PNAS RESEARCH ARTICLE | MEDICAL SCIENCES OPEN ACCESS

APOBEC2 deficiency disrupts hematopoietic lineage commitment, resulting in emergence of dual identity lymphocytes in mice and humans

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Edited by David G. Schatz, Yale University School of Medicine, New Haven, CT; received October 30, 2025; accepted April 30, 2026

APOBEC2 is a well-conserved member of the AID/APOBEC family of cytidine deaminases. Most members of the family catalyze the conversion of cytosine to uracil either in DNA or RNA, thereby acting as DNA mutators and/or RNA editors. APOBEC2 is the only family member that appears to be catalytically inactive. Instead, its ability to bind, but not deaminate DNA, has been co-opted into a transcription-factor-like functionality. APOBEC2 is highly expressed in skeletal muscle, where it functions to promote and maintain muscle identity by suppressing nonmuscle genes. APOBEC2 is also expressed in several cell types within the hematopoietic lineage. Here, we show that loss of APOBEC2 disrupts proper lymphoid lineage differentiation, resulting in the emergence of lymphoid cells expressing both TCRs and BCRs, as well as additional markers that define a mixed T and B cell identity in both mice with a depletion in *Apobec2* gene and humans with mutations in it. We further show that these T/B cells present dual functionality. Finally, we elucidate the molecular mechanisms associated with APOBEC2 deficiency that led to disruption of cell fate determination. Overall, our results establish APOBEC2 as a terminal repressor of B cell fates within the T cell lineage, whose loss results in disease outcomes in mice and humans.

APOBEC2 | terminal repressor | mixed identity lymphocytes | dual receptor T/B cells | BCR and TCR together

Significance

Here, we show that APOBEC2 preserves lineage fidelity within the lymphoid branch of the hematopoietic system. Loss or mutation of APOBEC2 in mice and humans leads to the emergence of lymphoid cells with mixed B and T cell features, termed T/B cells. This occurs partly through reactivation of B cell transcriptional programs within a subpopulation of the T cell lineage. Our findings challenge the assumption that cells coexpressing B and T cell receptors are experimental artifacts. Instead, they represent a genuine lymphocyte subset with dual identity. Their presence in infection, autoimmunity, and cancer suggests functional relevance, while highlighting APOBEC2's role in limiting lineage plasticity among subpopulations of lymphoid progenitors and maintaining proper immune cell differentiation.

APOBEC2 is a member of the evolutionarily conserved zinc-dependent deaminases of the apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide (APOBEC) family. It has been shown by several studies that members of this family directly facilitate the removal of an amino group from cytidine bases in polynucleotide chains, resulting in cytidine (C) to uridine/uracil (U) transition on DNA or/and RNA molecules (1–4). APOBEC2 is an outlier in this context, as it cannot catalyze the deamination reaction.

While APOBEC2 appeared to be catalytically inactive, its loss had functional consequences. Early work in mice showed that APOBEC2 depletion causes myopathy, reduction of muscle mass, and a shift from fast to slow muscle fiber formation (5, 6). Focusing on muscle (where APOBEC2 is highly expressed, especially during differentiation), Lorenzo et al. recently showed that APOBEC2's ability to bind DNA endows it with a nonconventional role as a transcriptional regulator. They found that loss of APOBEC2 resulted in the expression of nonmuscle genes during differentiation through a specific molecular mechanism that recruited chromatin remodeling complexes to the promoters of some of those genes. Consequently, it was proposed that APOBEC2 functions as a terminal repressor to safeguard muscle cell identity. These findings may help explain why APOBEC2 has also been linked to cancer development or progression, at least in a correlative fashion (7–9).

Surprisingly, Lorenzo et al. noted that many APOBEC2-silenced genes during muscle cell differentiation were T cell lineage specific (a cell type where APOBEC2 is reasonably well expressed, Dataset Accession number: GSE60101) (10). Here, we provide functional and molecular evidence of a specific role for APOBEC2 in T cell lineage commitment. We show that loss of APOBEC2 in mice results in the emergence of lymphoid cells with mixed T and B cell identity, which we call T/B cells. These cells express both T cell and B cell markers and can receive and process signals through either of these. These results are corroborated in human patients who carry biallelic *APOBEC2* mutations and support the suggestion that APOBEC2 is a terminal suppressor of B cell fate during T cell differentiation. This places *Apobec2* in a family of genes like *Pax5*, the guardian of identity,

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2531122123/-/DCSupplemental.

Published June 5, 2026.

development, and functionality in B cells (11–18), *Myt1l*, a terminal suppressor of alternative cell fates in the nervous system (19, 20) *Tcf7*, a key factor for T cell development and later functions (21–23), and *Prox1* which acts as active repressor of plasticity in liver cells and prevent tumorigenesis (24).

Results

APOBEC2 Deficiency Leads to the Emergence of Cells With Mixed T and B Cell Phenotypes. Apobec2 is well expressed in muscle but also in some immune cells, notably in $CD3^+$ T cells (10) (Lara-Astiaso et al., 2014 dataset, accession number GSE60103, and *SI Appendix*, Fig. S1 *A* and *B*). To assess the relevance of APOBEC2 in these subsets, we analyzed blood and spleen samples from wild-type (WT) and *Apobec2* knockout ($A2^{-/-}$) mice, using flow cytometry. Our analysis revealed the emergence of a novel cell population expressing both T cell (CD3) and B cell (CD19) markers ($CD3^+CD19^+$) in peripheral blood (*SI Appendix*, Fig. S1*C*) and spleens of $A2^{-/-}$ mice (Fig. 1*A*). To exclude the possibility that the identified $CD3^+CD19^+$ dual-expressing populations were artifacts resulting from cell doublets (for example, B and T cells stably interacting with one another), we performed fluorescence-activated cell sorting (FACS) to pre-enrich the population, followed by direct imaging using the ImageStream® Mk II imaging flow cytometer on live, presorted single cells (Fig. 1 *B* and *C*). As controls, we sorted single $CD3^+$ or $CD19^+$ cells and subjected them to the same analysis. While single sorted T or B cells retained either CD3 or CD19 positivity but not both (*SI Appendix*, Fig. S1*D*), the population of interest to us was demonstrably coexpressing both CD3 and CD19, confirming true coexpression rather than aggregation or doublet artifacts (Fig. 1*D*). Interestingly we found that in around 20% of cells CD3 and CD19 are colocalized (*SI Appendix*, Fig. S2*A*). Although this is only a snapshot observation in time (so that colocalization is more frequent than implied), this finding suggests that at least some of these cells may be capable of activating common signaling pathways in response to stimulation through either the BCR or the TCR.

To further characterize these $CD3^+CD19^+$ dual-expressing cells at the transcriptomic and clonotypic levels, we conducted 10× Genomics GEM-X Universal 5′ Gene Expression and V(D)J single-cell RNA sequencing. Transcriptomic profiling revealed simultaneous expression of both CD3 and CD19 (*SI Appendix*, Fig. S2*B*) in addition to canonical B-lineage markers, including Cd79a, Cd79b, and immunoglobulin (Ig) genes (Fig. 1*E*), as well as T-lineage markers such as CD3, CD4, CD8, and T cell receptor (TCR) genes (Fig. 1*E*), within the same cells. V(D)J sequencing of both TCR and BCRs further confirmed productive rearrangement of both immunoglobulin heavy (VH) and light (VK) chains (Fig. 1*F*), as well as TCR α and β chains (Fig. 1*G*) within the $CD3^+CD19^+$ population. Notably, paired clonotype analysis at the single-cell level revealed that individual cells harbored both VH/VK and TCRα/β chains concurrently (Fig. 1*H* and Dataset S1), indicating dual-receptor expression. Interestingly, the B cell receptor (BCR) repertoire exhibited markedly lower clonal diversity compared to the TCR repertoire, suggesting differential clonal expansion or selection pressures between the two lineages.

Next, we asked if we could identify the existence of these mixed T/B cell populations within the hematopoietic system using global unsupervised methods. Toward this, we performed 10× single-cell (sc)RNA-seq on spleen, bone marrow (BM), and thymus from WT and $A2^{-/-}$ mice. Our analysis identified distinct cell clusters in the $A2^{-/-}$ animals that exhibited dual T and B cell identities, which were transcriptionally distinct from conventional (e.g., wildtype) T cell subpopulations (Fig. 1*I*). Identity scoring, based on the expression of key T and B cell lineage factors, revealed the presence of cell populations in $A2^{-/-}$ mice that are typically clustered with T cells while also expressing B cell identity genes (Fig. 1*J*, circled). Notably, the dual-identity T/B cells are also characterized by transcription of T cell associated genes, like *Tcf7*, *Gata3*, *Bcl11b*, whose expression levels fall in between those from immunophenotypic B cells and T cells derived from the spleens of $A2^{-/-}$ mice (*SI Appendix*, Fig. S2*C*). Signatures suggesting mixed populations were also observable at the level of bulk RNAseq performed on $CD4^+$ and $CD8^+$ T cells isolated from the spleens of $A2^{-/-}$ and WT mice: differential gene expression analysis demonstrated a significant upregulation of B cell lineage–specific genes in $A2^{-/-}$ $CD4^+$ (*SI Appendix*, Fig. S3*A*) and $CD8^+$ T cells (*SI Appendix*, Fig. S3*A*), including *Pax5, Cd19, Cd79b, Ly6d,* and immunoglobulin light and heavy chain genes. Pathway enrichment analysis (PEA) and gene set enrichment analysis (GSEA) further revealed an enrichment of B cell identity programs and antigen presentation pathways within these $A2^{-/-}$ derived T cell populations (*SI Appendix*, Fig. S3 *B-E*). Taken together, these findings suggest that APOBEC2 deficiency disrupts lineage fidelity, leading to the emergence of lymphocytes with dual-lineage identity.

Dual-Identity $CD3^+CD19^+$ Cells Exhibit Functional Plasticity, Combining T Cell and B Cell Properties in $A2^{-/-}$ Mice. To assess whether the $CD3^+CD19^+$ (T/B) cells, which we identified both phenotypically and transcriptionally, possess functional characteristics of both T and B cells, we first evaluated the expression of the T cell receptor (TCR) and B cell receptor (BCR) on their surface. Indeed, and as suggested by the repertoire analyses (Fig. 1), we could confirm that these dual-identity lymphocytes express both TCR (as evidenced by staining with an anti-TCR-beta antibody, (Fig. 2*A*) and BCR, as evidenced by staining with an anti-IgM antibody (Fig. 2*B*), as well as CD3 and CD19 (*SI Appendix*, Fig. S4*A*).

Next, we examined the T cell functionality of these dual-expressor T/B cells ($CD3^+CD19^+$) by assessing their activation in response to stimulation with anti-CD3 and anti-CD28 antibodies (Fig. 2*C*). We analyzed $CD3^+$ T cells from splenic tissue of wild-type (WT) mice and $A2^{-/-}$ mice, as well as $CD3^+CD19^+$ cells from spleens of $A2^{-/-}$ mice. Using this antibody cocktail we demonstrated that we could activate $CD3^+$ T cells from both WT and $A2^{-/-}$ mice, as indicated by the upregulation of CD69, although the activation capacity was reduced in the $A2^{-/-}$ cells (Fig. 2*D*). Interestingly, the $CD3^+CD19^+$ ("T/B") $A2^{-/-}$ cells not only responded to activation stimuli but in fact exhibited an enhanced activation state, even in the absence of external stimulation (Fig. 2*D*).

To further explore the impact of Apobec2 loss on B cell–defining properties and to investigate whether $CD3^+CD19^+$ (T/B) cells could perform B cell functions, we cocultured immunophenotypic B cells ($CD19^+$ $B220^+$ $CD3^-$) from spleens of WT and $A2^{-/-}$ mice, and $CD3^+CD19^+$ (T/B) cells from spleens of $A2^{-/-}$ mice (Fig. 2*C*), with 40LB feeder cells under conditions that promote class switching from IgM to IgG1 (25). Three days after stimulation with IL-4, we could clearly observe that $A2^{-/-}$ B cells (Fig. 2*E*) were capable of performing class switch recombination almost as well as WT B cells (26). Interestingly, IgG1+ cells of the $A2^{-/-}$ B background appeared to be less well maintained in culture six days after stimulation (Fig. 2*E*), suggesting a loss in proliferative capacity relative to unswitched (IgM+) cells. Remarkably, the $CD3^+CD19^+$ (T/B) cells were also capable of undergoing class switching (Fig. 2*E*) while maintaining CD3

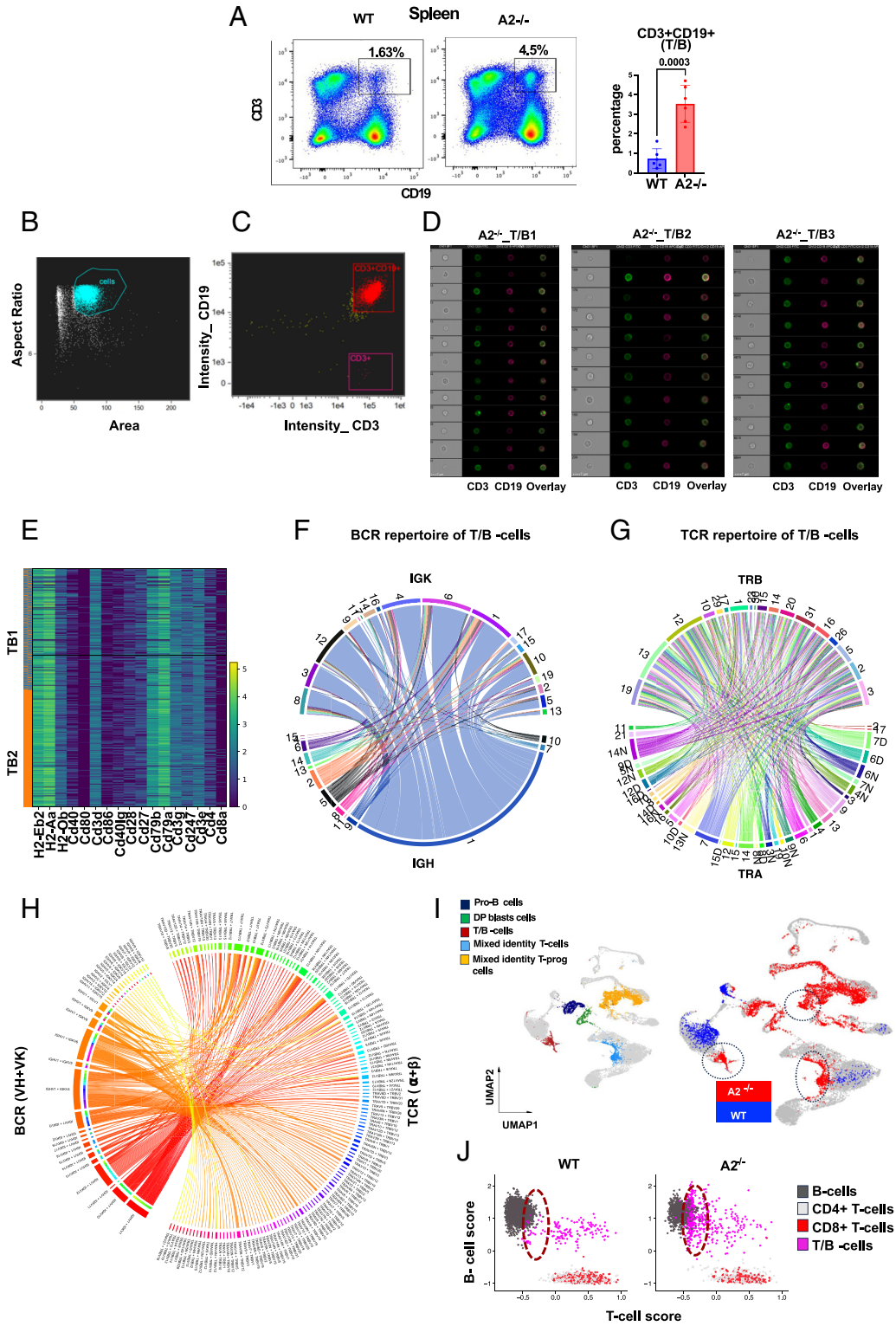

Fig. 1. APOBEC2 deficiency in mouse leads to the emergence of cells with a mixed B and T cell phenotype. (*A*) Flow cytometry analysis showing the fraction of $CD3^+CD19^+$ (T/B) cells in spleens of C57BL/6 WT and *Apobec2*$^{-/-}$ ($A2^{-/-}$) mice. (*B–D*) $CD3^+CD19^+$ T/B cells were isolated and analyzed using the ImageStream Mk II imaging flow cytometer. (*B*) Flow-based gating strategy identifying live, single cells and excluding doublets. (*C*) Flow cytometry plots showing gating and frequency of $CD3^+CD19^+$ double-positive cells, with percentages indicated. (*D*) Representative ImageStream images confirming reproducible coexpression of CD3 and CD19 within single cells across experiments. Data represent three biological replicates (n = 3 mice), with 10,000 events per sample. (*E*) Heatmap of B cell and T cell marker gene expression at the single-cell level from 10× scRNA-seq data. (*F–H*) TCR and BCR repertoire analysis of $CD3^+CD19^+$ T/B cells from $A2^{-/-}$ mice using 10× Genomics V(D)J sequencing. (*F*) Circos plots displaying paired usage of immunoglobulin heavy (VH) and light (VK) chain variable regions, with connector thickness reflecting clonal frequency. (*G*) Circos plots illustrating paired TCRβ and TCRα chain usage, with connector width indicating clonal abundance. (*H*) Chord diagram showing connectivity between BCR and TCR gene families, with links representing co-occurrence within individual cells. (*I*) Single-cell RNA sequencing of HSPCs from bone marrow and mature cells from spleen and thymus of WT and $A2^{-/-}$ mice. UMAP embeddings identify differentially abundant populations, with red indicating enrichment in $A2^{-/-}$ samples. (*J*) Scatter plots displaying the module scores for canonical T cell marker genes (*Cd3e, Tcf7, Gata3, Cd3d, Cd4, Cd8a, Bcl11b, Runx2, Ikzf2, Themis, Sell, Id2, Ccr7*) on the x-axis and B cell marker genes (*Cd19, Pax5, Spib, Cd72, Cd74, Cd79a, Blnk, Mef2c, Ms4a2, Ebf1, Ly6d, Bank1*) on the y-axis for each cell type, split by WT and $A2^{-/-}$ conditions. WT = Wild Type, $A2^{-/-}$ = *Apobec2*$^{-/-}$.

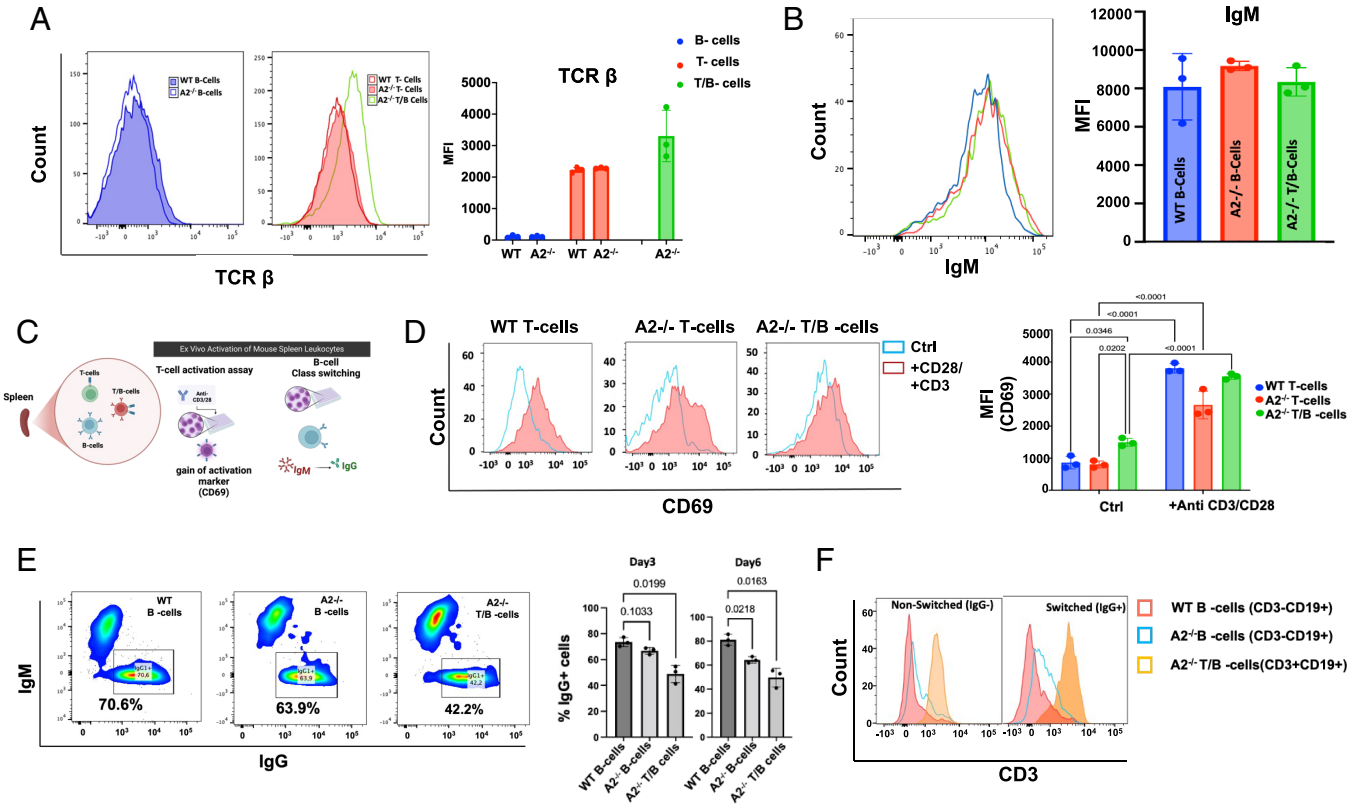

Fig. 2. Functional characterization of $CD3^+CD19^+$ (T/B) cells. (*A*) T cell receptor expression in WT and $A2^{-/-}$ T cells and B cells, alongside with $A2^{-/-}$ T/B cells. Staining with anti-TCR beta (TCR β), followed by flow cytometry analysis, demonstrates that $CD3^+CD19^+$ cells present TCR expression on their surface. (*B*) Expression of B Cell Receptor (anti-IgM) in WT and $A2^{-/-}$ B cells, alongside $A2^{-/-}$ T/B cells. Staining with anti-IgM followed by flow cytometry analysis demonstrates that $CD3^+CD19^+$ cells present also BCR expression on their surface. (*C*) Schematic graph showing the B and T cell functionality assays that were used to test the dual functionality of T/B cells. (*D*) Accessing T cell activation state by measuring CD69 intensity upon stimulation with anti-CD3 and anti-CD28. $CD3^+CD19^+$ cells from *Apobec2* knockout ($A2^{-/-}$) mice exhibit enhanced activation status even in the absence of external stimuli. (*E*) FACS plots showing the percentage of class-switching to IgG+ cells in WT B cells, $A2^{-/-}$ B cells, and T/Bcells ($CD19^+$ $CD3^+$) from *Apobec2*$^{-/-}$ after IL4/BAFF stimulation (splenocytes were isolated and cultured with 40LB feeder cells in the presence of IL4), quantification of IgG+ cells 3 and 6 d after culture. (*F*) Switched cells from (*E*) retain expression of CD3. WT = Wild Type, $A2^{-/-}$ = *Apobec2*$^{-/-}$.

expression postswitching (Fig. 2*F*). These findings suggest that $CD3^+CD19^+$ cells can respond to both T cell and B cell stimuli while retaining signaling capabilities associated with both cell types (*SI Appendix*, Fig. S4 *B* and *C*). This indicates that these cells exhibit functional properties characteristic of both T and B cells.

Loss of APOBEC2 During Early T Cell Lineage Commitment Is Causal to the Emergence of Dual-Identity T/B Lymphocytes. To assess whether loss of *Apobec2* affects commitment to the T cell lineage at an early differentiation stage, we measured the expression of key B cell identity genes and T cell factors in common lymphoid progenitors (CLPs), $Ly6d^-$ progenitors, and in $Ly6D^+$progenitors. CLPs comprise the first early progenitor population from which the T and B cell lineages arise (27–29). These progenitors are defined phenotypically by the expression of Flt3, Il7r, and low to medium expression of c-kit (30). The commitment to B cell lineage is associated with the differentiation of CLP into $Ly6d^+$ cells that is marked by the expression of Pax5 and B cell surface markers expression (31) and (*SI Appendix*, Fig. S5*A*) and down-regulation of T cell–specific genes (*SI Appendix*, Fig. S5*A*). In contrast, $Ly6d^-$ lymphoid progenitors that differentiate into T cells downregulate *Pax5* expression and other B cell markers (*SI Appendix*, Fig. S5*A*), upregulate the expression of T cell–specific transcription factors like *Tcf7*, *Bcl11b* (*SI Appendix*, Fig. S5*A*) and begin their transit to the thymus.

We found no changes in T or B cell identity genes in CLPs from WT or $A2^{-/-}$ animals (*SI Appendix*, Fig. S5*B*). Similarly, $Ly6d^+$ B cell progenitors also maintained similar expression levels of T cell and B cell marker genes in $A2^{-/-}$ compared to WT (*SI Appendix*, Fig. S5*C*). However, in $A2^{-/-}$ $Ly6d^-$ progenitors we detected increased expression of B cell factors such as *Ikaros*, *Irf4*, and *Pu1* compared to WT (*SI Appendix*, Fig. S5*D*). These findings suggest that loss of Apobec2 allows $Ly6D^-$ cells to differentiate to T cells but without properly shutting down B cell fate programs. The resulting "dual-identity" T/B cells and T cells from $A2^{-/-}$ appear to differentiate and mature properly within the thymus and exit to the periphery while still retaining expression of B cell identity factors (*SI Appendix*, Fig. S5 *E* and *F*) and as previously shown in Fig. 1*E*, suggesting a role for APOBEC2 in differentiation and perhaps also the maintenance of T cell fate.

To specify the developmental stage at which Apobec2 deletion (or mutation) disrupts lineage identity, we generated mouse models with Apobec2 deletion at various stages of lymphoid development (Fig. 3*A*). These include the deletion at B cell stages ($Apobec2^{flx/flx}$ $Mb1^{Cre/Cre}$), T cell stages ($Apobec2^{flx/flx}$ $CD4^{cre/cre}$), and at early stages of lymphoid development ($Apobec2^{flx/flx}$ $IL7r^{cre/cre}$). We then conducted FACS analyses to identify the models in which conditional loss of Apobec2 would result in a mixed lymphocyte population similar in features to the one we identified in the context of the full knockout model. Our analysis demonstrated that conditional deletion of *Apobec2* at the early lymphoid progenitor stage led to the emergence of a dual-identity T/B

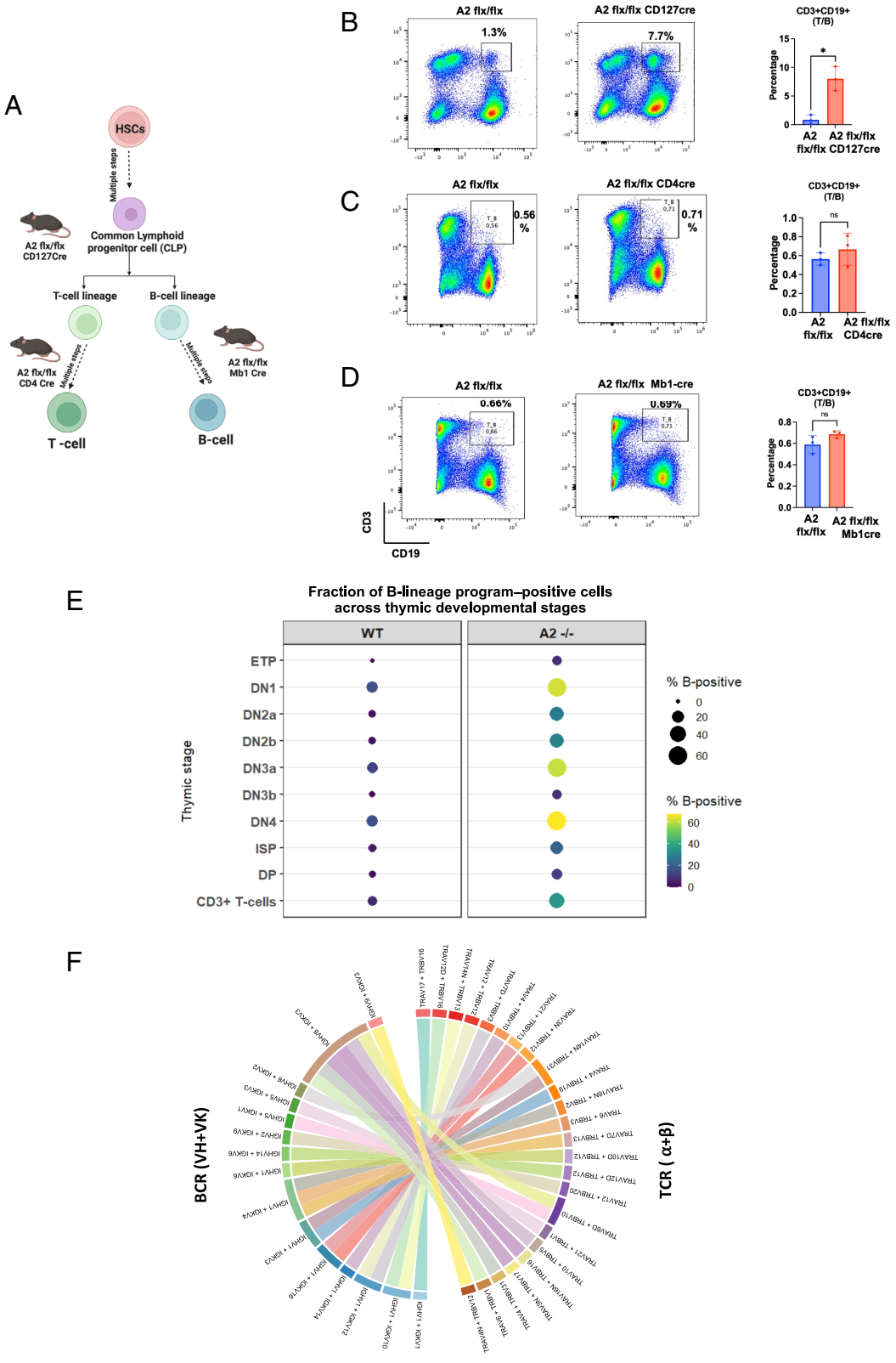

Fig. 3. *Apobec2* is pivotal to the suppression of B cell fates during early T cell lineage commitment. (*A*) Cre Cell-type specific deletion of *Apobec2* at different stages of the lymphoid branch. (*B*) FACS plots from *Apobec2* $^{flx/flx}$ and Apobec2$^{flx/flx}$ CD127$^{Cre+/Cre+}$ (*Left* and *Right*), the quantification of CD3^{+}CD19^{+} (T/B cells). (*C*) FACS plots from *Apobec2* $^{flx/flx}$ and Apobec2$^{flx/flx}$ CD4$^{Cre+/Cre}$ (*Left* and *Right*), the quantification of CD3^{+}CD19^{+} (T/B cells). (*D*) FACS plots from *Apobec2* $^{flx/flx}$ and Apobec2$^{flx/flx}$ Mb1$^{Cre+/Cre+}$ (*Left* and *Right*), the quantification of CD3^{+}CD19^{+} (T/B cells). (*E*) 10× scRNA-seq was performed on thymic populations and the different stages of thymic development were checked for fraction of cells that express key B cell Transcriptional program highlighted by (Pax5, Ebf1, Cd19, Ms4a1, Cd22, Cd79a, Cd79b). The graph shows the fraction of cells in each stage (ETP, DN1, DN2, DN3, DN4, ISP, DP, and CD3^{+} T cells that upregulate this program. (*F*) TCR and BCR repertoire analysis performed on thymic populations cells sorted from *Apobec2*$^{-/-}$ (A2$^{-/-}$) mice and subjected to 10× Genomics V(D)J single-cell immune repertoire sequencing. Chord diagram illustrating the connectivity between B cell receptor (BCR) and T cell receptor (TCR) gene families in individual cells that coexpress both TCR and BCR components. Each sector on the circle represents a distinct BCR (IGHV + light chain K) or TCR (TRA + TRB) V gene family pairing. The colored links (chords) indicate co-occurrence of BCR(VH+Vk) and TCR(α+β) gene families in the same cell. The color of each link corresponds to the BCR pair it originates from, highlighting the diversity of TCR associations per BCR.

lymphocyte pool (Fig. 3*B*), whereas deletion at later stages—specifically at the $CD4^+$ T cell stage (Fig. 3*C*) or the $Mb1^+$ early B cell stage (Fig. 3*D*) did not support further emergence of these cells. Our results indicate that cells with both T and B cell characteristics emerge between the early stages of lymphoid development in the bone marrow and the initial stages after T cell progenitors enter the thymus.

To investigate this further and to follow the dual-identity program during thymic development, we examined our scRNA-seq data to determine whether fraction of different thymic T cell progenitor populations (DN1–4, DP, SP CD8, and SP CD4) upregulate key B cell genes, including *Pax5*, *Cd79a*, *Cd79b*, *Cd20*, *Ebf1*, and Vpreb1. We found that there is a fraction of cells across all thymic populations that upregulate the B cell program at DN stages and that this is attenuated as differentiation progresses (DPs and spCD8 and SpCD4 cells; Fig. 3*E*).

To assess whether thymic populations that upregulate a B cell program can also express the B cell receptor (BCR) during development, we performed FACS staining for IgM across various thymic subsets, including DN, DP, CD8 single-positive (SP CD8), and CD4 single-positive (SP CD4) cells. IgM expression was detectable starting at the DN stage and persisted throughout subsequent stages (*SI Appendix*, Fig. S6*A*). To further assess this as the molecular level, we performed targeted 10× scRNA-seqV(D)J sequencing on thymic populations. This analysis revealed a distinct population exhibiting both BCR and TCR rearrangements (Fig. 3*F* and *SI Appendix*, Fig. S6 *B* and *C* and Dataset S2). We then analyzed genomic VDJ rearrangements at the immunoglobulin heavy-chain locus in different thymic populations from $A2^{-/-}$ and WT mice. In wild-type controls, heavy-chain V(D)J rearrangements were largely suppressed by the DP stage (*SI Appendix*, Fig. S6*D*), in contrast, in $A2^{-/-}$, we observed increased usage of V(D)J rearrangements during the transition from the DN stage to the DP stage (*SI Appendix*, Fig. S6*E*).

Together, these findings suggest that a dual-identity population emerges in the bone marrow and persists through thymic development. However, precisely defining the stage at which these cells first appear will require a reporter-based tracking system capable of recording Apobec2 expression during developmental transitions.

***Apobec2* Deletion Enhances Chromatin Accessibility of B Cell Identity Genes Within T Cells, Leading to Dual-Identity Lymphocytes With a Hybrid T/B Cell Molecular Signature.** Having demonstrated that the loss of *Apobec2* leads to the acquisition of B cell transcriptional identity within CD3+ T cells resulting in the emergence of cells exhibiting both T cell and B cell potential ($CD3^+CD19^+$ T/B cells) (Figs. 1 and 2), we wanted to explore this phenomenon at the molecular level. Previous work on muscle cell differentiation (32) used ChIP-seq to demonstrate that APOBEC2 occupies the promoter regions of genes that are silenced during differentiation. At the same time, these authors showed that transcription-start-site resident APOBEC2 recruited histone modification complexes, including factors like HDAC and CHD4, to further enforce gene silencing (32). Because of the small number of T/B cells available in comparison to muscle cell cultures, we opted against using ChIP-Seq in our study, but turned to an alternative technology (ATAC-seq) to assess whether the accessibility of B cell identity genes increases upon loss of Apobec2 in the T cell lineage.

We therefore conducted ATAC-seq on $CD3^+$ T cells and $CD3^+CD19^+$ (T/B) cells from $A2^{-/-}$ mice, as well as T cells and B cells from wild-type (WT) mice. We observed that the absence of APOBEC2 leads to increased chromatin accessibility at the regions within the regulatory sequences of key B cell identity genes in T/B cells from $A2^{-/-}$ mice. In fact, the chromatin accessibility profile of T/B cells is intermediate that of T cells and B cells, supporting the notion of a dual cellular identity at the epigenetic level. Interestingly, T cells from $A2^{-/-}$ mice exhibit a distinct phenotype compared to T/B cells, they also show partial chromatin reopening, albeit to a lesser extent (Fig. 4*A*).

We then investigated whether these accessible regions are associated with genes crucial for B cell identity regulation. Indeed, we found that many of these accessible regions regulate key factors essential for maintaining B cell identity, including *Ebf1*, *Spi1*, *Spi1B*, and *Pax* genes (Fig. 4*B*). An analysis of the top enriched accessible motifs revealed a significant enrichment for transcription factor motifs that are critical for identity specification within the hematopoietic system, such as motifs for Spi-B, Runx, EBFs, PU.1, among others (Fig. 4 *B* and *E* and *SI Appendix*, Fig. S7*A*). We next asked whether the expression of target genes regulated by these transcription factors correlates with increased chromatin accessibility at their corresponding regulatory motifs. To do this, we first identified the most enriched upstream transcription factors based on predicted downstream targets from our RNA-seq analysis (Fig. 4*B* and *SI Appendix*, Fig. S3*A*). This analysis revealed a significant enrichment of key regulators of B cell identity, including Spi1, EBF1, and EBF2, among others, in $A2^{-/-}$ T cells compared to WT (Fig. 4*C*). Furthermore, when examining the expression of their downstream target genes, we observed an upregulation of essential B cell identity genes and markers, such as *Pax5*, *Cd19*, and *Cd79a*, following Apobec2 deletion (Fig. 4*D*). A detailed analysis of the chromatin accessibility of these genes revealed that $CD3^+CD19^+$ cells exhibit the highest level of accessibility to B cell identity genes compared $A2^{-/-}$ T cells and WT T cells. However, it is again noteworthy that increased accessibility to B cell identity genes is already evident in $A2^{-/-}$ T cells (Fig. 4 *A*, *B*, and *E*). The data also reveal that $CD3^+CD19^+$ and $CD3^+$ cells of the $A2^{-/-}$ genotype have decreased accessibility of T cell–related genes but retain, to some extent, their T cell identity as they maintain the accessibility and expression of T cell identity genes (Fig. 1 *E–H* and *SI Appendix*, Fig. S7*B*).

To directly address a mechanistic involvement of APOBEC2 in establishing T cell identity at the transcriptional level, we performed antibody-guided chromatin tagmentation (ACT-seq) using antibodies against the activation mark H3K27ac on WTB cells, WTT cells, $A2^{-/-}$ T cells, and $A2^{-/-}$ T/B cells. We selected this mark because we previously demonstrated that in muscle, APOBEC2 suppresses genes in an acetylation-dependent manner through interaction with histone deacetylases (32), and we opted for ACT-seq because as a method it is transposase-based and thus directly comparable to ATAC-seq (33). Principal component analysis (PCA) of global H3K27ac profiles (n = 3 mice) revealed clear segregation of cell populations along PC1, which accounted for the largest proportion of variance (*SI Appendix*, Fig. S8*A*). Both $A2^{-/-}$ T cells ($CD3^+CD19^-$) and T/B cells ($CD3^+CD19^+$) formed distinct clusters from WT T cells and WT B cells, indicating a unique epigenetic landscape. Notably, the T/B cell population occupied an intermediate position between $CD3^+$ T cells and $CD19^+$ B cells, further supporting the coexistence of both lineage identities within this subset. Consistent with the ATAC-seq results shown in Fig. 4, loss of APOBEC2 led to derepression of B cell–related genes moderately in knockout T cells (Fig. 5*A* and *SI Appendix*, Fig. S8*B*) and more prominently in T/B cells (Fig. 5 *A* and *C*). Collectively, H3K27ac enrichment was observed at regulatory regions of key B cell genes in both $A2^{-/-}$ T cells and T/B cells compared with WT T cells, indicating that APOBEC2 mechanistically contributes to maintaining repression of B cell–specific chromatin regions. In addition, deletion of Apobec2 resulted in

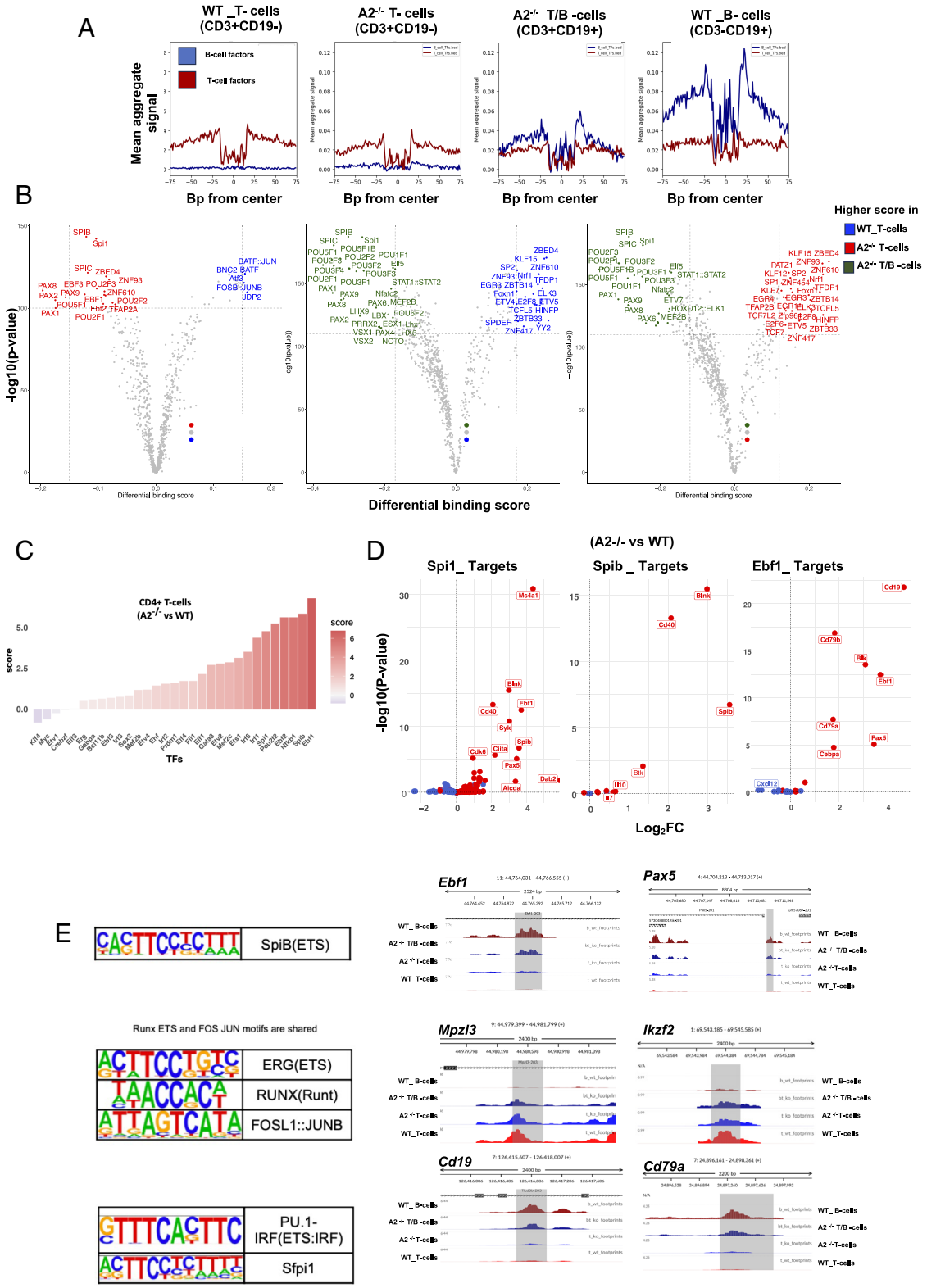

Fig. 4. Apobec2 deletion enhances chromatin accessibility of B cell identity genes in T cells, leading to a hybrid T/B cell molecular signature. (*A*) Top enriched binding sites of B cell transcription factors EBF1, SPIB, and OCT2 and for T -cell transcription factors BCL11B, GATA3, RUNX1, RUNX2, and TCF1 were selected to demonstrate the opening of chromatin in regulatory elements of genes that have motifs for those transcription factors. The panels illustrate the chromatin accessibility profiles of B cell identity genes (in blue) and T cell identity genes (in red) across various cell types: wild-type (WT) T cells, Apobec2$^{-/-}$(A2$^{-/-}$) T cells, T/B cells (CD3^{+}CD19^{+}) cells from Apobec2$^{-/-}$ mice, and WT B cells. Note here that while the T–B cells clearly have a mixed identity, the A2$^{-/-}$ T cells are also mixed (albeit in a lesser extent). (*B*) The volcano plots depict differentially accessible chromatin regions in the following comparisons: WT T cells vs. A2$^{-/-}$ T cells (*Left*), WT T cells vs. A2$^{-/-}$ T/B cells (*Middle*), and A2$^{-/-}$ T cells vs. A2$^{-/-}$ T/B cells (*Right*). Each dot represents a motif, with annotations indicating the transcription factors that bind to them. The x-axis shows the differential binding score, while the y-axis displays the -log10 adjusted *P*-value. (*C*) This panel shows the top enriched transcription factors predicted based on RNA-seq analysis, comparing gene expression between Apobec2$^{-/-}$ and WT T cells. (*D*) The volcano plots illustrate the differential expression of target genes for key transcription factors, whose motifs were found to be enriched in the ATAC-seq analysis. (*E*) shows the top enriched motifs in (enriched in T/B cells compared to the WT T cells) with its corresponding chromatin accessibility footprint in key target B cell identity genes such as *Pax5*, *Ebf1*, *Cd19*, and *Cd79a* among others. WT = Wild Type, A2$^{-/-}$ = *Apobec2*$^{-/-}$.

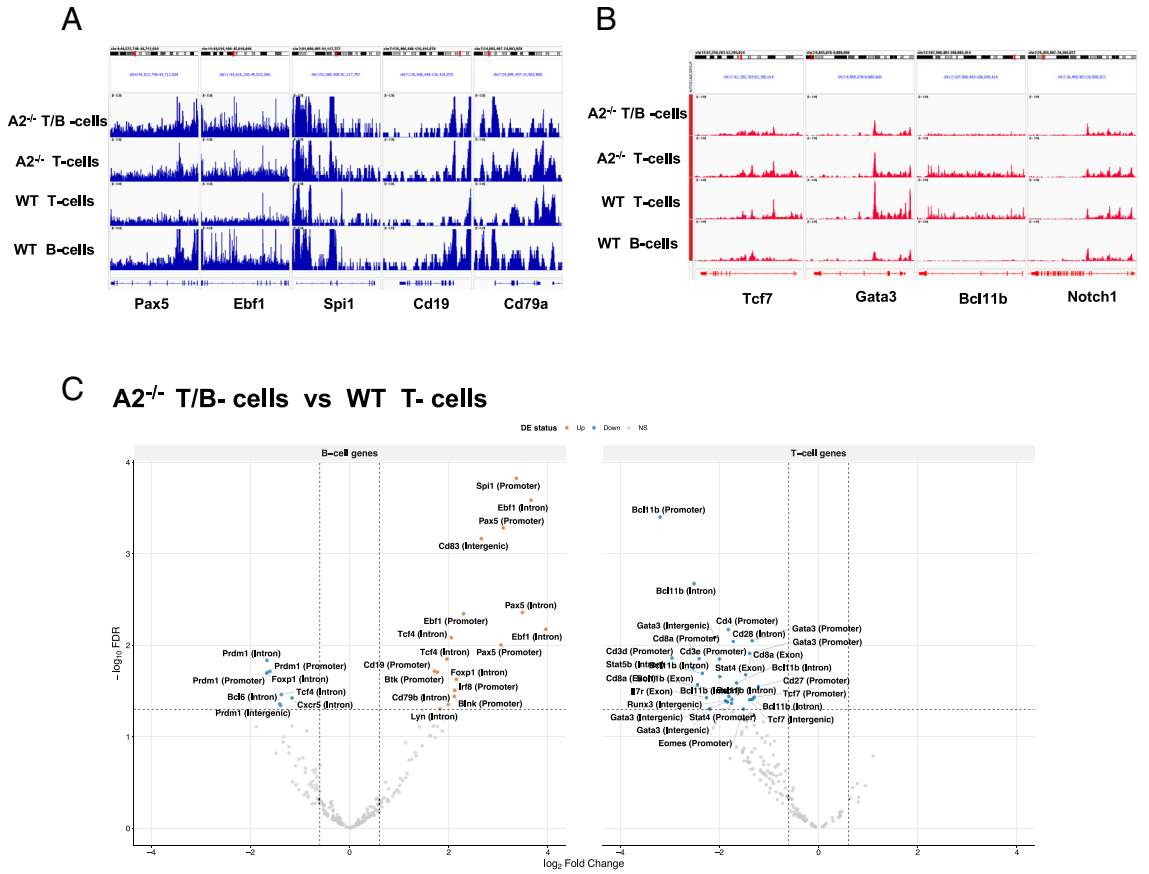

Fig. 5. APOBEC2 preserves T cell identity by restricting H3K27ac -associated activation of B cell lineage programs. Antibody guided Chromatic Tagmentation sequencing (ACT seq) was performed in sorted WT T and B cells, A2$^{-/-}$ T cells and A$^{-/-}$ T/B cells with an antibody against the activating histone mark H3K27ac was utilized. (*A* and *B*) Representative locus specific H3K27ac enrichment profiles of five key B cell identity genes (*A*) and four key T cell identity genes (*B*) in WT B cells, WT T cells, A2$^{-/-}$ T cells and A2$^{-/-}$ T/B cells. (*C*) Volcano plots depicting the B (*Left* part) and T (*Right* part) cell genes with enriched H3K27ac signal at regulatory elements in A2$^{-/-}$ T/B cells vs WT T cells

reduced H3K27 acetylation at loci encoding transcription factors critical for T cell identity in T cells (Fig. 5*B* and *SI Appendix*, Fig. S8*B*). This reduction may represent a secondary effect following activation of B cell–defining transcription factors such as Pax5, which has been shown to repress T cell lineage programs (34).

Human Patients With a Mutation in Apobec2 Also Exhibit the Emergence of T Cells With Perturbed Cell Identity. Given the distinct immunological phenotype we observed thus far, we wondered if it also existed in humans. Toward identifying individuals with variations in the *APOBEC2* gene, we queried the international registry of patients (35, 36) With inborn errors of immunity (IEI) to identify any potential genetically unsolved cases that also happened to have germline *APOBEC2* mutations. Specifically, we searched for rare coding variants of Apobec2 within whole-exome sequencing (WES) data following a filtering strategy that we have optimized and published previously (35, 37–41). We focused first on rare variants or private variants that would result in alterations within the protein sequence of APOBEC2, or which would be located in intronic regions predicted to impact the splicing of *APOBEC2*. In addition, we used in silico scores that have been developed to assess the functional impact of any such variants. 18 patients were identified to carry monoallelic or biallelic potentially deleterious *Apobec2* variants (*SI Appendix*, Table S1). The consensus negative selection score of *Apobec2* (CoNeS: 0.41) compared to the 483 known IEI genes (35, 36), would suggest an autosomal recessive model for APOBEC2 function in the immune system (as also supported by our findings in A2$^{-/-}$ mice).

We then filtered these 18 IEI cases for biallelic defects. Of note, only two siblings with homozygous *APOBEC2* variants were identified, and we reviewed the clinical presentations and immunologic profiling of these two siblings. The proband (P1) is a 15-y-old male born of consanguineous parents and with a normal history of vaccination. He presented with increasing tachypnea and weight stagnation from three weeks of age. At four months, an opportunistic infection with *Pneumocystis jiroveci* pneumonia was diagnosed. The boy underwent a detailed immunologic evaluation and was diagnosed with combined immunodeficiency due to low serum levels of IgA and IgM, normal lymphocyte counts, but an increased CD4/CD8 ratio due to borderline decreased cytotoxic T cell counts. Functional experiments also documented defects in the lymphocyte transformation test (LTT) to purified protein derivative (PPD) and Candida antigens. Except for oral trimethoprim-sulfamethoxazole, immunoglobulin treatment was initiated, first intravenously and later subcutaneously. During the follow ups the patient developed episodes of neutropenia and had elevated liver enzymes, mainly alanine transaminase ALT (*SI Appendix*, Table S2), an observation that is also present in *A2*$^{-/-}$ mice (*SI Appendix*, Fig. S9 *A–D*). P2 is the younger sister of P1. She is currently 10 years old and presents with similar clinical phenotypes (tachypnea with onset at one week of age, which underwent immunological investigation due to identification of IEI in the brother). Her evaluation confirmed that she shares her brother's immunological profile. Although hypogammaglobulinemia and defective LTT would suggest combined immunodeficiency in P2, the absolute CD8 counts were in the normal range

(*SI Appendix*, Tables S2 and S3). Both patients, at the time of the current study, were in good health under prophylactic antibiotic treatment and subcutaneous gamma globulin without HSC transplantation. Of note, at the latest follow-up, neither of them had developed musculoskeletal presentations.

Due to the close similarity of clinical presentations and immunological investigations, both patients underwent WES. As none of the known IEI genes were identified as causal, the patients were classified as unsolved cases. Our reanalysis of this family, focusing on *APOBEC2,* revealed that it is indeed the only gene with homozygous status (c.359C > A, p.T120N shared between the two patients—no other biallelic defect was common). Segregation analysis by Sanger sequencing confirmed the homozygous status of this rare missense variant (MAF=0.005) in the patients and the heterozygous status in their healthy parents (Fig. 6 *A*–*C*). Prediction of the impact of this mutation using the combined annotation-dependent depletion (CADD) score indicated the mutation is probably damaging (CADD:27.5 above the 95% CI mutation significant cutoff: 24.1). This was further supported by the DeepMind tool AlphaMissense, which was specifically evolved to merge sophisticated machine learning with structural biology. This evaluation indicated that T120N is a potentially deleterious variant affecting a highly conserved region of APOBEC2 protein (*SI Appendix*, Fig. S10*A*).

We then collected new samples from these two patients harboring the homozygous *APOBEC2*$^{T120N/T120N}$ mutation for lymphocyte subset analysis. Analysis revealed the emergence of CD3^{+}CD19^{+} cells within peripheral blood mononuclear cells (PBMCs) which was unrecognized in the routine hospital FACS analysis, mirroring observations in the A2$^{-/-}$ mouse model (Fig. 6*D*). Moreover, bulk RNAseq analysis from these CD3^{+}CD19^{+} cells demonstrated that at the transcriptional level, these cells exhibit downregulation of T cell differentiation pathways and upregulation of certain B cell–related pathways (*SI Appendix*, Fig. S10*B* and Datasets S3 and S4). Finally, this dataset was subjected to IPA pathway analysis which suggested that the CD3^{+}CD19^{+} cells downregulate critical genes involved in mammalian hematopoietic cell identity specification, including *STAT3* (42), *CEBPE* (43), and *IRF4* (44) (*SI Appendix*, Fig. S10*C*). Together, these findings in both mice and humans suggest a crucial role for APOBEC2 in regulating T cell identity within the lymphocyte lineage. In contrast, APOBEC2 dysfunction, such as through the inheritance of pathogenic variants, may have potentially harmful effects on immunity.

To understand the functional similarities between *Apobec2* -/- mice and in humans with the *APOBEC2* $^{T120N/T120N}$ variant, we transplanted hematopoietic stem and progenitor cells (HSPCs) from A2$^{-/-}$ mice, transduced with lentiviral particles carrying the coding sequence for Apobec2 (as a positive control), for the T120N variant or with ETV along with WT HSPCs (transduced with ETV). We found that *Apobec2* deletion impairs the repopulation capacity of donor cells, and this can be remedied with transduction with lentiviruses expressing the Apobec2 coding region. However, this impairment could not be rescued with lentiviruses expressing the T120N variant (Fig. 6*E*). Together, these findings in mice and humans suggest a key role for APOBEC2 within the

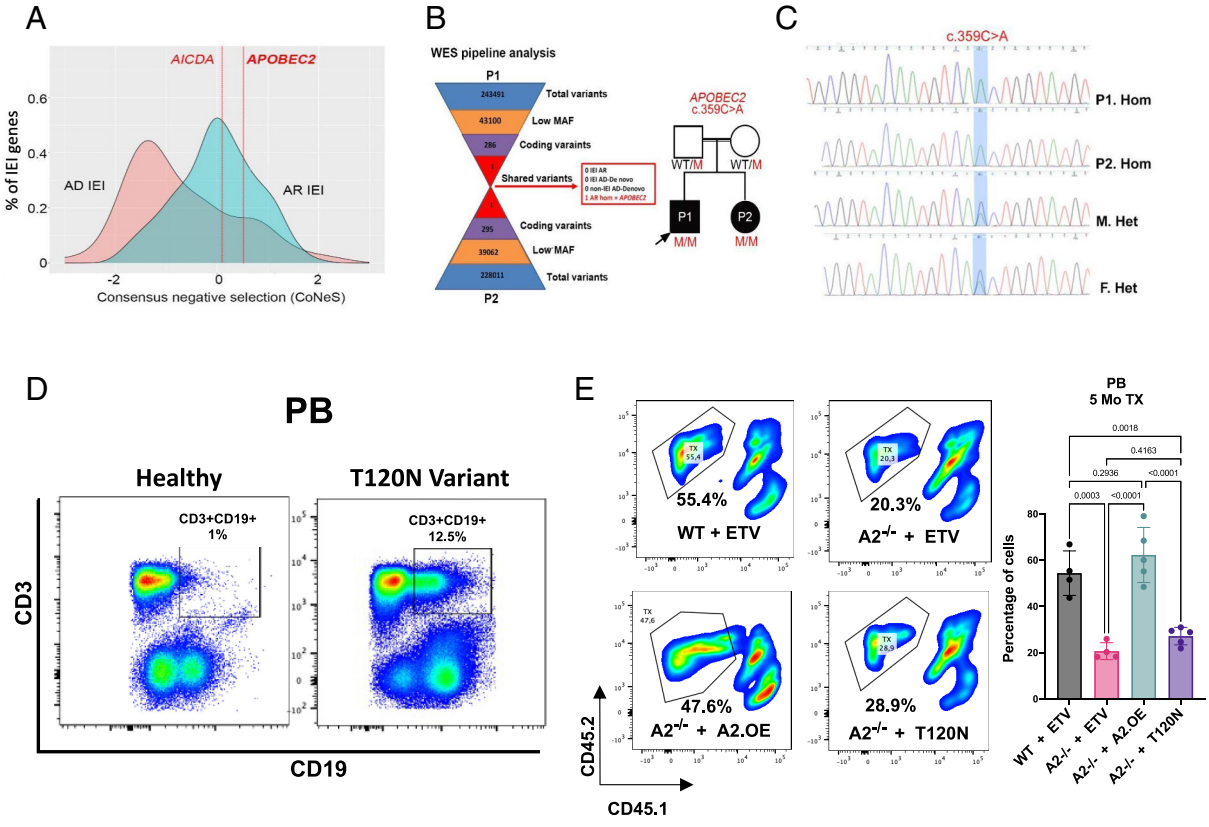

Fig. 6. Human patients with *APOBEC2* T120N mutation also exhibit the emergence of T cells with perturbed cell identity and functionality. (*A*) The distribution of consensus negative selection (CoNeS) scores for human known inborn errors of immunity (IEI) genes according to their dominant (AD), recessive (AR), mode of inheritance, indicates APOBEC2 deficiency should be a biallelic defect. (*B* and *C*) The whole-exome sequencing filtering for rare and damaging coding variants in two siblings indicated a homozygous APOBEC2 mutation confirmed by segregation analysis and Sanger sequencing. (*D*) FACS analysis of human mononuclear blood cells from a healthy mother and her daughter who carry the T120N variant. The data show the emergence of CD3^{+} CD19^{+} cells in the blood of T120N variants. (*E*) The frequency of donor transplanted cells (CD45.2+) in the blood of recipient mice 5 mo after transplantation of HSCs from WT and A2$^{-/-}$ mice that were transduced with empty vector (ETV), a vector containing the WT APOBEC2 (A2.OE) or the T120N variant.

T cell identity in the lymphocyte lineage, and that conversely, APOBEC2 dysfunction (through inheritance of pathogenic variants) has potentially deleterious effects on immunity.

Discussion

Recent work has established a role of APOBEC2 in muscle differentiation, demonstrating that it functions as a terminal repressor that binds to nonmuscle related genes, and silences their expression through the recruitment of chromatin remodeling factors. Interestingly, the silenced nonmuscle gene programs were reminiscent of T cell specification pathways, spurring our investigation, reported here, into the role of APOBEC2 in fate determination within cells of the lymphocyte lineage both in mouse and in human.

One of the key findings of the current study is the identification of a novel population of cells in the spleens of $A2^{-/-}$ mice which exhibit phenotypic characteristics of both T and B lymphocytes (namely, they express both T cell receptor and B cell receptor and can be activated through either – leading to their initial characterization as "dual-receptor expressors"). In addition to the dual-receptor phenotype, these cells are transcriptional "hybrids": They express genes that are key to the maintenance of a T cell fate (such as *Tcf7, Lef1, Gata3, and Bcl-11b*) but fail to shut down the expression of genes that are central to B cell identity (such as Pax5, Ebf1, Spib, and others). Here, we have designated these cells as "$CD3^+CD19^+$ T/B cells". This novel population was distinct enough from B or T cells to be detected as a separate set of cells in scRNA-seq data from $A2^{-/-}$ animals. Similar cells could also be detected in WT datasets, suggesting the existence of a population of dual-receptor lymphocytes whose further development requires proper APOBEC2 function (Fig. 1).

Lymphocyte populations with transcriptional characteristics similar to those of the T/B cells we report, have recently been identified as "dual expressors" by Zhang and colleagues (45). Similar to their findings, we also find that T/B cells make up ~1.5% of hematopoietic cells in the spleens of WT mice, suggesting that they represent an underexplored component of immune diversity. Loss of APOBEC2 leads to an expansion of this population to up to ~5% in $A2^{-/-}$ mice, however it is worth mentioning that even "proper" TCR^+CD3^+ T cells from $A2^{-/-}$ mice are of an intermediate phenotype with regard to their chromatin status (Figs. 4 and 5), suggesting that APOBEC2-derived deregulation broadly affects the T cell lineage (but that the transcriptomic phenotypes that result from this epigenetic deregulation are more stochastic). Similar cells are also detectable in human patients with mutations in the *Apobec2* gene (Fig. 6).

We hypothesize that APOBEC2 functions to promote T cell fate determination by shutting down B cell lineage-specific genes during development in the bone marrow. Low amounts of APOBEC2 might also be necessary for the maintenance of T cell fate (although this remains to be demonstrated at the transcriptional level). This hypothesis is supported by the heterogeneity observed within the proliferating $A2^{-/-}$ T/B cell population (Fig. 1 *I* and *J*). While some cells in this population retain the expression of T cell marker genes (and are likely the precursors of the T/B cells present in WT), the majority appear to lose substantial T cell marker expression and instead adopt a B cell–like identity (Fig. 1*J*). Thus, this T/B cell type that is present in the $A2^{-/-}$ might seem phenotypically similar to the T/B cells from the WT, but is mechanistically distinct and is predicated on loss of T cell fate maintenance either as a direct outcome of the Apobec2 depletion or as a secondary effect following B cell gene expression(Fig. 1).

Our analysis revealed substantial epigenomic alterations between WT and $A2^{-/-}$ cells, suggesting a profound influence of the APOBEC2 on chromatin accessibility. The clear separation between WT and A2–/– T cells in ATAC-seq and transcriptional datasets (Fig. 4) reflects significant chromatin structure differences, indicating that deletion of Apobec2 disrupts the overall chromatin landscape in T cells. Despite these widespread alterations, certain regulatory features appear conserved. The positional preference for transcription factor binding near transcription start sites remains largely unchanged between WT and $A2^{-/-}$ samples (Fig. 4*E* and *SI Appendix*, Fig. S7). This was further supported by global transcription factor footprinting analysis, which showed that, while certain motifs exhibited increased accessibility in $A2^{-/-}$ conditions, these changes were not uniformly distributed across all binding sites of enriched transcription factors (Fig. 4 and *SI Appendix*, Fig. S7). Therefore, the chromatin alterations in $A2^{-/-}$ cells may reflect localized, rather than global, disruptions in regulatory element accessibility.

Differential chromatin accessibility analysis in the absence of APOBEC2 revealed that T/B cells, compared with $A2^{-/-}$ T cells, adopt a more B cell–like chromatin landscape. This was evidenced by increased accessibility of motifs associated with B cell–defining transcription factors, including members of the PAX family, SPIB, SPI1, and POU2F2 (Figs. 4 *B–E* and 5 *A* and *C*). These findings suggest that loss of APOBEC2 promotes a shift of T/B cells toward a B cell–like epigenetic state, highlighting a role for APOBEC2 in maintaining T cell lineage integrity. However, comparison of $A2^{-/-}$ T/B cells with WT B cells revealed that $A2^{-/-}$ T/B cells still retain elevated accessibility at T cell–associated motifs, including TCF7, BCL11B, and GATA3 (Figs. 4 and 5 and *SI Appendix*, Fig. S7*B*). Thus, $A2^{-/-}$ T/B cells represent a bona fide hybrid population that exhibits both T and B cell epigenetic and transcriptional features. This dual identity likely reflects a unique or aberrant differentiation process that results to the generation of cells that possess properties of both B and T cells (Fig. 2). Collectively, these findings suggest that while APOBEC2 contributes to the regulation of a transitional T/B population during normal lymphocyte development, its deletion expands this population, enabling its more detailed characterization. These observations further highlight the importance of investigating the upstream mechanisms that trigger activation of the B cell program in the context of Apobec2 deletion. In particular, it will be important to determine whether lineage-specifying regulatory pathways, such as Notch signaling, are altered in the absence of APOBEC2. Elucidating how Apobec2 loss influences these regulatory networks may provide further insight into the mechanisms driving the observed shift toward a B cell–like transcriptional program.

Previous studies have reported the emergence of dual-identity T/B lymphocyte populations in various contexts. Studies of $CD3^+CD20^+$ cells (a population similar to $CD3^+CD19^+$) in humans have shown that these cells often possess memory-like characteristics and exhibit strong cytokine production capabilities (46–48). Additionally, dual-receptor-expressing cells have been reported to participate in responses to infection, producing both cytotoxic granules and cytokines such as IL-17 and TNF-a (49). Further support for a specific role in T/B cells in human immune responses also comes from studies in patients with type 1 diabetes, suggesting a maladaptive involvement of their dual receptors in the establishment or maintenance of autoimmunity (50). However, all these studies have been hampered by suspicion in the field that the cells in question were simply doublets, and therefore that the dual staining was an artifact (51, 52). Our studies suggest that this is not the case and that in fact, dual-identity T/B lymphocytes

are a previously unrecognized (and potentially only transiently present) subset in lymphoid organs that could have specific roles to play in tissue homeostasis or under conditions of infection or transformation.

Materials and Methods

Mice. All mice used in this study are of the C57BL/6J background. The mice were obtained from internal breeding or an external provider (JANVIER) and Cyagen.

Flow Cytometry. Flow cytometry and cell sorting were performed using BD instruments, with data analyzed in FlowJo v10. Cells were stained with antibody panels targeting major immune populations, including T cells (CD3, CD4, CD8), B cells (CD19, B220, IgM), and myeloid cells (CD11b, Gr-1), as well as TCRβ and BCR (IgM/IgG) markers. Donor and recipient cells in transplantation experiments were distinguished using CD45.1 and CD45.2 markers. Hematopoietic stem and progenitor cells (HSPCs) were identified by lineage depletion followed by staining for key markers. Additional assays included T cell activation (CD69), thymocyte characterization (CD44, CD25), and analysis of human PBMCs using CD3 and CD19 markers.

Human samples: Ethics Approval and Informed Consent. The full study protocol was reviewed and approved by the ethics committee of the German Cancer Research Center (DKFZ) in Heidelberg. All patient information and biological samples were collected and used in accordance with the principles of the declaration of Helsinki and approved by DKFZ. Written informed consent was obtained from all participants prior to inclusion in the study.

Bulk RNA-seq. Immunophenotypically defined T cells ($CD3^+$, $CD19^-$, $CD4^+$ or $CD8^+$) were sorted by fluorescence-activated cell sorting (FACS) into Eppendorf tubes (1×10^5 cells per sample). Total RNA was extracted according to the manufacturer's instructions. Libraries were prepared and sequenced on a NovaSeq 6000 platform using paired-end 50 bp reads. Differential gene expression analysis was performed using DESeq2 with thresholds of adjusted P-value ≤ 0.05 and |og2 fold change > 1.5. Data visualization included heatmaps and volcano plots.

Global 10× Single-cell RNA-seq. Bone marrow, spleen, and thymus cells from wild-type and knockout mice were isolated, enriched, and stained with lineage-specific antibodies prior to sorting into defined populations. Single-cell libraries were generated using the 10× Genomics platform. Doublets were identified and removed using DoubletFinder. Sequencing reads were aligned to the mm10 mouse genome using Cell Ranger. Downstream analysis was conducted in Seurat. Dimensionality reduction (PCA, UMAP) and Louvain clustering were used to identify cellular populations. T cell subsets were defined based on established marker genes and annotated using reference-based tools alongside manual curation.

10× Single-Cell RNA-seq and V(D)J Sequencing. Single-cell RNA-seq and V(D)J sequencing were performed on sorted spleen and thymus cells from wild-type and knockout mice using the 10× Genomics platform. TCR and BCR sequences were reconstructed and integrated with transcriptomic data to assess clonal diversity and repertoire overlap. Data were processed using Cell Ranger and analyzed in Scanpy, including filtering, normalization, and exclusion of receptor-related genes. PCA and UMAP were used for dimensionality reduction and visualization. T and B cell populations, including dual-receptor-expressing cells, were identified. Gene expression scoring, transcription factor activity inference, and repertoire analyses were performed, with selected rearrangements validated by PCR and amplicon sequencing.

ATAC-seq. Bulk ATAC-seq was performed on spleen-derived immune cells following nuclei isolation and tagmentation. Libraries were sequenced using paired-end reads. Data were processed using the nf-core/atacseq pipeline aligned to the mouse genome. Differential chromatin accessibility was assessed using DiffBind and DESeq2. Motif enrichment, genomic annotation, and functional enrichment analyses were performed, and transcription factor binding was evaluated using footprinting approaches.

ACT-seq. Bulk ACT-seq libraries were generated using antibody-guided chromatin tagmentation targeting H3K27ac-marked regions. Cells were permeabilized, incubated with a pA-Tn5 transposome complex, and subjected to Mg^{2+}-induced tagmentation. DNA fragments were purified, PCR-amplified, and sequenced. Peaks were called, filtered, and quantified, followed by genomic annotation. Differential accessibility between conditions was analyzed using DESeq2 with thresholds of adjusted P-value < 0.05 and |og2 fold change > 0.6.

Data, Materials, and Software Availability. The raw data from sequencing were deposited on the NIH submission portal with the following accession numbers: PRJNA1193500 (53) for CD4 and CD8 Bulk RNA-seq, PRJNA1187346 (54) for the scRNA-seq on the hematopoietic system, PRJNA1187471 (55) for ATAC sequencing, PRJNA1193856 (56) for Human Bulk RNA-seq, and ENA repository: PRJEB103940 (57) for 10× VDJ GEX scRNA-seq in spleen, PRJEB109736 (58) for 10× VDJ GEX scRNA-seq in thymus, PRJEB109743 (59) for the ACT dataset, and PRJEB110172 (60) for thymus and BM 10× scRNA-seq. This paper does not report original code.

ACKNOWLEDGMENTS. We thank the DKFZ Single-Cell Open Lab (scOpenLab) and genomics facility for assistance with all sequencing experiments. We also thank the flow cytometry facility of DKFZ for their support and Mr. Tobias Rubner for his guidance with Image stream experiments and data analysis. We also thank the animal facility and animal welfare officers at DKFZ for their support. Finally, we thank Prof. Hans-Reimer Rodelwald for providing the cre lines.

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