Lymph nodes link sex-biased immune aging to compromised antigen recognition

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Abstract

A diverse naive CD8 T cell repertoire is essential to provide broad protection against infection and cancer. Aging diminishes naive T cells, reducing potential diversity and leading to lymph node contraction. Here, we revealed that this decline occurs earlier in males, resulting in significant sex differences in immunity during middle age. Earlier in life, naive CD8 T cells in males become virtual memory cells prone to premature senescence. Due to androgen-driven thymic atrophy in males, naïve CD8 T cells are insufficiently replenished. Therapeutic thymus rejuvenation via testosterone ablation restored naive CD8 T cells in lymph nodes of middle-aged male mice, leading to enhanced tumor recognition. These findings show the crucial role of sex and age on lymph node T cell repertoires and suggest potential strategies to restore immune function in males during aging.

Main Text

Introduction

Age is a dominant risk factor for cancer, imposing a significant health burden on individuals over 50 years of age, with this demographic carrying 90% of the cancer mortality burden (*1*). Notably, males experience an almost twofold higher incidence and risk of mortality compared to females (*2*, *3*). While recent work has begun to clarify how tumor-targeting T cell responses are influenced by sex (*4–6*) and by age (*7–10*), relatively few studies address how these factors intersect, leaving critical gaps in understanding their combined effect on immune surveillance, elimination of dysplastic lesions by the immune system and the efficacy of immunotherapies. Therapies that reverse the effects of sex and age on immune function will improve healthy longevity.

The immune system's ability to recognize and respond to a wide array of antigens is directly linked to the number of naive T cells and their clonotypic diversity. Naive T cells gradually decline with age due to diminished de novo production in the involuted thymus and ongoing peripheral attrition through differentiation into memory cells and senescence-prone effector cells **(Fig.1A)** (*11–14*). The decline of naive T cells causes insufficient repopulation of lymph nodes (LNs), which are essential hubs that facilitate encounters between antigen-presenting cells (APCs) and a diverse population of naive T cell clonotypes that are critical to generating adaptive immune responses (*15*). Although age-related loss of naive T cells—and with it maximal clonotypic diversity - has been implicated in impaired vaccine responses and tumor surveillance (*11*, *16*, *17*), whether and how this process differs by sex - particularly during the critical transition from young adulthood to middle age - remains unclear.
In this study, we bridge this gap by analyzing naive CD8 T cell dynamics across various ages in females and males to measure the impact on effective antigen recognition in LNs. Accelerated thymic involution and LN contraction in males reduces naive T cell availability and impairs tumor recognition, emphasizing the critical role of clonal diversity of naïve T cells in LNs. We show that temporarily reversing thymus atrophy rescues naive T cell numbers and tumor-targeted T cell responses in middle-aged males—emphasizing the potential of immune rejuvenation strategies to mitigate age and sex-related differential treatment responses. This understanding will help to enhance recent immune rejuvenation efforts (*18–21*).

Age and sex are critical factors for tumor draining LN-derived T cell responses to melanoma

Analysis of data from the Surveillance, Epidemiology, and End Results (SEER) program indicates that males have higher overall cancer incidence rates, including melanoma, a tumor type that is drained by LNs in the skin, with these differences becoming apparent in middle age **(Fig. 1B,C)**. In addition to the well-documented age-related T cell impairments—such as exhaustion or the development of suppressor phenotypes (*4–6*, *9*), a notable proportion of tumor-infiltrating lymphocytes (TILs) in older individuals retain a naive state. Consequently, these TILs may not actively engage in, and could even remain bystanders to, antitumor immune responses (*22–24*). Despite similar levels of tumor-infiltrating T cells (TILs) in melanoma between sexes across age groups **(fig. S1A)**, published scRNA-seq data (24–27) from melanoma patients **(fig. S1B-D)** revealed a higher proportion of naive-like CD8 T cells within tumors from male patients, whereas female tumors contained TILs with a more cytotoxic and exhausted phenotype, suggesting antigen specific activation **(Fig. 1D-E)**.

In mice, tumor control declined dramatically from young adult to middle age in males, whereas females have a more modest decline in CD8 T cell-dependent tumor control **(Fig. 1F)**. In young adult mice, tumor growth dynamics were similar between sexes; however, middle-aged females exhibited a CD8 T cell-dependent delay in tumor growth compared to males and a stronger response to anti-PD-1 treatment, supporting the notion of a T cell-dependent sex disparity in limiting cancer progression **(Fig. 1F; fig. S2A-H)**. At an early stage of tumor growth—when significant sex-related differences in tumor size were already apparent **(Fig. 1G)**—we performed flow cytometric analysis of tumor-infiltrating lymphocytes (TILs) in B16-OVA tumors in middle-aged mice. Consistent with patient data **(Fig. 1D-E),** tumors in middle-aged females contained a higher proportion of antigen-experienced T cells ($CD44^+PD1^+$) and T cells with functional effector phenotype ($GzmB^+$), while males exhibited a predominance of naive CD8 T cells **(Fig. 1H; fig. S3A,B)**. This disparity occurred despite comparable overall CD8 T cell infiltration and intratumoral proliferation of effector T cells **(fig. S3C)**. The number of exhausted T cells ($TIM3^+PD1^+$) was higher in females and regulatory T cells similar between sexes **(fig. S3D,E)**.The higher frequency of $CD44^+PD1^+$ CD8 cells in the blood circulation in female mice suggests an extra-tumoral source for these cells **(Fig. 1I)**. We performed splenectomy (SpX) and utilized lymphotoxin alpha-deficient ($LTa^{-/-}$) mice—which lack functional LNs (*25*)—and found that LNs, rather than the spleen, are critical for supporting the superior tumor recognition capacity in females **(Fig. 1J)**. Consistently, tumor-draining LNs (tdLNs) from female mice contained significantly higher numbers of antigen-experienced ($CD44^+PD1^+$) and tumor specific ($Tetramer^+$) CD8 T cells, independent of the proliferation capacity of activated cells **(Fig. 1K, fig. S3E,F)**. These data suggest a higher probability of naive T cells recognizing tumor antigens in tdLNs of females. Together, these findings suggest that females contain a higher proportion of tumor-targeting CD8 effector TILs originating from the tdLNs. Of note, while sex disparities of intradermal APCs have been reported for adult mice (*26*), in middle-aged, LNs did not contain dysregulated proportions of APCs or show differences in antigen presentation between the sexes in human or murine LNs **(fig. S4A-E)**.

Age-related decline of naive CD8 T cells and contraction of LNs occur earlier in males

Volume segmentation of full-body computed tomography (CT) scans of humans from a publicly available database revealed larger spleen volumes in males, whereas LNs were significantly smaller **(Fig. 2A; fig. S5A,B)**. With age, LNs can accumulate fat due to lipomatosis, disrupting the correlation between volume and cellularity and potentially lead to an underestimation in the decline in cellularity (*27*). In middle-aged mice, LNs did not occur with a dysregulated stromal structure **(fig. S6A)**. LN size and cellularity declined with age, occurring earlier in males, leading to a significant sex disparity in middle-aged mice **(Fig. 2B,C)**. The differences were more pronounced in skin-draining LNs compared to mesenteric LNs and were independent of reproductive status and genetic background **(fig. S6B-D)**. LN weight strongly correlated with cellularity during physiological aging and under lymphopenic conditions, emphasizing the co-dependence of LN size with the number of lymphocytes **(Fig. 2D; fig. S6E)**, which suggests that, despite potential confounding factors, imaging-based LN volume measurements in humans reflect underlying cellularity.

The overall T cell pool is considered to be relatively stable, with only the naive-to-memory ratio declining due to an accumulation of memory T cells over time (*11*, *28*). However, our data indicate that after being established in young adults, absolute numbers of central memory T cells (Tcm) did not increase with age. Instead, naive CD8 T cells declined and declined faster in males **(Fig. 2E)**. A decline in the naive CD8 T cell frequency was also evident in the blood, spleen, and bone marrow (BM) **(Fig. 2F)**. Published multimodal datasets of human T cells from blood and LNs (*29*, *30*) corroborated these findings, showing a decline of naive CD8 T cells determined by applying a gene expression "*Naive score*" on scRNA-seq and flow cytometry data **(Fig. 2G,H; fig. S5C-F)**. To examine TCR diversity in LNs, we performed TCR sequencing of CD8 T cells from LNs of middle-aged mice and found a maximally diverse TCR repertoire with almost only unique clonotypes per LN, mirroring observations in humans (*31*), with over 96 % of unique T cell clonotypes in non-reactive LNs independent of sex **(fig. S7A-E)**. Thus, we hypothesized that the absolute number of naive T cells within a LN at any given time is directly correlated with the number of unique TCRs present and is therefore a rate-limiting factor for the probability of antigen recognition. Supporting this hypothesis, immunization of middle-aged mice with inactivated H1N1 virus particles or B16-OVA tumor cell lysates resulted in similar frequencies of CD8 T cells expressing the general activation marker CD69. However, significantly more CD8 T cells in females exhibited TCR-specific activation, as indicated by Nur77 expression (*32*) **(Fig.2I; fig. S7F)**. We attribute this sex imbalance to the partial depletion in males of naive CD8 T cells and the concomitant contraction of LNs, which are central to locoregional antigen processing and presentation. These data highlight the functional impact of higher naive T cell numbers in middle-aged females on the generation of antigen specific immune responses.

Enhanced propensity for an antigen-agnostic loss of T cell naivety in males

Continuous exposure to pathogens drives the transition from naive to central memory T cells (Tcm) in humans (*11*, *17*, *33*). Our data demonstrate that naive CD8 T cells, which are potentially capable of long-term survival, decrease with age in mice raised in low-pathogen environments **(Fig. 2E-F)**. Interestingly, adult male mice exhibit a higher proportion of $CD44^+CD62L^+$ cells even before absolute naive CD8 T cell numbers begin to differ between sexes in middle age **(Fig. 3A; Fig. 2E)**. We hypothesized that the early emergence of $CD44^+CD62L^+$ cells may contribute to the reduced naive CD8 T cell pool observed in middle-aged males. Consistent with previous studies (*34–36*), not all $CD44^+CD62L^+$ cells represented true Tcms ($CD49^+$); rather, the majority were virtual memory T cells (Tvm) characterized as $CD122^+CD49^-$ **(fig. S8A)**. The proliferation and survival of peripheral naive T cells depend on homeostatic mechanisms involving cytokines such as IL-7 and IL-15, as well as low-affinity TCR interactions with self-antigens (*14*). These processes can lead to antigen-agnostic differentiation of naive T cells into Tvm cells, particularly in cells with elevated tonic TCR signaling/presence of CD5 (*37*). The TCR affinity for self-antigens drives Tvm formation, with IL-15 serving as a co-stimulatory factor that defines the affinity activation threshold for self-TCR recognition (*37–39*) **(Fig. 3B)**. In murine lymph nodes (LNs), IL-15/IL-15 receptor alpha complexes (IL-15 Cx) are lower in adult males compared to females and decrease with age in both sexes. Thus, reduced IL-15 Cx levels are unlikely to explain the early Tvm formation in males **(fig. S8B)**. However, experimentally lowering the affinity activation threshold of naive T cells by treating adult female mice

with recombinant IL-15 Cx for four weeks caused a significant increase in Tvm cells within LNs. This expansion of Tvm cells subsequently led to a substantial reduction in the absolute number of naive CD8 T cells seven months later when the mice reached middle age **(Fig. 3C)**, making them similar to male mice. OT-I TCRs, specific for OVA, are homogeneously expressed on all CD8 T cells in OT-I mice and exhibit relatively higher recognition of endogenous self-antigens (*37*, *40*). This property enabled us to investigate aging-related changes in a model where all naive T cells express a TCR prone to antigen-agnostic differentiation into Tvm cells. As expected, naive OT-I mice displayed accelerated Tvm formation in LNs and spleen, accompanied by a significant reduction in naive CD8 T cells and LN cellularity between adulthood and middle age in both sexes (**Fig. 3D; fig. S8C)**. To extend these findings to the diversity of endogenous TCRs in wild-type (WT) mice, we used CD5 as a surrogate marker for TCR self-reactivity. Naive $CD5^{high}$ T cells are more prone to Tvm differentiation during aging than cells with intermediate levels of CD5 ($CD5^{int}$) (*37*, *41*). Consistent with this, we observed a higher proportion of $CD5^{high}$ naive T cells in OT-I LNs compared to WT LNs in young mice and a greater frequency of $CD5^{high}$ cells in Tvm populations than in naive T cells **(Fig. 3E, left)**. Notably in WT mice, $CD5^{high}$ cell proportions were higher in adult males but decreased with age, while females exhibited the opposite trend **(Fig. 3E, right)**. In middle-aged males, the decline in $CD5^{high}$ cells is likely a result of their conversion to Tvm cells when the mice were younger.

While the transition from naive to Tvm cells does not appear to impair the immediate ability of T cells to suppress tumor growth **(Fig. S8D)** or respond to infections (*42*), the tendency of Tvm cells to undergo apoptosis limits their longevity compared to naive T cells (*34*, *43*).Furthermore, the tropism of Tvm cells to relocate from LNs to the BM **(fig. S8E,F)** or liver (*37*) limits the number of T cells in LNs. As a result, the accelerated formation of Tvm cells differentially in male mice contributes to a sex-biased decrease of naive CD8 T cells in the LNs of male mice in middle age.

Accelerated thymus involution contracts the CD8 T cell pool in males across species

Given the significant impact of the reduction of naive T cells through memory differentiation and apoptosis, we investigated the reasons behind the lack of systemic replenishment. Mimicking the aging effect on LNs with experimental lymphodepletion, naive CD8 T cells recovered faster in middle-aged females than males **(Fig. 4A)**. The lymphopenia induced temporary increase in homeostatic proliferation in LNs was similar between sexes **(fig. S9A,B).** However, the majority of naive CD8 T cells during recovery was comprised of $CD103^{high}$ recent thymic emigrants (RTEs) (*44*, *45*), with a higher prevalence in females **(fig. S9C,D)**. The thymus is essential for T cell production early in life but its importance in human adults across all age groups is less certain (*12*). Studies have suggested negative health consequences, including an increased risk of cancer, for people who have undergone thymectomy (*46–48*). We found significant sex differences in thymus cellularity of mice **(Fig. 4B; fig. S10A-C)** and thymus tissue degeneration in humans, particularly in young adults and middle-aged individuals **(Fig. 4C)**, matching the decline in LN size and naive CD8 T cell numbers in mice and humans. These observations are corroborated by data from Drabkin *et al.* (*49*) that show age- and sex-related thymus involution in an independent cohort using the same method **(fig. S11A,B)**. The presence of RTEs correlated with the frequency of true naive CD8 T cells, reflecting the pronounced thymus atrophy in middle-aged human males **(Fig. 4D; fig. S11C,D)**. Interestingly, CD4

RTE cells in humans undergo a similar decline with age as CD8 RTEs, including sex-bias in middle-age, but only had a minor impact on naive CD4 T cell levels (**fig. S11E)**. Moreover, thymectomies (TmX) performed on young adult mice significantly reduced RTEs in LNs (detected by signal joint T cell receptor excision circles, sjTREC) and eliminated the sex differences found in sham-TmX animals **(Fig. 4E)**. Interestingly, TmX also abrogated the female advantage in LN cellularity, number of naive CD8 T cells **(Fig. 4F,G; fig. S12A-C)**, and equalized functional sex-disparities in tumor recognition and T cell-mediated growth suppression in middle aged mice **(Fig. 4H,I; fig. S12D,E)**. Collectively, these results demonstrate that the supply of LNs with de novo produced naive CD8 T cells from the thymus remains relevant even later in life in mice and suggest a continued role in humans during middle age.

Therapeutic thymus revitalization replenishes naive T cells and invigorates tumor-recognition

Thymus atrophy is not only linked to age-related deficiencies in the supply of bone marrow-derived lymphoid progenitors and stromal senescence but also to detrimental effects of testosterone (*50*). Although a direct impact on thymopoiesis in humans remains unclear, testosterone administration during gender-affirming therapy in individuals assigned female at birth negatively affects naive CD8 T cell levels (*51*). Further, data show that sex steroid ablation (SSA) partially reverses thymus atrophy and rejuvenates the T cell compartment in middle-aged mice but has minimal effect on aged LNs (*52–54*). We achieved long-term (young adults to middle-aged) and short-term (in middle-aged) SSA through gonadectomy (GonX). We also administered the gonadotropin-releasing hormone (GnRH) modulator Degarelix (DeX) to middle aged mice to achieve SSA **(fig. S13A,B,E,F,I,L)**. Our data show a higher proportion of double negative (DN) thymocytes, primarily in males, in middle aged mice **(fig. S10A-C)**. Performing GonX or DeX treatment increased thymus cellularity, normalized the ratio of double positive (DP)/double negative (DN) cells, and boosted mature single positive (SP) CD8 cell numbers ready to be released into the periphery as *de novo* naive T cells. **(Fig. 5A-C; fig. S13C,G)**. SSA led to repopulated LNs with sjTREC-carrying RTEs (**Fig. 5D,E**) and restored naive CD8 T cell numbers in LNs of middle-aged males to levels comparable to young adults and middle-aged females. In aged LNs, naive CD8 T cell numbers also increased after DeX treatment but remained low compared to middle-aged mice (**Fig. 5F; fig. S13A-M**). Consequently, SSA eliminated sex differences in tumor recognition in tdLNs and the presence of antigen-experienced ($CD44^+PD1^+$or $CD39^+$) and tumor-antigen specific ($tetramer^+$) CD8 TILs, resulting in similar tumor growth dynamics between middle-aged females and males **(Fig. 5G,H; fig. S14A-J)**. These findings indicate that SSA effectively revitalizes thymic function and enhances antitumor immunity in middle-aged male mice, eliminating sex-based differences in immune responses against tumors. However, the benefits of SSA on LNs are markedly reduced in aged males, highlighting an age-dependent limitation of this intervention.

Discussion

Our study revealed significant sex- and age-related disparities in the size of the naïve T cell populations and subsequent immune response to tumors, highlighting the critical roles of LNs and

naive $CD8^+$ T cells in antitumor immunity. Although T cell infiltration in tumors is often considered a positive prognostic indicator, the mere presence of T cells does not necessarily correlate with effective antitumor responses (*22–24*, *55*). We demonstrated that the frequency of "tumor-ignorant" naive TILs is higher in middle-aged males— a disadvantage that has been reported to become more pronounced in older age of both sexes (*8*). The increased prevalence of naive TILs in males suggests a relative decrease in activated, tumor-recognizing T cells, which alters the effector-to-naive ratio within the tumor microenvironment. Our data attributes this imbalance to the partial depletion of naive CD8 T cells and the concomitant contraction of LNs in middle-aged males. While aged mice exhibit a significant reduction in T cell receptor (TCR) diversity — up to 80% — leading to measurable deficits in immune functionality, it remains less clear whether a reduction in T cell clones has equivalent consequences in humans (*58–61*). Notably, LNs in aged humans preserve their proportional T cell diversity (*62*). However, our data suggest that a functional TCR repertoire depends not only on the relative diversity but also on the absolute number of unique T cell clones at the locoregional level of draining LNs. The strong interdependence between LN stromal cells and lymphocytes implies a reciprocal enhancement of aging effects: declining numbers of naive T cells contribute to stromal dysfunction and senescence, which in turn exacerbates lymphocyte reduction (*11*, *27*, *63–66*).

In line with recent publications (*48*), our findings underscore the importance of thymic functionality for CD8 T cell homeostasis in mice and demonstrate its continued significance in human adults, while other reports highlight thymus-independent CD4 T cell maintenance in humans (*12*). The dependency on the thymus for replenishment is particularly crucial for CD8 T cells but less so for CD4. CD4 T cells exhibit a lower propensity to lose their naivety through antigen-agnostic memory differentiation in mice (*37*, *39*, *67*) and are less susceptible to phenomena like excessive memory formation by CD8 T cells during chronic infections with cytomegalovirus or Epstein-Barr virus (CMV/EBV) in humans (*68*). Consequently, while the systemic population of CD4 T cells remains relatively stable over time, independent of age and sex, naive CD8 T cells are more prone to Tvm formation, senescence and apoptosis, making maintaining an abundant and diverse repertoire of naive CD8 T cells critically dependent on de novo synthesis in the thymus.

Our findings indicate that age-related changes in the T cell compartment and LNs are at least partially reversible through therapeutic rejuvenation of the thymus in middle-aged animals using SSA. SSA effectively restored thymic output and repopulated LNs with naive CD8 T cells, reducing sex-biased disparities in immune responses. However, this approach was less successful in older animals. While the thymus in aged mice still retains some plasticity and can respond to SSA, advanced age-related remodeling—such as fibrosis and defective lymphocyte recruitment in aged LNs—appears to hinder LN rejuvenation (*53*, *66*). The relatively low response rates to immune checkpoint blockade (ICB) therapies and the limited success of TIL-derived TCR therapies—partly due to low tumor reactivity of TILS—underscore the need for strategies aimed at increasing the naive T cell pool, particularly in middle aged males (*23*, *69*). Augmenting lymphoid progenitor cells in the BM by depleting competing myeloid-biased hematopoietic stem cells has shown promise in improving adaptive immune responses but results in only modest increases in naive T cells (*19*), presumably due to the lack of thymus-based T cell maturation in advanced age. Rejuvenating the thymus itself or combining thymic rejuvenation with bone marrow-targeted interventions, may be

more effective in enhancing naive T cell numbers during aging. Among other approaches with cytokine and growth factor infusion, SSA – like treatment with Degarelix – is currently being evaluated in clinical trials, particularly for improving immune regeneration after radiation-induced immunodeficiency (*18*, *54*, *70*). SSA has shown potential in increasing thymopoiesis and TIL presence in melanoma and advanced prostate cancer (*6*, *71*, *72*). Beyond pharmacological interventions, lifestyle factors like maintaining a healthy weight and engaging in moderate caloric restriction can prevent accelerated thymic atrophy and improve thymopoiesis (*73*, *74*).

In conclusion, our data highlight that the therapeutic reinvigoration of thymopoiesis can reinstate a functional repertoire of diverse naive T cells and counteracts age-related impairments of antigen recognition in LNs in mice. We underscore the importance of considering both sex and age in understanding immune system decline and developing therapeutic interventions. Future research should explore the molecular mechanisms of thymic involution and rejuvenation to identify targeted therapies capable of mitigating thymic atrophy without necessitating long-term SSA. Personalized approaches that account for individual hormonal profiles and age-related changes could optimize immune rejuvenation therapies. Ultimately, enhancing thymic function and naive T cell production holds promise for improving immune responses to infections, vaccinations, and cancer, particularly in the aging male population.

Figures

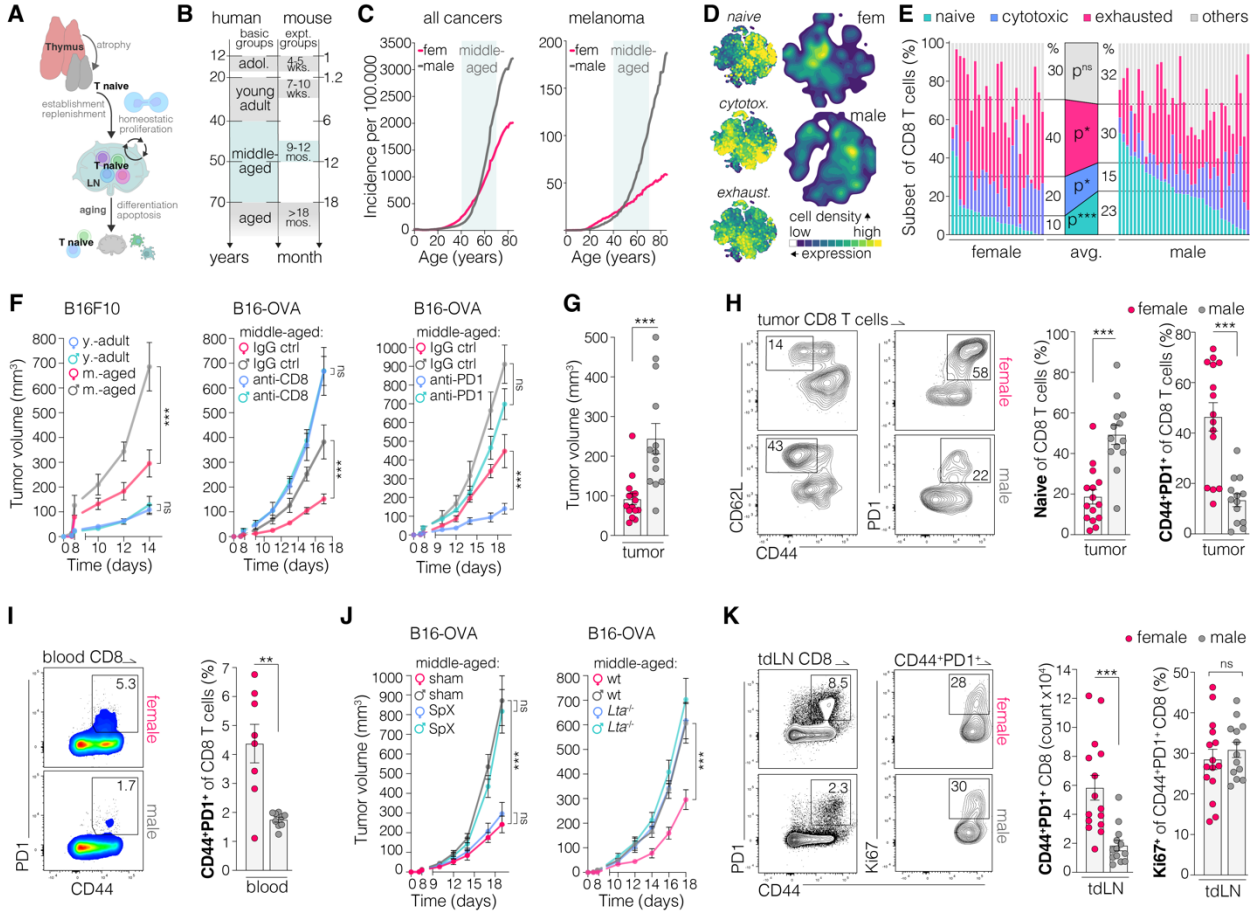

Fig. 1, Age and sex are critical factors for tdLN-derived T cell responses to melanoma. (A) Schematic illustration of naive T cell homeostasis. **(B)** Age categories in humans and mice. **(C)** Incidence per 100.000 individuals, data from SEER (n=1.8-17.8x10^6 cases per year and sex group) (REF). **(D)** Cell density projections and expression levels of module scores of CD8 T cells isolated from melanomas from patients, integrated public data sets (*4–7*). Corresponding data in fig. S1C,D. **(E)** Frequency of CD8 T cell subsets in individual patients and in average per sex (females n=25, males n=35). **(F)** B16F10 or B16-OVA tumor growth in, *left*, untreated young adult (y.-adults, n=5) or middle-aged (m.-aged, n=8) mice, *middle*, after CD8 T cell depletion (n=5/group), and *right*, with anti-PD1 checkpoint inhibition (n=5/group). **(G)** B16-OVA tumor at the day of flow cytometry analysis, group matched 13-15 days after implantation (corresponding to G,H,J) **(H)** Flow cytometry analysis of CD8 T cells isolated from B16-OVA tumors**.** Gating strategy in fig. S7A. **(I)** CD8 T cells from blood of middle-aged mice with B16-OVA tumors. **(J)** B16-OVA tumor growth in middle-aged mice, *left*, after sham surgery or splenectomy (SpX, n=8/group), and *right*, in wt or *Lta*$^{-/-}$ mice (n=8-10/group). **(K)** Flow cytometry analysis of CD8 T cells from tdLNs of middle-aged mice with B16-OVA tumors 13-15 days after implantation**.** Gating strategy in figure S3A. Data shown as mean ± SEM with data points representing individual animals or persons. Statistics calculated with MWU test (G,H,I,K), 2-way ANOVA (F,J), ns=nonsignificant, *p<0.05, **p<0.01, ***p<0.005.

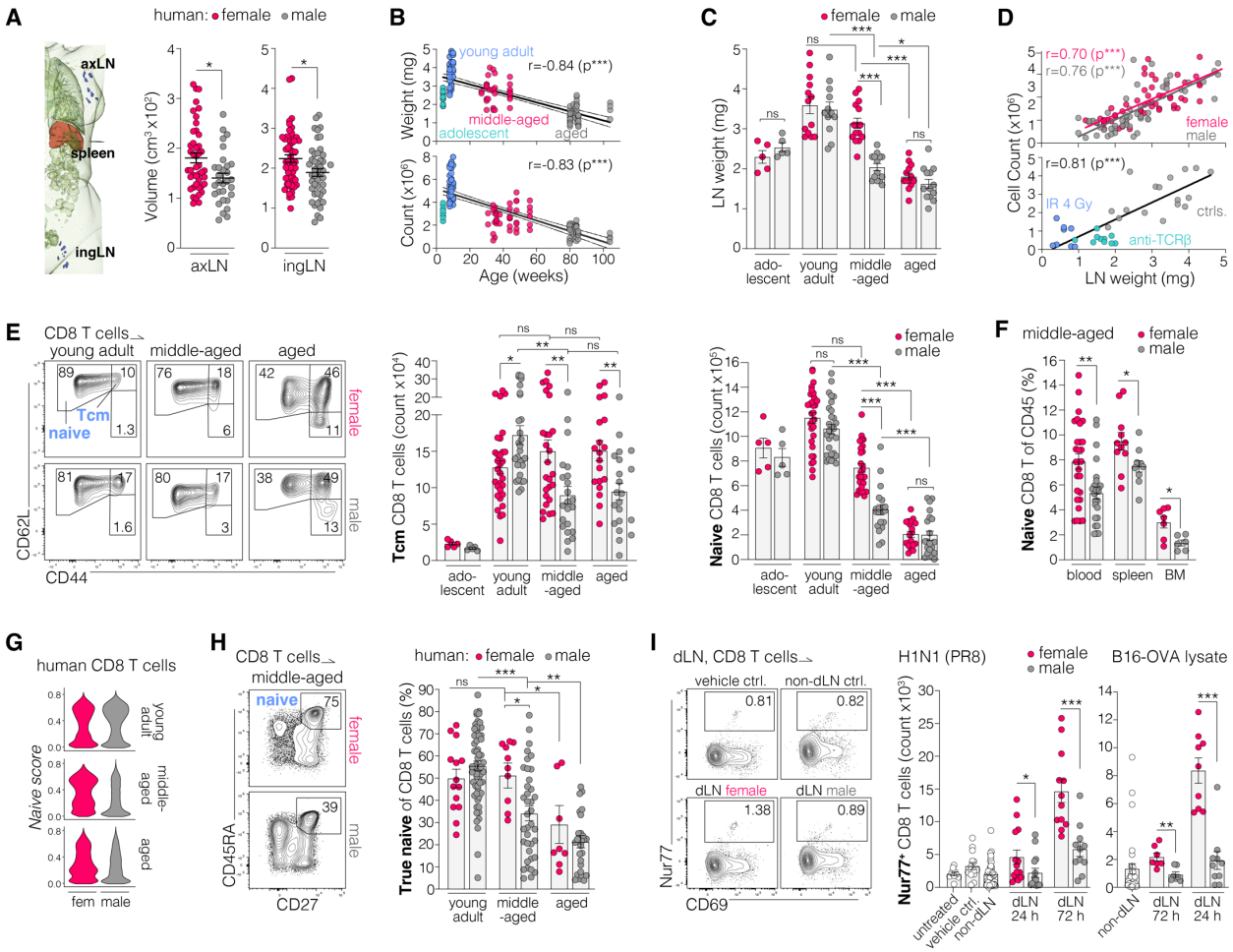

Fig. 2, Age-related naive CD8 T cell decline and LN contraction occurs earlier in males. (A) Average axillary (axLN) and inguinal (ingLN) LN volume per human individual, segmented from CT scans (*20*, *21*), corresponding data in fig. S5A,B. **(B)** Correlation of LN weight or cellularity with age of mice, including female and male, adolescents excluded from correlation calculation. **(C)** LN weights in age-groups of female/male mice. **(D)** *Top*, correlation of LN weight to LN cellularity across all ages, *bottom*, and after irradiation (IR 4 Gy) or anti-TCRß antibody mediated T cell depletion. **(E)** Flow cytometry analysis of naive, central memory (Tcm) and effector memory (Tem) CD8 T cells from ingLNs of mice. Gating strategy in fig. S6F. **(F)** Frequency of naive CD8 T cells in the blood, spleen and bone marrow (BM). **(G)** Expression levels of naive T cell genes (*Naive score*) in human CD8 T cells, ABF300 study (*2*). Naive score described in fig. S5C. **(H)** Flow cytometry quantification of true naive CD8 T cells from human PBMCs, public data set (*3*). Gating Strategy in fig. S5F. **(I)** Nur77 expression in CD8 T cells from the dLN of middle-aged mice 24 or 72 h after immunization with UV inactivated H1N1 influenza virus or B16F10-OVA melanoma lysate. Data shown as mean ± SEM with data points representing individual animals or persons. Statistics calculated with MWU test (A,C,E,F,H,I), Pearson correlation (B,D), ns=nonsignificant, *p<0.05, **p<0.01, ***p<0.005.

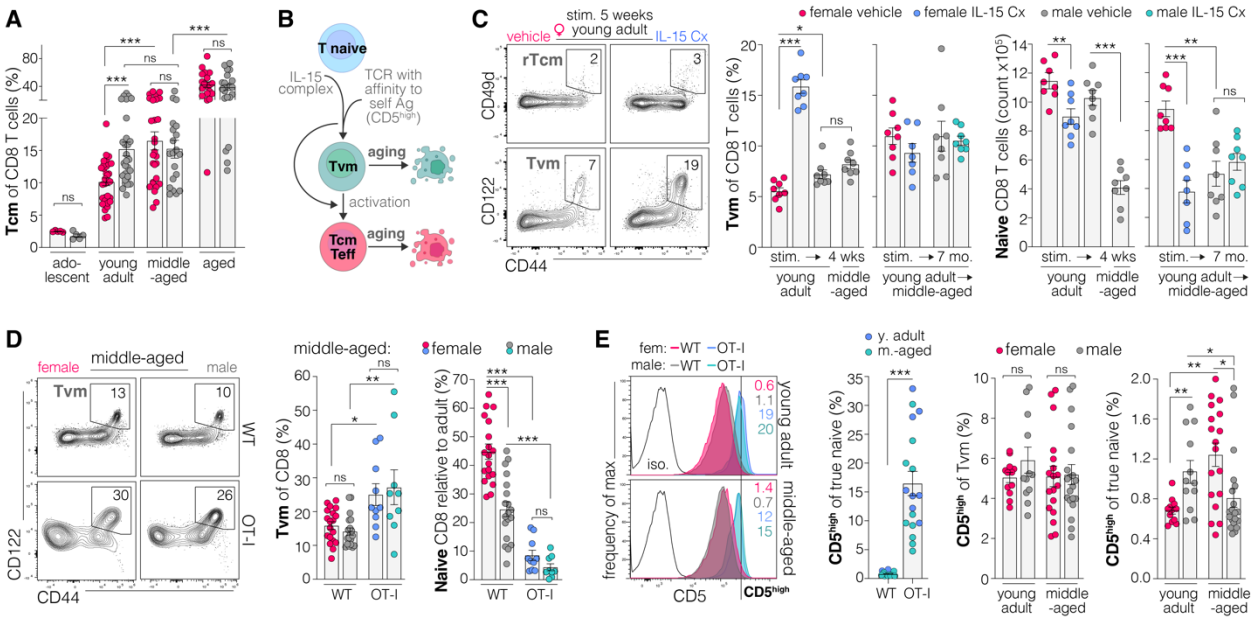

Fig. 3, Enhanced propensity for antigen-inexperienced loss of T cell naivety in male mice. (A) Frequency of Tcm relative to CD8 T cells in ingLNs of mice. Gating strategy in figure S6F. **(B)** Schematic illustration of naive T cell differentiation into Tvm or into Teff/Tcm. **(C)** Flow cytometry analysis of CD8 T cells in ingLNs of adult and middle-aged mice treated with vehicle or IL-15 Cx directly after the acute stimulation phase (stim. → 4 wks.) and 7 months later (stim. → 7 mo.), n=8/group. **(D)** Tvm percentage of CD8 T cells and naive CD8 T cells in middle-aged ingLNs relative to frequency level in adults, wild type (WT) or OT-I mice. **(E)** Representative flow cytometry histograms of CD5 expression in female and male naive CD8 T cells from ingLNs of WT or OT-I mice and true naive and Tvm WT females and males. Data shown as mean ± SEM with data points representing individual animals. Statistics calculated with MWU test (A,C,D,E), ns=nonsignificant, *p<0.05, **p<0.01, ***p<0.005.

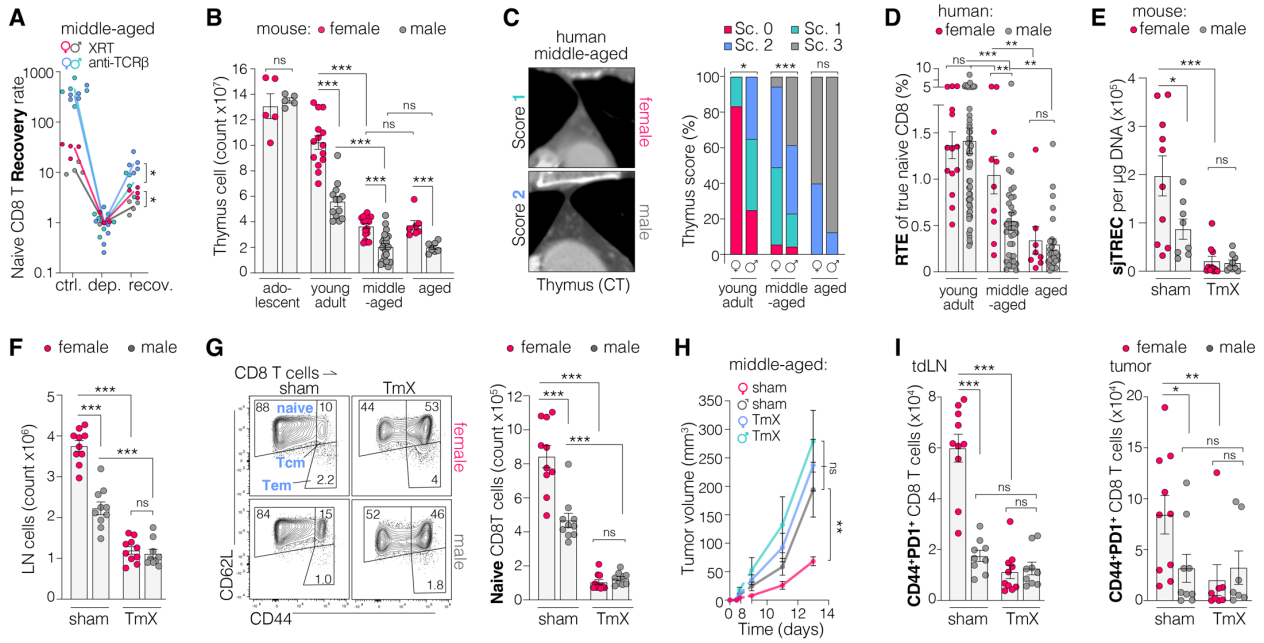

Fig. 4, Accelerated thymus involution predisposes males to a contracting T cell population. (A) Recovery rate of naive CD8 T cells 7 days after irradiation (XRT) or anti-TCRb T cell depletion (depl.) and after a period of recovery (recov.) 28 days for XRT and 19 days for anti-TCRb. Recovery rate of cell numbers normalized to average level during depletion. **(B)** Total count of thymocytes in mice of different age groups. **(C)** Analysis of human thymus degeneration in CT scans, score 0: predominantly soft tissue; score 1: parity of fatty and soft tissue; score 2: predominantly fat; score 3: complete fatty replacement, Scoring system details in fig. S10A. **(D)** Flow cytometry analysis of human PBMCs for recent thymic emigrants (RTE, CD38^{high}) in true naive CD8 T cells (data from (3)). Gating strategy in fig. S5F. **(E)** T cell receptor incision cycle (TREC) quantification in lymphocytes of LNs from middle-aged mice after sham surgery or thymectomy (TmX) performed when young. **(F)** Inguinal LN cellularity in middle-aged mice after sham surgery or TmX performed when young. **(G)** Flow cytometry of CD8 T cells and quantification of true naive CD8 T cells in middle-aged mice after sham surgery or TmX performed when young. Gating strategy figure S6F. **(H)** B16-OVA tumor growth in middle-aged mice after sham surgery or TmX performed when young, n=9-10/group. **(I)** Flow cytometry analysis of CD8 T cells from the tdLNs or the primary tumor, from (H). Data shown as mean ± SEM with data points representing animals or persons. Statistics calculated with MWU test (A,B,D,E,F,G,I), Fisher's exact test (C), 2-way ANOVA (H), ns=nonsignificant, *p<0.05, **p<0.01, ***p<0.005.

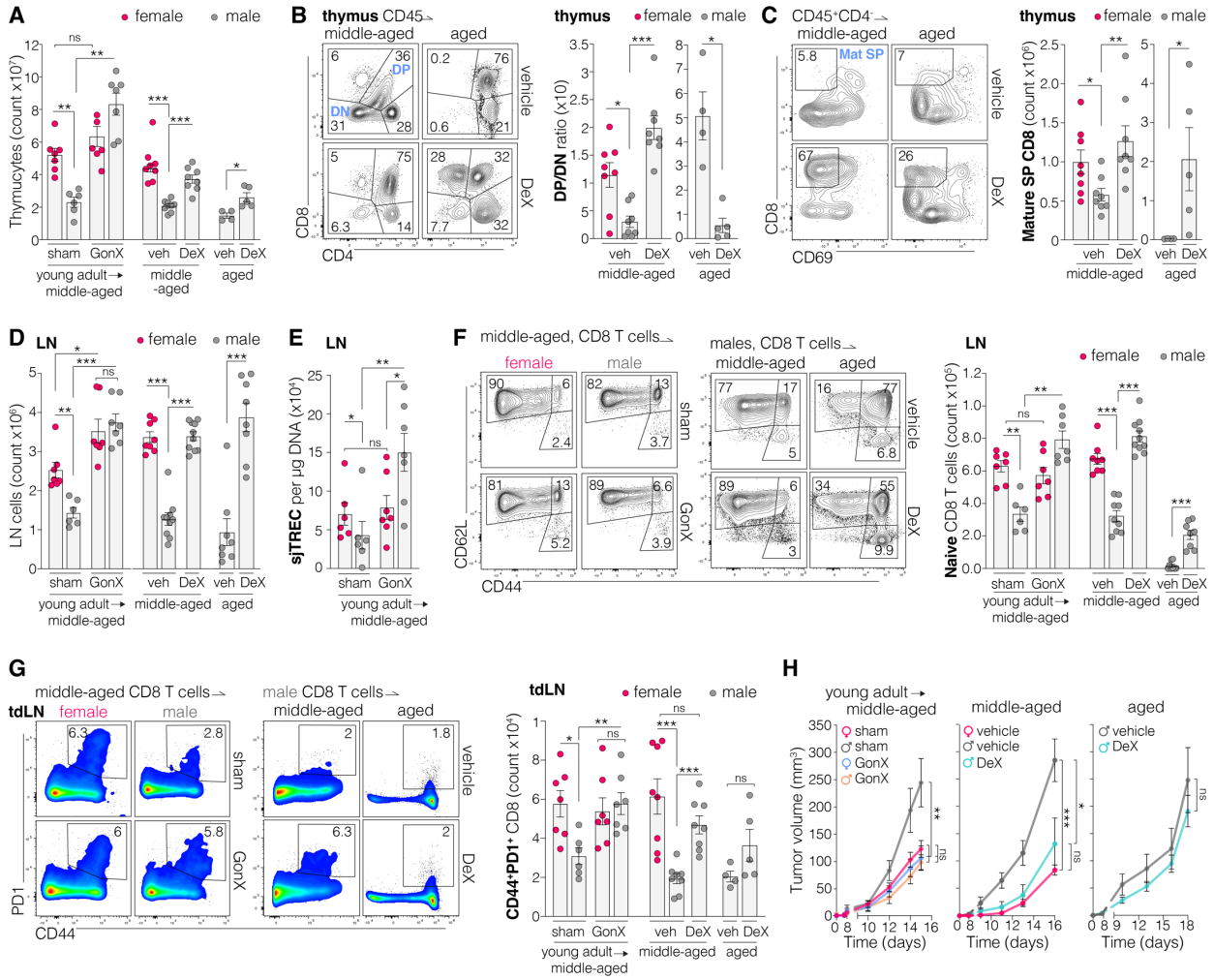

Fig. 5, Therapeutic thymus rejuvenation replenishes naïve T cells and invigorates tumor-recognition. (A) Total count of thymocytes in mice after sham surgery or gonadectomy (GonX) and vehicle (veh) or Degarelix (DeX) treatment in middle-aged and aged mice. **(B)** Flow cytometry analysis of thymocytes and double positive (DP) to double negative (DN) ratio. Gating strategy fig. S10A. **(C)** Representative flow cytometry plot of CD45⁺CD4⁻ thymocytes and quantification of mature (Mat) single positive (SP) CD8 cells. **(D)** Inguinal LN cellularity. **(E)** TREC quantification in lymphocytes of LNs after sham surgery or GonX. **(F)** Flow cytometry analysis of CD8 T cells from ingLNs of mice. Gating strategy in fig. S6F. **(G)** Flow cytometry analysis of CD8 T cells from tdLNs. Gating strategy in figure S3A. **(H)** B16-OVA tumor growth in, *left*, sham and GonX (n=6-7) middle-aged mice, *middle*, vehicle or DeX treated middle-aged mice (n=8/group), and *right*, vehicle or DeX treated aged mice (n=4-5/group). Data shown as mean ± SEM with data points representing individual animals. Statistics calculated with MWU test (A,B,C,D,E,F,G), 2-way ANOVA (H), ns=nonsignificant, *p<0.05, **p<0.01, ***p<0.005.

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