Paracrine FGF21 dynamically modulates mTOR signaling to regulate thymus function across the lifespan

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Body

Main

T lymphocytes are critical mediators of the adaptive immune response that require continuous replenishment. New T cells are generated in the ***thymus*** by periodic recruitment of ***thymus*** seeding progenitors,. Mutually inductive ***signaling*** in the unique stromal microenvironment of the ***thymus*** directs these multi-potent progenitors along a program of differentiation, proliferation, and selection to generate a self-tolerant, self-restricted T-cell population, –. The stromal microenvironment comprises various types of thymic epithelial cell (TEC) as well as mesenchymal, neural, vascular, and non-lymphoid hematopoietic populations. TECs generate most of the known inductive ***signals*** required for T-cell differentiation in the ***thymus***, as well as a variety of factors that ***regulate*** the spatial migration of progenitors through the ***thymus***,.

Rearrangement of T-cell receptor (TCR) gene segments during T-cell development allows the generation of a panoramic spectrum of TCR specificities to facilitate the recognition of diverse antigens. This diversity depends on the number of T cells generated in the ***thymus***. However, thymic output in humans is estimated to peak during the first few years of life but decline by around 75% by middle age, , , , –, resulting in impaired production of new, naive T cells, and a diminished TCR diversity driven by the compensatory proliferation of memory T cells, , –. Resulting immunodeficiencies include decreased vaccine responsiveness and tumor surveillance, as well as diminished response to infection, (reviewed in ref.). Not only does a smaller pool of naive T cells compromise the T-cell response to new infections, but memory cells generated from aged naive CD4 (cluster of differentiation 4) T cells also show defective responses, and thymic atrophy is likewise associated with impaired negative selection and increased release of autoreactive T cells into the periphery and subsequent autoimmune disease, , , , , , –.

Although ***thymus*** dysfunction begins relatively early in life, a recent study showed that it retains substantial protective capacity in adults. When outcomes were compared between cardiac surgery patients who underwent complete thymectomy at the time of surgery and those in whom a thymic “remnant” was retained, complete thymectomy increased all-cause mortality, cancer risk, cancer severity, and autoimmune disease risk 5 years after surgery, strongly supporting the notion that increasing ***thymus function*** holds strong potential for improving T-cell ***function*** in older adults.

The ***thymus*** retains regenerative capacity with age,, and previous studies suggest that thymic regeneration increases immunity to microbial infections and tumor surveillance. Approaches to induce ***thymus*** regeneration have so far only resulted in transient success, are limited by age, and do not completely restore thymic ***function*** or

transcriptional programs, –. Improving our understanding of thymic growth and regeneration could inform better translational strategies for extending the healthspan. The most substantial barrier to identifying effective approaches for ***thymus*** regeneration is a limited understanding of the mechanisms that ***regulate*** ***thymus*** growth. Although these mechanisms are not fully resolved, it is clear that the primary targets of both age-induced atrophy and experimental thymic regeneration are stromal rather than lymphoid cells,, particularly cortical stromal cells. Stromal cells represent less than 1% of the total thymic cellularity, and their isolation requires enzymatic digestions known to induce important changes in their biology. To overcome these barriers, we applied a computational deconvolution approach to estimate global stromal gene expression signatures,. Application of this approach over the course of aging and experimentally induced ***thymus*** regeneration revealed that cortical thymic epithelial cell (cTEC) size and morphology are primary determinants of overall ***thymus*** size, and therefore new T-cell generation, ***across*** the ***lifespan***.

These analyses also suggested a critical role for ***mTOR*** (mammalian target of rapamycin) ***signaling*** (through two distinct complexes) in ***regulating*** the size and morphology of cTECs, consistent with the observation that high-dose rapamycin administration, which inhibits both mTORC1 (***mTOR*** complex 1)/mTORC2 (***mTOR*** complex 2) activity, has been shown to decrease ***thymus*** size. We found that the expression patterns of soluble ligands capable of activating the ***mTOR*** pathway were either absent or unchanged during aging and regeneration in cortical stromal cells, making autocrine ***signaling*** unlikely. However, expression of known TEC regulator ***FGF21*** was diminished with age and ***dynamically*** ***regulated*** in medullary stromal cells during regeneration. ***Fgf21*** expression has previously been reported in thymic stromal cells and medullary thymic epithelial cells (mTECs) in particular, and expression of its obligate coreceptor βKlotho (Klb,) has been reported in cTEC, but not in other thymic subsets. Expression of ***Fgf21***, but not Klb, declines with age in the ***thymus***, and transgenic overexpression of ***Fgf21*** in the liver delays thymic atrophy in mice.

These findings led us to hypothesize that ***FGF21*** expression by mTECs promotes direct or indirect ***paracrine*** activation of mTORC1/mTORC2 in cTECs to ***regulate*** cell proliferation, individual cell size, and morphology, thereby controlling overall ***thymus*** size. To test this hypothesis, we designed a knock-in (KI) mouse model in which overexpression of ***FGF21*** is driven broadly in most mTECs. Here we report that ***paracrine*** ***FGF21*** ***signaling*** activates temporally distinct patterns of mTORC1 and mTORC2 ***signaling*** in cTECs, which is sufficient to increase ***thymus*** size and ***function*** in aging mice, resulting in increased T-cell responsiveness to influenza infection as well as decreases in indicators of autoreactivity, relative to age-matched controls.

Results

Expression of ***Fgf21*** by mTECs declines with age

Because our previous studies indicated a role for ***Fgf21*** expressed by medullary stromal cells in the ***regulation*** of ***thymus*** growth, we sought to characterize the stromal cell subsets expressing ***Fgf21*** ***across*** the ***lifespan***. Analysis of a previously published single-cell RNA-sequencing (scRNA-seq) dataset revealed that among the nine subsets of TECs identified, ***Fgf21*** expression was found at the highest levels in mature mTECs (Airehi, Cd52hi), as well as proliferating TECs, which is a rarer population resembling mature mTECs (previously described as mTEC II (MHC-II+, Ly6d−)) seen predominantly in the early postnatal period, (Fig.). ***Fgf21*** expression in mature mTECs was highest during the early postnatal period and declined after 4 weeks of age, persisting at lower levels through 52 weeks (Fig.). By contrast, ***Fgf21*** expression in proliferating TECs remained high through middle age. However, as the overall abundance of this population declined significantly following the postnatal period (Fig.), the total amount of ***FGF21*** produced by this subset of cells would be expected to decline during aging. ***Fgf21*** expression was detected at minimal levels in mature cTECs (Prss16hi, Cxcl12hi) throughout the first year and in a small population of structural TEC (sTEC: Cd177hi, Car8hi) likely to be found in the medulla based on expression of Enpp2 (ref.) (Fig.). Thus, single-cell transcriptomic evidence confirmed mature mTECs as a primary source of ***Fgf21*** within the ***thymus*** and also confirmed age-associated declines in ***Fgf21*** expression, consistent with previous reports,.

Characterization of ***Fgf21*** expression in TECs and identification of Lpo as an appropriate candidate for driving broad, constitutive ***Fgf21*** expression in mTECs throughout the ***lifespan***.

a, ***Fgf21*** relative expression levels (log-normalized within population, indicated by color) and frequencies of cells with non-zero expression values within each subset (indicated by symbol size) among nine distinct TEC populations (proliferating TEC (MHC-II+, Ly6d−), tuft-like mTEC (Avilhi, Trpm5hi), post-Aire mTEC (Krt80hi, Spink5hi), mature mTEC (Airehi, Cd52hi), intertypical TEC (Ccl21ahi, Krt5hi), perinatal cTEC (Syngr1hi, Gper1hi), mature cTEC (Prss16hi, Cxcl12hi), neural TEC/nTEC (Sod3hi, Dpthi), and structural TEC/sTEC (Cd177hi, Car8hi) were obtained from the thymi of mice aged 1, 4, 16, 32, and 52 weeks using a previously published scRNA-seq dataset. b, Changes in relative abundance of TEC populations described in a present in the mouse ***thymus*** from the early postnatal period (1–4 weeks) to 1 year of age. c, Schematic diagram outlining the selection criteria for prioritizing candidate pan-mTEC genes. Top candidates were selected using a published transcriptional database consisting of global transcriptional profiles generated from cortical and medullary lymphocytes and microdissected whole cortical and medullary tissue (GEO: GSE132136). Aire-dependent genes were excluded based on previous published Aire-***regulated*** gene lists,. The candidate genes were ranked using gene expression levels (gcrma values) and intensity skew (based on BioGPS expression values), followed by immunofluorescence microscopy validation to identify expression patterns in ***thymus***). IF, immunofluorescence. d, Frozen 5 μm sections from 3-month-old and 12-month-old C57BL/6 mouse thymi (n = 3 per group) were co-stained with DAPI, anti-LPO/Goat anti-Rabbit Cy5 (red), and anti-EpCAM (CD326) A488 (green). Representative images are shown at ×20 magnification. Scale bars, 50 μm. e, Schematic diagram of the Lpo–***Fgf21***–mCherry KI allele. A bicistronic vector was designed allowing for the insertion of ***Fgf21*** and mCherry cDNA at the end of exon 13 of the Lpo gene through incorporation of P2A and T2A peptide sequences. Ex, exon; UTR, untranslated region; HA, homology arm.

Identification of Lpo as a candidate mTEC gene

To begin identifying the role of mTEC-derived ***FGF21*** in the ***regulation*** of ***thymus*** size, we designed a mouse model to drive gene expression broadly, but specifically, in mTECs in the ***thymus***. Using the previously described transcriptional datasets, we applied a series of selection criteria to prioritize broadly expressed mTEC genes (Fig.). The top candidate genes were ranked based on confidence of medullary stromal expression and relative tissue specificity in a published database of gene expression ***across*** a broad range of tissue types (BioGPS GeneAtlas MOE430, gcrma (guanidine-cytosine robust multiarray average). To avoid selecting Aire-dependent genes, which are expressed by only a subset of mTECs, typically at low levels, we excluded Aire-***regulated*** genes using two previously published gene lists,. Among the top candidate genes was Lpo (lactoperoxidase), which was predicted to have highly specific expression in the thymic medullary stroma, lacrimal, and salivary glands. Immunofluorescence microscopy confirmed that within the ***thymus***, LPO expression was preferentially expressed in the medulla, with a high degree of co-localization with TECs expressing the highest levels of EpCAM (epithelial cell adhesion molecule), which is characteristic of mTECs (Fig.), and, importantly, remained highly expressed in the ***thymus*** at 12 months of age (Fig.). Similar to ***Fgf21***, Lpo expression was highest in mature mTECs and proliferating TECs and remained high during the first year of life (Extended Data Fig.). To further confirm that Lpo expression was not Aire dependent, we analyzed a previously published microarray database comparing gene expression between FACS (fluorescence-activated cell sorting)-sorted wild-type and Aire-deficient mTECs, which revealed that expression of Lpo appears to be Aire independent (Supplementary Table). However, expression of Lpo was modestly but significantly lower in mTECs of Fezf2−/− mice compared to wild-type mice, suggesting Lpo expression, or the population of cells expressing Lpo, may be partially ***regulated*** by Fezf2 (Supplementary Table). Upon validation of Lpo as a candidate for driving gene expression broadly in most mTEC, we designed a bicistronic vector allowing for the insertion of ***Fgf21*** and the monomeric red fluorescent protein mCherry complementary DNA at the end of exon 13 of the Lpo gene by incorporating P2A and T2A sequences (Fig. and Extended Data Fig.). We then used CRISPR–Cas9 to generate founder mice expressing the Lpo–***Fgf21***–mCherry KI allele (Fig.). Sanger sequencing and PCR verification confirmed expression of the KI mutation in founders (Extended Data Fig.), which we subsequently backcrossed with C57BL/6 mice to create heterozygous LPOFGF21/WT and homozygous LPOFGF21/***FGF21*** experimental mice. Unless otherwise indicated, heterozygous mice (LPOFGF21) were used for experiments.

We first performed flow cytometric evaluation of mCherry reporter expression among thymic stromal subsets to confirm expression of the KI allele in mTEC subsets (Fig. and Extended Data Fig.). We found mCherry to be expressed specifically in mTEC and broadly among various mTEC subsets, including UEA1+ (Ulex Europaeus

Agglutinin 1) mTEC (CD45.2−, EpCAM+, Ly51−, UEA1+), mTEClo (CD45.2−, EpCAM+, Ly51−, major histocompatibility complex II (MHCII)lo, CD80lo), and mTEChi (CD45.2−, EpCAM+, Ly51−, MHCIIhi, CD80hi) (Fig.). mCherry was not detected in cTEC (CD45.2−, EpCAM+, Ly51+, UEA1−), cDC (conventional dendritic cells, CD45.2+, CD11c+, B220−), pDC (plasmacytoid dendritic cells, CD45.2+, CD11c+, B220 (CD45R)+), endothelial cell (EC) (CD45.2−, EpCAM−, CD31+), pericyte (PC) (CD45.2−, EpCAM−, CD31−, PDGFR (platelet-derived growth factor receptor) αβ+, CD146+, gp38 (podoplanin)−), or fibroblast (CD45.2−, EpCAM−, CD31−, PDGFRαβ+, CD146−, gp38+) subsets (Fig.), consistent with publicly available datasets. Expression of mCherry ranged from 50% to 75% of all mTEC and was detected in a proportion of all mTEC subsets evaluated (Fig.). The frequency of mCherry positive cells was slightly diminished with age in most mTEC subsets in LPOFGF21 KI mice (Extended Data Fig.); however, the average mCherry mean fluorescence intensity (MFI) was generally maintained, except among mTEClo (Extended Data Fig.). To further evaluate mCherry expression, we performed immunofluorescence microscopy. Thymic sections from 1-month-old LPOFGF21 and LPOWT littermate controls were stained with fluorescent antibodies targeting K5, K8, DEC 205, Ly51, UEA1, and Aire. Co-localization with medullary markers K5, UEA1, and Aire, but not with cortical markers K8, DEC 205, and Ly51 indicated broad and specific medullary expression of the KI allele in the ***thymus*** (Fig. , Extended Data Fig. , and Supplementary Fig.).

Flow cytometric evaluation of mCherry expression in the LPOFGF21 KI ***thymus*** confirms broad and specific expression of the KI allele in mTEC subsets.

a, Flow cytometry gating strategy (pre-gated on viable singlets) used for the identification of cTEC (CD45.2−, EpCAM+, Ly51+, UEA1−), total mTEC (CD45.2−, EpCAM+, Ly51−, UEA1+), mTEClo (CD45.2−, EpCAM+, Ly51−, MHCIIlo, CD80lo), mTEChi (CD45.2−, EpCAM+, Ly51−, MHCIIhi, CD80hi), cDC (CD45.2+, CD11c+, B220−), pDC (CD45.2+, CD11c+, B220+), endothelial cell (EC) (CD45.2−, EpCAM−, CD31+), pericyte (PC) (CD45.2−, EpCAM−, CD31−, PDGFRαβ+, CD146+, gp38−), and fibroblast (Fb) (CD45.2−, EpCAM−, CD31−, PDGFRαβ+, CD146−, gp38+) subsets from thymi of the indicated mice after enzymatic digestion and stromal cell enrichment. FSC-A, forward scatter area; I-A/I-E, MHCII. b, Representative histograms of mCherry expression among mTEC, DC, endothelial, and mesenchymal subsets of 1-month-old LPOWT (n = 4) and LPOFGF21 mice (n = 5). c, The frequency of mCherry+ cells among cTEC, mTEC, DC, endothelial, and mesenchymal subsets was quantified in LPOFGF21 KI mice (n = 5). Data among mTEC subsets were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Horizontal lines indicate significant comparisons among mTEC subsets. NS, $P \geq 0.05$. Mean and s.e.m. indicated by horizontal lines. Each symbol represents an individual mouse. Data are representative of three independent experiments. d, Representative confocal microscopy images of thymic mCherry expression (red) at ×10 (left) and ×20 (right, magnified view of the indicated ×10 region) magnification from LPOFGF21 (n = 5) 1-month-old mice. Thymic sections were stained with anti-cytokeratin 5 A647. Scale bars, 100 μm (×10 magnification) and 50 μm (×20 magnification).

To quantify gene expression in the LPOFGF21 KI ***thymus***, we measured Lpo, ***Fgf21***, and mCherry messenger RNA in FACS-sorted mTECs as well as whole salivary gland, liver, and lung tissue from 1-month-old LPOFGF21 and LPOWT littermate controls (Extended Data Fig.). We detected robust expression of Lpo in the salivary glands (maximally expressing tissue predicted by Biogps.org database) and mTEC of both LPOWT and LPOFGF21 mice (Extended Data Fig.), suggesting KI alleles do not disrupt Lpo expression. ***Fgf21*** was significantly increased and mCherry was robustly expressed in the salivary glands of LPOFGF21 mice (Extended Data Fig.). These results were confirmed with immunofluorescence microscopy showing robust expression of LPO and mCherry in salivary gland epithelium of LPOFGF21 mice (Extended Data Fig.). ***Fgf21*** expression was significantly increased, by about fourfold, in LPOFGF21 mTECs compared to LPOWT littermate controls (Extended Data Fig.). In the liver, ***Fgf21*** was expressed at similar levels in LPOWT and LPOFGF21 mice, while Lpo and mCherry mRNA were undetectable (Extended Data Fig.). Expression of Lpo, ***Fgf21***, and mCherry were not detectable in the lungs of either LPOWT or LPOFGF21 mice (Extended Data Fig.). ***FGF21*** can ***signal*** via FGFR1 or FGFR3 but requires the βKlotho co-receptor, and expression of βKlotho by CD45− thymic stromal cells has been shown to increase with aging. To address which thymic stromal subtypes may express these co-receptors, we measured expression of Klb, Fgfr1, and Fgfr3 in FACS-sorted thymic macrophages (CD19−, CD11b+, F4/80+), fibroblasts (CD45−, EpCAM−, CD31−, gp38+), and mTECs (CD45−, EpCAM+, Ly51−) from 6-month-old C57BL/6 mice (Extended Data Fig.). Klb was undetectable in thymic fibroblasts and macrophages but was partially expressed in mTECs at levels similar to white

adipose tissue and pancreas (Extended Data Fig.). Fgfr1 expression was significantly higher in mTECs and was detected at low levels in macrophages (Extended Data Fig.). Fgfr3 was expressed at about the same level as Klb in mTECs and was undetectable in macrophages and fibroblasts (Extended Data Fig.). We were unable to purify cTECs in sufficient numbers to evaluate ***FGF21*** receptor expression in this population by quantitative real-time PCR (qRT-PCR). In agreement with Youm et. al., these data suggest that mTECs and other thymic stromal cells not yet identified may respond to ***FGF21***. mTEC-driven ***FGF21*** overexpression did not increase circulating ***FGF21*** levels, as serum levels of ***FGF21*** did not significantly differ between age-matched LPOWT and LPOFGF21 mice when measured in 1-month-old and 13-month-old mice (Extended Data Fig.). While circulating ***FGF21*** levels increased slightly with aging, consistent with previous reports, no differences were detected among age-matched LPOWT and LPOFGF21 mice. However, whole ***thymus FGF21*** production was found to be significantly higher in both young (1-month-old) and older (13-month-old) LPOFGF21 KI mice compared to LPOWT age-matched controls when measured by enzyme-linked immunosorbent assay (ELISA) (Extended Data Fig.). Taken together, these results indicate faithful expression of the Lpo–***Fgf21***–mCherry KI allele in mTECs and in salivary gland.

FGF21 overexpression increases ***thymus*** size ***across*** the ***lifespan***

To determine the effects of constitutive mTEC-mediated ***FGF21*** overexpression on ***thymus*** size during aging, we compared ***thymus*** somatic index (***thymus*** weight to body weight ratio) in LPOWT and LPOFGF21 littermates from 1 to 14 months of age (Fig.). While ***thymus*** size declined in both LPOWT and LPOFGF21 mice with age, the ***thymus*** somatic index remained significantly increased in LPOFGF21 mice relative to age-matched controls (Fig.). Body weights did not significantly differ up to 18–21 months in LPOWT and LPOFGF21 mice (Extended Data Fig.). ***Thymus*** cellularity was increased in both male and female LPOFGF21 mice at 1, 3, and 12–14 months of age relative to control littermates (Fig. and Extended Data Fig.), and the average ***thymus*** cellularity of 12- to 14-month-old LPOFGF21 KI mice was similar to that of 3-month-old LPOWT littermates in male and female mice (Fig. and Extended Data Fig.). We next analyzed the thymic CD4/CD8 T-cell subset frequencies and total numbers in 1-month-old and 12- to 14-month-old LPOFGF21 mice. The relative frequency of each subset (DN, DP, CD4+ SP, CD8+ SP) did not significantly differ between LPOWT and LPOFGF21 mice at any age tested (Fig. and Extended Data Fig.). The frequency of cTECs was increased in 1-month-old LPOFGF21 mice relative to controls but was not significantly different in 12- to 14-month-old mice (Fig. and Extended Data Fig.). The 12- to 14-month-old LPOFGF21 mice had increased total numbers of DN, DP, and CD4+ SP lymphocytes (CD8+ SP numbers trended up, but increase was not statistically significant), as well as increased cTECs and mTECs, highlighting the effect of ***FGF21*** overexpression on preserving the total number of TEC as well as supporting lymphocyte development (Fig. and Extended Data Fig.). We observed no difference in proliferation or apoptosis (Extended Data Fig.) in cTECs and mTECs of 3-month-old LPOWT and LPOFGF21 mice, suggesting that the increased TEC numbers found in young LPOFGF21 mice relative to controls were maintained during aging without impacts on proliferation or survival at later ages.

Overexpression of ***FGF21*** in LPOFGF21 KI mTECs results in increased ***thymus*** size and diminishes age-associated thymic atrophy.

a, ***Thymus*** weight to body weight ratios (somatic index) of LPOWT and LPOFGF21 KI female mice at 1 month (n = 7 per group), 3 months (n = 5 per group), and 12 months of age (n = 6 per group). b, Total ***thymus*** cellularity was determined in LPOWT and LPOFGF21 KI female mice at 1 month (n = 8 LPOWT, n = 7 LPOFGF21), 3 months (n = 9 LPOWT, n = 8 LPOFGF21), and 12 months of age (n = 9 LPOWT, n = 7 LPOFGF21). c,d, Thymocyte CD4−CD8− DN, CD4+CD8+ DP, CD4+ SP, and CD8+ SP subset frequency (c) and total cell number (d), as well as the frequency and total number of cTEC (CD45.2−, EpCAM+, Ly51+) and mTEC (CD45.2−, EpCAM+, Ly51−), were quantified in 1-month-old (n = 6 LPOWT, n = 10 LPOFGF21) (top) and 13-month-old (bottom) (n = 5 LPOWT, n = 6 LPOFGF21) LPOWT and LPOFGF21 KI female mice. Data were analyzed via two-sided Student's t-tests in a–d. NS, $P \geq 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$; ****$P < 0.0001$. Mean and s.e.m. are indicated by horizontal lines. Each symbol represents an individual mouse. Data are representative of three or more experiments.

Kinetics of mTORC1 and mTORC2 ***signaling*** in cTECs

Our previous work and that of others suggested ***mTOR*** complexes as potential downstream mediators of ***FGF21 signaling*** in cTECs, and transcriptional ***mTOR*** targets were ***dynamically regulated*** in cortical stroma upon castration-induced regeneration of the ***thymus***. To characterize changes in ***mTOR*** pathway activation at the protein level, we first investigated changes in mTORC1/mTORC2 ***signaling*** during aging using antibodies measuring phosphorylation of p4E-BP1 (phospho-eukaryotic translation initiation factor 4E-binding protein 1) and pAktS473 (phospho-AKT1 at serine 473), respectively, by flow cytometry in cTECs, mTECs, and CD45+ lymphocytes from C57BL/6 mice (Fig.). We compared activation of p4E-BP1 and pAktS473 in 3-week-old mice, representing a period of active ***thymus*** growth, and 6-month-old mice, representing a period of age-associated atrophy. We observed a significant age-associated decline in mTORC1 as well as mTORC2 activation in cTECs (Fig.). We also observed a small but significant decline in p4E-BP1 and pAktS473 levels in mTECs, but not in CD45+ lymphocytes (Fig.). The high ***mTOR*** activity in young TEC followed by declines in aging are consistent with previous work indicating roles for ***mTOR signaling*** in TEC growth, , –. We next evaluated the impact of mTEC-driven ***FGF21*** overexpression on mTORC1/mTORC2 activity during aging. At 2.5 weeks of age, when the ***thymus*** is actively growing, LPOFGF21 KI cTECs in male and female mice showed evidence of significantly increased mTORC1, but not mTORC2 activity, compared to LPOWT controls (Fig. and Extended Data Fig.). At 3 months of age, representing a shift from peak growth to a period of atrophy, LPOFGF21 mice had significantly increased pAktS473, but not p4E-BP1, levels (Fig. and Extended Data Fig.). This trend continued at 12 months of age, with mTORC2 but not mTORC1 activity significantly increased in cTECs of LPOFGF21 mice (Fig. and Extended Data Fig.). However, in mTECs there appeared to be only dynamic ***regulation*** of mTORC1, with increased p4E-BP1 in mTECs of 2.5-week-old LPOFGF21 mice, but not mTORC2, activity (Extended Data Fig.). In CD45+ lymphocytes we observed no effect of LPOFGF21 KI allele expression on mTORC1/mTORC2 activity (Extended Data Fig.), suggesting that ***FGF21*** overexpression driven by mTECs ***signaled*** primarily in a ***paracrine*** manner to cTECs to affect mTORC1 and mTORC2 activation at distinct times during tissue growth and maintenance. The shift from increased mTORC1 to increased mTORC2 ***signaling*** during the transition from early ***thymus*** growth to later phases is consistent with our previous observation that expression of the mTORC1 regulator Tsc1 is upregulated after the first few days of ***thymus*** growth during regeneration. To test whether Tsc1 expression was similarly upregulated during later phases of postnatal thymic growth, we compared TSC1 expression in cTECs of 3-week-old, 6-week-old, and 4-month-old LPOWT mice by flow cytometry. Consistent with the kinetics of ***thymus*** growth during regeneration, we found that TSC1 expression was low at early growth stages and steadily increased during aging (Fig.). To further understand the kinetics of ***mTOR***/TSC1 ***signaling*** in the cortical stroma in the actively growing/regenerated ***thymus***, we used a previously published stromal transcriptome database collected during thymic regeneration to evaluate expression of a manually curated set of genes that are targets of transcription factors known to be ***regulated*** by mTORC1 (reviewed in refs. ,). These included targets of transcription factors positively ***regulated*** by mTORC1, sterol-regulatory-element-binding proteins, Fasn and Acaca (AKA Acc1) in the same published dataset described above (Fig. , green). The expression of these genes increases during early stages of thymic regrowth (peaking around day 10 of regeneration), consistent with positive ***regulation*** by mTORC1. Also consistent with increased mTORC1 activity during early phases of thymic regrowth, expression of target genes ***regulated*** by a transcription factor inhibited by mTORC1, transcription factor EB, Sqstm1 and Lamp1, declines during early phases of thymic regrowth (Fig. , red). Repression of genes important for lipid oxidation (Sqstm1, Lamp1), in favor of those involved in lipid biogenesis (Fasn, Acaca), is consistent with the notion that thymic regrowth requires re-extension of cTEC cellular projections, which would require anabolic metabolism increasing lipid incorporation into extending cellular membranes. Fewer transcriptional targets of mTORC2 ***signaling*** have been identified; however, recent work identified several members of the Hedgehog pathway as targets positively ***regulated*** at the transcriptional level by mTORC2 activity. Of those identified in this study, Gli3 was included in our high-confidence cortical stromal gene list (Fig. , blue circles). Consistent with the notion that mTORC1 activity is increased early during cTEC growth phases, followed by Tsc1-mediated down-***regulation*** that would favor mTORC2 activity promoting cytoskeletal rearrangements and maintenance of cTEC labyrinth morphology, we find that mTORC1-dependent changes in gene expression begin to revert after about day 10 during regeneration. Conversely, we find that expression of the mTORC2 target Gli3 begins to increase around day 10 (Fig.). Together, these results are consistent with the hypothesis that ***FGF21 signaling*** predominantly mediates mTORC1 ***signaling*** in cTECs during early phases of ***thymus*** growth, while mTORC2-mediated ***signaling*** predominates at later stages. However, it is likely that other upstream ligands or metabolites also contribute to changes in mTORC1/mTORC2 ***signaling across*** the ***lifespan***.

mTORC1 and mTORC2 activity in cTECs are distinctly and ***dynamically*** ***regulated*** by ***paracrine*** ***FGF21*** ***signaling*** during ***thymus*** growth and atrophy.

a,b, Representative histograms (top) and MFI (bottom, arbitrary units) of p4EBP1T36,T45 (a) and pAktS473 (b) in cTECs (CD45.2−, EpCAM+, Ly51+), mTECs (CD45.2−, EpCAM+, Ly51−), and CD45.2+ lymphocytes from thymi of 3-week-old (n = 4) and 6-month-old (n = 6) C57BL/6 mice. Single-cell suspensions of enriched thymic stromal cells were stained with fluorescent antibodies targeting p4EBP1T36,T45 and pAktS473 following fixation and permeabilization. wk, weeks; mo, months. c,d, Representative histograms (top) and MFI (bottom, arbitrary units) showing p4EBP1T36,T45 (c) and pAktS473 (d) activation in cTEC of 2.5-week-old (n = 5 LPOWT, n = 6 LPOFGF21), 3-month-old (n = 4 LPOWT, n = 4 LPOFGF21), and 12-month-old (n = 4 LPOWT, n = 6 LPOFGF21) LPOWT and LPOFGF21 KI female mice. e, Representative histograms (left) and MFI (right) of TSC1 expression in cTECs from LPOWT mice at 2.5 weeks, 6 weeks, and 4 months of age (n = 4 per group). Data were analyzed via two-sided Student's t-test in a–d or one-way ANOVA with Tukey's multiple comparisons test in e. NS, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Mean and s.e.m. indicated by horizontal lines. Each symbol represents an individual mouse. Data are representative of three or more experiments. f, Expression of manually curated mTORC1-(red/green) and mTORC2-(blue) ***regulated*** genes throughout course of post-castration regeneration is normalized to 12-month-old untreated ***thymus***, which also represents day 0 of the regeneration sequence.

We next examined whether pharmacological ***mTOR*** inhibition mitigates the impact of ***FGF21*** overexpression on ***thymus*** size, CD4+CD8+ DP thymocyte number, and ***mTOR*** activity in cTECs. Three-week-old LPOWT and LPOFGF21 littermates were injected with 4 mg kg−1 rapamycin or vehicle via intraperitoneal injection every other day for 2 weeks, as previously described, (Fig.). After 2 weeks of treatment, rapamycin induced a significant decline in the ***thymus*** somatic index in both LPOWT and LPOFGF21 mice (Fig.). Total ***thymus*** cellularity was also significantly reduced in rapamycin-treated LPOWT and LPOFGF21 mice (Fig.), abrogating the effects of ***FGF21***-mediated increases in ***thymus*** cellularity. We observed a significant decrease in the frequency of CD4+CD8+ DP lymphocytes and a significant increase in the frequency of CD4−CD8− DN lymphocytes of rapamycin-treated LPOWT and LPOFGF21 mice (Fig. , top panel), consistent with previous studies,. In LPOFGF21 KI, but not LPOWT mice, we also observed a significant increase in the frequency of CD8+ SP lymphocytes (Fig. , top panel). However, when we compared the total cell number of each of these thymocyte subsets, rapamycin only induced a significant decrease in the total number of CD4+CD8+ DP lymphocytes and did so in both LPOWT and LPOFGF21 mice (Fig. , bottom panel). The total number of DP lymphocytes were reduced to similar levels in LPOWT and LPOFGF21 rapamycin-treated mice, despite LPOFGF21 vehicle-treated mice having a significantly higher number of DP lymphocytes compared to LPOWT vehicle-treated mice (consistent with Fig.). We also observed a decreasing trend in the total number of CD4+ SP lymphocytes in rapamycin-treated LPOFGF21 mice (Fig.), which may reflect increased autophagy following relief of mTORC1 inhibition, leading to more MHCII-mediated negative selection of CD4+ SP lymphocytes relative to CD8+ SP lymphocytes.

Pharmacological ***mTOR*** inhibition mitigates the impact of ***FGF21*** overexpression on DP thymocyte number and mTORC1 ***signaling*** in cTECs.

a, Schematic of experimental approach. LPOWT and LPOFGF21 KI mice were treated with 4 mg kg−1 rapamycin or vehicle via intraperitoneal injection every other day for 2 weeks, beginning at the time of weaning (3 weeks of age). At the time of euthanasia, the ***thymus*** was collected, and subsequently ***thymus*** weight to body weight ratios, total ***thymus*** cellularity, thymocyte subset frequencies, and mTORC1/mTORC2 activation in cTECs, mTECs, and lymphocytes were determined. b, ***Thymus*** weight to body weight ratio was determined in LPOWT vehicle-treated (n = 4), LPOWT rapamycin-treated (n = 4), LPOFGF21 KI vehicle-treated (n = 4), and LPOFGF21 KI rapamycin-treated (n = 4) mice. c, Total ***thymus*** cellularity was estimated in LPOWT vehicle-treated (n = 4), LPOWT rapamycin-treated (n = 4), LPOFGF21 KI vehicle-treated (n = 4), and LPOFGF21 KI rapamycin-treated (n = 4) mice. d, Thymocyte CD4−CD8− DN, CD4+CD8+ DP, CD4+ SP, and CD8+ SP subset frequency (top) and total cell number (bottom) were estimated in LPOWT vehicle-treated (n = 4), LPOWT rapamycin-treated (n = 4), LPOFGF21 KI vehicle-treated (n = 4), and LPOFGF21 KI rapamycin-treated (n = 4) mice. e,f, MFI (arbitrary units) of pS6S235–236 (e) and pAktS473 (f) in cTEC, mTEC, and CD45.2+ lymphocytes were measured in LPOWT vehicle-treated (n = 4), LPOWT rapamycin-treated (n = 4), LPOFGF21 KI vehicle-treated (n = 4), and LPOFGF21 KI rapamycin-

treated (n = 4) mice. Data were analyzed via two-way ANOVA followed by Tukey's multiple comparisons test in b–f. NS, P ≥ 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Mean and s.e.m. are indicated by horizontal lines. Each symbol represents an individual mouse. Data are the result of two independent experiments. Schematic in a created in https://BioRender.com.

When we compared mTORC1/mTORC2 ***signaling*** in cTECs, mTECs, and lymphocytes in vehicle-treated and rapamycin-treated LPOWT and LPOFGF21 mice (Fig.), we found that rapamycin treatment induced a significant decrease in pS6 (indicator of mTORC1 activity) ***signaling*** in cTECs, mTECs, and lymphocytes of LPOFGF21 mice (Fig.). Rapamycin treatment promoted a trend toward decreased pS6 levels in cTECs of LPOWT mice but was not statistically significant, while pS6 significantly declined in mTECs and CD45+ lymphocytes of LPOWT mice (Fig.). pAktS473 only decreased in rapamycin-treated cTECs of LPOFGF21 mice (Fig.). Rapamycin treatment has direct negative effects on DP thymocyte survival,, but our data are nonetheless consistent with the hypothesis that the effects of ***FGF21*** overexpression on ***thymus*** size are mediated through ***mTOR*** pathway activation in TECs.

FGF21 overexpression improves T-cell responses to influenza

Based on the observation that ***FGF21*** overexpression increased ***thymus*** size, we next evaluated peripheral T-cell changes induced by mTEC-driven ***FGF21*** overexpression. We first compared the ratio of naive (CD62L+, CD44−) to memory (CD62L+, CD44+) CD8+ T cells in the spleens of 2-month-old and 12-month-old LPOWT and LPOFGF21 KI mice (Fig.). While we found no significant difference in the CD8+ naive to memory ratio of 2-month-old mice, we found a significant increase in 12-month-old LPOFGF21 mice compared to LPOWT controls (Fig.), suggesting improved export of naive T cells into the periphery of aged LPOFGF21 mice. We also found similar increases in the naive to memory ratio of CD4+ splenocytes in aged LPOFGF21 mice, consistent with increased export of thymic CD4+ SP T cells seen during aging (Figs. and and Extended Data Fig.).

FGF21 overexpression in mTECs mitigates waning naive T-cell frequency and T-cell responsiveness to influenza infection in older mice.

a,b, Representative gating strategy showing isolation of CD62L+, CD44lo naive T cells and CD62L+, CD44hi memory T cells from CD3+, CD8+ (a) and CD3+, CD4+ (b) splenocytes in 2-month-old and 12-month-old LPOWT and LPOFGF21 mice. c,d, CD8+ (c) and CD4+ (d) naive to memory ratios were quantified in splenocytes of 2-month-old and 12-month-old LPOWT and LPOFGF21 mice (n = 6 per group). Data were analyzed via Student's t-tests. NS, P ≥ 0.05; **P < 0.01. e, Schematic diagram showing influenza infection timeline; 12-month-old LPOWT (n = 6) and LPOFGF21 (n = 6) mice were infected with 1,000 p.f.u. influenza A X31 virus via intranasal challenge. Body weights were recorded from 0 to 10 d.p.i. Upon euthanasia, the ***thymus***, spleen, MLN, and BAL were collected for staining with NP366-374-specific tetramers. Spleen and ***thymus*** total size were estimated via cell counting. Lung tissue was also collected for influenza mRNA quantification. f, Overall percentage of body weight lost throughout the course of infection was determined by comparing body weight at 0–10 d.p.i. from baseline body weight in influenza-infected LPOWT and LPOFGF21 mice (n = 6 per group, 3 independent experiments). g, ***Thymus*** size was determined 10 d.p.i. in LPOWT and LPOFGF21 mice (n = 6 per group) via counting with hemocytometer. h, Influenza viral mRNA was quantified in lung tissue collected 10 d.p.i. in LPOWT and LPOFGF21 mice (n = 6 per group). i,j, The relative frequency (i) and total number (j) of NP366-374-specific CD90.2+, CD8+ T cells in BAL, MLN, and spleen in influenza-infected LPOWT and LPOFGF21 mice (n = 6 per group) were quantified 10 d.p.i. via flow cytometry. Data were analyzed via two-sided Student's t-tests in c–j. NS, P ≥ 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Mean and s.e.m. are indicated by horizontal lines. Each symbol represents an individual mouse. Data are the result of two independent experiments. Schematic in a created in https://BioRender.com.

The decline in T-cell immunity due to decreased thymic output during aging is thought to narrow the TCR repertoire, for instance, by diminishing the frequency of T cells specific for some flu epitopes,, which can be rescued upon ***thymus*** regeneration. To test the functional capacity of peripheral T cells in LPOFGF21 mice, we challenged 12-month-old LPOWT and LPOFGF21 mice with 103 p.f.u. (plaque-forming units) of influenza A X31 via intranasal infection (Fig.) and monitored changes in body weight over the course of 10 days. X31-infected LPOFGF21 mice lost significantly less weight relative to control littermates (Fig.). Similarly, when older 18-month-old LPOFGF21

mice were challenged with influenza A PR8 (103 p.f.u.), we observed a significant reduction in the amount of body weight lost compared to PR8-infected age-matched LPOWT controls at 7 d.p.i. (days post infection), suggesting improved control of infection (Extended Data Fig.). Because short-term stressful stimuli such as infection induce rapid thymic atrophy, –, we compared total ***thymus*** cellularity at 10 d.p.i. (Fig.). We found that infected aged LPOFGF21 mice had significantly greater ***thymus*** cellularity than infected LPOWT controls (Fig.). Infected LPOFGF21 mice had significantly decreased influenza mRNA in the lung compared to infected LPOWT mice at 10 d.p.i., suggesting improved viral clearance (Fig.). At 10 d.p.i., bronchoalveolar lavage (BAL), spleen, and mediastinal lymph nodes (MLN) were also collected to measure the frequency and total number of influenza-specific CD8+ T cells via NP366-374 tetramer staining, as previously described (Fig.). Both the frequency and total number of NP-specific CD8+ T cells were significantly increased in the BAL of infected LPOFGF21 mice relative to controls, while no difference was observed in the spleen or MLNs of infected mice (Fig.). These results suggest that ***FGF21*** overexpression increases the number of influenza-specific T cells available to respond to infection in the BAL. Taken together, these observations suggest that ***FGF21*** overexpression in mTECs mitigates waning naive T-cell frequency and T-cell responsiveness to influenza infection in older mice.

The observations that aged LPOFGF21 mice have increased export of naive CD4+ and CD8+ T cells, as well as improved T-cell responsiveness to viral infection, led us to consider the impact of increased thymic ***FGF21 signaling*** on TCR repertoire diversity. Aging induces monoclonal and oligoclonal expansion of CD8 T cells, resulting in dampened immune responses to infection, –. To address the impact of increased intrathymic ***FGF21 signaling*** on CD8 TCR repertoire diversity during aging, we measured the frequency of Vβ2+, Vβ7+, Vβ8+, and Vβ11+ CD8 T cells in peripheral blood lymphocytes of 2-month-old and 12-month-old LPOWT and LPOFGF21 mice (Extended Data Fig.). Although previous studies showed expansion of Vβ8+ and Vβ11+ CD8 T cells at >15–20 months of age, we found no significant difference in Vβ frequency between LPOWT and LPOFGF21 mice during the first year of life (Extended Data Fig.). These data suggest that mTEC-driven ***FGF21*** overexpression may not impact age-associated CD8 T-cell clonal expansion. However, differences may not be apparent until later in life, and this approach may not be sensitive enough to detect subtle differences.

FGF21 overexpression mitigates impairment of T-cell tolerance

With the observation that ***FGF21*** overexpression improved the age-associated decline in naive T cells and improved outcomes to influenza infection, we next evaluated age-associated declines in clonal deletion among ***signaled*** (CD5+ TCRβ+) thymocytes using flow cytometry, as previously reported, (Fig.). We compared both early clonal deletion, occurring among CCR7− ***signaled*** thymocytes, and later clonal deletion, occurring among CCR7+ thymocytes, in 2-month-old and 12-month-old LPOWT and LPOFGF21/***FGF21*** homozygous KI mice (Fig.). As we and others have previously reported,,,, we observed an age-associated decline in the frequency of both early and late clonally deleted (cleaved caspase 3+) thymocytes in LPOWT mice (Fig.). Clonal deletion of early and late ***signaled*** thymocytes were both significantly higher in the LPOFGF21 mice at both time points compared to LPOWT controls (Fig.). Surprisingly, while we observed an age-associated decline in the clonal deletion of late (CCR7+) thymocytes in LPOFGF21 mice (although this was less pronounced than the decline in LPOWT mice), early (CCR7−) clonal deletion was preserved at the level of young mice in 12-month-old LPOFGF21 mice (Fig.), suggesting potential indirect effects of ***FGF21***-mediated ***paracrine signaling*** to preserve earlier stages of clonal deletion.

Persistent medullary TEC-driven overexpression of ***FGF21*** protects against age-associated impairments in clonal deletion and development of peripheral autoimmunity.

a, Gating strategy used to detect clonal deletion among early (CCR7−) and late (CCR7+) lineage− (CD19−, CD25−, TCRγδ−, NK1.1−) ***signaled*** (CD5+, TCRβ+) T cells. b,c, The frequency of cleaved caspase 3+ cells was quantified among early (b) and late (c) ***signaled*** T cells in 2-month-old LPOWT and LPOFGF21/***FGF21*** homozygous mice (n = 5 per group) and in 12-month-old LPOWT and LPOFGF21/***FGF21*** homozygous mice (n = 5 per group). Data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Mean and s.e.m. are indicated by horizontal lines. Significant comparisons are indicated (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). d, The presence of ANAs was assessed in serum from 12-month-old LPOWT and LPOFGF21 mice (n = 8 per group). Serum was incubated on HEp-2-coated slides, followed by immunostaining with anti-mouse IgG FITC. The number

of animals that tested positive for ANAs is indicated in the IgG channel image. Images are at ×20 magnification. Scale bars, 50 μm. e, Liver, lung, and salivary tissue were collected from 12-month-old LPOWT and LPOFGF21 (n = 7 per group) and evaluated for the presence of lymphocytic infiltrates via H&E staining (indicated via arrows). The number of mice testing positive for infiltrates are indicated in each tissue. Scale bars, 50 μm (×20 magnification). f, Representative plots showing the gating strategy determining the frequency of CD4+ Treg cells (CD4+ CD8− CD25+ Foxp3+) (top) and Helios+/Helios− CD4+ Treg cells (bottom) among splenocytes in 2-month-old and 12-month-old LPOWT and LPOFGF21 mice (n = 3 per group). g–i, The frequency of CD4+ Treg cells (g), Helios+ CD4+ Treg cells (h), and Helios– CD4+ Treg cells (i) were compared between 2-month-old (n = 3 per group) and 12-month-old (n = 3 per group) LPOWT and LPOFGF21 mice by one-way ANOVA followed by Tukey's multiple comparisons test. Mean and s.e.m. are indicated by horizontal lines. Significant comparisons are indicated (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

We next evaluated 12-month-old LPOFGF21 mice for the presence of indicators of peripheral autoimmunity. LPOFGF21 mice had a lower frequency of antinuclear antibodies (ANAs) in the serum, with only 25% of KI animals testing positive for the presence of antinuclear immunoglobulin G (IgG) (compared to 75% of LPOWT animals) (Fig.). We further investigated the impact of ***FGF21*** overexpression on development of peripheral autoimmunity by evaluating lung, liver, and salivary gland tissue from 12-month-old LPOFGF21 mice for the presence of inflammatory lymphocytic infiltrates (defined as >20 lymphocytes per foci). LPOFGF21 mice had decreased incidence of lymphocytic infiltrates in the liver and salivary gland, while incidence of infiltrates in the lung generally did not differ from LPOWT mice (Fig.). As LPO is expressed in salivary gland, it is possible that changes in local salivary gland expression of LPO may contribute to differences in lymphocytic infiltrates in this tissue.

To address whether these observed differences in peripheral autoreactivity could be partially explained by differences in regulatory T (Treg) cell frequencies, we measured the frequency of CD4+ Treg cells (CD25+ Foxp3+) in splenocytes from 2-month-old and 12-month-old LPOWT and LPOFGF21 mice (Fig.). Consistent with previous work, –, we observed an age-associated increase in CD4+ Treg cells in both LPOWT and LPOFGF21 mice as well as an age-related increase in both genotypes of CD25lo CD4+ Treg cells (Fig.). In both groups of aged animals, we noted an apparent increase in the population of CD25lo CD4 Treg cells, which is also consistent with previous reports. It is worth noting that 12-month-old LPOFGF21 mice had a higher frequency of CD4+ Treg cells compared to age-matched LPOWT controls (Fig.). Expression of the transcription factor Helios has been used to distinguish thymic-derived tTreg cells from peripherally derived pTreg cells,. Consistent with reduced thymic output in the aged ***thymus***, we observed a decrease in the frequency of Helios+ Treg cells and an increase in the frequency of Helios− Treg cells in splenocytes of 12-month-old LPOWT mice, while the frequencies of Helios+/Helios− CD4 Treg cells were not significantly different between 1-month-old and 12-month-old LPOFGF21 mice (Fig.). These results, together with our peripheral naive T-cell and infection data, support the conclusion that mTEC-derived ***FGF21*** overexpression improves ***thymus*** ***function*** in older mice to preserve T-cell output while maintaining both generation of Treg cells and negative selection of autoreactive T cells during aging.

Paracrine ***FGF21*** ***signaling*** increases cTEC size during aging

cTECs form networks of finely branched processes which create the structural niches required for T lymphocyte development and limit the overall size of the ***thymus***. We have previously demonstrated that aging is associated with a marked contraction of the cortical compartment, predominantly resulting from contraction of cTEC size and cortical projections. To address whether ***paracrine*** ***FGF21*** ***signaling*** to cTECs would protect cell size during aging, we compared cTEC size and morphology between 1-month-old and 6-month-old LPOWT and LPOFGF21 KI mice crossed with FoxN1Cre R26Confetti reporter mice, which allows for labeling of individual TEC,. Qualitative analysis of cTEC morphology in 3D projections from 60 μm optical stacks revealed a significant contracture of cTEC cell size in 6-month-old LPOWT mice, with the most striking changes occurring in the subcapsular zone as we have previously reported (Fig. and Supplementary Videos and). By contrast, cTECs in 6-month-old LPOFGF21 mice retained more of their labyrinth morphology, more closely resembling that seen in 1-month-old mice (Fig. and Supplementary Videos and). Quantification of cTEC cell area revealed that while aging induced a significant reduction in average cTEC size in 6-month-old FoxN1Cre R26Confetti LPOWT thymi, age-matched FoxN1Cre R26Confetti LPOFGF21 thymi were protected from decreases in cTEC size (Fig.). These results, in agreement with our observed increased activation of pAktS473 in cTECs of aged LPOFGF21 KI mice (Fig. and Extended Data Fig.

), indicate a potential role for ***FGF21***-mediated activation of mTORC2 in maintenance of cTEC cell morphology and overall size.

Paracrine thymic ***FGF21*** ***signaling*** increases cTEC size during aging.

a–d, Representative maximum intensity Z-Stack 3D projections taken from 60 μm optical stacks (×20 magnification) from 1-month-old FoxN1Cre R26Confetti LPOWT (n = 4 biological replicates), 1-month-old FoxN1Cre R26Confetti LPOFGF21 (n = 4), 6-month-old FoxN1Cre R26Confetti LPOWT (n = 3), and 6-month-old FoxN1Cre R26Confetti LPOFGF21 (n = 5) thymic sections. 3D animations can be found in Supplementary Movies –. e, cTEC area (μm2) was quantified from Z-Stack projections from thymic sections from 1-month-old FoxN1Cre R26Confetti LPOWT (n = 4), 1-month-old FoxN1Cre R26Confetti LPOFGF21 (n = 4), 6-month-old FoxN1Cre R26Confetti LPOWT (n = 3), and 6-month-old FoxN1Cre R26Confetti LPOFGF21 (n = 5) mice via blinded analysis with ImageJ software. Each symbol represents a single cTEC. Red horizontal lines represent mean ± s.e.m. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons. NS, $P \geq 0.05$; ****$P < 0.0001$.

Discussion

Thymus size is dynamic over the ***lifespan*** and is ***regulated*** by both reversible responses to stimuli including steroid hormones, infections, and stressors, as well as progressive age-associated factors, , –. In this study, we found that ***FGF21*** overexpression in the ***thymus*** resulted in a larger ***thymus*** size early in life, which persisted during aging. This is consistent with the view that common ***signaling***, environmental, and/or epigenetic, mechanisms may ***regulate*** both developmental processes and aging outcomes. For instance, rapamycin treatment restricted to developmental windows extends ***lifespan*** in crustaceans, flies, and mice,, and larval crowding extends ***lifespan*** in flies, , , –, as does litter crowding in mice. Similarly, short exposure to growth hormone during early postnatal periods restricts ***lifespan*** in long-lived Ames dwarf mice. Together, these findings support the notion that developmental processes, even during the earliest periods in life, have functional consequences on the ***regulation*** of ***lifespan*** and healthspan. Given that the ***thymus*** begins to atrophy relatively early in life, it is possible that it may be especially sensitive to such developmental effects on peak organ size and subsequent age-associated declines in size and ***function***. Therefore, the increased ***thymus*** cellularity we find in older LPOFGF21 mice may result strictly from increased ***FGF21*** activity during ***thymus*** development, during later periods, or both. Future studies designed to ***modulate*** ***FGF21*** and ***mTOR*** ***signaling*** at distinct periods in the ***lifespan*** using inducible, tissue-specific genetically modified mouse models will be required to distinguish these possibilities.

Although the steady state size of the ***thymus*** progressively declines with age, even that can be temporarily reversed in all tested species, including mice, non-human primates, and humans (reviewed in ref.). Great progress has been made toward regeneration of ***thymus*** ***function*** in otherwise healthy aging adults, and after exogenous insults, such as radiation, , –, but long-term, durable regeneration remains to be achieved. It is worth noting that a population of "age-associated" TECs linked with defective recovery of ***thymus*** cellularity following sublethal total body irradiation has recently been described and have been linked with reduced expression of epithelial regenerative factors following acute injury (including ***Fgf21***), presenting further barriers to reconstituting thymic ***function***.

The transient nature of regeneration after castration is consistent with the fact that the gene expression signature of cortical stromal cells at the peak of regeneration is largely unchanged relative to the atrophied state and with work showing that accumulation of relatively large amounts of oxidative damage early in life in cTECs occurs as a result of the naturally low levels of the hydrogen peroxide quenching enzyme catalase that are necessary to drive the conspicuously high levels of basal autophagy that is required for antigen presentation and induction of T-cell tolerance,.

Together, these observations suggest that an optimal approach for durable regeneration may require protection from oxidative damage and sustained growth factor ***signaling*** to cTECs. Here we report that sustained ***paracrine*** ***FGF21*** ***signaling*** does indeed partially mitigate atrophy and sequelae (Fig. and Fig.). The kinetics of mTORC1/mTORC2 ***signaling*** and TSC1 expression in cTECs are consistent with a role for mTORC1 in ***regulating*** cell proliferation during early phases of ***thymus*** growth in young animals, followed by a shift toward mTORC2

activity during the steady state, and then declines in both ***FGF21*** and ***mTOR signaling*** during atrophy (Fig. and Extended Data Fig.). Indeed, although mTORC1 ***signaling*** promotes cell growth, a TSC1-mediated shift away from mTORC1-mediated repression of autophagy has been shown to be important for TEC survival and ***function***. It is worth noting that ***FGF21*** overexpression, unlike antioxidant overexpression, was protective against indicators of peripheral autoimmunity (Fig.). Supporting a role for mTORC2 in preserving cellular morphology in cTECs during later stages of ***thymus*** maintenance, ***paracrine FGF21*** also promoted increased cTEC cell size during aging (Fig.). Together, the observations that overexpression of ***FGF21*** increased individual cTEC size and early (cortical) T-cell progenitor number, but not cTEC number, in older mice (consistent with Youm et. al.), suggest that individual cTEC size is a key regulator of the number of niches available for T-cell progenitors and therefore overall ***thymus*** size. Because ***FGF21*** is not a mitogen and has a strong safety profile,, it may represent an ideal candidate for meeting the growth ***signal*** criterion described above. Thus, an optimal approach for durable and safe ***thymus*** regeneration in adults may involve a combination of sustained ***FGF21 signaling***, distinct periods of selective mTORC1/mTORC2 stimulation, and appropriate strategies for reducing oxidative damage in stromal cells.

In addition, in this study we report the validation of Lpo as a broadly expressed mTEC gene (Figs. and and Extended Data Figs. , , and). While Lpo expression is not predicted to be Aire dependent, its expression may be partially ***regulated*** by Fezf2, as evidenced by comparing Lpo expression between wild-type and Fezf2−/− mTECs in a previously published microarray dataset (Supplementary Table). Outside the ***thymus***, Lpo is highly expressed in secretory structures including the salivary and lacrimal glands. While the physiological ***function*** of Lpo in the ***thymus*** is currently unclear, it is known to oxidize thiocyanate ions to neutralize thiols produced by foreign microbes in the saliva, tears, and milk, –. Emerging evidence supports the importance of maintaining an environment of low-grade sterile inflammation in the thymic medulla to promote self-tolerance, potentially through expression of type II and III interferons,. As inflammation is associated with increased oxidative stress, it is tempting to speculate that LPO's oxidizing activity may be beneficial for self-tolerance induction through the generation of oxidized self-peptides, as a mimic of oxidation events that may occur in the periphery during inflammation. In support of this concept, a study characterizing germline Lpo knock-out mice revealed widespread organ autoimmunity and systemic inflammation. Thus, Lpo expression in the ***thymus*** medulla may promote antimicrobial defenses and/or promote an oxidative environment to allow induction of tolerance to oxidized self-peptides.

Limitations of the study

Due to the direct effects of rapamycin on lymphocytes, it is difficult to directly assess the effects of pharmacological mTORC1/mTORC2 inhibition on thymic stromal cell ***function***. Similarly, it is not yet clear whether ***FGF21*** mediates changes in ***mTOR signaling*** in cTECs directly or indirectly. Future studies incorporating cTEC-specific and mTEC-specific gene ablation or overexpression will be required to more comprehensively define the cellular and molecular mechanisms ***regulating paracrine FGF21 signaling*** in the ***thymus***. Further studies will also be required to determine the downstream effects of ***FGF21 signaling*** on cTEC cytoskeleton organization and proliferation. These are important considerations, as age-associated contraction of the cortical epithelium would be expected to reduce the capacity of the ***thymus*** to support selection of developing T cells,. It is important to note that it also remains to be seen whether increased thymic ***FGF21*** expression has the capacity to reverse thymic atrophy after it has already occurred in aged animals.

Methods

Mice

C57BL/6 (JAX: 000664) mice were purchased from The Jackson Laboratory at 4–6 weeks of age and subsequently used as breeders. Offspring were subsequently used for experiments between 3 weeks and 12 months of age. Sanger sequence-verified LPOFGF21 KI founders were paired with C57BL/6 mice as breeders to generate LPOFGF21/WT heterozygous or LPOFGF21/***FGF21*** homozygous KI mice for experiments. Non-KI littermate controls were used as WT controls. Mouse genotypes were determined using real-time PCR with specific probes designed for the LPOFGF21 KI mutation (Transnetyx). Male and female LPOWT and LPOFGF21 KI mice between 2.5 weeks and 14 months of age were used for experiments. All mice used in experiments were bred and maintained at The University of Texas Health Science Center at San Antonio animal facility under specific

pathogen-free conditions. Mice were kept with a 14 h light/10 h dark cycle (7 a.m. to 9 p.m.), and all mice were provided with food and water ad libitum. Mice were housed at a daily temperature between 22–25 ºC, and relative humidity was maintained between 30% and 70%. Animal studies and all procedures were approved by the Institutional Animal Care and Use Committee, under protocol number 20190095AR.

Generation of LPOFGF21 KI mice

Guide RNAs targeting Lpo gene stop codon at exon 13 were identified using CRISPOR.Tefor.net guide RNA search tool. Double-strand break of the CRISPR guide RNA (crRNA), ACCATGGACATGTTCGATGT, was located 29 bp from the foreign DNA KI locus before the stop codon. The plasmid donor DNA was synthesized from GenScript; 1,443 base pair foreign DNA composed of T2A–mCherry–P2A–***Fgf21*** cDNA, flanked by 700 bp 5′ homology arm and 702 bp 3′ homology arm, was cloned into pUC-57 backbone plasmid. PAM (protospacer adjacent motif) sequence was modified from AGG to AGa in donor DNA to silence Cas9 activity in it. Single-guide RNAs (32 ng µl−1), donor DNA (15 ng µl−1), and eSpCas9 Nuclease (50 ng µl−1) were mixed in injection buffer (10 mM Tris–NaCl, 0.25 mM EDTA) and incubated at room temperature for 20 min before zygote microinjection session. sgRNAs were synthesized from Synthego (sgRNA with chemical modification), and eSpCas9 was purchased from Millipore-Sigma. PCR verification was performed to confirm successful KI mutation insertion in Sanger sequence-verified founders.

Quantification of ***Fgf21*** and Lpo expression in TEC subsets

Fgf21 and Lpo expression were quantified in nine TEC populations described in a previously published single-cell transcriptome profiling dataset (ArrayExpress, E-MTAB-8560). scRNA-seq data obtained from the dataset (relative ***Fgf21***/Lpo expression, TEC population frequencies from 1–52 weeks) were analyzed using the Seurat package in R (v4).

Analysis of Lpo ***regulation*** expression by Aire and Fezf2

Regulation of thymic Lpo expression by Aire and Fezf2 was studied by measuring the log2 fold change in Lpo expression between FACS-sorted mTECs of wild-type mice compared with Aire−/− and Fezf2−/− mice using two previously published microarray datasets (Aire−/−: GSE:14365, Fezf2−/−: GSE:69105 (ref.)). The datasets indicated were analyzed using the GEO2R R script (v4.2.2) with default of options and all available replicates.

Stromal cell isolation

For thymic stromal cell isolation, thymi were collected and minced into small pieces with scissors. Fragments were passed several times through a P1000 pipette. ***Thymus*** pieces were allowed to settle, after which the supernatant containing lymphoid cells was removed. The remaining fragments were digested with 0.125% collagenase D (Roche) for 10 min at 37 °C. Three additional rounds of mechanical mixing, sedimentation, and removing lymphoid cells were performed, followed by a final digestion in 0.05% trypsin (Sigma). After the final digestion, cells were washed and pelleted by centrifugation (440 × g), then resuspended in FACS buffer (HBSS (Hanks' balanced salt solution), 5% FBS (fetal bovine serum), 0.05% DNAse) containing a cocktail of fluorescent antibodies recognizing Alexa 700-conjugated anti-CD45.2 (Ly-5.2) (Clone 104; Biolegend; 1:150 dilution), PECy-7-conjugated anti-EpCAM (CD326) (Clone G8.8; Biolegend; 1:600 dilution), and biotinylated anti-Ly-51 (Clone 6C3; Biolegend; 1:100 dilution)/PerCPCy5.5-conjugated streptavidin (Biolegend; 1:500 dilution). For dendritic cell isolation, after the final digestion, all the collected lymphoid cells and stromal cells were combined in one tube, washed, pelleted by centrifugation, and then stained with PerCPCy5.5-conjugated anti-CD45.2 (Clone 30-F-11; eBioscience; 1:200 dilution), APC-conjugated anti-CD11c (Clone HL3; BD Pharmingen; 1:150 dilution), FITC-conjugated anti-B220 (Clone RA3-6B2; Biolegend; 1:400 dilution), and PECy7-conjugated anti-EpCAM. For endothelial and mesenchymal (pericyte, fibroblast) cell isolation, stromal cells were stained with biotin-labeled anti-PDGFRα and anti-PDGFRβ (Clones APA5, APB5; Biolegend; 1:100 dilution), followed by incubation with a cocktail containing BV650-conjugated Streptavidin (Biolegend; 1:500 dilution), FITC-conjugated anti-CD31 (Clone W18222B; Biolegend; 1:300 dilution), APC-conjugated CD146 (Clone ME-9F1; Biolegend; 1:150 dilution), PE-conjugated gp38 (Clone 8.1.1; Biolegend; 1:200 dilution), Alexa 700-conjugated CD45.2, and PE-Cy7-conjugated anti-CD326. Dead and/or dying cells were excluded by DAPI staining (0.1 µg ml−1 final concentration). For intracellular flow cytometry experiments,

cells were stained with Zombie Violet viability dye (Biolegend catalog number 423113; 1:1,000 dilution), then subsequently fixed and permeabilized using BD Cytofix/Cytoperm kit. Fixed and permeabilized cells were then stained with a cocktail of fluorescent antibodies recognizing APC-conjugated anti-Phospho-AktS473 (Cell ***Signaling*** Technology catalog number 11962; dilution 1:50), PE-conjugated anti-Phospho-4EBP1T36/45 (Clone V3NTY24; eBioscience; dilution 1:30), and PE-conjugated anti-Phospho-S6S235/236 (Clone cupk43k; eBioscience; dilution 1:30). To measure TSC1 expression, fixed and permeabilized cells were incubated with pure anti-TSC1 (Clone A6F1; Invitrogen; dilution 1:200), followed by secondary staining with FITC-conjugated Goat Anti-Mouse IgG (Jackson catalog number 115-095-146; dilution 1:200). Flow cytometry analysis was performed on the Cytek Aurora spectral flow cytometer running SpectroFlo v3.3.0. Experiments were analyzed using FlowJo software (v10.9).

TEC proliferation/apoptosis

To measure the frequency of TECs undergoing apoptosis and/or proliferation, thymic stromal cells were enriched with collagenase as described above. Following surface staining, samples were washed with annexin binding buffer containing 10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2 (pH 7.4), then incubated with APC-conjugated Annexin V (Invitrogen, catalog number A35110; dilution 1:30) at room temperature for 10 min. Cells were washed with binding buffer and resuspended with Foxp3 fixation/permeabilization buffer (eBioscience, catalog number 00-5523-00). After being fixed for 30 min on ice, samples were washed with permeabilization buffer, followed by incubation with FITC-conjugated anti-Ki67 (Clone 11F6, Biolegend; dilution 1:200) at room temperature for 30 min. Apoptotic cells were distinguished from dead cells via staining with LIVE/DEAD Blue (Invitrogen, catalog number L23105; dilution 1:1,000) before fixation/permeabilization.

Thymocyte subset isolation

Thymocyte CD4/CD8 populations were stained by preparing single-cell suspensions from whole thymi crushed against a mesh filter and subsequently filtered. Five million cells per sample were stained with a cocktail of fluorescent antibodies containing APC-Cy7-conjugated anti-CD4 (Clone RM4-5; Biolegend; dilution 1:250) and BUV 395-conjugated anti-CD8 (Clone 53-6.7; BD Biosciences; dilution 1:250). DAPI staining was used to exclude dead/dying cells. Flow cytometry analysis was performed on the Cytek Aurora spectral flow cytometer running SpectroFlo v3.3.0. Experiments were analyzed using FlowJo software (v10.9).

TCR Vβ staining

To measure CD8 TCR repertoire diversity, peripheral blood lymphocytes were obtained from 2-month-old and 12-month-old mice. Briefly, blood was obtained via cardiac puncture and collected into 1.5 ml microcentrifuge tubes containing 50 µl EDTA (pH 8.0) kept on ice to prevent clotting. Blood samples were then transferred to a 15 ml conical tube, and red blood cell lysis was performed with ACK lysis buffer. To measure the frequency of Vβ2+, Vβ7+, Vβ8+, and Vβ11+ CD8 T cells, samples were incubated with a cocktail of fluorescent antibodies containing APC-Cy7-conjugated anti-CD4, BUV395-conjugated anti-CD8, PE-conjugated anti-TCR Vβ2 (Clone B20.6; BD Biolegend; dilution 1:200), FITC-conjugated anti-TCR Vβ7 (Clone TR310; Biolegend; dilution 1:100), BV510-conjugated anti-TCR Vβ8 (Clone F23.1; BD Biosciences; dilution 1:200), and BV650-conjugated anti-TCR Vβ11 (Clone RR3-15; BD Biosciences; dilution 1:200). DAPI staining was used to exclude dead/dying cells before performing flow cytometry analysis.

Treg cell analysis

To measure the frequency of peripheral CD4+ Treg cells, single-cell suspensions were prepared from spleens collected from 2-month-old and 12-month-old LPOWT and LPOFGF21 mice. Briefly, after removing red blood cells with ACK lysis buffer, approximately 5 × 106 splenocytes were incubated with a cocktail of fluorescent antibodies containing APC-Cy-7-conjugated anti-CD4, BUV 395-conjugated anti-CD8, PE-conjugated anti-CD25 (Clone PC61; BD Biosciences; dilution 1:200), and BV650-conjugated anti-CD44 (Clone IM7; Biolegend; dilution 1:200). Samples were then washed and incubated with the Foxp3 fixation/permeabilization buffer described above. Following fixation and permeabilization, samples were incubated with PE-Cy7-conjugated anti-Foxp3 (Clone FJK-16s; eBioscience; dilution 1:250) and FITC-conjugated anti-Helios (Clone 22F6; Invitrogen; dilution 1:250). Flow cytometry analysis

was performed on the Cytek Aurora spectral flow cytometer running SpectroFlo v3.3.0. Experiments were subsequently analyzed using FlowJo software (v10.9).

Gene expression qRT-PCR

Following enrichment of thymic stromal subsets with collagenase as described above, single-cell suspensions were enriched for mTECs by staining with FITC-conjugated anti-CD45.2 (Clone 104; Biolegend; dilution 1:300), PE-Cy7-conjugated anti-EpCAM, and A647-conjugated anti-Ly51 (Clone 6C3; Biolegend; dilution 1:100). To enrich for thymic macrophages, single-cell suspensions were stained with APC-conjugated anti-CD19 (Clone 6D5; Biolegend; dilution 1:200), FITC-conjugated anti-CD11b (Clone M1/70; Biolegend; dilution 1:500), and PE-conjugated anti-F4/80 (Clone BM8; Biolegend; dilution 1:200). To enrich for thymic fibroblasts, single-cell suspensions were stained with APC-conjugated anti-CD45.2 (Clone 104; Biolegend; dilution 1:150), PE-Cy7-conjugated anti-EpCAM, FITC-conjugated anti-CD31 (Clone MEC13.3; Biolegend; dilution 1:200), and PE-conjugated anti-gp38 (Clone 8.1.1; Biolegend; dilution 1:200). Thymocyte subsets (CD4+ SP, CD8+ SP, CD4+ CD8+ DP, and CD4− CD8− DN) were isolated by staining with FITC-conjugated anti-CD8 (Clone H35-17.2; Invitrogen; dilution 1:200) and PE-conjugated anti-CD4 (Clone RM4-4; Biolegend; dilution 1:200). Thymic stromal or thymocyte populations of interest were subsequently sorted using a BD FACS Aria Fusion cell sorter running FACS Diva v8.0.1 (BD Biosciences) using the single-cell sorting mode with a 100 µm nozzle. qRT-PCR was performed as previously described. Briefly, RNA was isolated using the RNAqueous Micro Kit for FACS-purified samples (Invitrogen, catalog number AM1931) or the E.Z.N.A. HP Total RNA Kit (Omega, catalog number R6812) for homogenized whole salivary, liver, lung, pancreas, and white adipose tissues per manufacturer's instructions. cDNA synthesis was performed using a SuperScript VILO cDNA synthesis kit (Invitrogen) per manufacturer's instructions. cDNA preamplification was performed using the TaqMan PreAmp Master Mix Kit (catalog number 4384267, Thermo Fisher Scientific) per manufacturer's instructions. Quantitative PCR (cycle 1: 95 °C for 10 min; cycle 2, ×40: 95 °C for 15 s and 60 °C for 1 min) was performed using a BioRad CFX96 Real-Time System/C1000 Touch Thermal Cycler using presynthesized FAM-MGB TaqMan Gene Expression Assay Probes (Thermo Fisher Scientific) to amplify the following genes: Lpo (assay identification (ID), Mm00475466_m1), ***Fgf21*** (assay ID, Mm07297622_g1), mCherry (assay ID, Mr07319438_mr), Klb (assay ID, Mm00473122_m1), Fgfr1 (assay ID, Mm00438930_m1), Fgfr3 (assay ID, Mm00433294_m1), and pan_Influenza A (assay ID, Vi99990011_po). qPCR results were analyzed using Bio-Rad CFX Manager software (v3.1.1517.0823). Cq values were normalized to Hprt ($2^{-\Delta Cq}$).

Serum/whole thymic tissue analysis of ***FGF21***

Analysis of circulating ***FGF21*** from the serum and whole ***thymus*** tissue was performed using the Mouse/Rat FGF-21 Quantikine ELISA Kit (catalog number MF2100, R&D Systems) according to the manufacturer's recommendations. Briefly, whole blood was collected by cardiac puncture at the time of euthanasia and allowed to clot at room temperature for 30 min. Blood samples were spun at 1,000 × g for 10 min, then serum was aliquoted and stored at −80°C. Whole protein extracts were obtained from homogenized whole pieces of thymic tissue (5 mg) using the EZLys Tissue Protein Extraction reagent (Abcam, catalog number ab286872). Samples were kept on ice during homogenization and incubated with protease inhibitors (Thermo Scientific, catalog number 78429) and DNAse to prevent tissue degradation. After homogenization, samples were pelleted by centrifugation (1,000 × g, 10 min, 4 °C) to remove debris. Supernatant was aliquoted and stored at −80°C. Samples were diluted according to the manufacturer's instructions before beginning the ELISA. An Agilent Bio-Tek Synergy LX plate reader was used to obtain the optical density set to 450 nm. Wavelength correction was applied by subtracting readings at 540 nm. A standard curve was created using provided standards and subsequently used to determine the relative concentration of ***FGF21*** (pg ml−1) in each sample.

Confocal/immunofluorescence imaging

To obtain LPO costaining images in C57BL/6 mice, fresh frozen thymic sections from 3- to 12-month-old mice were fixed in acetone followed by co-staining with anti-LPO (Proteintech catalog number 10376-1-AP; dilution 1:75)/Goat anti-Rabbit Cy5 (Thermo catalog number A10523; dilution 1:100) and anti-EpCAM A488 (Clone G8.8; Biolegend; dilution 1:200). To detect mCherry colocalization in LPOFGF21 mice, thymi from 1-month-old mice were fixed overnight in 2% paraformaldehyde while rocking at 4°C, followed by a 10–20% sucrose gradient overnight. About

10 μm sections were cut on a cryostat and blocked with 5% FBS in PBS before costaining with anti-Ly51 A647 (Clone 6C3; Biolegend; dilution 1:150), anti-DEC 205 A647 (Clone NLDC-45; Biolegend; dilution 1:200), anti-cytokeratin 8 A488 (Clone EP1628Y; Abcam; dilution 1:500), anti-cytokeratin 5 A647 (Clone EP1601Y; Abcam; dilution 1:200), anti-Aire A647 (Clone MM-525; BD Biosciences; dilution 1:150), and UEA1 FITC (Thermo catalog number L32476; dilution 1:200). Sections were stained for 1 h at room temperature, then washed and coverslipped with Prolong Diamond Antifade Mountant (Thermo catalog number P36961). All images were captured using a Zeiss LSM710 confocal microscope installed with ZEN 2011 software. Endogenous mCherry ***signal*** was captured using a 561 nm HeNe1 laser. DAPI was excited using the 405 nm diode laser; A488 ***signal*** was detected using a 488 nm argon laser, and A647 ***signal*** was captured using a 633 nm HeNe2 laser. Images were captured at ×10 and ×20 magnification. Identical laser settings were used to capture images from LPOWT and LPOFGF21 tissues. Images were subsequently processed using ImageJ software (v1.54d, National Institutes of Health (NIH)).

Confetti cTEC image analysis

Thymic sections from LPOWT and LPOFGF21 mice crossed with FoxN1Cre R26Confetti reporter mice were imaged using a three-stage sequential scan, as previously described. This method allows for maximum detection of three Confetti fluorescent proteins (cytoplasmic YFP and RFP, and membrane CFP) and simultaneous capture of age-associated autofluorescent pigments that can be removed from reporter images. Briefly, the first scan used 405 nm and 458 nm lasers to excite both CFP and autofluorescent pigments, with collection windows of 463–509 nm (for CFP) and 566–628 nm (for autofluorescent pigments). The second scan used a 514 nm laser to excite YFP with a collection window of 519–556 nm, and the third scan used a 561 nm laser to excite RFP with a collection window of 566–628 nm. Some 20× Z-Stacks were acquired on a Zeiss LSM710 confocal microscope. For full-color displays, CFP is presented as blue, YFP as yellow, and RFP as red. For qualitative analysis of cTEC morphology, 3D maximum projections were created in ImageJ with a 0.2 pixel minimum filter using the “3D Project” tool. Following acquisition, cTEC area quantification in this study was performed blinded using ImageJ software. Z-Stack images were processed before cTEC area quantification using the Stacks -> Z Project -> Average Intensity ***function***. Z-Stack images were then separated into the three acquired reporter channels (RFP, YFP, CFP) and the channel capturing autofluorescent pigments, which was removed from the composite image. Channel ***signal*** brightness/contrast were optimized for each image using the Image -> Adjust -> Color balance tool. After ***signals*** among the three channels were equalized within the image, individual cells (defined by any single color surrounded by cells of other colors) contained wholly within the image volume were identified for quantification. cTEC cell area from freehand selections was measured via the Analyze -> Measure tool. The criteria for selection excluded cell areas <400 μm2 or >3,000 μm2. At least 10–20 cTECs were measured per Z-Stack for a total of 20–40 cTECs quantified per individual mouse. Final quantitation represents one or two distinct Z-Stacks from at least three independent sex-matched thymi per group at each age.

Rapamycin-mediated ***mTOR*** inhibition

At the time of weaning, 3-week-old LPOWT and LPOFGF21 KI mice were injected with 4 mg kg−1 rapamycin (Thermo Scientific catalog number J62473.EX3) or vehicle via intraperitoneal injection every other day for 2 weeks as previously described,. At the time of euthanasia, the ***thymus*** was collected, and mTORC1/mTORC2 activation was measured in cTECs, mTECs, and lymphocytes via intracellular flow cytometry as described above. Mouse ***thymus***/body weights and total ***thymus*** cellularity via hemocytometer were also determined. Thymocyte CD4/CD8 subsets were also separately stained as described above.

Influenza infection

The 12-month-old LPOWT and LPOFGF21 KI mice were transferred to a BSL-2 facility and allowed to habituate overnight before infection. The following day, mice were anesthetized with light isoflurane anesthesia, and 1,000 p.f.u. of mouse-adapted H3N2 influenza (strain A/X-31 H3N2) or H1N1 (strain A/PR8) suspended in 50 μl PBS was intranasally administered to the left nare. Mouse body weights were recorded daily until 10 days post infection.

BAL and analysis of influenza-specific T cells

Upon euthanasia of influenza-infected and/or naive control mice, the trachea was exposed, and a 51-mm-long (75% polyester, 25% cotton) thread was passed underneath the trachea using curved tweezers. An 18-gauge Insyte Autoguard shielded I.V. catheter (BD) was inserted into the upper trachea and secured using the thread. BAL was removed by injecting and aspirating 1.0 ml of PBS using a 1 ml PP/PE syringe (Sigma-Aldrich). BAL was placed in a separate tube on ice, and the injection and aspiration procedure was repeated another time. Cells were centrifuged (440 × g, 5 min, 4 °C) and resuspended in ice-cold FACS buffer (HBSS, 5% FBS, 0.5% DNAse (1 mg ml−1), pH 7.2). Dasatinib (Tocris Bioscience) was added to the cells at a concentration of 50 nM, and cells were incubated 30 min at 37 °C. After incubation, cells were cooled on ice for 5 min. Fc receptors were blocked with CD16/CD32 (eBioscience, catalog number 14-0161-81; dilution 1:130) for 10 min on ice. Cells were then stained with 0.5 µg of NP366-374/Db (NP) tetramers and incubated protected from light for 1 h at 25 °C. Cells were then washed with sorting buffer and then stained with a cocktail containing fluorescent antibodies conjugated against CD8-V500 (Clone 53-6.7; BD Biosciences; dilution 1:100), CD4-FITC (Clone RM4-4; Invitrogen; dilution 1:200), CD90.2-PerCPeFluor710 (Clone 30-H12; Invitrogen; dilution 1:200), and CD44-BV510 (Clone IM7; Biolegend; dilution 1:200) on ice for 30 min. Cells were washed and suspended in sorting buffer for FACS analysis. NP-tetramer+ cells were analyzed on CD90.2+CD8+ singlet lymphocytes using a Cytek Aurora flow cytometer running SpectroFlo v3.3.0 and FlowJo v10.9 flow cytometry analysis software (Tree Star).

ANA evaluation

The presence of ANAs was evaluated in serum of 10- to 12-month-old LPOWT and LPOFGF21 mice. About 25 µl serum (1:100 dilution) was added to HEp-2 slides (MBL International, AN-1012) and incubated at room temperature for 30 min. Slides were washed twice, and then sample wells were incubated with FITC-conjugated anti-IgG (poly4060, Biolegend; dilution 1:100) for 30 min. Slides were washed and coverslipped with Vectashield Vibrance with DAPI (Vector Laboratories). Images were captured at ×10 and ×20 magnification using a Zeiss LSM710 microscope. Samples were coded, and evaluation was performed blinded. Laser settings for the FITC channel were kept identical between each sample.

Tissue lymphocytic infiltration studies

Lung, liver, and salivary gland tissue collected from 10- to 12-month-old LPOWT and LPOFGF21 mice were fixed for 24 h in 10% NBF and blocked in paraffin. Slides were cut and stained with hematoxylin and eosin (H&E). Sections from at least three different anatomic areas were examined for lymphocytic infiltration in each tissue. Samples were coded and evaluated for the presence of lymphocytic infiltrates blinded.

Statistics and reproducibility

All data were analyzed using Excel (Microsoft, v2410) and GraphPad Prism (v10.0.2) software. Statistical significance ($P < 0.05$) was computed using two-tailed unpaired Student's t-test, ordinary one-way ANOVA (analysis of variance), or two-way ANOVA, as indicated in the figure legends. Tukey's multiple comparisons test was performed following significant one-way and two-way ANOVA tests. No statistical method was used to predetermine sample size, but our sample sizes are similar to those reported in previous publications,. Sample sizes for each experiment are indicated in the figure legends. At least three biological replicates per group were used for all experiments. No animals or data were excluded from our analyses. No randomization method was used. Except where noted (evaluation of ANA positivity, glymphocytic infiltrates, and cTEC size analysis), investigators were not blinded to group allocation during analysis. Data distribution was assumed to be normal, but this was not formally tested.

Reporting summary

Further information on research design is available in the linked to this article.

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Notes

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