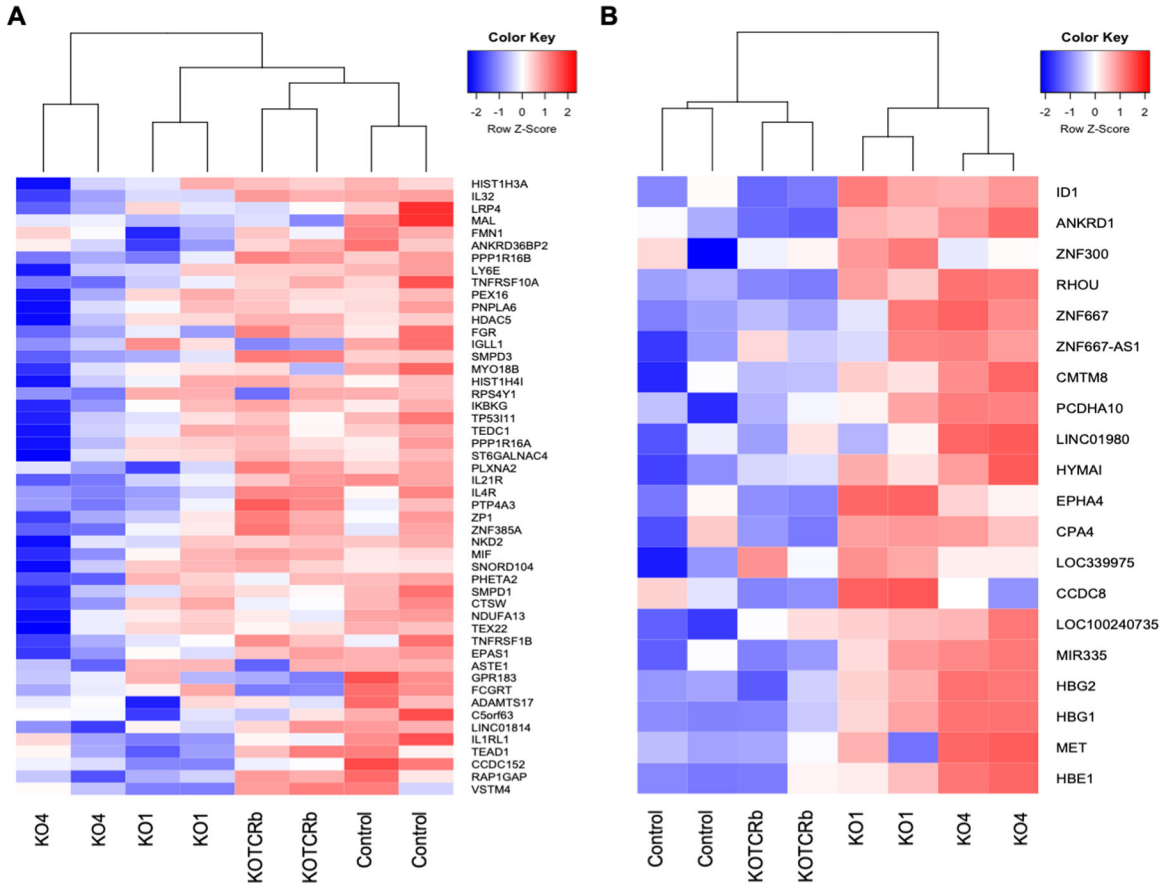

Figure 3. Transcriptomic analysis of Control WT, RAG2-KO, and RAG2-KO TCRβ-transduced $CD4^+CD8^+$ DP cells.

RNA-seq analysis of Control WT, RAG2-KO1/4, and $TCR\beta^+$ RAG2-KO1/4 $CD4^+CD8^+$ DPs. Shown are genes that are differentially highly expressed in Control WT DPs compared to RAG2-KO1/4 DPs (A) and differentially highly expressed in RAG2-KO1/4 DPs compared to Control WT DPs (B). (n=2 for Control WT, RAG2-KO-1, and RAG2-KO-4, and n=1 for RAG2-KO-1 TCRβ-transduced and RAG2-KO-4 TCRβ-transduced of one independent experiment).