**An early decline in ETPs reflects reduced pre-thymic progenitors and altered signals from the thymus microenvironment**

**Short title: Early ETP decline reflects BM and thymus changes**

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**Abstract**

Age-associated thymus involution results in decreased generation and export of T cells, with an associated increase in susceptibility to pathogens. Elucidating mechanisms underlying diminished T-cell production during involution will inform strategies to restore thymopoiesis with age. T-cell development depends on periodic thymic colonization by circulating bone marrow (BM)-derived thymus seeding progenitors (TSPs). Upon thymic entry, TSPs differentiate into early T-cell progenitors (ETPs), which give rise to all downstream thymocyte subsets. Here, we find a significant decline in ETP cellularity between 1 and 3 months (3MO) of age, mirrored by reduced total thymocyte cellularity. This early thymic involution could be driven by changes in pre-thymic progenitors and/or thymic stromal cells. Using a multicongenic progenitor transfer approach, we determined that the number of TSP-supportive niches does not diminish through 12MO. However, the number of lymphoid progenitors in BM and blood declines substantially by 3MO of age, although their cell-intrinsic capacity to seed and differentiate in the thymus is unimpaired, indicating that pre-thymic hematopoietic changes contribute to the early decline in ETPs. Moreover, sustaining thymic epithelial cells maintains ETP cellularity with age, despite the reduction in circulating TSPs, supporting the idea that age-associated defects in ETP niche quality also contribute to early involution. Consistent with this possibility, Notch signaling, which is essential for T-lineage differentiation, diminishes in 3MO BM progenitors and in ETPs. Together, our findings suggest that a reduction in both BM lymphopoiesis and thymic stromal support contribute to the initial age-associated decline in ETPs, setting the stage for progressive thymic involution.

## Introduction

T cells develop in the thymus which provides a unique and complex stromal microenvironment essential for T-cell lineage commitment, differentiation and selection[1,2]. Since thymocytes do not have the ability to self-renew, the thymus periodically recruits bone marrow (BM)-derived thymus seeding progenitors (TSPs) which are essential to maintain T cell production throughout life[3]. TSP entry is a well-orchestrated multistep process mediated by the adhesion molecules P-selectin, ICAM-1, and VCAM-1, the chemokines CCL25 and CCL21, and other niche factors, like KITL, supplied by thymic endothelial cells[4-8]. After entering the thymus via vasculature at the cortico-medullary junction, TSPs differentiate into highly proliferative $CD4^{-}$ $CD8^{-}$ $CD44^{hi}$ c-$kit^{hi}$ early T-cell progenitors (ETPs) in the thymic cortex. Commitment of developing thymocytes to the T-cell lineage depends on inductive signals provided by cortical thymic epithelial cells (cTECs)[1,2,9,10]. In particular, activation of Notch signaling in ETPs via Delta-like Notch ligand 4 (DLL4) expressed by cortical thymic epithelial cells (cTECs) is indispensable for T-cell lineage commitment and differentiation[11-13]. Since ETPs give rise to all downstream T cell subsets, thymopoiesis and T cell development depend on both continued influx of TSPs into the thymus and the availability of functional stromal niches that support TSP entry and downstream ETP differentiation.

During age-associated thymus involution, thymus size and cellularity undergo a progressive decline accompanied by reduced output of naïve T cells. Thymus involution is initiated early in life, by ~7 weeks of age in mice[14,15]. Thus, we first asked if ETP cellularity is adversely affected during the earliest stages of thymus involution. Here, we find that the decline in ETP cellularity is initiated early, between 1MO and 3MO of age in mice, and further declines with advancing

age. Thymus involution involves not only a reduction in thymocyte cellularity, but also a decline in the number, composition, proliferative capacity, and organization of the TEC compartment[16-20]. TEC degeneration is a key driver of age-associated thymic involution, as sustaining or restoring TECs prevents or reverses involution, respectively[14,21-24]. Given that thymic stromal niche factors play a key role in recruitment of TSPs, and thymic stromal signals are required for ETP survival, proliferation, and T-lineage commitment, we anticipated the early age-associated decline in ETPs would be driven by changes in the thymic microenvironment.

Here, we used several approaches to determine whether the early loss of ETPs is due to age-related changes in availability of functional TSP/ETP niches and/or a decline in pre-thymic lymphoid progenitors. First, we used a multicongenic progenitor transfer approach to quantify the number of functional TSP niches from the onset of involution through middle-age[25]. These studies revealed that the number of TSP niches is sustained, despite substantial involution, through 12MO of age. We thus considered whether the reduction in ETP cellularity could be due to a decline in the hematopoietic progenitors that seed the thymus. Surprisingly, we observe a significant decline in the number of lymphoid progenitors with T-lineage potential in the BM and blood at MO of age, although their cell-intrinsic ability to seed and differentiate in the thymus remains intact. Because Notch signaling plays a key role in promoting T-lineage specification and progenitor differentiation, we also interrogated niche function by evaluating *Dll4* expression by cTECs and assessing Notch signaling activity in ETPs and BM progenitors. Notably, we find reduced Notch signaling in T-lineage precursors in both the BM and thymus by 3MO of age. Collectively, these findings demonstrate a surprisingly early decline in BM lymphoid progenitors and circulating TSPs, along with diminished Notch signaling in the BM and

thymus microenvironments, implicating both pre-thymic and thymic changes in the early decline of ETP cellularity at the outset of thymus involution.

## Methods

### Mice

C57BL/6J (CD45.2, Thy1.2), B6.PL-Thy1a/CyJ 9 (CD45.2, Thy1.1), B6.SJL-*Ptprc*$^{a}$ *Pepc*$^{b}$/BoyJ (CD45.1, Thy1.2), C57BL/6-Tg(CAG-EGFP)1Osb/J (GFP)[26], Tg(Cp-HIST1H2BB/Venus)47Hadj/J (Notch-Venus reporter)[27], and K5.cyclin D1[28] (Conti lab, MD Anderson Cancer Center, Smithville, TX) mice were obtained from Jackson Laboratories, except as specified, and bred in-house. F1 mice for multicongenic barcoding experiments were bred in-house from CD45 congenic, Thy1 congenic, and EGFP strains (supplemental Figure 2B). C57BL/6J recipient mice for multicongenic barcoding experiments were obtained from the National Institute of Aging. All mice were maintained under specific pathogen-free conditions at the animal facilities at the University of Texas at Austin and the University of Texas MD Anderson Cancer Center, Smithville. All experimental procedures were performed in accordance with the Institutional Animal Care and Use Committees.

### Multicongenic barcoding progenitor transfer assay

BM cells were obtained from long bones of 4-6-week-old mice of 8 different congenic backgrounds (supplemental Figure 2B). Bones were crushed and single cell suspensions were prepared in FACS wash buffer (PBS+ 2% FBS), followed by RBC lysis (RBC lysis buffer, BioLegend). Cells were stained with lineage (Lin)-specific antibodies against CD11b, Gr-1, Ter-119, B220, CD19, CD3 and CD8 (all from BioXCell), and Lin$^{+}$ cells were depleted with sheep anti-

rat IgG immunomagnetic beads (Dynabeads, Invitrogen). Cells were then immunostained and $Lin^-Flk2^+CD27^+$ BM progenitors from F1 congenic strains were FACS purified (supplemental Figure 2A). Progenitors from each of the 8 congenic strains were mixed at equal ratios, and a total of 100,000 cells were retro-orbitally injected into non-irradiated 1MO, 3MO, 6MO and 12MO C57BL/6J recipient mice. Thymocytes were analyzed after 21 days by flow cytometry to distinguish the contributions of distinct congenic donor strains. 2.5 million events were recorded per recipient thymus, and successful thymic colonization was scored if a minimum of 40 cells of a given donor strain was detected. Quantification of niches was performed using a multinomial sampling algorithm (see Statistical Analysis).

**Competitive heterochronic progenitor seeding assay**

$Lin^-Flk2^+CD27^+$ progenitors were isolated from the long bones of 1MO and 3MO congenic donor mice as described above. 1MO and 3MO progenitors were mixed at 1:1 ratio and 100,000 cells were retro-orbitally injected into 1MO and 3MO non-irradiated C57BL/6J recipient mice. After 21 days, donor chimerism was analyzed in the recipient thymi by flow cytometry.

**Statistical analyses**

Bayesian estimation to quantify the number of TSP niches was performed using R (The R Foundation for Statistical Computing) (see Supplemental Methods). Statistical tests were done using Prism (V9.2.0; GraphPad Software), and significance was determined using an unpaired Student *t*-test, 1-way analysis of variance (ANOVA) with Kruskal-Wallis multiple comparisons test or 2-way ANOVA with Sidak's multiple comparisons test, as indicated in the figure legends.

**See supplemental methods for additional experimental detail**.

## Data Sharing Statement

For original data, please contact lehrlich@austin.utexas.edu or erichie@mdanderson.org

## Results

### *ETP and total thymocyte cellularity decline by 3MO of age*

We first quantified age-associated changes in ETPs and downstream thymocyte subsets between 1 and 12MO of age (Figure 1; supplemental Figure 1A). Total thymus cellularity declines with age, dropping significantly by 2MO, consistent with previous reports that thymus involution begins early in life (Figure 1A)[14,15,20]. Notably, ETP cellularity also declines progressively, starting at 1.5MO, which precedes, but otherwise mirrors the reduction in total thymocytes (Figure 1A). There is a strong correlation between ETP and total thymocyte numbers through 12MO of age (Figure 1B), and this correlation extends to subsequent thymocyte subsets (supplemental Figure 1B), showing that increased numbers of downstream thymocytes do not compensate for the loss of ETPs with age. Moreover, the consistent DN2 to ETP ratio over the first year of life further suggests that ETP differentiation is not impaired with age (Figure 1C).

### *The number of available thymic niches for TSPs does not decrease with age*

The age-associated decline in ETP cellularity could be a function of changes in the thymic stromal microenvironment and/or defects in pre-thymic lymphoid progenitors. Given the important role of thymic microenvironment in supporting all stages of developing T cells and its known impact on involution, we first tested whether the reduction in ETPs is due to an age-

related decline in the number of functional progenitor niches that support early stages of thymopoiesis. We used a modification of a previously reported multicongenic progenitor transfer approach to quantify available, functional TSP niches from 1MO-12MO of age. This approach previously revealed that a young thymus contains ~10 available niches that can be colonized by thymocyte progenitors at any one time[25]. We FACS sorted $Lin^{-}Flk2^{+}CD27^{+}$ BM progenitors, which encompass all thymus seeding and T cell progenitor activity in the BM[29], from 8 different congenic strains, mixed them at equal ratios, and transplanted them into non-irradiated recipient mice of different ages (Figure 2A; supplemental Figure 2A-B). Recipient thymuses were analyzed after 21 days to allow sufficient time for donor progenitors to complete one wave of T-cell differentiation[29]. To determine the number of missing congenic tags, we quantified the number of different donor strains present in each recipient thymus by flow cytometry (supplemental Figure 2C), and then used mathematical modeling to estimate niche numbers. If the number of available niches that support TSP seeding declines with age, then fewer donor strains will be detected in thymuses of older recipients. However, we found that the number of available, functional TSP niches does not decline with age; to the contrary there is a trend toward an increased number in 6 and 12MO recipients (Figure 2B-C). Therefore, the age-associated decline in ETP cellularity is not due to a reduction in the number of functional niches available to thymus seeding progenitors.

***1MO and 3MO T-cell progenitors seed and thrive comparably in the thymus environment***

We next asked whether the decline in ETPs reflects intrinsic defects in 3MO TSPs, compromising their ability to seed and differentiate in available thymic niches. Thus, we

performed competitive heterochronic progenitor transfer assays in which isolated $Lin^-$ $Flk2^+CD27^+$BM progenitors obtained from 1MO and 3MO congenic donor strains were mixed at an equal ratio and injected i.v. into 1MO versus 3MO non-irradiated recipients. If BM progenitors from 3MO donors have intrinsic defects, then we would expect to detect fewer 3MO compared to 1MO donor-derived cells in recipient thymuses. However, comparable percentages and numbers of 1MO and 3MO donor cells were recovered from recipient thymuses of both ages (Figure 3A-B), indicating that TSPs from 3MO donors are fully competent to seed and thrive in the thymus. Moreover, there is no apparent defect in the differentiation capacity of 3MO TSPs, as a similar percentage of DP thymocytes are generated from 1MO versus 3MO donor cells (Figure 3C).

We next considered the possibility that changes in proliferation or survival could account for the decline in ETPs at 3MO. However, a comparable percentage of 1MO and 3MO ETPs incorporated BrdU (Figure 3D-E), and analysis of intracellular cleaved caspase-3 showed that apoptosis is negligible in ETPs at both 1MO and 3MO of age (supplemental Figure 3). Taken together, the data suggest that the decline in ETP numbers at 3MO of age is not a consequence of defects in the ability of 3MO thymus seeding progenitors to thrive in the thymus.

***Lymphoid progenitors with T-lineage potential decline in the BM and blood of 3MO mice***

As we found no apparent defects in either the availability of functional TSP niches with age or the ability of 3MO TSPs to enter and thrive in those niches, we asked whether the number of BM or circulating lymphoid progenitors decreases by 3MO of age. A significant reduction in the number of TSPs could play a key role in the age-associated decline in ETP cellularity. To assess

this possibility, we used flow cytometry to quantify circulating Lin$^-$Flk2$^+$CD27$^+$ cells, which comprise all thymic seeding activity in the blood[29] (Figure 4A). We also quantified upstream Lin$^-$Flk2$^+$CD27$^+$ lymphoid progenitors in the BM (Figure 4C). Interestingly, the frequency and number of Flk2$^+$ CD27$^+$ lymphoid progenitors in both blood and BM undergoes a significant decline between 1MO and 3MO of age (Figure 4B, D). Flk2$^+$ MPPs and Ly6d$^-$ CLPs are the lymphoid progenitors with T-lineage potential within the Lin$^-$Flk2$^+$CD27$^+$ compartment, and both of these subsets also decline significantly by 3MO of age (Figure 4E-F). These results demonstrate a surprisingly early reduction in the number of BM lymphoid progenitors and circulating TSPs, indicating that the decrease in ETP cellularity by 3MO is likely due, at least in part, to a reduction in the number of progenitors available to enter the thymus and differentiate therein.

***Sustaining TEC proliferation prevents the age-associated decline in ETP cellularity***

cTECs provide a critical supportive niche for ETP development, suggesting the age-associated deterioration of the TEC compartment could also contribute to the reduction in ETPs by 3MO of age. This scenario is supported by analysis of thymocyte and ETP cellularity in K5.D1 transgenic mice in which a keratin 5 promoter drives Cyclin D1 transgene expression in TECs, but not in hematopoietic or non-TEC stromal cells. The K5.D1 transgene promotes cell cycle progression and sustained growth of the TEC compartment, which in turn, prevents age-associated thymus involution[21]. Sustaining the TEC compartment in K5.D1 mice prevents the decline in ETP and total thymocyte cellularity with age (Figure 5A-B). However, the number of circulating Flk2$^+$CD27$^+$ progenitors is comparable in 1MO K5.D1 versus non-transgenic wild-type

(WT) control mice, and these cells undergo a similar decline by 3MO of age in both genotypes (Figure 5C). Moreover, the number of Ly6d$^-$ CLPs declines comparably in K5.D1 and WT mice by 3MO, with a similar trend for BM Flk2$^+$ MPPs (Figure 5D-E). These results indicate that despite a decrease in BM lymphoid progenitors and circulating TSPs, sustaining the TEC compartment is sufficient to prevent the decline in ETPs. Thus, both a decline in lymphoid progenitors and reduced TEC function could contribute to the age-associated reduction in ETPs in WT mice.

***Notch signaling is downregulated by 3MO in ETPs and BM lymphoid progenitors***

Activation of the Notch signaling pathway in ETPs is required for their development and T-cell lineage commitment. Our previously reported gene expression data show that expression of the Notch ligand *Dll4* is downregulated in cTECs between 1 and 3MO of age[30]. Quantitative RT-PCR analysis of isolated 1MO and 3MO cTECs confirms reduced *Dll4* expression by 3MO of age (Figure 6A). In addition, the level of NOTCH1 expression on ETPs declines significantly by 3MO (Figure 6B). To determine if the decreased levels of NOTCH1 on ETPs and reduced *Dll4* expression by cTECs at 3MO of age are associated with diminished Notch signaling in ETPs, we used the CBF-H2B-Venus strain in which expression of an H2B-Venus fusion protein is driven by Notch activation[27]. Due to perdurance of the H2B-Venus fluorescent signal, this reporter identifies cells that are currently undergoing or have recently undergone Notch signaling. We first tested whether this reporter is faithfully expressed in immature thymocyte subsets known to undergo Notch-signaling. As expected, reporter levels were high in ETPs and DN2s and dropped in the DN3 and DN4 subsets (supplemental Figure 4). Notaby, the frequency of ETPs undergoing Notch signaling declines between 1MO and 3MO of age (Figure 6C-D). Also, the

lower relative mean fluorescence intensity (RFI) of the Venus reporter in 3MO ETPs indicates a reduced level of Notch signaling.

Notch signaling in lymphoid progenitors in the bone marrow also plays a critical role in generation of T-lineage progenitors[31,32]; thus, we considered the possibility that changes in Notch signaling could contribute to the reduction in BM lymphoid progenitors at 3MO of age. Although the frequency of Notch-signaled Flk2$^{+}$ MPPs and Ly6d$^{-}$ CLPs was comparable at 1MO and 3MO (Figure 6E-H), there was a notable enrichment of Ly6d- CLPs expressing low levels of the Venus reporter in 3MO relative to 1MO mice (Figure 6I-J). This suggests that by 3MO of age, Notch signaling wanes in Ly6d- CLPs in the BM. Taken together, these data indicate that the early decline in ETP cellularity is associated with diminished Notch signaling activity in ETPs and in BM lymphoid progenitors.

**Discussion**

It is well established that thymus cellularity and T-cell output decline during age-associated thymus involution[17]. While prior studies focused on comparative analysis of thymuses from young mice (1-4MO of age) versus severely involuted thymuses from middle-aged and old mice (9-28MO of age), the present investigation focuses on changes that occur during the initial stages of thymus involution between 1 and 3MO of age. In agreement with other reports[14,15,20], we find a significant decline in thymus cellularity by 3MO of age. Furthermore, the progressive reduction in total thymocyte numbers with age is highly correlated with a reduction in the number of ETPs, which contain progenitors that give rise to all subsequent stages of developing T cells. These results indicate that the early decline in ETPs plays a key role in initiating age-

associated thymus atrophy. Given that TSPs, ETPs and all subsequent stages of developing T cells rely on inductive and supportive signals from the thymic microenvironment, which undergoes profound age-associated changes that have been shown to be critical in driving thymus involution, we first tested whether a reduction in the thymic niches that support TSPs and ETPs are diminished by 3MO of age. Unexpectedly, we did not find an age-related decline in the number of functional, available TSP niches between 1 and 12MO of age. In fact, there was a slight increase in these niches by 12MO of age. Thus, we subsequently investigated whether the early decline in ETP numbers reflects a reduction in BM and/or circulating T-cell progenitors. Our studies reveal a dramatic decline in circulating Flk2$^{+}$CD27$^{+}$ progenitors, which contain TSPs, as well as a reduction in BM lymphoid progenitors by 3MO of age. Furthermore, expression of the Notch ligand DLL4 by cTECs is reduced by 3MO, as is Notch signaling activity in ETPs and BM-resident CLPs. Overall, our results suggest that a decline in pre-thymic lymphoid progenitors in the BM and blood, along with changes in the BM and thymic stromal microenvironments, contribute to the early onset of thymus involution by 3MO of age.

It has previously been established that hematopoietic progenitors in the BM become myeloid-biased with age. For example, transplantation experiments revealed that HSCs from aged mice are biased towards a myeloid fate[7,33-36]. Moreover, fate mapping studies have shown that MPPs from old mice transit more efficiently into common myeloid progenitors (CMPs) than CLPs[36]. Nevertheless, the differentiation potential of lymphoid-biased HSCs on a per cell basis does not decline with age[37,38]. These prior studies assessed the decline in BM lymphoid potential between young (1-4MO) and much older mice (10-28MO). Since we find that ETP numbers decline by 3MO of age, we explored the possibility that a reduction in BM lymphoid

progenitors contributes to the early decline in ETPs. Importantly, we found a striking decrease in the number of circulating TSPs, and the number and percentage of the BM progenitors Ly6d$^-$ CLPs and Flk2$^+$ MPPs at 3MO. Thus, diminished production and/or egress of T-lineage progenitors from the BM likely contribute to the early reduction in ETPs. Interestingly, a fetal-derived lymphoid-biased HSC subset persists in the BM until at least 2 weeks of age before being lost in adult mice[39]. Also, embryonic-derived hematopoietic progenitors contribute to thymopoiesis through 7 weeks of age[40]. These studies raise the possibility that the early decline in BM lymphoid progenitors and ETPs could reflect a reduced contribution of embryonically derived hematopoietic progenitors. Of note, competitive heterochronic progenitor transfer assays, in which equivalent numbers of congenic 1MO and 3MO Flk2$^+$CD27$^+$ BM progenitors were injected into non-irradiated recipients of either age, showed that there is no cell-intrinsic defect in the ability of 3MO lymphoid progenitors to seed the thymus or differentiate therein. To our knowledge, the present study is the first to show a substantial reduction in BM lymphoid progenitors and circulating TSPs by 3MO of age.

As lymphoid progenitor activity is regulated by niche factors in the thymus and BM microenvironments, age-associated changes in stromal cells could also play a role in the early decline of ETPs and BM lymphoid progenitors. In this regard, heterochronic BM transplantation experiments showed that successful T-cell reconstitution depends on the age of the recipient thymus rather than on the age of donor hematopoietic progenitors[41,42], consistent with the notion that thymic stromal support of thymopoiesis wanes with age. Alterations in the composition, organization and/or function of the thymus microenvironment could impair seeding and/or differentiation of TSPs, which enter the thymus on a periodic basis to occupy a

limited number of stromal cell niches[25,43,44]. Therefore, we asked whether the availability of functional TSP niches diminishes with age. Using multicongenic barcoding progenitor transfers, combined with mathematical modeling, thymuses from 1MO and 3MO old mice were found to contain a comparable number of available TSP niches. Thus, the early decline in ETP numbers is not a function of reduced TSP niche availability. To the contrary, our results showed an upward trend in the number of TSP niches with age. Previous investigations have reported evidence for a feedback loop in which key TSP niche factors and T-cell progenitor entry are restricted by the number of intrathymic T-cell precursors[7,25,45]. Since we find a substantial reduction in the number of circulating TSPs and intrathymic ETPs as early as 3MO of age, it is possible that the observed increase in available TSP niches with advancing age is due, at least in part, to a decline in feedback from TSPs and/or ETPs.

After TSPs transition to the ETP stage, cTECs play a critical role in supporting their survival, proliferation and T-lineage commitment by providing niche factors, like DLL4, KITL and IL-7[2,3,46]. Given that the TEC compartment deteriorates during thymus involution, we considered the possibility that ETP niche factors become diminished with age. The essential role of TECs in sustaining thymopoiesis was demonstrated by reversing the age-dependent loss of FOXN1, a key transcription factor required for TEC differentiation and proliferation. Upregulating *Foxn1* in the TEC compartment of old, severely involuted thymuses partially reversed thymus involution by restoring TEC cellularity, which resulted in increased numbers of both ETPs and total thymocytes[23]. Consistent with these findings, we show that the number of ETPs and downstream thymocyte subsets do not decline with age when TEC deterioration and thymus involution are prevented by expression of a Cyclin D1 transgene in TECs of K5.D1 mice.

Together, these data support the concept that TEC-derived signals play an essential role in ETP homeostasis and suggest that age-related changes in the TEC compartment contribute to the decline in ETPs.

It is well established that Notch signaling plays a key role in T-lymphopoiesis[2,11,13,31,32,47]. NOTCH1 is expressed on lymphoid progenitors in the BM and on ETPs in the thymus, while the critical Notch ligand DLL4 is expressed by vascular endothelial cells in both BM and thymus as well as on cTECs[2,31,32]. Several observations in the present report suggest that diminished Notch signaling contributes to the early decline in BM lymphoid progenitors and ETPs between 1MO and 3MO of age. We found that lower levels of NOTCH1 are expressed on ETPs at 3MO of age, when *Dll4* expression is reduced on cTECs. Subsequent analysis of Notch signaling using the CBF-H2B-Venus Notch reporter strain[27] demonstrated an age-related decline in Notch signaling in ETPs, as well diminished Notch signaling in BM-resident CLPs. This decline may be due to deterioration of functional vascular and/or TEC niches. It is interesting to note that while the number of available TSP niches does not decline by 3 months, the ETP niche in the cTEC compartment may not be fully capable of supporting the progenitors that seed the thymus and enter the parenchyma, resulting in a lower number of detectable ETPs and downstream progeny. We did not find an increase in apoptosis of ETPs in the 3mo thymus, which might be expected in this scenario, but it is possible that clearance of ETPs undergoing apoptosis is quite rapid.

Altogether, our collective results indicate that an early age-associated decline in Notch signaling in T-cell progenitors could result in a reduced number of BM lymphoid progenitors, circulating TSPs and thymic ETPs at the outset of thymic involution. This potential link will be

further evaluated in future studies. In summary, this study shows that an early age-associated decline in BM lymphoid potential and alterations in thymic stromal cells during the first 3MO of life synergize to initiate the earliest stages of thymus involution.

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**Authorship contributions**

J.S., A.V., E.R. and L. E. designed the experiments and wrote the manuscript; J.S., A.V., H.S., E.P., performed experiments and analyzed data; B.L. and S.S. performed statistical analyses; A.K., E.R. and L.E. edited the manuscript.

**Disclosure of conflict of interest**

The authors declare no conflicts of interest.

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**Figure 1**

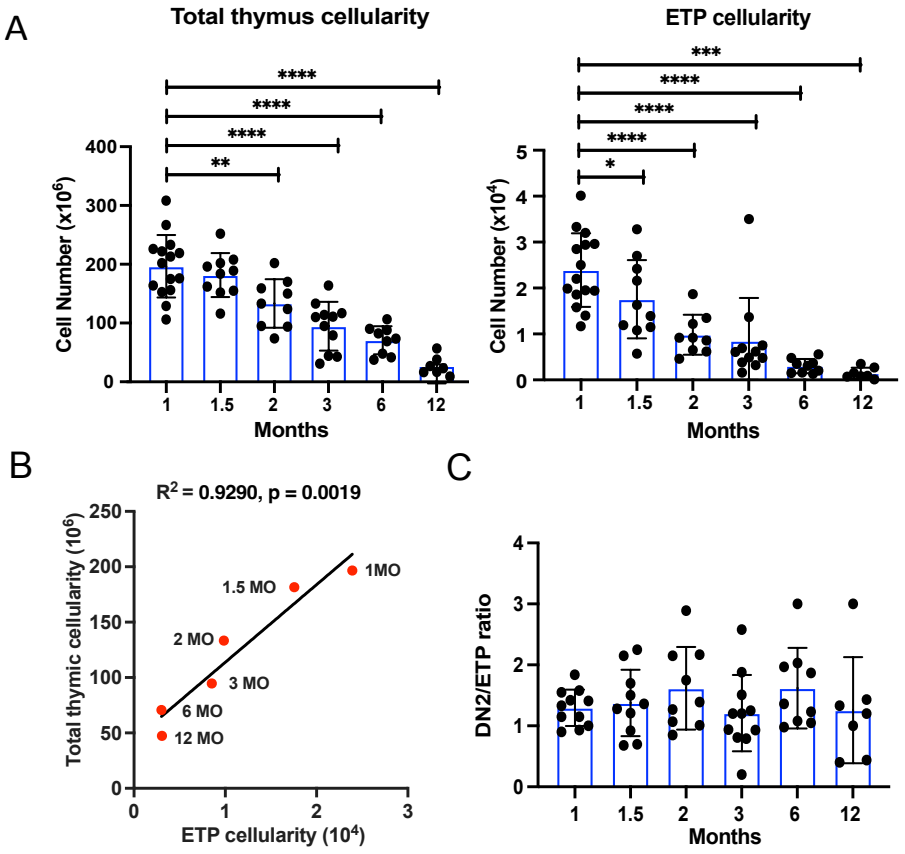


**Figure 1. ETP and total thymocyte cellularity declines by 3MO of age.** (A) Quantification of total thymocytes and ETPs in C57BL/6J mice at the indicated ages. (B) Linear regression analysis of total thymic cellularity and ETP cellularity at each age. $R^2$ = coefficient of correlation (C) Ratio of DN2 to ETP cellularity at each age. Symbols represent A) and C) data from individual mice and B) an average of 7-14 mice at each age. Bars represent means +/- SEM from 3-4 independent experiments for each age group. Statistical analysis in (A) was performed using one-way ANOVA with Dunnett's multiple comparisons test where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 2**

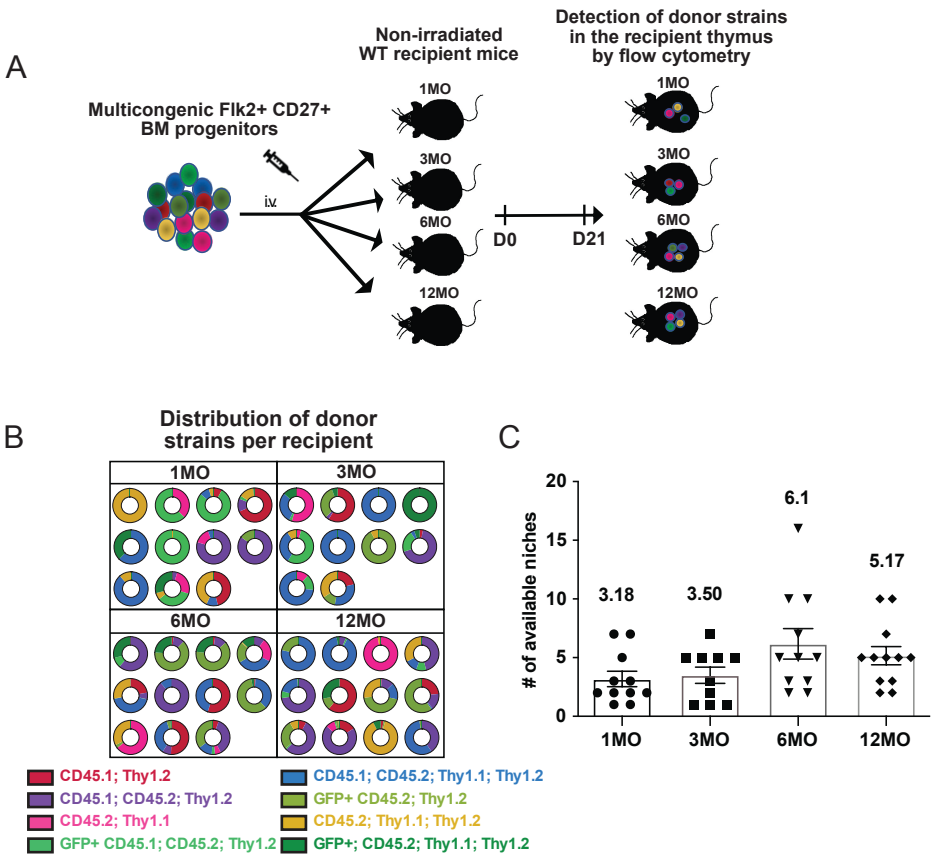


**Figure 2. The number of available, functional TSP niches does not decline with age.** (A) Schematic of the multicongenic barcoding experiment to quantify available niches in mice of different ages. $Lin^{-}Flk2^{+}CD27^{+}$ BM progenitors were isolated from 8 different congenic strains (supplemental Figure 2B), mixed at equal ratios and i.v. injected into non-irradiated 1MO, 3MO, 6MO and 12MO mice. After 21 days, the thymi from recipient mice were analyzed by flow cytometry to quantify the number of detectable donor strains that underwent T-cell differentiation. (B) Pie charts illustrate the number and relative frequency of distinct color-coded donor strains in individual recipients of the indicated ages. (C) The estimated number of available functional niches in each age recipient as determined by multinomial sampling. Symbols indicate data from individual mice; bars represent means +/- SEM compiled from 4 independent experiments (n=10-12 mice per recipient age).

**Figure 3**

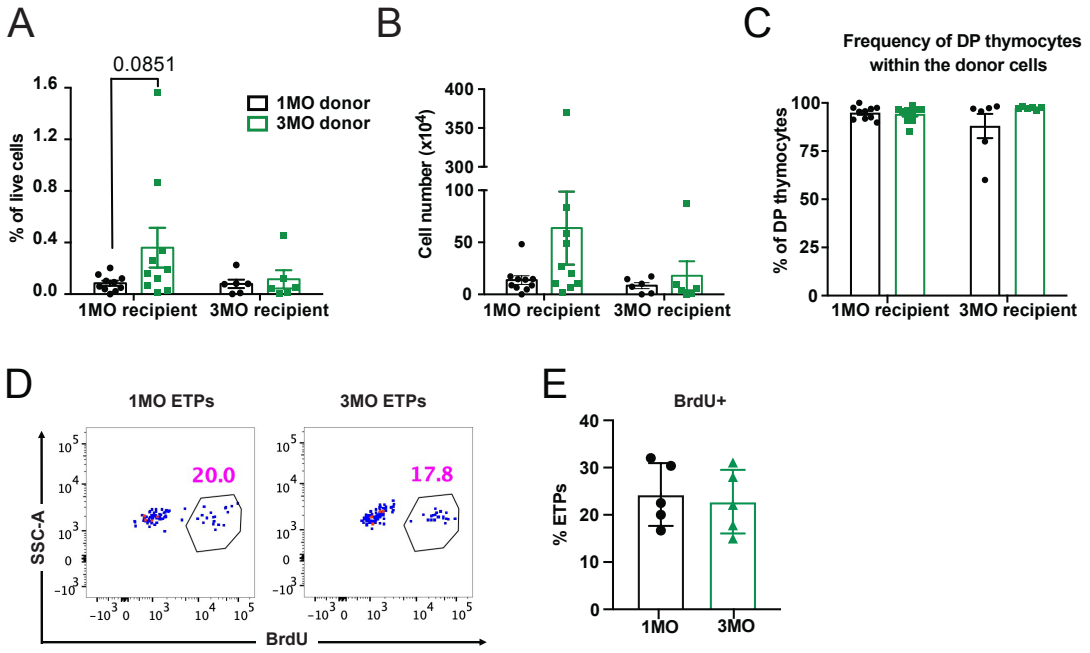


**Figure 3. 3MO T-cell progenitors show comparable seeding and differentiation capacity relative to 1MO progenitors.** (A-C) Donor chimerism in 1MO and 3MO recipient thymuses 21d after transplantation of an equal number of 1MO and 3MO $Lin^{-}Flk2^{+}CD27^{+}$ BM progenitors into 1MO or 3MO non-irradiated recipients. Quantification of the (A) frequency and (B) number of 1MO and 3MO donor-derived thymocytes in recipients of the indicated ages. (C) Quantification of the percentage of DP thymocytes derived from 1MO and 3MO donors detected in recipient mice of the indicated ages. (D) Representative flow cytometry plots and (E) quantification of the percentage of ETPs that incorporated bromodeoxyuridine (BrdU) 8 hours after i.p. injection in mice of the indicated ages. (A-C,E) Symbols represent data from individual mice. Bars represent mean +/- SEM from 4 independent experiments. p value for (A) was obtained by two-way ANOVA with Sidak's multiple comparison test.

**Figure 4**

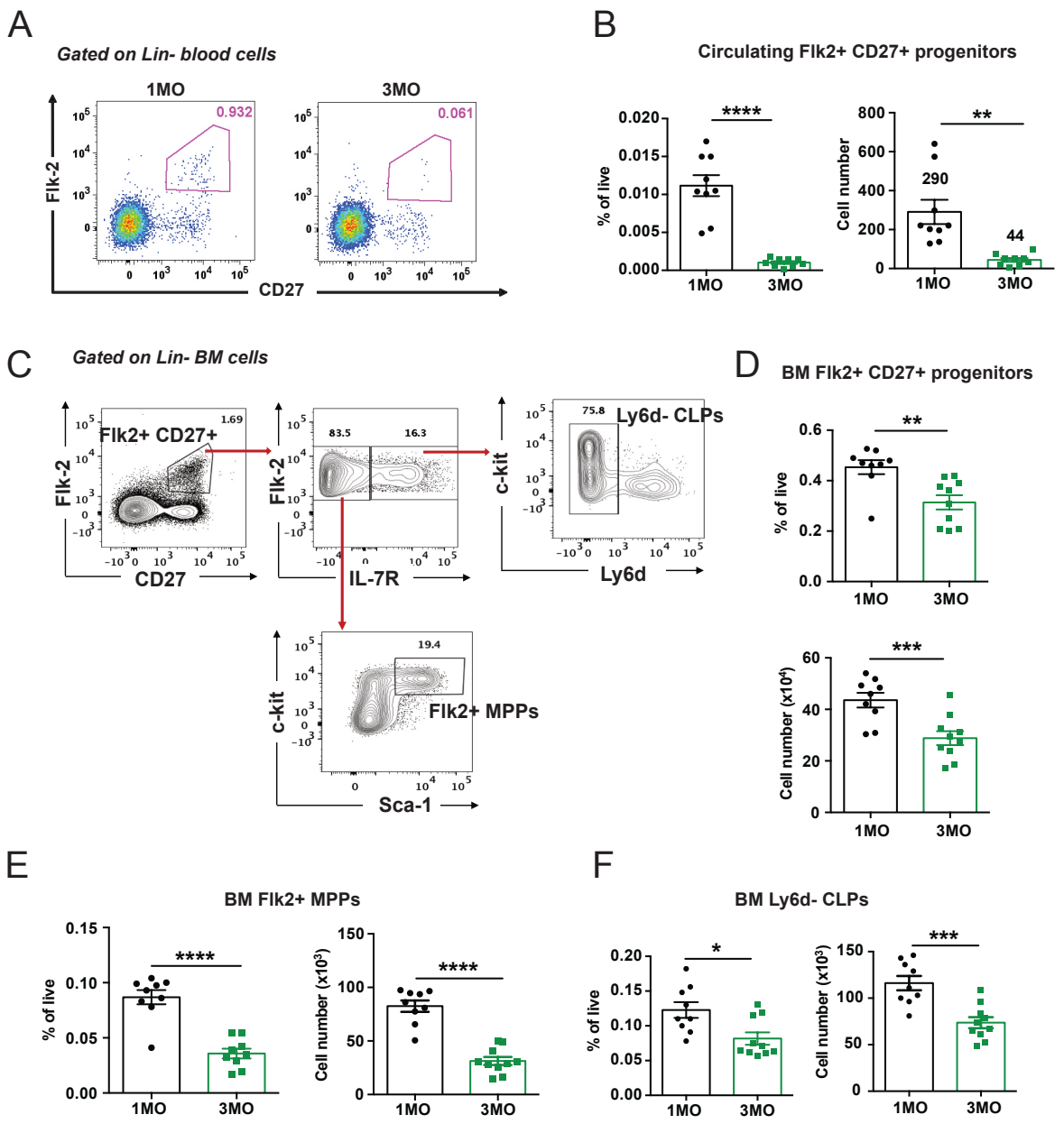


**Figure 4. The number of circulating TSPs and BM lymphoid progenitors declines between 1MO and 3MO of age.** (A) Representative flow cytometry plots showing the percentage of $Flk2^{+}CD27^{+}$ progenitors within the Lin- compartment of the blood in 1MO and 3MO mice. (B) Frequency and absolute cell numbers of circulating lymphoid progenitors in the blood of 1MO and 3MO mice. (C) Representative gating strategy for quantification of $Lin^{-}Flk2^{+}CD27^{+}$ progenitors, $Ly6d^{-}$ common lymphoid progenitors (CLPs), and $Flk2^{+}$ multipotent progenitors (MPPs) in the BM. (D-F) Frequency and absolute numbers of (D) $Flk2^{+}CD27^{+}$ progenitors, (E) $Flk2^{+}$ MPPs and (F) $Ly6d^{-}$ CLPs in BM of 1MO and 3MO mice. Symbols represent data from individual mice at each age. Bars represent mean +/- SEM of data compiled from 3 independent experiments. Statistical analysis was performed using Student *t*-test *p < 0.05, **p <0.01, ***p<0.001, ****p<0.0001.

**Figure 5**

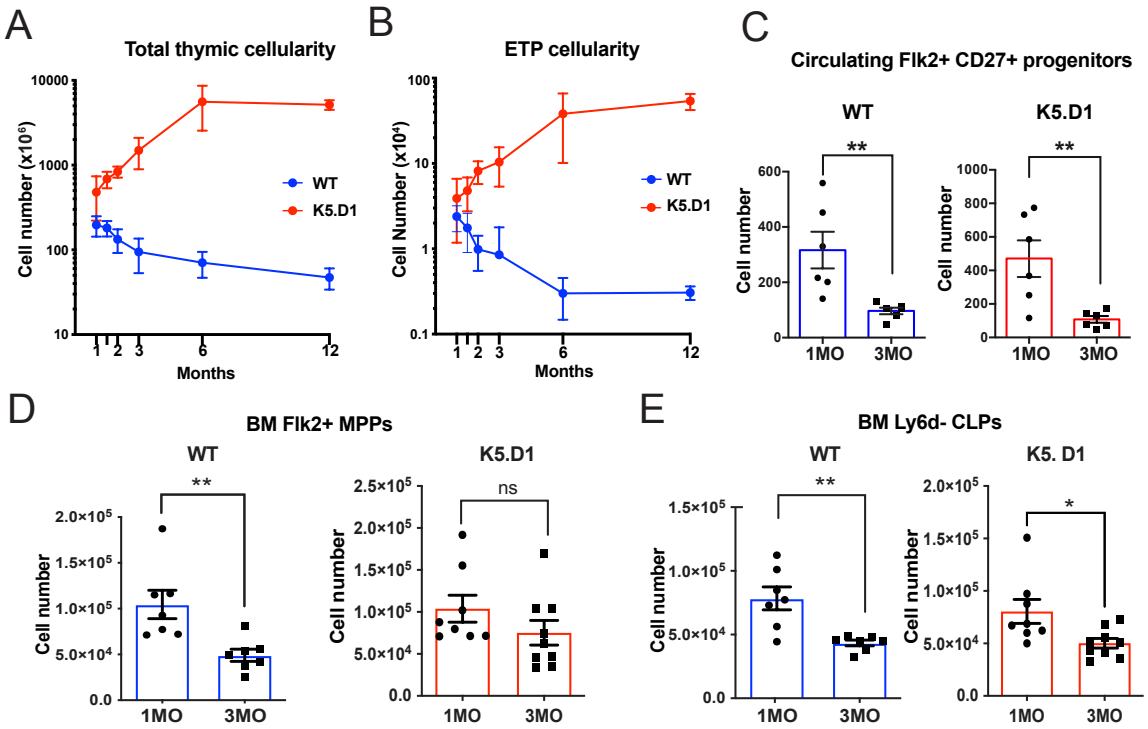


**Figure 5. Sustaining TEC proliferation prevents the age-associated decline in total thymocyte and ETP cellularity, despite a reduction in circulating progenitors by 3MO.** Quantification of (A) total thymocyte and (B) ETP cell numbers in WT versus K5.D1 transgenic littermates at the indicated ages. (C) Absolute numbers of circulating lymphoid progenitor cells in the blood of 1MO and 3MO WT and K5.D1 littermates. Quantification of (D) Flk2$^{+}$ MPP and (E) Ly6d$^{-}$ CLP cells in the BM of mice at 1MO and 3MO of age. (A-B) symbols indicate the average cell numbers of 7-14 mice pooled from 3-4 independent experiments per age. Error bars show mean +/- SD. (C-E) Symbols represent data from individual mice. Error bars show mean +/- SEM for data pooled from 3 independent experiments. Statistical analysis was performed using Student *t*-test **p <0.01, ***p<0.001.

Figure 6

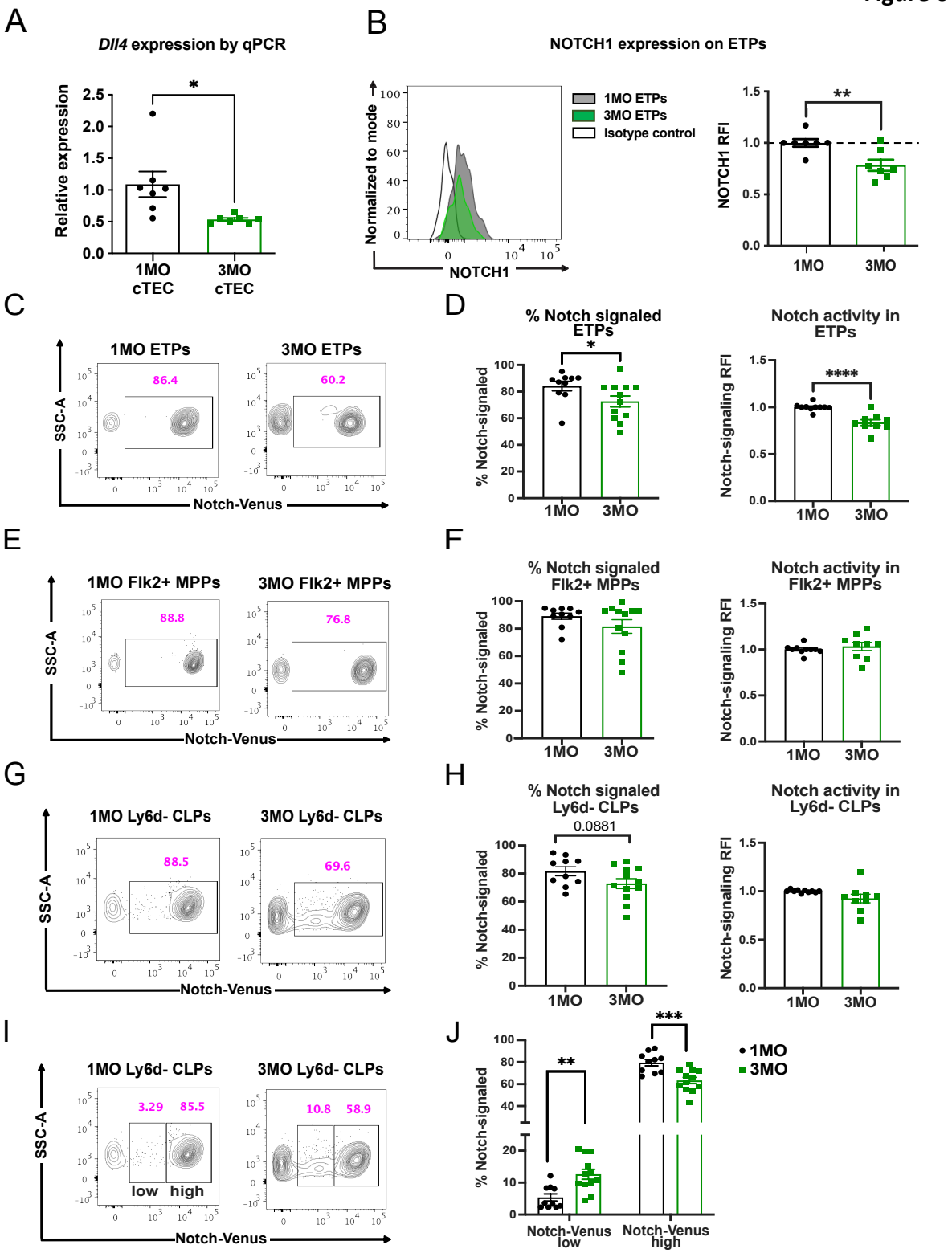


**Figure 6. Age-associated changes in Notch signaling correlate with the decline in ETPs at 3MO.** (A) qPCR analysis showing relative *Dll4* expression by EpCAM$^{+}$ Ly51$^{+}$ cTECs from 1MO and

3MO C57BL/6J mice. Data are pooled from 2 biological experiments with 3-4 technical replicates per experiment, and expression levels were normalized to those at 1MO of age. (B) Representative histogram (left) and quantification of relative fluorescence intensity (RFI) (right) of NOTCH1 cell surface expression on ETPs. Data are normalized to the average NOTCH1 MFI of 1MO mice in each individual experiment. Data are pooled from 4 independent experiments. (C-H) Representative flow cytometry plots, as well as the frequencies and RFIs of Notch-Venus reporter expression in (C, D) thymic ETPs, (E,F) BM Flk2$^{+}$ MPPs and (G,H) BM Ly6d$^{-}$ CLPs. (I) Representative flow cytometry plots showing gating to distinguish Notch-Venus low from Notch-Venus high cells. (J) Frequencies of Notch-Venus low and Notch-Venus high Ly6d$^{-}$ CLPs in 1MO and 3MO BM. Symbols represent data from individual mice at each age. Bars represent mean +/- SEM of data pooled from 5 independent experiments. Statistical analysis was performed using Student *t*-test *$p < 0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.