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Age-related thymic atrophy results in dysfunctional αβ-T cell selection, exhibited by reduced output of naïve conventional T (Tcon) cells coupled with a contraction of the T cell receptor (TCR) repertoire. This opens "holes" or "windows" in the effector T (Teff) repertoire allowing for infection. However, the impact of the aged thymus on the $CD4^{+}FoxP3^{+}$ regulatory T (Treg) TCR repertoire, important for self-tolerance, is insufficiently understood. Given evidence that thymic Treg (tTreg) cell generation is relatively enhanced in the aged, atrophied thymus, and that the aged periphery accumulates peripheral Treg (pTreg) cells, we investigated why these Treg cells are unable to effectively attenuate the increased auto-reactivity-induced chronic inflammation (inflammaging) in the elderly. We designed a mock self-antigen (membrane-bound ovalbumin, mOVA) chimeric mouse model, bearing a *FoxN1*-floxed gene for induction of conditional thymic atrophy, that received OVA-specific (OT-II) TCR transgenic progenitor cells. We observed a significant decrease in OVA-specific tTreg and pTreg cells, but not polyclonal (pan)-Treg cells, in mice with thymic atrophy compared to controls. Further, the OVA-specific pTreg cells from mice with thymic atrophy demonstrated significant reduction in the ability to suppress OVA-specific stimulation-induced proliferation *in vitro*, and exhibited lower FoxP3 expression. In our TCR repertoire diversity sequencing for Treg cells among recent thymic emigrants (RTEs) from Rag^{GFP}-$FoxP3^{RFP}$ dual reporter mice, we observed a trend for decreased TCR diversity in mice with thymic atrophy compared to littermates with normal thymus. These data indicate that although the effects of age-related thymic atrophy do not affect pan-Treg generation, certain tissue-specific Treg clones may experience abnormal agonist selection, likely

creating TCR repertoire holes in the aged T cell regulatory system. Taken together, our findings suggest that altered Treg cell development in the atrophied thymus and subsequent functional defects likely contribute to age-related chronic inflammation, even in the absence of acute autoimmune disease in the elderly.

THYMIC ATROPHY IMPAIRS THE

DEVELOPMENT AND FUNCTION

OF AN ANTIGEN-SPECIFIC

TREG CLONE

Rachel R. Thomas, B.A.

APPROVED:

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Major Professor

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Committee Member

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Committee Member

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Committee Member

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University Member

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Chair, Department of Microbiology, Immunology & Genetics

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Dean, Graduate School of Biomedical Sciences

THYMIC ATROPHY IMPAIRS THE DEVELOPMENT AND FUNCTION OF AN ANTIGEN-SPECIFIC TREG CLONE

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

By

Rachel R. Thomas, B.A.

Fort Worth, Texas

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LIST OF ABBREVIATIONS

Ag: antigen

Aire: autoimmune regulator

APC: antigen presenting cell

APECED: autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy

BM: bone marrow

$CD4^{SP}$: $CD4^{+}CD8^{-neg}$ single positive

$CD8^{SP}$: $CD4^{-neg}CD8^{+}$ single positive

cKO: conditional knock-out

CMJ: cortico-medullary junction

CMV: cytomegalovirus

CDRs: complementarity-determining regions

$CreER^{T}$: Cre-recombinase and estrogen receptor fusion protein

cTEC/mTEC: cortical/medullary thymic epithelial cells

CTL: cytotoxic T lymphocyte

DC: dendritic cell

ETP: early T cell progenitor

FC: floxed-*FoxN1* gene with CreER^{T}

FEZF2: FEZ family zinc finger protein 2

FF: floxed *FoxN1* gene

FCM or FFM: FCmOVA or FFmOVA

FoxN1: forkhead box N1

FoxP3: forkhead box P3

GFP: green fluorescent protein

HSC: hematopoietic stem cell

IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

IRBP: interphotoreceptor retinoid-binding protein

iTEC: induced thymic epithelial cell

i.v. intravenous(ly)

MEF: mouse embryonic fibroblast

MHC: major histocompatibility complex

NK: natural killer cell

OT-II: MHC Class-II restricted transgenic T cell receptor recognizing ovalbumin peptide

pTreg: peripheral Treg

RAG1, 2: recombination activating genes-1, 2

RFP: red fluorescent protein

RIP-mOVA: rat insulin promotor-driven membrane-bound ovalbumin

RQ-MFI: relative quantitative (geometric) mean fluorescence intensity

RTEs: recent thymic emigrants

TCR: T cell receptor

(t)Tcon: (thymic) conventional T cell

Teff: effector T cell

TF: transcription factor

Tg: transgenic

TM: tamoxifen

(t)Treg: (thymic) regulatory T cell

tTreg/pTreg: thymic Treg/peripheral Treg

SASP: senescence associated secretory phenotype

WT: wild-type

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ATTRIBUTION OF PUBLISHED WORK

Permission was obtained to utilize my previously published work as part of this Dissertation as long as it was clearly indicated. Therefore, here are the sections taken directly from my published manuscripts:

CHAPTER I. Sections 1.2, 1.3 and 1.4 including Figures 2 and 3 are adapted from my published book chapter:

Rachel Thomas and Dong-Ming Su (May 17th 2019). Age-Related Thymic Atrophy: Mechanisms and Outcomes, Thymus, Nima Rezaei, IntechOpen, DOI: 10.5772/intechopen.86412. Available from: https://www.intechopen.com/books/thymus/age-related-thymic-atrophy-mechanisms-and-outcomes

CHAPTER I. Sections 1.5 and 1.6 including Figure 4 are adapted from my published review manuscript:

Thomas R, Wang W, Su DM. Contributions of Age-Related Thymic Involution to Immunosenescence and Inflammaging. Immun Ageing. 2020 Jan 20;17:2. doi: 10.1186/s12979-020-0173-8. PMID: 31988649; PMCID: PMC6971920.

CHAPTER II. All sections and figures (Fig. 16 is slightly modified) are published in:

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CHAPTER I

INTRODUCTION TO THE STUDY

1.1 Introduction to the Immune System

The immune system is primarily responsible for the protection of the host organism from pathogen-induced infections. Pathogens are characterized as bacteria, viruses, parasites, or any other foreign agent that enters the body and causes harm to the host. The immune system is also responsible for the regulation of host cells, which have become damaged or pathogenic, such as injured, senescent, or malignant cells. There are specialized immune cells that are uniquely capable of targeting malignant, infected, or dying cells, such as natural killer cells and cytotoxic T lymphocytes (CTLs) [1].

There are two primary arms of the immune system: innate and adaptive immunity. Innate immunity begins with the basic physical and chemical barriers that discourage pathogens from entering the body as well as molecular factors, such as complement, which provide rapid clearance of and/or responses against pathogens [2]. On a cellular level, innate immunity is typically associated with antigen non-specific mechanisms by which innate immune cells, such as macrophages and neutrophils, can recognize pathogen-associated molecular patterns or pro-inflammatory cytokines secreted by damaged or infected cells. Innate immune cells have enhanced ability to phagocytize

pathogens and cell debris, as well as upregulate the production of pro-inflammatory cytokines in order to activate the adaptive immune system. Because innate immune cells do not require antigen-recognition and typically reside in various tissues or in circulation, the innate immune response can be activated very rapidly [1,2].

The antigen presenting cells (APCs), such as classical dendritic cells, are the bridge between the innate and adaptive responses, since they process various antigens from a site of inflammation and take these antigens to the secondary lymphoid organs, such as spleen and lymph nodes, for the activation of adaptive immune cells [3]. This activation occurs through the complex process of antigen presentation. First, depending on the original source of the antigen (intra- or extracellular), the antigen will be presented on either major histocompatibility complex (MHC) Class-I or II, respectively [3,4]. Importantly, the type of MHC molecule utilized for antigen presentation helps to tailor the adaptive immune response, since $CD4^+$ T cells must be activated by MHC-II presentation, while $CD8^+$ T cells require MHC-I presentation [1]. Further, APCs produce various cytokines, further facilitating activation and tailoring of the adaptive immune response.

Since the adaptive immune system is characterized by an antigen-specific response, it requires several days to undergo full activation, the goal being to generate T and B lymphocytes that are highly specific for the antigens of interest [5]. The adaptive immune cells also exhibit extremely tailored immune responses depending on the type of pathogen that is causing infection. These responses are primarily driven by various cytokines produced by APCs, which are able to facilitate the differentiation of lymphocytes into different phenotypic subtypes. For example, T cells can be differentiated into helper cells, such as $CD4^+$ Th1, Th2, and Th17 cells, as well as cytotoxic cells, such as $CD8^+$ CTLs [5]. Additionally, B cells can under tailoring in the class of antibodies they generate, whether IgD, IgM, IgG, IgA or IgE [5]. Moreover, the immune system is

a highly specified and regulated system that is responsible for the overall homeostasis of an organism.

One final important endpoint of the adaptive immune system is the ability to generate immunological memory against pathogens that have been encountered [5]. A subset of B and T cells will differentiate into memory cells, which are long-lived and typically reside in the bone marrow or various tissues of the body [6,7]. The protective nature of memory cells is the ability to rapidly respond to reinfection by an already encountered pathogen. Immunological memory is the foundational concept behind the advent of immunizations and therefore, robust adaptive responses are essential for long-term protection of the host (recently the concept of innate contributions to immunological memory has also been proposed) [8].

Finally, dysregulation of the immune system is responsible for many diseases including allergic diseases, autoimmune diseases, and chronic inflammatory diseases. The problem of malignancy can also be understood as insufficiency of the immune system to effectively target these pathogenic host cells. Many aspects of immune dysfunction occur with the process of aging [9], and this is the primary focus of the work discussed herein.

1.2 Thymus, Thymopoesis, TCR Repertoire, and Central Tolerance Establishment

The thymus is the central lymphoid organ responsible for T cell development and selection of conventional T (Tcon) and regulatory T (Treg) cells[10-12]. Thymocyte development begins after double negative (referring to absence of CD4 and CD8 expression) T cell progenitors from the bone marrow (BM) enter the thymic cortex and begin to express CD4 and CD8 co-receptors [13,14]. The first stage of thymocyte development is termed positive selection. This occurs when

thymocytes that are double positive (DP) for CD4 and CD8 co-receptors are selected for continued development. This is dependent on the ability of a given T cell receptor (TCR) to recognize self-matched major histocompatibility complex (MHC) with the potential to recognize self or foreign antigen (Ag). It is through this process that CD4 or CD8 single positive (SP) lineage and MHC-restriction for either MHC Class I (MHC-I) or MHC Class II (MHC-II) is determined [10,15]. Next, the positively selected thymocytes migrate from the thymic cortex to the thymic medulla for the latter stages of thymocyte development where negative selection of self-reactive thymocytes [10,16,17], and generation of self-Ag recognizing Treg cells occurs [18]. These latter stages of T cell development comprise central tolerance establishment, which is the primary focus of our study (discussed further in section 1.1.2).

1.2.1 TCR Rearrangement for Selection of a Diverse TCR Repertoire

The T cell receptor (TCR) is the antigen-recognizing receptor that dictates the antigen specificity for which a given T cell can respond. In order to generate productive TCRs, thymocytes undergo somatic recombination of the genes encoding the variable (V), diversity (D) and joining (J) segments of TCR β-chains or variable (V) and joining (J) segments of TCR α-chains. Portions of these segments, called the hypervariable regions or complementarity-determining regions (CDRs), will become the antigen-recognizing component of the TCR [19,20].

First, the TCR β-chain undergoes recombination, during the latter part of the double negative stage 3 (DN3) [21]. At this time, the TCRβ is paired with a surrogate or pre-T-cell α-chain, which is expressed with the cell membrane receptor CD3 [22] in order to provide constitutively active signaling independent of MHC:Ag interaction. If the rearrangement process fails to generate a

productive TCRβ, it is possible for the thymocyte to undergo an additional rearrangement, since there are numerous V, D, and J gene segments, although this is limited by the number of D genes (two) present for the beta chain. Ultimately, the vast majority of thymocytes will not achieve a productive TCR and will undergo death by neglect [14]. Once the TCR β-chain is successfully rearranged, the thymocyte will undergo TCR α-chain rearrangement, which again can include multiple recombination events, if needed [19,20].

The most noteworthy enzymes involved in TCR rearrangement are the recombination activating genes RAG1 and RAG2. These enzymes recognize the recombination signal sequences that flank each gene segment and facilitate recombination events. Therefore, diversity in the TCR repertoire initially comes from the various pairings of V, D, and J regions [19,20]. Further diversity is also introduced to both chains in a non-template encoded manner in which the enzymes such as Artemis, terminal deoxynucleotidyl transferase (TdT), and exonucleases add or remove nucleotides to the joining regions [23]. After the V, J, (and D for β-chains) are recombined, there is an additional layer of diversity generated by the random pairing of a given TCR β- and TCR α-chain.

It is of great advantage to have a high diversity of TCRs in the T cell repertoire because this allows for the recognition of a diverse pool of potential pathogens. The theoretical αβTCR repertoire diversity is estimated to be around 10^{15} and 10^{20} in healthy adult mice and humans, respectively [24-26], while the functional diversity is more difficult to determine. One study has demonstrated a functional splenic naïve αβTCR repertoire diversity of 1-2 x 10^6 in healthy adult mice, asserting that although much lower than the theoretical, this level is sufficient for robust immune T cell responses [27]. Important to note, in this peripheral naïve T cell pool, there are likely to be 10 or fewer of any given TCR clone [27].

Given the importance of TCR diversity, it is not surprising that skewing of TCR repertoire is associated with many diseases, such as rheumatoid arthritis [28]. The advent of next-generation TCR repertoire sequencing is quickly becoming a tool for assessing T cell diversity across many disease states, since many diseases exhibit expansion of certain pathogenic T cells, which become dominant clones [29]. Further, the TCR repertoire diversity decreases with age [30,31], which underlies the inefficient responses to new pathogens associated with the elderly through introduction of "holes" in the Tcon cell repertoire [32]. The impacts of the aged thymus on Treg cell repertoire diversity is even less understood.

1.2.2 Central Tolerance Establishment via Two Mechanisms: Negative Selection and tTreg Agonist Selection

After thymocytes complete the positive selection process, resulting in a diverse pool of T cells, they migrate to the thymic medulla where central immune tolerance is established [33,34]. Here it becomes apparent that sufficient TCR diversity is essential not only for a robust immune response to various infections, but also for suppression of uncontrolled immune response to self-tissues. The final stages of thymocyte selection, therefore, are primarily dependent on both the affinity and avidity (affinity multiplied by the number of interactions) between self-Ag/peptide, which binds to the MHC groove (self-pMHC) on medullary thymic epithelial cells (mTECs), and TCRs on developing $CD4^+$ thymocytes. The overall signaling strength produced by the interaction(s) determines the fate of $CD4^+$ thymocytes [35,36].

Central tolerance is established via two mechanisms: (1) negative selection, referring to the deletion of highly self-reactive thymocytes [17], and (2) thymic regulatory T (tTreg) cell generation, also called agonist selection [18]. Under the currently accepted paradigm, both of these activities

utilize the same self-pMHC and TCR interaction, but differences in overall signaling strength decide between the two fates. Importantly, transcriptional regulators, such as the autoimmune regulator (Aire) and more recently FEZ family zinc finger protein 2 (FEZF2), are implicated in facilitating the promiscuous expression of self-antigen by mTECs for the purposes of central tolerance induction [37-40]. Indeed genetic mutations resulting in defects in Aire function cause severe autoimmunity, such as autoimmune polyendocrineopathy-candidiasis-ectodermal dystrophy (APECED) [41].

The second arm of central tolerance (Treg cell agonist selection) is closely connected with the first (negative selection). It is accepted that negative selection is an imperfect system [42], and studies of both humans and animals with Treg cell defects, such as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) or other mutations in Treg-associated genes [43], clearly demonstrate that Treg cells play an important role in maintaining immune tolerance in the periphery against any self-reactive Teff cells that escape the negative selection process. Additionally, Aire has recently been shown to facilitate Treg agonist selection, highlighting how central this regulator is to both aspects of central tolerance establishment [44]. The overall balance of these two arms of central tolerance induction underlies T cell immune homeostasis [11].

The current paradigm of CD4 single positive ($CD4^{SP}$) thymocyte fate determination (after positive selection) is summarized in Fig. 1, during which interactions between self-pMHC and TCRs in $CD4^{SP}$ culminate in TCR signal transduction, where (1) strong signaling results in negative selection of these $CD4^{SP}$ thymocytes, (2) intermediate signaling results in tTreg agonist selection, and (3) weak signaling results in thymic conventional T (tTcon) cell development (potential Teff cells in the periphery) [36,45]. This paradigm also illustrates how the thymus

establishes central immune tolerance for the T cell compartment to ensure that highly self (auto)-reactive thymocytes are not released as Tcon cells, which would have the capacity for autoimmune reactions in the periphery [17]. In addition, if a self-reactive Tcon cell is aberrantly released to the periphery, a self-reactive Treg clone should be present to suppress any autoimmune reactions induced by that Teff cell.

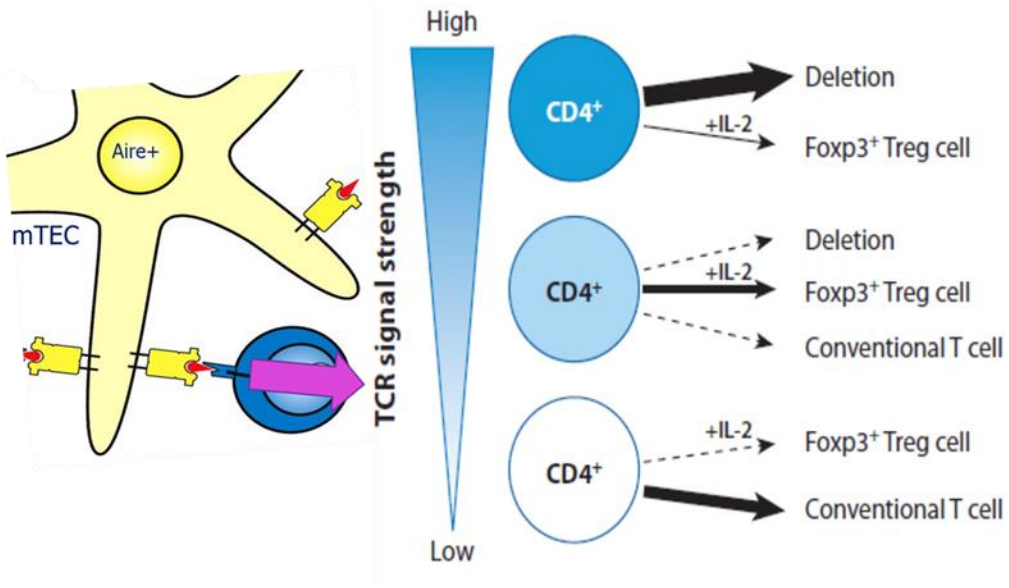

Figure 1: Current paradigm of TCR signaling strength for self-reactive CD4^{SP} thymocyte fate determination. Illustration showing interactions between self-pMHC on mTECs and self-reactive TCRs on CD4^{SP} thymocytes that can produce three levels of signaling strength (avidity). A strong signal leads to negative selection, resulting in thymocyte deletion. An intermediate signal leads to tTreg agonist selection/generation. A weak signal results in thymocyte differentiation into tTcon cells.

1.3 Age-Related Thymic Involution/Atrophy

As part of the aging process, the thymus undergoes progressive involution or atrophy in most vertebrates, exhibiting not only morphological changes, but also functional decline [46,47] and significantly lowered thymic output [48]. The theoretical causes of this age-related diminishment of thymopoesis are two-fold. First, a hematopoietic exclusive defect, which stems from the observations that there are reduced numbers of hematopoietic stem cell (HSC) progenitors produced by the bone marrow with age [49] causing a reduction in early T-cell progenitors (ETP) entering the thymus [50]. Secondly, a non-hematopoietic exclusive defect, suggesting that the primary age-related atrophy of the thymus is derived from HSC niche [51,52] and thymic stromal cells, which are fashioned as stem niches or ETP niches [53,54]. The myriad changes which characterize thymic atrophy first occur within this niche, then extend to hematopoietic progenitors as a result of age. We believe that these substantial age-related alterations in thymic microstructure and microenvironment, which provides various important thymic factors, contribute more heavily to the diminished thymopoesis observed in the elderly [47,53]. The chief thymic stromal cells are thymic epithelial cells (TECs), including two sub-populations distinct in their localization, function, and molecular expression patterns, namely medullary TECs (mTECs) and cortical TECs (cTECs) [13]. Compelling evidence show that age-related thymic atrophy is tightly associated with postnatal TEC homeostasis, which is regulated by TEC autonomous transcription factors (TFs), such as forkhead box N1 (*FoxN1*) [55].

In addition to reduction in thymic mass (size and thymocyte numbers), there is substantial remodeling of the thymic microstructure. The thymus is characterized by two primary compartments, namely the cortex and the medulla. In between the cortex and medulla, there is a zone termed the cortico-medullary junction (CMJ) (Fig. 2A). These two compartments contain

specialized thymic epithelial cells (TECs), cortical (cTECs) or medullary (mTECs), and these cellular compartments are responsible for different stages of thymocyte development and selection [10,56]. Regarding thymic microstructure, the aged, involuted thymus, in addition to an overall decline in TEC-associated markers, such as keratin and major histocompatibility complex class-II (MHC-II), also manifests altered ratios of cTECs to mTECs, and an overt change in microstructure due to disrupted CMJ, resulting in a disorganized medullary region (Fig.2B). A decline in MHC-II^{hi} expressing TECs is a sign of reduction of mature mTECs [57,58]. Additionally, increased numbers of fibroblasts [57] and accumulation of adipose tissue in the thymus is also observed [58].

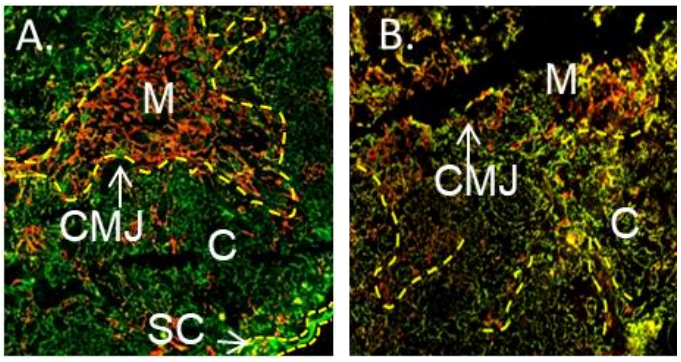

Figure 2: Thymic microstructural changes characterized by K8 and K5 fluorescent staining. In the aged thymus, the CMJ is not clear, because the medulla is disorganized and medullary TECs are dispersed and do not form a distinct compartmental region. Normally, $K8^+$ TECs (green) are primarily localized in the cortical region, while $K5^+$ TECs (red) are primarily localized in the medullary region. A) Young (~2 months old) murine thymus; B) Aged (>18 months old) murine thymus. C = cortex, M = medulla, CMJ = cortico-medullary junction, SC = sub-capsule.

Increased senescent cells (β-Gal$^+$, p21$^+$, and TAP63$^+$ cells) [59] in the aged thymus are also present, and it has been demonstrated that TECs contribute to the senescence observed in the aged thymus [57,59,60]. This possibly contributes to an increased inflammatory environment (increased levels of IL-6, IL-1β, etc.) within the involuted thymus [61,62]. Additionally, there is augmented apoptosis in TECs of the atrophied thymus, contributing to diminished stromal cellularity in the aged thymus [57].

1.4 Mechanisms of Age-Related Thymic Atrophy

In order to fully understand age-related thymic atrophy, it is necessary to discuss the mechanisms underlying various biological events that contribute to thymic atrophy. Generally, two mechanisms are proposed. One is of hematopoietic origin, focusing on the primary hallmarks of diminished thymic input and output. The second is of non-hematopoietic origin, focusing on thymic stromal cell-mediated structural disparities.

1.4.1 Diminished Hematopoietic Input and Defective Hematopoietic Cell Development Associated with Thymic Atrophy

Perhaps the most noted outcome of age-related thymic atrophy is diminished thymic output and thymopoesis. This attracts attention and has led many groups to examine whether the bone marrow (BM) derived hematopoietic stem cell (HSC) lymphoid progenitors are sufficiently able to seed into the thymus during aging, because HSCs are reduced [49] with a myeloid biased development in advanced age [63]. There have been many studies investigating this aspect of thymopoesis and it is suggested that age-related HSCs contain defects [49] that could contribute to insufficient ETPs entry into the aged thymus [50]. Thus, this result could explain decreased thymic output with age [64].

Mechanisms of diminished thymic input resulting in thymic involution and declined thymic output are mainly based on bone marrow transplantation (BMT) experiments via mouse model. In this model, transferring aged HSCs into young mice could not rejuvenate the thymic involution induced by irradiation prior to bone marrow transplantation [65]. Additionally, the HSC progenitors have been shown to exhibit an age-related skewed proportion within the HSC pool towards myeloid lineage versus lymphoid lineage [63,66-68]. It has also been observed that early stage thymocytes, defined as the ETPs in the triple negative-1 (TN1) thymocytes, from aged mice demonstrated decreased differentiation after *in vitro* fetal thymic organ culture [50]. This group also reported declined proliferation and enhanced apoptosis of these early thymocytes taken from aged animals compared to young controls. The overall assertion was that the deficiency in thymocyte differentiation and development past this early stage was attributed to the production of the HSCs in the aged bone marrow [50]. Therefore, aged HSCs and ETPs were regarded as having an intrinsic defect [69].

Given the comprehensive microenvironments in young and aged animals, and the vulnerability of HSCs or ETPs during *in vitro* preparation, these experiments using BMT and ETP culture may not provide the necessary rigor for the conclusions drawn from them, and certainly do not adequately reflect physiological conditions. Therefore, we designed an age-mismatched experimental system with less *in vitro* preparation to reexamine these biological events [53,70]. One design was to utilize young or aged IL-7R knockout mice as recipients [71], in which their BM niche is relatively open and available to accept exogenous BM cells without irradiation [72]. After grafting young BM cells into young and aged IL-7R knockout mice, the young BM cells produced a young profile in young recipients, but the same young BM cells produced an old profile in aged recipients [53], which implies that microenvironment, constructed by niche cells, directs BM cell aging, rather

than the HSCs themselves [54]. The other design was to utilize mouse fetal thymus kidney transplantation into young or aged mice, in which BM progenitors from young or aged recipients seed the grafted young thymus *in vivo* [70]. After grafting fetal thymic lobes into young and aged wild-type recipient mice, the thymic seeding BM progenitors from young and old BM were able to grow equally well in the fetal thymus (young thymic microenvironment) [70]. In addition, aged HSCs seeding the fetal thymus did not demonstrate any intrinsic defects [53,73]. These comprehensive experiments provided solid evidence that the non-hematopoietic constructed microenvironment, rather than aged-related defects of HSCs, directs hematopoietic progenitor aging [54], thereby mediating the kinetics of thymic involution [47].

An important fact linking these potential mechanisms is the unique cross-talk or interaction that occurs between the developing hematopoietic progenitors (such as thymocytes) and the stromal microenvironment (such as TECs) in the thymus [13]. For example, there are reports that several key thymic factors involved in this cross talk are adversely impacted by age-related thymic atrophy. One such factor is IL-7, secreted by TECs, which is important for thymopoesis and has been shown to be declined in the aged thymus [74]. Interestingly, direct exogenous supplementation of IL-7 helped to improve aged thymopoesis [75]. On the other hand, thymocytes provide signals to TECs to promote TEC development, at least during thymic organogenesis [76,77], but the dynamics of this phenomenon during thymic aging remain unknown.

In general, adult organ size is governed by the tissue-specific stem cell pool [78,79]. It is known that there are two types of tissue-specific stem pools: infinite pool, such as the liver, and restricted pool, such as the pancreas. For example, if the liver is injured, its infinite stem pool can expand at a high capacity; whereas, if the pancreas is injured, the expansion of its tissue-specific stem cell pool is very limited due to its restricted and finite epithelial progenitor pool [79]. The thymic

epithelial progenitor pool has characteristics of the restricted, finite epithelial progenitor pool [79]. Therefore, it is conceivable that aging TECs exhibit limited turnover compared to mobile thymocytes, which are periodically entering from the BM [80,81].

Taken together, deficiencies in thymocyte-TEC interactions in the thymus [13] promote thymic atrophy during aging. However, given the fact that thymocytes are mobile with a relatively short period of thymic residency, while TECs have permanent residency in the thymus, experimental evidence [53,70] and the "seed and soil" theory describing how the soil (stem niche) directs seed (HSC) fate [82-84], lead us to conclude that age-related thymic involution begins with defects in the TEC compartment.

1.4.2 Disruption of Thymic Stromal Cell Homeostasis Mediates Thymic Atrophy

In light of the aforementioned evidence of age-related TEC defects and the decline in total TEC numbers in the aged, atrophied thymus, we now move to discuss the underlying mechanisms of these alterations. Many studies have been conducted to identify factors involved in the cellular and molecular aspects of TEC aging (cytokines, transcription factors, microRNAs, sex steroids, etc.). The single most predominant factor currently accepted as significantly contributing to this phenomenon is TEC autonomous transcription factor FoxN1. This idea was based on the athymic nude mouse phenotype [85,86]. FoxN1 is expressed mainly in epithelial cells of the thymus and skin to regulate epithelial cell differentiation in these organs [85]. It is thereby responsible for thymic organogenesis and subsequent T cell development in the thymus [55], as well as hair follicle development in the skin [87,88]. Many past and current studies utilize nude mice, which exhibit a null mutation in *FoxN1* resulting in lack of hair and athymia, resulting in a lack of T cells [89,90].

FoxN1 is noted to be declined in expression in the age-related atrophied thymus and has even been described as one of the first markers of the onset of thymic involution [91,92]. The question is whether this declined FoxN1 expression is due to TEC aging, which results in a decline in many TEC-associated genes, or if primary FoxN1 decline with aging induces a TEC defect that then results in age-related thymic involution. This cause-and-effect relationship, which had been substantially debated prior to generation of a conditional knock-out (cKO) of *FoxN1* mouse model [93]. In this model, the murine *FoxN1* gene is *loxP*-floxed and the uCreER^{T} is introduced through crossbreeding [94]. In this model, the tamoxifen (TM)-inducible ubiquitous Cre-recombinase (uCreER^{T}) transgene has a low level of spontaneous activation, even without TM induction [95,96], causing gradual excision of the *FoxN1*$^{flox/flox}$ gene over time. This results in progressive loss of *FoxN1* with age and thymic involution that is positively correlative with declined FoxN1 levels [97]. Supplying exogenous FoxN1, such as via plasmid [97] or transgene [98,99], into the aged thymus greatly improves thymic atrophy and function. Additionally, use of FoxN1 reporter mice have enabled further elucidation of the timeline and kinetics of thymic atrophy with age [100]. For example, one group recently published a study demonstrating that FoxN1 decline initiates the onset of thymic involution, beginning predominantly in the cTEC compartment [100]. Therefore, a decline in FoxN1 expression with aging causally induces flaws in TEC homeostasis, thereby resulting in age-related thymic atrophy, as opposed to the notion that age-induced thymic atrophy causes FoxN1 decline in the thymus.

1.5 Outcomes of Age-Related Thymic Atrophy

1.5.1 Decreased Functional Naïve Conventional T Cell Output and Reduced Tcon Repertoire Diversity

As stated previously, the most readily observed outcome of age-related thymic involution is the decline in thymic output, which has been characterized to include reduced naïve conventional T (Tcon) cell output over time [101] and fewer recent thymic emigrants (RTEs) [48]. However, the peripheral T cell numbers are not decreased in aged individuals [102-104]. The actual effect is an overall diminished TCR repertoire diversity observed in the aged peripheral T cell pool [48,73,105,106], which is due to oligoclonal expansion of memory T cells along with insufficient RTE output. This is suggested to contribute to the decreased capacity for new immune responses to infection, such as to influenza virus [32] and poor vaccination efficacy, which are typical phenotypes of immunosenescence [107-110], observed in the elderly [111].

This phenotype has been recapitulated in *FoxN1* cKO mice, which have an accelerated aging in the thymus, but maintain a young periphery, as they exhibit impaired peripheral T cell responses in infection with influenza virus [112]. This group demonstrated a direct role of thymic atrophy in contributing to Tcon cell impairment associated with aging.

1.5.2 Increased Self-Reactive Conventional T Cells Output Due to Perturbed Negative Selection

In light of the alterations in thymocyte number and diminished naïve T cell output with age-related thymic atrophy, it is of paramount importance to understand the effects of the altered thymic microenvironment on central tolerance establishment of the thymocytes that are still being developed in the atrophied thymus.

Under the current paradigm, negative selection is the process by which thymocytes, or T clones, with too high an affinity for self are deleted from the developing thymocyte repertoire via apoptosis [10,16,17]. Studies also show that when these high affinity TCRs receive strong signaling, negative selection takes place [35,45]. However, the TCR signaling strength is not based on TCR

affinity alone, but it is produced by the interaction between self-antigen/peptide presented on MHC-II (self-pMHC-II) and cognate TCR affinity (Fig. 1). Therefore, if the conditions (affinity and avidity, etc.) of self-reactive T clones are unchanged, the TCR signaling strength varies entirely based on ability for effective self-pMHC-II expression. In other words, if self-antigen can be normally presented in the MHC-II groove, the reciprocal TCR signaling can be produced through a strong interaction. We know that MHC-II is expressed on mTECs, however, aging induces mTEC defects (Fig. 2B), resulting in reduced capacity for self-pMHC-II ligand expression. Therefore, a strong signaling strength shifts either to an intermediate strength, which favors $CD4^{SP}FoxP3^{+}$ tTreg cell generation (Fig. 3, arrow-a), or to a low strength, which results in the generation of self-reactive T clones (Fig. 3, arrow-b). The self-reactive T clones via this pathway are neither depleted nor shift to tTreg cells, but become Tcon cells that are released to the periphery. If they encounter specific self-tissues, they will become effector T (Teff) cells that can attack self-tissues and induce pathological inflammation.

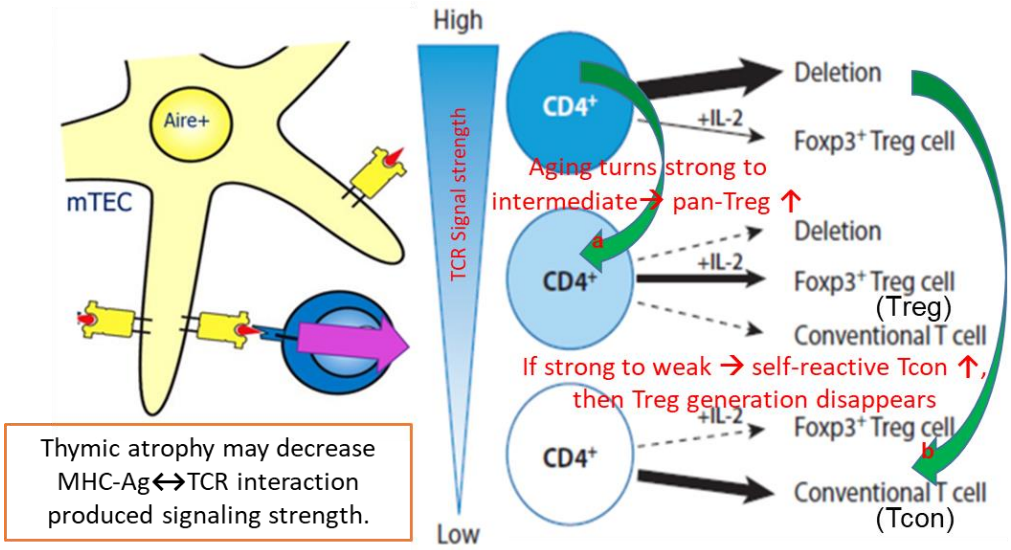

Figure 3: Alterations in signaling strength decide self-reactive CD4^{SP} T clone fates. Illustration of proposed changes to thymocyte signaling strength and subsequent fate determination due to thymic aging where (Green arrow-a) shifts signaling strength from strong to intermediate and relatively enhances polyclonal tTreg generation, while some antigen-specific interactions exhibit an extremely weak signal, resulting in diminished antigen-specific tTreg cells and increased antigen-specific tTcon cells (Green arrow-b).

The *FoxN1* cKO mouse model is a typical model for studying the capacity for efficient self-pMHC-II ligand expression, because it has a defect in non-hematopoietic lineage TECs, but maintains intrinsically normal hematopoietic lineage cells and a young periphery. We demonstrated that *FoxN1* cKO-induced thymic involution perturbs negative selection, as increased numbers of autoreactive Tcon cells, such as interphotoreceptor retinoid-binding protein (IRBP)-specific T cells, were released from the atrophied thymus of *FoxN1* cKO mice compared to young normal thymus controls [113]. This is due to decreased capacity for self-pMHC-II ligand expression, confirmed via assessment of mock self-antigen, membrane-bound ovalbumin (mOVA), expression in normal versus atrophied thymus in transgenic mice expressing mOVA as a self-antigen with or without induced thymic atrophy [114].

1.5.3 Changes in Thymic-Derived Regulatory T cell Generation

As mentioned earlier, central tolerance establishment encompasses two mechanisms. The first is negative selection. However, negative selection is not entirely perfect [42] causing some self-reactive T clones still escape negative selection and are release to the periphery as Tcon cells. Therefore, the second defense against self-reactivity is $CD4^{SP}FoxP3^{+}$ peripheral Treg (pTreg) cell-mediated autoimmune suppression. pTreg cells are reportedly comprised of 80 - 95% of the thymic Treg cell repertoire [34,115,116], in other words, most Treg cells are generated within the thymus, as thymic-derived T regulatory (tTreg) cells. Under the current paradigm, activities of both negative selection and tTreg generation in the thymus utilize the same agonist self-antigens with synonymous presentation [12,34]. Whether self-reactive T clones developing in the thymus are negatively selected or develop into tTreg cells depends on TCR signaling strength when all other variables, such as TCR affinity, IL-2, etc., are fixed. Put simply, strong signaling induces self-reactive T clone death, an intermediate signal leads to tTreg generation, and a weak signal results

in T clone survival to differentiate into Tcon cells (Fig. 3). This paradigm implies that depletion or survival for thymocytes is dependent on overall TCR signaling strength [10,11].

There are two scenarios that introduce changes in TCR signaling strength. One is on the thymocyte side; the other is on the TEC side. As we know, when the TCR responds to antigen recognition, the immunoreceptor tyrosine-based activation motifs (ITAMs) are activated and the signaling kinase Zap70 is subsequently phosphorylated. A mouse model with a knock-in allele of TCRζ chain gene with tyrosine-to-phenylalanine mutations in 6 out of 10 ITAMs led to a 60% decrease in TCR signaling potential [117]. This mouse model exhibited a defect in negative selection, but an increase in tTreg generation [117]. The second scenario occurs in the TEC compartment. Transgenic expression of a microRNA targeting MHC class-II transactivator (CIITA) resulted in declined MHC-II on mTECs [56], which leads to insufficient mTECs presentation of self-antigens dampening the interaction with reciprocal TCRs. This also resulted in an enhancement of tTreg generation at the expense of negative selection [56].

In the aged thymus, as we mentioned earlier, mTECs are flawed and self-antigen cannot be normally presented in the MHC-II groove, which results in a diminished interaction with TCRs on developing thymocytes. This is similar to the second scenario described above, in which a defect exists in the TEC compartment and causes reduced TCR signaling strength. We observed a relatively enhanced tTreg generation in the atrophied thymus, exhibiting no change in overall tTreg numbers, but an increased ratio of tTreg to tTcon cells in the aged, atrophied thymus compared to young controls [114]. This is probably a demonstration of the atrophied thymus attempting to compensate for defective negative selection [113] in order to maintain central T cell tolerance in the elderly.

If self-reactive TCR signaling strength is too low, which is possible for certain self-antigen specific T clones, these T clones may neither be depleted nor form tTreg cells, but rather may directly differentiate into self-reactive Tcon cells. As an artifact of impaired promiscuous self-antigen expression in mTECs through an autoimmune regulator (*Aire*) knock-out model, the *Aire*-dependent TCAF3 epitope of prostate antigen cannot be promiscuously expressed on mTECs [118]. This resulted in prostatic-specific T clones, which should be negatively selected, but in contrast were redirected into prostatic-reactive Tcon cells. The authors observed loss of prostate-specific tTreg cells for this same epitope, and heightened prostate-reactive Tcon cells that infiltrated the prostate of these mice causing auto-inflammatory lesions [44,119]. Defects in self-peptide expression on mTECs due to protein knock-out [120], are beginning to suggest that some of the same impairments exhibited by the atrophied thymus, may impact antigen-specific (monoclonal) tTreg generation, meanwhile increasing this same self-antigen specific Tcon generation, despite an unchanged or increased total (polyclonal) tTreg population [121]. It will be interesting to see what further subtle implications the aging thymus has on central tolerance establishment via potentially altering certain self-tissue specific tTreg populations and altering the overall aged Treg TCR repertoire, in spite of a relatively increased aged polyclonal Treg population [114].

1.6 Intersection of Aged T Cell Immune System, Immunosenescence, and Inflammaging

The aged immune system has various characteristics. One of which is immunosenescence, which describes the vast and varied changes in the structure and function of the immune system as a result of age [107-110]. Many of the early observations, such as reduced ability to fight new infections, diminished vaccine immunity, [122] and reduced tumor clearance [123,124] are generally categorized as immune insufficiencies. Immunosenescence is not due to the lack of immune cells,

but due to reduced immune repertoire diversity, attributed to insufficient production of naïve immune cells and amplified oligo-clonal expansion of memory immune cells. Immunosenescence is therefore linked to the thymus.

The second characteristic of aged immunity is termed inflammaging. Inflammaging describes the elevated self-reactivity in the elderly, resulting in the typical chronic, low-grade, but above baseline, systemic inflammatory phenotype observed in the absence of acute infection [62,113,125-130]. Inflammaging was originally attributed to somatic cell senescence-associated secretory phenotype (SASP) [131-133] and chronic innate immune activation. In recent years, however, the contribution of aged adaptive immune components and specifically self-reactive T lymphocytes has been realized [113,134], as a probable primary contributor to the age-related development of subclinical autoimmune predisposition. Although immunosenescence and inflammaging appear to be opposing phenotypes, they comprise two sides of the same coin [135] when attempting to holistically understand age-related immune dysfunction [109,110,135,136]. It has been proposed that the basal inflammatory state in the elderly, defined by inflammaging, greatly contributes to many age-related degenerative diseases [134], including metabolic diseases, such as Type-II Diabetes (as a complication of pancreatitis), neurodegenerative diseases, such as Alzheimer's disease, and cardiovascular diseases, such as atherosclerosis [62,134,137-139].

When discussing hallmarks of biological aging, seven overarching pillars [140] are thought to collapse, namely: decreased adaptation to stress, loss of proteostasis, exhaustion of stem cells, derangement of metabolism, macromolecular damage, epigenetic dysregulation, and intercellular communication disorder. These changes are intricately linked through the crossroads of immunosenescence and inflammaging [134,141], which characterize immunology of aging.

Conventional senescence is a general term usually denoting somatic cellular senescence, referring to permanent or durable cell-cycle arrest first observed in cultured fibroblasts. The original observations leading to the discovery of senescence were not fully acknowledged by the scientific community because the initial observations were described in *in vitro* cultured cells, although this group believed there to be cell intrinsic factors leading to the observed "degeneration" of the cells [142]. It was later demonstrated that senescence occurs *in vivo* and has since been more adequately defined as cells exhibiting permanent cell cycle arrest, lack of proliferation, expression of corresponding anti-proliferation markers, such as $p16^{INK4a}$ and senescence-associated β galactosidase (SA-β-gal), shortened telomeres, and activation of DNA-damage signaling cascades. The characteristics of somatic cell senescence have recently been significantly reviewed elsewhere [143,144].

Somatic cellular senescence is believed to be advantageous as an evolutionary protection against cancer development [143]. However, senescence of somatic cells during aging is thought to significantly contribute not only to degeneration of aged tissue function if SSCs are accumulated in certain organs, but also to the systemic inflammatory milieu via induction of SASP [62,130-134,145]. This largely pro-inflammatory cellular secretion pattern induces increased basal levels of serum IL-6 and IL-1, as well as matrix metalloproteinases (MMPs) [62,143]. SASP has therefore been cited as a major contributor to inflammaging [62,130,134,145]. Some of the mechanisms suggested to trigger cellular senescence are prolonged or chronic insults that accumulate over time, such as oxidative stress, gradual telomere shortening, and chronic infections. One additional characteristic of senescent cells is that they actively resist apoptosis [143]. The anti-apoptotic pathways involve many factors including downregulation of Capsase-3 and increased Cyclin-dependent kinase inhibitors, p16 and p21 [146]. More recently, histone modification studies have implicated altered expression

ratios of Bcl-2 and Bax family genes in mediating the anti-apoptotic phenotype of senescent fibroblasts [147].

Immunosenescence is a much broader term that encompasses all age-related changes to the immune system, both innate and adaptive [138,148]. The primary hallmarks of immunosenescence are dampened immune responses to new infection or vaccination, and diminished anti-tumor immunosurveillance, including altered immune response phenotypes in activated T cells, increased memory T cell accumulation, and an inverted T lymphocyte subset ratio [148]. Immunosenescence in T cells [149] is commonly termed "cellular exhaustion". This is usually characterized as loss of co-stimulatory surface molecule CD28 and expression of Tim-3, in addition to the other features of cellular senescence [150]. T cell exhaustion differs from conventional senescence because of upregulation of surface markers such as PD-1 and Tim-3. Additionally, this type of growth arrest is not permanent, as blocking PD-1 can reverse T cell exhaustion, as demonstrated by recent clinical trials [150,151]. This unique type of growth arrest in T cells is primarily due to prolonged or chronic TCR/antigen stimulation.

Recently, a link between immunosenescence and somatic cellular senescence has been established [152,153], in which the SSCs are no longer homeostatically reduced by the immune response. This results when natural killer (NK) cells, macrophages, astrocytes, and T cells undergo diminished chemotaxis toward accumulated SSCs for targeted depletion [152-154]. The mechanisms by which T cells deplete accumulated SSCs could include $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ Th1-like cells producing cytotoxic inflammatory cytokines (such as IFN-γ), and Th2-like cells producing IL-4 and TGF-β [152,153]. In addition to diminished chemotaxis, there is also dampened phagocytosis by neutrophils and macrophages associated with age that facilitates SSC accumulation [155,156]. This ultimately results in increased production of SASP [132], which

significantly contributes to inflammaging and subsequent development of age-related diseases [133,157]. This intersection of inflammaging and immunosenescence with age-related diseases remains unclear, but many groups are currently exploring various models to further elucidate the impact of inflammaging and immunosenescence on age-related disease progression [134,158].

There are several proposed components underlying immunosenescence and inflammaging etiology. In addition to cellular SASP secretions which contribute to inflammaging as discussed above, chronic innate immune activation due to long-term latent or persistent viral infection, for example, with members of the *Herpesviridae* family, have been proposed to contribute to low level pro-inflammatory cytokine production [129]. Most notably, cytomegalovirus (CMV), infection has been explored as a potential biomarker in aging human patients [129,159-161]. For example, several longitudinal studies of aging adults saw correlations with CMV sero-positivity and increased morbidity [162,163]. Importantly, the role of the aged adaptive immune responses to self-tissues (in the absence of acute infection), primarily induced by the T cell compartment, has been found to be a major player in the onset and progression of inflammaging [113,125] and associated with immunosenscence [126,164]. The aged, atrophied thymus continues to select T cells throughout the lifetime of the individual. However, the atrophied thymus is less able to negatively select self-reactive T cells, releasing these harmful, self-reactive T cells to the periphery, thereby, increasing subclinical autoimmune predisposition in the elderly [126]. Additionally, age-related thymic atrophy results in reduced output of functional naïve T cells, or recent thymic emigrant cells (RTEs) [48], over time [101]. Since peripheral T cell numbers remain unchanged or relatively elevated in aged individuals [102-104], the reduced thymic output in combination with peripheral oligo-clonal expansion of memory T cells, which occupy immunological space in the periphery [165-167], results in an overall contracted TCR repertoire diversity [48,73,105,106] thereby inducing immune insufficiency

(immunosenescence). Figure 4 shows the intersection of thymic atrophy, immunosenescence and inflammaging.

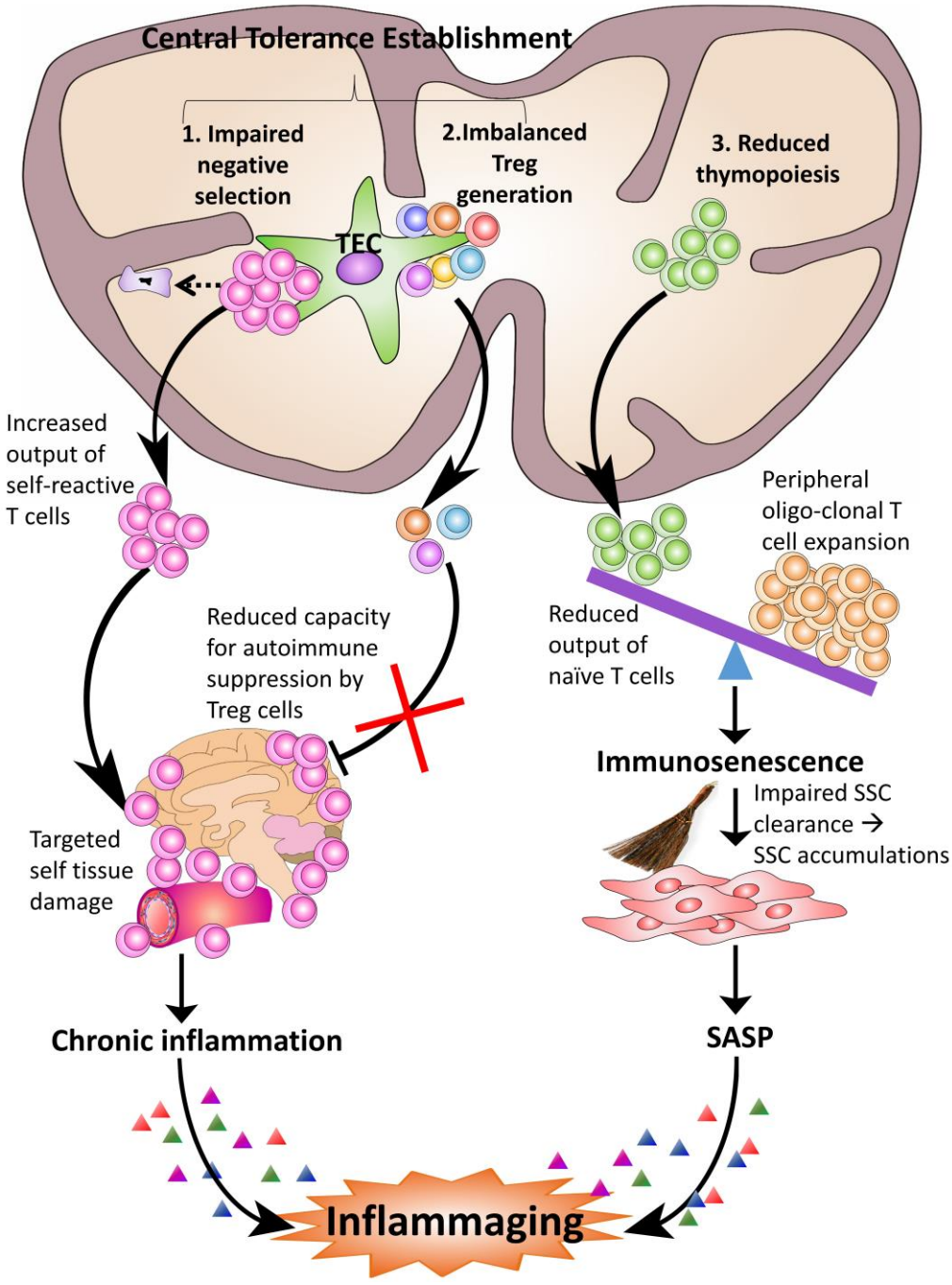

Figure 4: Intersection of immunosenescence and inflammaging is associated with age-related thymic involution. The aged, involuted thymus exhibits ineffective central tolerance and declined thymopoiesis. The ineffective central tolerance includes (1) impaired negative selection, which

leads to the increased output of self-reactive T cells that attack self-tissues/organs, and (2) imbalanced generation of tTreg TCR repertoire, which fails to sufficiently suppress self-reactive T cell–mediated autoimmune responses. Autoimmune responses lead to tissue damage and thus cause chronic inflammation, which is one of the contributors to inflammaging. Reduced thymopoiesis leads to decreased output of naïve T cells for the clearance of senescent somatic cells (SSCs) and the expansion of oligo-clonal T cells in the aged periphery lack sufficient clearance capacity, which allows for SSC accumulation. SSCs are an important source of SASP, another contributor to inflammaging.

1.7 Trends for Rejuvenation of Age-Related Thymic Atrophy

Since the T cell compartment is implicated in so many aspects of inflammaging and immunosenescence, we believe that one potential strategy for ameliorating the effects of inflammaging is via rejuvenation of the aged, involuted thymus. By restoring thymic function, we would repair the defects in negative selection and rebalance tTreg generation. Currently, there are several strategies for rejuvenation of thymic involution in the literature, some of which target systemic T cell immunity and others focus on the thymus itself.

1.7.1 FOXN1-TEC Axis

Since the TEC-autonomous factor *FOXN1* is heavily implicated in onset and progression of age-related thymic involution, several strategies attempt to target the *FOXN1*-TEC axis to specifically restore TEC function.

(1.) Cellular therapy: First, some TEC stem cell-based strategies include utilization of human embryonic/pluripotent stem cells [168-170], *FOXN1*$^{eGFP/+}$ knock-in epithelial cells [171], and young TEC-based [172] or inducible TEC-based [173] strategies. These all involve engraftment of exogenous *FOXN1* producing cells into thymic tissue. One such group directly transplanted TECs from newborn mice intrathymically into middle-aged recipients and observed renewed growth of the thymus as well as enhanced T cell generation [172].

Another group generated induced TECs (iTECs) from exogenous *FOXN1*-overexpressing mouse embryonic fibroblasts (MEF) cells by initiating the exogenous *FOXN1* expression that converted MEF cells into epithelial-like cells *in vitro* [173]. Engraftment of these iTECs under the kidney capsule of syngenic adult mice created a *de novo* ectopic thymus. Host T cell progenitors seeded the *de novo* thymus-like organ generated by the transplant and normal thymocyte

distributions were observed after 4 weeks. Additionally, typical thymus microstructure was seen in the *de novo* thymic engraftment [173].

(2.) Cytokine therapy: There are also some cytokine-to-TEC based therapies, such as keratinocyte growth factor [174,175] and IL-22 [176-178]. Many of these animal studies observed thymic regrowth and improved thymopoiesis, however, they largely used models of acute thymic insult, such as irradiation. As for chronic age-induced thymic atrophy, IL-22 may offer more benefits for improved thymic microenvironment since one study saw correlative up-regulation of IL-22 and FOXN1 after acute thymic insult in mice [179]. Though promising, the extent of crosstalk between IL-22 and FOXN1 within the thymus remains to be determined.

Another cytokine under investigation is IL-7, which is normally secreted by TECs, and helps mediate thymopoesis. IL-7 is reduced in the aged thymus [74] but its role in other aspects of immune system development and proliferation presents a challenge in approaching IL-7 supplementation as a systemic therapy. One such example is a study administering recombinant IL-7 to aged rhesus macaques, which demonstrated little effect of thymic function, but did result in enhanced peripheral T cell proliferation [180]. Several clinical studies have been conducted with systemic IL-7 treatment to boost peripheral T cell proliferation after chemotherapy or after infection or vaccination to amplify immune responses, but these were more focused on peripheral expansion (reviewed [181]). Importantly, peripheral T cell subsets express differing levels of the IL-7 receptor, effecting the extent of IL-7-induced expansion (i.e. more $CD8^+$ T cells expand compared to $CD4^+$ T cells with minimal expansion of Treg cells) [181].

However, IL-7 targeting to the aged thymus may restore more balanced T cell development in the elderly. For example, one study generated a plasmid-delivered IL-7 fusion protein that combined IL-7 with the N-terminal extracellular domain of CCR9 to target this protein to the

thymus and reduce adverse systemic effects of increased IL-7 [75]. They observed restoration of thymic architecture and enhanced cellularity, similar to that of young animals, in the thymus of aged animals that received fusion protein treatment compared to unaltered IL-7 and control plasmid groups [75]. This study holds great promise as a targeted cytokine therapy.

Finally, since TCR repertoire contraction is a contributor to immune insufficiency in aging, it is interesting to note that systemic treatment with recombinant IL-7 resulted in increased TCR diversity in patients who had undergone bone marrow transplant [182]. Again, given the other effects of systemic IL-7, this may not present a realistic therapy for thymic atrophy alone, but it does compel further study into how some of these cytokines and circulating factors may impact T cell development and selection independently and/or synergistically with age-related thymic involution.

(3.) Gene therapy: Similar to the TEC-based cellular therapy, some groups have utilized genetically-based methods to enhance exogenous *FOXN1* expression, either with *FOXN1* cDNA plasmid or *FOXN1* transgenes) [97-99]. One group intrathymically injected plasmid vectors carrying *FOXN1*-cDNA into middle-aged and aged mice and observed partial rescue of thymic size and thymocyte numbers compared to empty vector controls [97]. Another group, utilizing an inducible *FOXN1* overexpression reporter gene system, showed *in vivo* upregulation of *FOXN1* expression in middle-aged and aged mice resulted in increased thymic size and thymocyte numbers [99]. They also observed enhanced ETP cell numbers, and the mTECs:cTECs ratio was restored to normal levels [99]. Moreover, these targeted *FOXN1* gene therapies also show great promise for rejuvenation of aged thymic structure and function.

1.7.2 Periphery-Thymus Axis

(1.) Growth hormones: Decline in growth hormone during aging has been suggested to contribute to age-related thymic involution and animal studies using growth hormone supplementation show rescue of thymic atrophy, increased T cell progenitor recruitment into the thymus, as well as enhanced thymic microenvironmental cytokine production [183-185]. Studies of growth hormone date back to the early 1999s after the observations that TECs express growth hormone receptors and that insulin-like growth factor is expressed in the thymus [186-188]. Studies of insulin-like growth factor 1 (IGF-1), which is closely related to growth hormone, show similar thymic functional and structural improvements upon increased IGF-1 levels in aged mice [183,189]. Although, the effects of crosstalk between growth hormones and many other neuroendocrine hormones with thymocytes and TECs are under investigation, these systemic pathways are extremely interwoven and thus difficult to compartmentally delineate [183,189].

(2.) Sex hormones: The effects of sex hormones on the thymus have long been characterized, with the earliest reports of thymic atrophy correlating with adolescence and reproductive hormones dating back to a 1904 study in cattle [190]. Early studies using castration and sex steroid antagonists in both male mice and male patients receiving androgen blockade for prostate cancer therapy demonstrated phenotypes varying from delayed onset of thymic involution to complete thymic regeneration [191-194]. Most of these early studies, however, focused primarily on phenotypic data, such as an increase of thymopoiesis, with insufficient mechanistic results. Generally, the rejuvenation is thought to occur in the TEC compartment because androgen receptors are expressed by TECs [195]. One of the potential mechanisms reported was that sex steroids inhibit cTEC expression of Notch ligand Delta-like 4 (DLL4), shown in one study utilizing a luteinizing hormone-releasing hormone blockade that saw enhanced thymopoesis after blockade in mice [196]. DLL4 is an important factor for promoting T cell differentiation and development. It

remains unclear whether Notch ligands (there are four types) are decreased in the aged thymus and how this might play a role in decreased thymopoiesis with age.

In contrast, other studies of thymic rejuvenation through sex steroid ablation exhibited in the least, only a short-lived rejuvenation, and at most no influence whatsoever on thymic involution in mice [197]. Others suggest that the observable thymic restoration can be transient (only 2 weeks) but harmful, asserting that the "rejuvenated" thymus potentially produces more harmful T cells and increasing self-reactivity [198]. In support of the opinion that sex hormone ablation may cause detrimental autoimmune implications, a human study, which used medical castration resulted in a declined % $CD4^+CD25^+$ Treg cells and increased NK cells, which may compromise immune tolerance [199].

Recently, studies on sex hormones and their impact on thymocyte selection of the TCR repertoire via *AIRE* gene expression by TECs in the thymus demonstrate that there are differences in males and females in both mouse and human samples [200-202]. Androgens from males promote *AIRE* expression in mTECs to enhance thymocyte negative selection, while estrogens reduce *AIRE* expression, dampening thymocyte negative selection and potentially increasing autoimmunity [201,202]. Therefore, these hormones may mediate thymic functionality to a greater extent than simply structural atrophy. In light of this, sex steroid antagonists or castration-based rejuvenation of thymic aging may have more disadvantages (inducing autoimmune predisposition in the elderly) than advantages.

(3.) Blood-borne factors: Of note, there are likely circulating factors that impact age-related thymic involution, including proteins, mRNAs, microRNAs and other signaling molecules. One method to test this is a heterochronic parabiosis model, in which young and aged mice are surgically conjoined resulting in mutual influence of blood-borne factors. These experiments,

however, have not demonstrated rejuvenation of the aged thymus [203-211]. Conversely, when serum-derived extracellular vesicles, which carry cellular factors throughout the body, were taken from young mice and given to aged hosts, partial thymic rejuvenation with increased negative selection signaling was observed [212]. Interestingly, we also observed decreased levels of circulating pro-inflammatory IL-6, suggesting rescue from inflammaging following treatment with these young serum-derived extracellular vesicles [212]. Further work to elucidate the mechanism of ameliorated inflammaging phenotype is necessary, as it could be due to increased targeted deletion of senescent cells in the periphery causing less SASP secretion, enhanced Treg production, or other unknown mechanisms.

(4.) Life-Style/Physical Exercise: Finally, life-style habits should not be overlooked pertaining to immune health and healthy aging. Indeed, CT scans of patient thymus tissue demonstrate that advanced fatty degeneration of the thymus is positively correlated with increased BMI and with smoking [213]. Additionally, physical exercise has demonstrated countless benefits for immune health, some of which have recently been reported. One such study has documented an intriguing correlation between physical exercise and improved thymic function in elderly patients. This in-depth study compared numerous aspects of immunosenescence and thymic output in aged adults who participated in high levels of regular exercise for much of their adult lives and aged adults who had been inactive [214]. This study found that the aged individuals who maintained physical exercise regimens exhibited reduction in typical decline in thymic output, decreased markers of inflammaging, such as reduced serum IL-6, and increased serum IL-7 and IL-15, which may foster thymic health and function [214]. The age-associated increase in Th17 phenotype was also significantly lessened in the aged cohort with physical exercise and lower peripheral Treg cell numbers were observed in these individuals compared to the inactive aged cohort [214]. Though not

all aspects of immunosenescence were lessened in the exercising cohort, as both groups maintained the age-related accumulation of senescent T cells, this study does present some compelling findings. This group published a recent review and discussed the direct cross-talk between skeletal muscles during exercise and the immune compartment, even describing exercise as a potential adjuvant to immunizations, as some studies have also shown enhanced T cell priming and increased naïve T cell frequency [215]. Therefore, it is significant to mention the effects of physical exercise and overall healthy life-style habits on immune health and directly on thymic health over the lifespan.

In sum, there are many varied avenues for restoration of aged thymic structure and function as well as its influences on inflammaging. Many of these rejuvenation strategies focus on the TEC compartment, since decline in TECs and TEC-associated factors are implicated in thymic involution onset and progression, however, the role of other systemic players are still under investigation. Additionally, each strategy has disadvantages. For example, intrathymic injection of newborn TECs can rejuvenate middle-aged thymus [172], but the source of newborn TECs is limited and may not be ideal as a translational therapy. Additionally, generation of an ectopic *de novo* thymus under the kidney capsule [173] can generate naïve T cells, but this does not remedy the increased self-reactive T cells released by the original atrophied thymus remaining in the host. Also, the use of thymus-targeted cytokines may be beneficial, but caution is needed, as systemic cytokine therapies usually encompass adverse effects. Moreover, continued investigation is required for future development of practical and effective interventions for age-related thymic involution and inflammaging.

1.8 Regulatory T (Treg) Cells and Regulation of Self-Reactivity

Treg cells are classically characterized as $CD4^+CD25^+FoxP3^+$ T cells, with expression of Forkhead Box P3 (FoxP3) as the primary phenotypic identifier. FoxP3 is the transcriptional regulator that endows Treg cells with suppressive capacity, and it is highly epigenetically regulated, in both a tissue-dependent[216] and in an age-dependent manner (for example, FoxP3 expression increases with age[217]). Treg cell generation primarily occurs in the thymus, (tTreg generation). They can also be induced in the periphery from $FoxP3^{neg}$ $CD4^+$ T cells, termed iTreg, however, the majority (80-95%) of Treg cells are thymus-derived with TCR repertoires that are generated via thymic selection [34,115,116]. The current paradigm for $CD4^{SP}$ thymocyte development (Fig. 1) asserts that compared to tTeff cells, tTreg cells have a relatively higher affinity for self-antigens and lower affinity for foreign antigens, as demonstrated by numerous studies[11,16,18,120]. It has also been suggested through one study that tTreg cells have a higher level of TCR diversity and Tcon cells [218]. This is justified by the fact that Treg cells primarily function as anti-inflammatory (immunoregulatory) T cells with the capacity to dampen autoimmune and chronic inflammatory responses in the periphery, in order to normalize immune homeostasis [35].

There are several mechanisms by which Treg cells exert their immunoregulatory function in order to preserve and/or restore immune homeostasis [219,220]. First, their high level of CD25 (IL-2Rα) expression has long been thought to act as a competitive sink for IL-2 in the peripheral microenvironment, since IL-2 is the primary driver of activation and proliferation for effector T (Teff) cells[221,222]. There has been some dispute as to whether this is the primary mechanism of Treg-mediate immunosuppression [223], or whether IL-2 signaling via the high-affinity IL-2 receptor CD25 is simply upstream of Treg expansion and enhanced function via upregulation of FoxP3 and other molecules associated with Treg function [224]. Importantly, IL-2 in the thymus is essential for Treg generation [224,225].

Another, perhaps more accepted means of immunosuppression mediated by Treg cells is the downregulation of CD80/CD86 expression on dendritic cells (DCs). This occurs via CTLA-4 expressed on Treg cells, which has been shown to remove CD80/CD86 from the surface of DCs and thereby inhibit the necessary co-stimulation of Teff cell activation [226]. Treg cells have also been shown to deplete MCH-II:Ag complexes from DCs to further reduce pro-inflammatory immune cell activation [227]. Additionally, Treg cells secrete IL-10 and TGF-β, both of which are anti-inflammatory cytokines that can dampen local immune responses [220]. Further, recent findings suggest that Treg cells can regulate immune responses on a metabolic level via delivery of cAMP to neighboring Teff cells or APCs [220], however, more investigation into these pathways is needed.

An additional significant mechanism of Treg-mediated suppression is via PD-1/PD-L1 signaling. PD-1, usually expressed on activated T cells, gives a negative signal when interacting with PD-L1. Therefore, PD-1 on an activated Treg cell can exert immunosuppression against Teff cells [220]. This particular surface molecule is of interest in in the sphere of immunotherapy, not only for autoimmune and inflammatory disease therapies, but especially in cancer patients, since Treg cells more often than not are detrimental during cancer by suppressing necessary anti-tumor immune responses [228,229].

It is worth noting that Treg cells are thought to function primarily in an Ag-specific manner, however, bystander suppression is another means by which Treg cells can exert suppressive function in an Ag-independent manner [230]. One demonstration of this ability is in a set of Treg transfer experiments in which Treg cells expanded as islet-specific Treg cells were able to ameliorate Type 1 Diabetes in NOD mice after onset of disease (at which time most of the islet cells had already been killed) [231]. Bystander suppression is thought to occur as a result of cytokine signaling within an active microenvironment and therefore is proposed as a secondary tactic that

could provide longer lasting immunoregulation at the site of inflammation. Other studies show that both Ag-specific and Ag-independent (bystander) mechanisms of Treg cell suppression are likely important for ameliorating active autoimmune disease [230,232].

Pertaining to aging, pTreg cells accumulate in the periphery and demonstrate comparable or even increased suppressive capacity to Treg cells in young individuals. Treg cell accumulation has been attributed to decreased Bim expression [167], a pro-apoptotic molecule that regulates the normal homeostatic reduction of immune cells after each completed immune response. The quantitatively increased and functionally enhanced pTreg cell population can pose several complications in the elderly, most notably by interfering with anti-tumor responses [233,234]. Indeed, there is a negative correlation between Treg cells in the tumor microenvironment and peripheral blood with prognosis of cancer patients[235]. Therefore, the seemingly protective roles of Treg cells introduce a double-edged sword to immune regulation in the aged immune system [236].

Like many other cell types, Treg cells demonstrate functional instability and plasticity [237]. Specifically, emerging evidence shows that pTreg cells can exhibit instability, referring to a decline of FoxP3 expression and suppressive function, and plasticity [238], referring to acquired interferon-gamma (IFN-γ) or IL-17 expression, during autoimmune diseases such as Type I Diabetes and Multiple Sclerosis [239-241]. Treg cells that have lost FoxP3 expression are termed “ex-FoxP3” cells and Treg cells that have altered cytokine profiles are termed “Th1-like Tregs” or “Th17-like Tregs”, depending on the cytokines produced [237]. Therefore, these cells potentially contribute to autoimmune pathology not only by inhibited suppression, but also in the case of Treg plasticity, the acquisition of pro-inflammatory cytokine production. This modulation of Treg cell function has also been proposed as a potential target for cancer immunotherapy [234].

Because age-related thymic atrophy also contributes to a holistic contraction of the TCR repertoire in the peripheral aged T cell pool [28,31,106,111], there is the possibility that the TCR repertoire diversity of the Treg cell population is also contracted by the atrophied thymus. If so, this could create "holes" in the Treg compartment allowing for reduced suppression of certain self-reactive Teff cells (and increased self-reactivity). This could help explain the increased autoimmune proneness in the elderly despite an apparently normal polyclonal (pan) Treg population. The generation of tTreg cells is decisively dependent on the reactivity of their TCR with self-antigens presented in MHC class-II on medullary thymic epithelial cells (mTECs) [115]. The atrophied thymus is unable to properly present self-antigens due to defects of the thymic medulla and mTECs [97,125,242-244], which profoundly affects promiscuous self-antigen expression for negative selection [38,40,245,246]. This also influences tTreg generation [114,247]. Therefore, age-related thymic atrophy could impair the generation of certain self-Ag-specific tTregs [114,247] causing "holes" in the Treg TCR repertoire.

1.9 Project Significance

1.9.1 Problem and Central Hypothesis

In an era when people are living longer lives, the goal for healthy aging is emerging as a priority in many fields of biomedical research, and our lab specifically investigates the effects of age-related thymic atrophy on T cell immunity during both homeostasis and disease states. Since we have demonstrated that the atrophied thymus contributes to increased self-reactive Teff cell release with the potential to influence inflammaging[113], it is important to understand the effects of thymic atrophy on tTreg generation. We have recently published data showing that thymic atrophy does not impair overall tTreg generation[114], but this, coupled with the observation of pTreg

accumulation with age[248], raises the ***question***: why is this increased total (pan) Treg population unable to adequately suppress self-reactivity associated with inflammaging?

We ***hypothesized*** that despite an overall increased pan Treg population, the atrophied thymus results in reduced generation of certain self-antigen-specific Treg cells. This could lead to self-antigen-specific "holes" in the Treg repertoire, allowing for specific self-tissue autoimmune reactivity.

1.9.2 Project Innovation

In addition to potentially defective generation of some antigen-specific tTreg cells, we believe the atrophied thymus may also contribute to the observed pTreg cell instability (decline of FoxP3 expression and suppressive function) and plasticity [238] (acquired IFN-γ or IL-17 production), during autoimmune diseases [239,240]. During aging, pTreg cells accumulate [248], due to a decrease in pro-apoptotic *Bim* activation [165-167]. Since the majority (80-95%) of this pTreg pool is generated by the thymus [34,115,116], age-related pTreg accumulation could exacerbate an altered Treg TCR repertoire in the aged periphery and possibly enhance detrimental phenotypes of Treg instability and plasticity, further contributing to inflammaging-associated self-tissue damage.

Therefore, it is of ***clinical significance*** to determine how age-related thymic atrophy impacts antigen-specific tTreg generation and how this altered Treg pool may potentiate differences in suppressive function in the periphery. Uncovering this mechanism is an important step towards developing creative solutions to normalize aged immunoregulatory function and thereby reduce the risks associated with inflammaging. This project was ***innovative*** because we investigated age-related hyper-self-reactivity resulting from potential "holes" in the aged

immunoregulatory system, by assessing the contribution of defective thymocyte negative selection and tTreg generation in the atrophied thymus, which has been largely overlooked.

Specifically, we suggested a *novel concept*, which is that age-related thymic atrophy induces a contracted Treg TCR repertoire creating "holes" in the $CD4^{+}$FoxP3 Treg cell repertoire that contribute to self-reactivity. We attempted to uncover a *novel mechanism* to address why relatively enhanced tTreg generation and accumulated pTreg cells cannot sufficiently suppress self-reactive Teff cell-induced inflammaging through a systematic study of tTreg TCR repertoire diversity, antigen-specific tTreg cell generation, and antigen-specific Treg cell suppressive function. We *used comprehensive novel mouse models*: a *FoxN1* conditional knockout (cKO) model to induce thymic atrophy and mOVA-Tg host mice (OVA as a mock self-Ag) reconstituted with OT-II TCR-Tg T progenitors for two types of TCR-Tg chimeric mouse models. Additionally, we generated dual reporter chimeras allowing us to sort newly-generated (thymic-derived) Treg cells for TCR repertoire diversity analysis to determine whether the atrophied thymus is capable of introducing "holes" in the tTreg repertoire.

1.10 Research Strategy and Approach

During aging, T cell-mediated immune functions are impaired resulting in immunosenscence [109,110], exhibited by poor immune responses to infection, tumor cell clearance, and vaccination [249], but coupled with increased self-reactivity [113,250-252]. This coexisting condition of diminished response to pathogens and higher response to self is potentially attributed to several age-related changes, such as contraction of the αβ-T lineage T cell receptor (TCR) repertoire [26,28,30,32,53,253], and compartmental imbalances in naïve versus memory T cells[53,105] and effector T (Teff) cells versus $CD4^{+}FoxP3^{+}$ regulatory T (Treg) cells [254]. It is accepted that these changes open "holes" or "windows" for infection [32], but we believe these changes also create "holes" during the generation of aged central immune tolerance that promote self-reactivity, partially contributing to the age-related, low-grade, chronic inflammation termed "inflammaging". Inflammaging is induced from chronic activation of both innate and adaptive immune reactions, and is implicated as a risk for many age-related degenerative diseases in the elderly [62,127-130].

Importantly, it remains largely unclear how holes in aged central tolerance, which permit some self-reactive conventional T (Tcon) cells to avoid regulatory immune suppression are introduced [255]. The generation of central tolerance includes two major mechanisms: thymocyte negative selection and thymic Treg (tTreg) generation. Both processes utilize the same agonist self-antigens (Ags) interacting with TCRs on developing thymocytes[12,34]. It is already known that the atrophied thymus demonstrates dysfunction [113,125,251], inducing an imbalance of thymocyte negative selection [113,125,126] versus polyclonal (termed pan) tTreg cell selection [114]. However, the generation of various tissue-specific tTreg cells during aging is unclear, largely due to the complexities involved in the generation of certain tissue-specific tTreg cells. Therefore, uncovering the mechanism(s) by which the atrophied thymus alters the antigen-specific

immunoregulatory Treg compartment, it could explain why enhanced polyclonal tTreg generation in the aged thymus [114] and accumulated peripheral (pTreg) cells [165-167,248] are unable to sufficiently suppress some self-tissue-specific Teff cell-mediated inflammaging [113]. By assessing other models of defective negative selection, albeit some more severe than in the aged thymus, such as autoimmune regulator (*Aire*) gene knock-out, defects in the generation of certain tissue-specific Treg cells are apparent [44,119,256]. These types of observations have further informed our project rationale in which the defects in atrophied thymus may also negatively influence tTreg cell selection.

In order to investigate this minimally understood topic, we ***hypothesized*** that age-related thymic atrophy causes a contracted TCR repertoire diversity in the Treg compartment [111] which, in combination with accumulated pTreg cells [165-167,248], biases antigen-specific tTreg cell selection [115] and increases Treg cell instability [238] in the aged microenvironment, especially upon autoimmune stimulation [257]. To this end, we proposed three specific aims outlined in the following sections.

1.10.1 Aim 1. Identify an extrinsic defect in generation of a mock self-antigen-specific Treg cell clone in the atrophied thymus.

Scientific Premise: A contraction of total T cell TCR repertoire diversity in the elderly [26,28,30,32,53,253] is not only attributed to expansion of memory and pTreg cells, but also due to the aged, atrophied thymus, resulting in reduced output of naïve T cells. This causes insufficient immune responses to infection and cancer in the elderly [105,258]. However, the generation of new tTreg cells is not reduced in the atrophied thymus [114], nor is tTreg output declined [248]. In addition, FoxP3 expression is relatively enhanced in aged pTreg cells [217]. Why, then, is the increased Treg population unable to suppress increased self-reactivity in the elderly? [82,83]

We *postulated* that this is due to an age-related biased selection of Treg cells, which could result in reduction or absence of certain self-tissue-specific Treg cells. Normally Treg TCRs recognizing self-antigens are selected in order to combat self-reactive Teff cells (with few Treg TCRs recognizing foreign-/allo-Ags [259-261]). If thymic selection is perturbed, the consequence is a reduction in suppression of self-responses. For example, in the *Aire*$^{-/-}$ thymus, the Aire-dependent TCAF3 epitope of a prostate antigen cannot be promiscuously expressed on mTECs [118], and therefore prostate-specific Treg cells could not be selected [44]. Since Aire expression is reduced in the aged thymus, similar influences on tissue-specific Treg cell selection could occur. Because tTreg TCRs are selected via their interaction with endogenous self-antigens presented by MHC-II (self-pMHC) on mTECs [18,115,262], different signaling strengths produced by the interaction between self-pMHC and TCR lead to the development of distinct tTreg subsets [263,264] each arising from different Treg thymocyte precursors. The current paradigm describing how TCR signaling strength determines CD4^{+} thymocyte fate is illustrated in Figure 1 and modified to reflect our project rationale in Fig. 16 (see Chapter II).

Here, we focused on the shift away from self-reactivity by determining how the atrophied thymus contributes to a reduced generation of tissue-specific tTreg cells[255]. We took advantage of a mock self-antigen, ovalbumin (OVA), and designed two comprehensive chimeric mouse models (Fig. 5). First, lethally irradiated rat insulin promoter (RIP)-driven ovalbumin transgenic (Tg) (mOVA-Tg) mice [265] served as hosts, and OT-II TCR-Tg (specific $CD4^+$ TCR to $OVA_{323-339}$ Ag) mouse [266] bone-marrow (BM) cells were transplanted intravenously (i.v.) into host mice for immune system reconstitution. The mOVA-Tg mice carry a *FoxN1*-floxed gene with ubiquitous tamoxifen (TM)-inducible Cre-recombinase ($uCreER^T$) Tg [93,97] (termed FCmOVA or FCM mice) or without $uCreER^T$ Tg (termed FFmOVA or FFM mice). The *FoxN1*-floxed gene can be conditionally knocked out (cKO) via $uCreER^T$ Tg activation with intraperitoneal (i.p.) injection(s) with tamoxifen (TM**)** to induce thymic atrophy, mimicking thymic aging [93,97]. Second, newborn FFM or FCM mice served as thymus donors for transplantation under the kidney capsule of OT-II TCR-Tg host mice who received TM to induce thymic atrophy of the transplanted FCM thymi[113]. This thymic transplant chimera circumvented potential side effects of irradiation to the thymus and allowed us to look specifically at thymic Treg and Tcon cell development.

Experimental Design: The mouse strains utilized for both Aim 1 (and Aim 2) are illustrated in Figure 5. The first type of model is a BM chimeric mouse model. We chose mOVA (a mock self-Ag) as a Treg TCR ligand and OT-II TCR-Tg T cells (OVA-reactive $CD4^+$ T clone), because most natural Treg cell TCR ligands are currently unknown, and reactions of a given tissue-specific Treg clone within a heterogeneous polyclonal T cell pool are difficult to study *in vivo*[263]. In our RIP-mOVA host mice, Tg tissue-specific Ag, is expressed in the pancreas due to the RIP-promoter and is recognized by OT-II TCR-Tg T cells, which express a $CD4^+$ $OVA_{323-339}$-specific TCR, restricted to I-A(b) (murine MHC Class II). In the chimera, the OT-II TCR-Tg (monoclonal

due to *Rag*$^{-/-}$ background) T cells can infiltrate into the pancreas [267]. However, due to co-transfer of wild-type (WT) BM cells, this host immune system maintains a polyclonal TCR repertoire (more physiologically relevant), so the developmental effects of thymic encounters with a relatively rare "self-antigen" can be assessed *in vivo*. Also, the BM cells from donor mice (OT-II TCR-Tg), express both CD45.1 and CD45.2 congenic markers, from a congenic F1 generation produced through cross-breeding between CD45.1/WT/C57BL6 mice and CD45.2/OT-II^{+}/C57BL6 mice. Based on CD45.1 or CD45.2 markers, we can easily distinguish the original source of the cells. In the chimera, WT (CD45.2^{+}CD45.1^{-}) and OT-II TCR-Tg (CD45.2^{+}CD45.1^{+}) BM cells intraclonally compete for thymic selection.

Figure 6 illustrates a schematic of how the OT-II Tg CD4^{SP} T cells develop in the mOVA-Tg thymus. In the mice without an atrophied thymus, most OT-II thymocytes should be depleted via negative selection in the mOVA-Tg thymus, while some OT-II-specific Tcon and Treg cells are still selected and released to the periphery due to mOVA presentation [259], confirmed by our [114] and other groups [268]. We have also demonstrated that the mOVA mice with induced thymic atrophy (FCM) have decreased expression of mOVA by mTECs[114], which supports our project rationale of decreased signaling strength for this particular self-Ag under conditions of thymic atrophy.

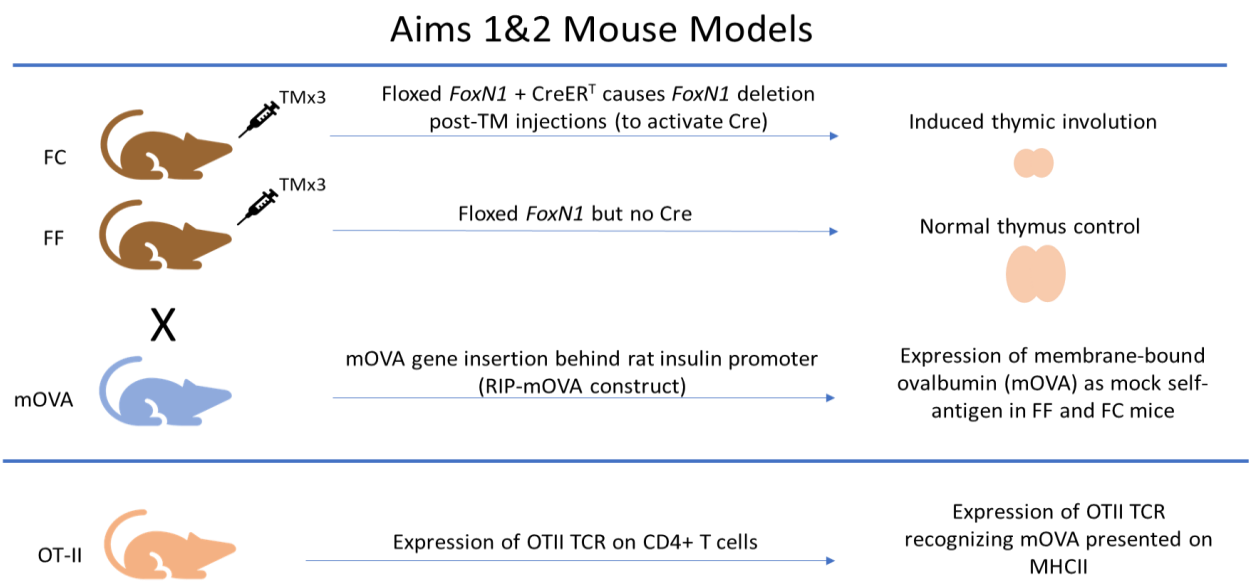

Figure 5: Illustration of Aim 1 & 2 Mouse Strains. Mice will be crossbred as illustrated to generate the different groups utilized in the experimental workflows outlined in Aims 1 and 2.

Immune Reconstitution (Chimera) Model Rationale

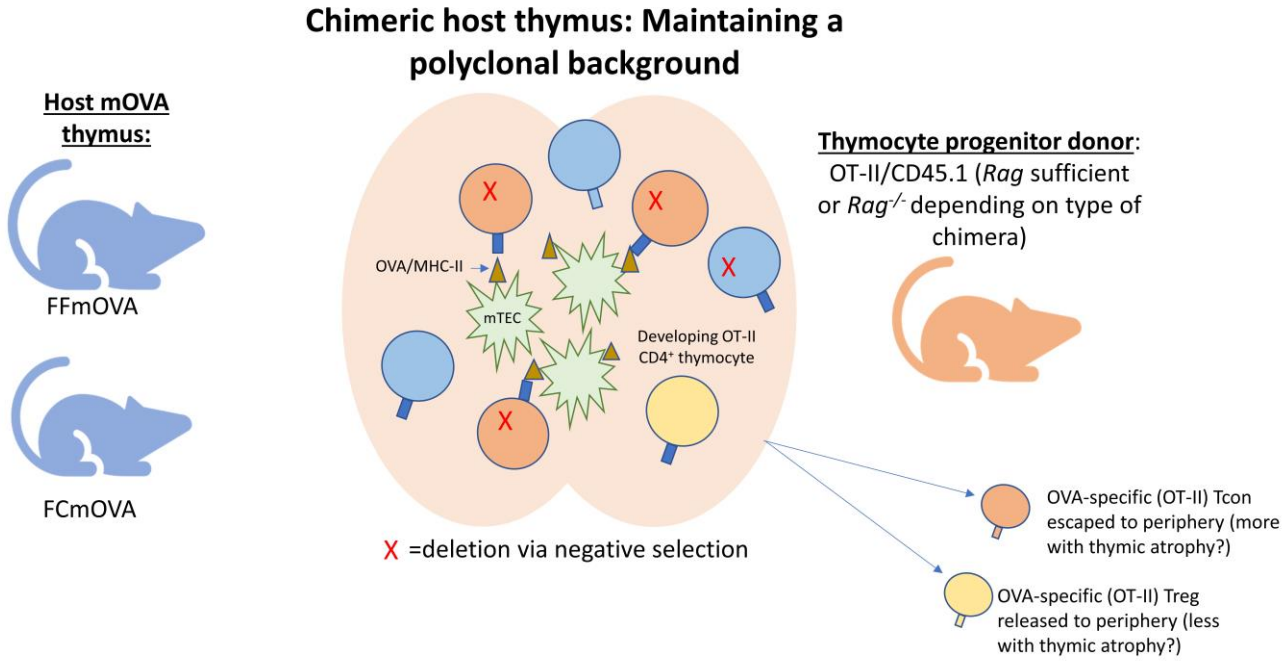

Figure 6: Schematic of T cell central tolerance induction in our TCR transgenic chimera mouse model. Illustration of the mock-self-Ag (mOVA)-Tg thymus and the development of $CD4^{SP}$ OT-II-specific thymocytes. For either the BM chimera or thymic transplant chimera models, the OVA-specific thymocytes will undergo central tolerance induction for mOVA expressed by mTECs in the mOVA-Tg thymus. Based on the current understanding of pMHC-TCR signaling strength for central tolerance induction, the fates of the mock-self-Ag-specific T cells will either be directed to negative selection, tTreg agonist selection, or escape as self-reactive tTcon cells.

The second animal model we used for Aim 1 was the newborn thymic transplantation chimera model in which thymi from either FFM or FCM newborn mice were transplanted under the kidney capsule of young (4-6 week old) OT-II/CD45.1&.2 hosts (see Fig. 9A workflow and our previous publication for more detailed procedure[113]). TM was administered twice to host mice after transplantation to induce thymic atrophy for FCM (but not induced for FFM that lack Cre) of the transplanted thymi. After 2 weeks, the thymi were removed and analyzed for pan and specific Treg and Tcon populations. This confirmed our BM chimera model findings as well as provided more information regarding the Ag-specific T cell populations observed by our lab in a previous project that did not distinguish Treg from Tcon when assessing the number of OT-II-specific T cells generated by the engrafted mOVA thymus[113].

To assess whether OVA-specific pTreg cells were decreased in mice with thymic atrophy, we performed flow cytometry to measure proportions of pan pTreg (i.e. $CD45.1^-$ or TCR $V\alpha2^+\beta5^+$ negative population) and OVA-specific pTreg (i.e. $CD45.1^+$ or TCR $V\alpha2^+\beta5^+$ positive population) cells. Absolute cell numbers, ratios of specific-to-pan Treg cells, and ratios of specific Treg-to-specific Tcon cells in the peripheral lymphoid organ (spleen) and the non-lymphoid organ, pancreas, were analyzed.

1.10.2 Aim 2. Evaluate intrinsic defects in a mock-self-antigen-specific Treg clone developed in the atrophied thymus.

Scientific Premise: Treg instability and plasticity were first observed in Treg cell biology in the context of autoimmune inflammatory conditions via cell-fate reporter mice [269], which showed that the expression of FoxP3 in a proportion of mature Treg cells could become reduced or lost. These Treg cells also could acquire an effector-like phenotype, termed "ex-FoxP3 cells" [269]. In addition, Treg cells could also produce pro-inflammatory cytokines IFNγ (called Th1-like Treg) or IL-17 (called Th17-like Treg), and were able to induce, rather than suppress, autoimmunity in NOD mice and diabetic patients [239,240], as well as in multiple sclerosis (experimental autoimmune encephalomyelitis (EAE) mouse model) [270,271].

Several studies have confirmed that Treg plasticity is an inherent property primarily controlled by epigenetic regulation [272], which can result in increased pathology and reduction of suppressive function [238,272]. Although the phenotypes of FoxP3 instability and Treg plasticity have been widely observed in inducible Treg (iTreg) cells [273-275], tTreg cells have also been shown to exhibit instability in FoxP3 expression, particularly under inflammatory autoimmune conditions in target tissues [269,276,277]. For example, FoxP3 expression is lost in tTreg cells specific for an epitope of myelin oligodendrocyte glycoprotein (MOG) (amino acids 38–49) during the development of EAE, with an increased frequency of ex-Foxp3 cells in the central nervous system at the preclinical and peak stages of EAE, and decreasing during EAE resolution [277].

We therefore asked whether self-Ag-specific tTreg cells generated from the normal and atrophied thymus possessed the same properties of Treg instability/plasticity upon encountering autoimmune inflammatory stimuli in the periphery. This alteration was observed in aged NOD mice, in which Treg cell numbers were not changed but the Treg suppressive function was reduced

[278]. Age-related thymic atrophy is associated with a wide range of autoimmune phenotypes, such as lympho-infiltration of non-lymphoid organs, increased anti-nuclear antibody, etc. [125]. This is termed autoimmune "proneness", similar to the autoimmune proneness in NOD mice. Therefore, we ***postulated*** that there would be differences in the functional phenotype of tissue-specific Treg cells generated from the atrophied thymus due to perturbed tTreg agonist selection, which could be exacerbated upon autoimmune stimulation [114,279]. We investigated this with using our BM chimera mouse model from Aim 1 which experiences low-level Ag-specific (OVA) stimulation *in vivo,* due to RIP-driven mOVA expression on the pancreas[265]. Since pTreg cells are relatively stable in the peripheral lymphoid tissues spleen and LNs [280], we expected to observe functional changes predominantly in Treg cells found at the inflammatory sites (pancreas and/or pancreatic LNs) [238,281,282].

Experimental Design: First, we utilized flow cytometry to evaluate the protein expression level of FoxP3 and CD25, the primary phenotypic markers of classical Treg cells, which are closely associated with Treg suppressive capacity. We utilized the same BM and thymic transplant chimera mouse models from Aim 1 (Fig. 5).

Next, to assess whether thymic atrophy results in OVA-specific pTreg cells that have reduced suppressive function against OVA-induced specific Teff responses, we performed a Treg functional assay. Equal numbers of OVA-specific Treg cells ($CD4^+CD25^+CD45.1^+$) were sorted from BM chimeric mice with/without thymic atrophy, while Teff cells ($CD4^+CD25^-$) were sorted from unmanipulated OT-II TCR-Tg mice. Dendritic cells (DCs) prepared from the WT mouse BM served as APCs, and $OVA_{323-339}$ peptide was used for stimulation of proliferation. Detailed culture conditions and measurements are also reported in our previous publication [113,114], and experimental group design and expected results are outlined as Table 1. By culturing equal numbers of sorted

Tregs from both host mice groups, we sought to determine whether or not the Tregs from mice with atrophied thymus had intrinsic defects in suppressive capacity.

Teff & APC	Treg Cells	OVA	αCD3+αCD28	Expected results/reaction	
$CD4^+CD25^-$ $OT\text{-}II^+$ + DCs from BM	none	+	-	+++ (no suppression)	Negative Control
	none	-	+	+++ (no suppression)	
	FFmOVA Chimera $OT\text{-}II^+$ Treg Cells	+	-	+/- (some suppression)	
	FCmOVA Chimera $OT\text{-}II^+$ Treg Cells	+	-	++/- (less suppression)	
	WT Treg Cells	+	-	++ (little suppression)	Positive Control
		-	+	--- (strong suppression)	

Table 1. Design of Treg Suppression Assay (Functional Testing)

1.10.3 Aim 3. Determine the potential mechanism for these changes in a mock-self-antigen-specific Treg clone, namely: thymic contraction of tTreg TCR repertoire diversity.

Scientific Premise: A key function of the thymus is thymocyte selection, including positive selection of thymocytes that recognize self-matched MHC with potential to recognize self or foreign Ag [10,15], negative selection of self-reactive thymocytes [10,16,17], and tTreg cell agonist selection [18], through which a diverse T cell repertoire is generated and central immune tolerance is established [33,34]. Sufficient TCR diversity is essential not only for a robust immune response to various pathogens, but also for suppression of uncontrolled immune response to various self-Ags/self-tissues. Thymocyte selection is primarily dependent on both the affinity and avidity (affinity x number of interactions) between self-Ag/peptide which binds to the MHC groove (self-pMHC) on mTECs and TCRs on developing $CD4^{SP}$ thymocytes. The signaling strength produced by the interaction(s) determines the fates of $CD4^{SP}$ thymocytes [35,36].

The current paradigm shown in Figure 1 is adapted to illustrate our experimental rationale in Figure 3, where 1) strong signaling results in negative selection, 2) intermediate signaling results in tTreg selection [36,45], and 3) weak signaling results in thymic conventional T (tTcon) cell development (potential Teff cells in the periphery). Because thymic aging reduces the capacity for self-pMHC ligand expression, we suggest that this globally shifts strong signals for negative selection to an intermediate strength so that a relatively enhanced polyclonal tTreg generation takes place (Fig. 3, Green arrow-a) [114]. During thymic dysfunction, such as *Aire* deficiency [39,283], strong signaling for certain self-tissue specific thymocytes, such as *Aire*-dependent TCAF3 epitope of a prostate Ag [118], may become extremely weak, causing these thymocytes, which should be negatively selected, to become Tcon cells (Fig. 3, Green arrow-b). Loss of prostate-specific Treg

cells, and enhanced prostate-specific Tcon cells in *Aire* knockout mice is a typical example of this pathway [44,119].

Given that 1) the entire TCR repertoire diversity is contracted in the aged T cell pool [28,31,106,111], partially due to functional defects of the involuted thymus [111], and 2) the repertoire of Treg cells is also shaped in the thymus[18,115,284,285], and 3) our mock self-Ag specific Treg TCR selection is altered in a manner directly associated with thymic atrophy (shown in Aims 1 and 2), we ***hypothesized*** that these changes will contribute to TCR repertoire diversity contraction in the aged tTreg pool. This hypothesis is also supported by the fact that a declined Treg repertoire diversity was found in autoimmune-prone non-obese diabetic (NOD) mice [286], whose underlying autoimmune proneness is similar to that of aged individuals [113,125].

The inability to generate certain tissue-specific tTreg cells potentially manifests in an age-related contraction of the TCR repertoire. Ample evidence shows that the entire TCR repertoire diversity is contracted in an aged T cell pool [28,31,106] partially due to thymic atrophy [111], though to our knowledge no studies have directly assessed tTreg TCR diversity as a result of thymic aging. We ***postulated*** that in conjunction with a reduction of the entire TCR diversity, TCR diversity of the tTreg compartment in aged individuals could also be reduced as a result of thymic atrophy. Therefore, we investigated newly generated (with a Rag-GFP reporter) FoxP3$^+$ (with a RFP reporter) Treg TCR diversity via TCRβ CDR3 sequencing analysis comparing inducible thymic atrophied (*FoxN1*-flox) [93] mice with their normal thymus control counterparts using a BM chimera model.

Experimental Design: CD4$^+$FoxP3$^+$ Treg cells are characterized as αβ-TCR T lineage cells. Although the vast majority of TCRs are heterodimers composed of two distinct subunits (α and β), both of which contain variable domains, the β chain has been the primary target of many

studies [25] due the presence of the D gene component which contributes to its higher combinatorial potential. The CDR3 regions on each chain are the most hypervariable region for Ag binding, and therefore possess the most sequence variation. This is why we chose to focus on this region of the β chain for our Treg cell sequencing analysis. Additionally, when deciding between DNA- and RNA-level sequencing, we chose DNA sequencing for TCR repertoire analysis because it would not be impacted by possible variation in the copy number of mRNA transcripts on a cell-to-cell basis. As for the diversity introduced by non-template encoded P and N nucleotides: during thymocyte development, these are added to the DNA (albeit in a "random", non-template encoded manner) and are therefore present in the genomic DNA (gDNA) collected from the mature T cells that were sorted from the mice for sequencing. Therefore, the diversity metrics performed with DNA level sequencing does account for the entire CDR3 region of the TCR-β chain.

We analyzed changes in tTreg TCR diversity using the Adaptive ImmunoSEQ TCR sequencing approach. The newly-generated tTreg cells were isolated for sequencing of the CDR3 regions of the TCR β-chain using a DNA-sequencing method and analyzed with bioinformatics software for various TCR repertoire diversity metrics, [287]. This newly-generated, thymic-derived subset was identified by a green fluorescent protein (GFP) reporter system driven by the recombination-activating gene *rag2*-promoter, i.e. Rag-GFP [288-290], while FoxP3 was identified by a red fluorescent protein (RFP) in FoxP3-RFP reporter mice (Jackson Lab #008374) [291]. The crossbred dual reporter mice were bone marrow donors for the generation of BM chimeras with our inducible thymic atrophy (*FoxN1*-flox cKO) [93] mice as hosts. After chimerization, GFP and RFP double positive $CD4^+$ $CD25^+$ (Treg enriched) cells were sorted for TCR sequencing analysis. See Figure 7 for illustration of the mouse model for Aim 3.

Animal groups and methods: We performed flow cytometry-assisted cell sorting (FACS) to collect newly-generated Treg cells from mice with inducible thymic atrophy (*FoxN1*-flox homozygous carrying uCreER^{T} Tg, termed FC, or littermates without uCreER^{T} Tg, termed FF, as controls [93], based on RAG-GFP and FoxP3-RFP reporters, and $CD4^{+}(CD8^{-})$, $CD25^{+}$ markers from the spleen and lymph nodes (LNs). gDNA from these cells were sent for TCR repertoire sequencing by Adaptive ImmunoSEQ.

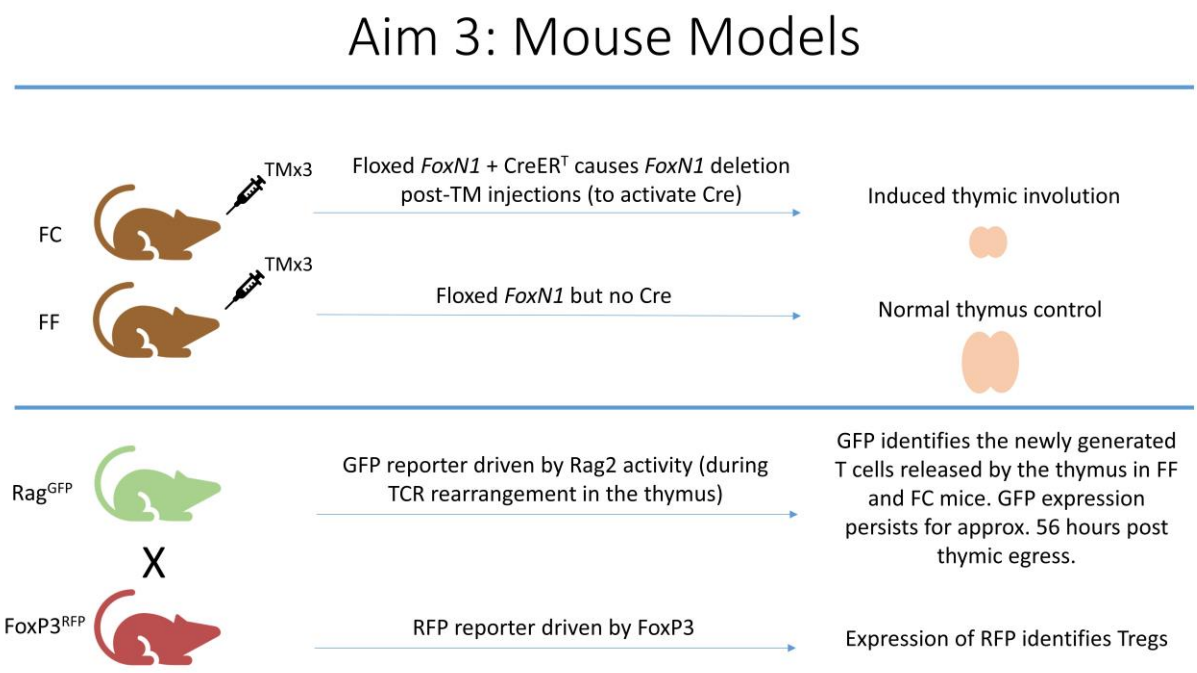

Figure 7: Illustration of Aim 3 Mouse Strains. Mice will be crossbred as illustrated to generate the different groups utilized in the experimental workflows outlined in Aim 3.

CHAPTER II

Thymic Atrophy Creates Holes in Treg-Mediated Immuno-Regulation via Impairment of An Antigen-Specific Clone

By:

Rachel Thomas, Jiyoung Oh, Weikan Wang, and Dong-Ming Su

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2.1 ABSTRACT

Age-related thymic atrophy results in reduced output of naïve conventional T (Tcon) cells. However, its impact on regulatory T (Treg) cells is insufficiently understood. Given evidence that thymic Treg (tTreg) cell generation is enhanced in the aged, atrophy thymus and that the aged periphery accumulates peripheral Treg (pTreg) cells, we asked why these Treg cells are unable to effectively attenuate increased auto-reactivity-induced chronic inflammation in the elderly. We designed a mock self-antigen chimera mouse model, in which membrane-bound ovalbumin (mOVA) transgenic mice, bearing a *FoxN1*-floxed gene for induction of conditional thymic atrophy, received OVA-specific (OT-II) T cell receptor (TCR) transgenic progenitor cells. The chimeric mice with thymic atrophy exhibited a significant decrease in OVA-specific tTreg and pTreg cells but not polyclonal (pan)-Treg cells. These OVA-specific pTreg cells were significantly less able to suppress OVA-specific stimulation induced proliferation *in vitro*, and exhibited lower FoxP3 expression. Additionally, we conducted preliminary TCR repertoire diversity sequencing for Treg cells among recent thymic emigrants (RTEs) from Rag^{GFP}-$FoxP3^{RFP}$ dual reporter mice and observed a trend for decreased diversity in mice with thymic atrophy compared to littermates with normal thymus. These data indicate that although the effects of age-related thymic atrophy do not affect pan-Treg generation, certain tissue-specific Treg clones may experience abnormal agonist selection. This, combined with enhanced pan-pTreg cells, may greatly contribute to age-related chronic inflammation, even in the absence of acute autoimmune disease in the elderly.

2.2 INTRODUCTION

Age-related thymic involution or atrophy is a progressive condition observed in most vertebrate animals, resulting in the obvious reduction of naïve conventional T (Tcon) cell output [48]. This contributes to the systemic dysfunction of the aged immune system termed immunosenescence via the broad dampening of T cell-mediated immune responses [107,292]. Mounting evidence shows that age-related thymic atrophy contributes to not only diminished immunity to foreign antigens (Ags), but also enhanced reactivity to self, due to increased output of self-reactive Tcon cells [113]. Therefore, age-related thymic atrophy contributes to both aspects of aged immune dysfunction [135,292]. This dysregulation associated with age-related thymic involution also affects the generation and function of regulatory T (Treg) cells [114,217]. However, it is insufficiently understood how Treg cells generated by the atrophied thymus contribute to the age-related disruptions of immune homeostasis.

Age-related thymic atrophy is characterized by a primary decline in expression of the thymic epithelial cell (TEC)-autonomous transcription factor, forkhead box N1 (*FoxN1*) [93,97]. Thus, the vast majority of the TECs, specifically in the medullary TEC (mTEC) compartment, retract, resulting in functional decline and influencing the establishment of central tolerance. Central tolerance includes reciprocal mechanisms of negative selection (depletion) of self-reactive T clones and agonist self-antigen (Ag) selection (generation) of regulatory T (Treg) clones during late stages of thymocyte development [11,293]. Our previous studies demonstrated that the atrophied thymus had impaired negative selection, as shown by increased numbers of self-reactive Tcon cells in the periphery [113], but relatively enhanced Treg cell generation, as shown by an increased proportion of newly-generated thymic Treg (tTreg) cells to Tcon cells in the atrophied thymus [114]. Ample evidence also shows that the proportion of peripheral Treg (pTreg) cells accumulate [167,248]

with enhanced suppressive function [217] in the elderly. We therefore wondered why these relatively increased Treg cells are unable to effectively counteract self-reactive Tcon cell-driven subclinical autoimmune proneness [113] of inflammaging, referring to a chronic, systemic inflammatory condition in the absence of acute infection observed with advanced age [294].

Thymocyte negative selection of Tcon or agonist selection of Treg TCR repertoires is dependent on presentation of self-Ags, mainly by mTECs [244,295], mediated partially by the expression of autoimmune regulator (*AIRE*) gene [283] or others, such as *FEZF2* [38]. Evidence shows that Treg TCR selection in the *Aire*$^{-/-}$ thymus results in defects in certain tissue-specific [44] but not polyclonal (pan-) Treg cells [11,293,296]. However, these observations may be different for a given self-antigen clone. For example, one group showed that prostate TCAF3-specific Treg cells are missing from the repertoire in mice with *Aire*$^{-/-}$ thymus [44,119], while another group demonstrated that tissue-specific (such as TRP-1/TYRP1, melanocyte-specific) Treg cells are not affected in numbers [256]. Aging results in mTEC dysfunction, including reduced *Aire* levels [113,125,212]. Thus, by aligning the studies of the aged thymus with *Aire*$^{-/-}$ thymus, we hypothesized that Treg generation in the aged thymus (or age-mimicking atrophied thymus via an inducible floxed-*FoxN1*-knockout mouse model) [93,97] follows a similar paradigm as observed in *Aire*$^{-/-}$ mice. Specifically, we postulated that relatively enhanced generation of tTreg clones in the aged thymus [114] are polyclonal Treg TCRs, while certain tissue Ag-specific Treg clones may not be agonist selected (missing), or even if selected, may experience an intrinsic functional defect, allowing for low and persistent inflammaging, but not outright autoimmune disease.

In order to address this hypothesis, we generated two mock self-Ag chimera mouse models [113,114], in which membrane-bound ovalbumin (mOVA) transgenic mice [265,297], received mixed wild-type (WT) and MHC-II restricted, OVA-recognizing T cell receptor (TCR) transgenic

(termed OT-II TCR-Tg) progenitor cells [266]. These models circumvent the limitations of attempting to identify a single T cell clone within a polyclonal pool. Additionally, each system provided polyclonal precursors along with TCR-Tg precursors so that our reconstitution models provided a more physiologically relevant intraclonal competition during thymocyte development [298], and the thymic and peripheral stromal cells expressed mOVA-Tg (driver by the rat insulin promoter, RIP). In addition, these mouse thymuses carried a *FoxN1*-floxed gene with (FC) or without (FF) CreER^{T} [93,97] for conditional thymic atrophy induction, termed FCmOVA (or FCM) or FFmOVA (or FFM), respectively. These mice experience the hallmarks of thymic aging (age-mimicking atrophy due to induced *FoxN1* knockout), but possess a young periphery since our experiments are performed on mice 2-3 months of age. This allows us to observe impacts on the Treg cells resulting from thymic involution rather than from aged peripheral conditions.

We observed that with thymic atrophy, this OVA-specific (OT-II TCR-Tg) Treg population was significantly decreased in both the thymus and periphery. Further, we found that the Treg-enriched cells from mice with thymic atrophy exhibited impaired suppressive capacity against OVA-specific T effector (Teff) cell proliferation after OVA peptide stimulation *in vitro*. Finally, these OVA-specific Treg cells displayed lower FoxP3 expression.

Lastly, we postulated that reduction/absence of certain self-antigen specific Treg clones in the atrophied thymus is potentially due to altered agonist selection of these Treg cells within the Treg repertoire. Indeed, we saw decreased Treg diversity after TCR repertoire sequencing analysis of newly-generated Treg cells (based on *Rag*^{GFP}-*FoxP3*^{RFP} reporters) in mice with thymic atrophy compared to controls. In sum, these data indicate that although the effects of age-related thymic involution do not affect pan-Treg cell generation, agonist selection of certain tissue-specific Treg clones is impaired, altering the tTreg repertoire of the aged T cell regulatory system. This,

combined with the enhanced pan-pTreg cells, this could greatly contribute to age-related chronic inflammation.

Throughout our study, we refer to pTreg cells based on their physiological location, but based on the current Treg terminology, we need to emphasize that our "pTreg" population does include both tTreg cells (generated in the thymus) and peripherally-induced pTreg cells described in the current update on Treg nomenclature [299]. The primary reason for our description of pTreg cells based on location is that the current understanding is that at least 80% of peripherally circulating Treg cells are derived from the thymus [115,116]. Therefore, describing the Treg cells based on their location seemed most appropriate for this study.

2.3 MATERIALS & METHODS

2.3.1 Ethics Statement

All experiments were performed in compliance with the protocols (#IACUC 2016-0037 and 2019-0028) approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Texas Health Science Center (UNTHSC) in accordance with the NIH guidelines for animal welfare.

2.3.2 Mouse Models

All mice had a C57BL/6 genetic background, which express CD45.2 or CD45.1/.2 congenic markers. *loxp*-floxed *FoxN1* knock-in mice (Jackson Lab #012941) were generated per our previous publications [93,97], and either do not carry Cre recombinase, termed FF mice, and will maintain a normal thymus after tamoxifen (TM) injection, or carry CMV promoter-driven $CreER^T$ and have induced thymic atrophy after TM injection, termed FC mice, i.e. *FoxN1* conditional

knockout (cKO) mice. RIP-mOVA (rat insulin promoter-driven membrane-bound ovalbumin) mice (Jackson Lab #005431) were crossbred with our FF or FC mice to generate FFmOVA (FFM) and FCmOVA (FCM) mice with inducible *FoxN1* knockout. OT-II TCR-transgenic (Tg) mice (expressing a TCR that recognizes mOVA in the context of the MHC-II molecule, I-A^b) (Jackson Lab #004194) were kept on a *Rag* sufficient background or crossbred with *Rag1* knockout ($Rag^{-/-}$) mice (Jackson Lab #002216). These were crossbred with CD45.1 mice (Charles River #NCI B6-Ly5.1/Cr) to generate OT-II/CD45.1/.2 (F1) mice. Rag^{GFP} reporter mice (Jackson Lab #005688) were crossbred with $FoxP3^{RFP}$ mice (Jackson Lab #008374) to generate dual-reporter mice, whose newly generated Treg cells express both GFP and RFP in the CD4 single positive ($CD4^{SP}$) population for live fluorescence-assisted cell sorting (FACS). All animals were housed in our specific pathogen-free animal facility

2.3.3 Bone Marrow Chimera Construct

Bone marrow (BM) progenitor cells were harvested from the femur and tibia of young (4-6 week old) OT-II/CD45.1/.2 (with $Rag^{-/-}$) or WT (CD45.2) mice. Cells were depleted of erythrocytes via ACK lysis buffer and depleted of T cells via anti-CD4, anti-CD8 magnetic bead-assisted cell sorting (MACS) (Miltenyi Biotech LS Columns and PE-conjugated microbeads). Approximately 10 x 10^6 cells/mouse were injected intravenously (i.v.) at a ratio of 1:5 WT:OT-II into lethally (900 Rad) irradiated FFM or FCM recipient mice. One week after immune reconstitution, recipient mice received two doses (1mg/10g body weight/day) of tamoxifen (TM) to induce thymic atrophy. Mice received antibiotic water for one week prior to irradiation until one week post irradiation (2g/L neomycin). A total of 6-8 weeks after immune reconstitution, the mice were sacrificed for further analysis. The workflow was identical for the reporter chimeric mice, except that the host mice were FF and FC mice and each received 10 x 10^6 BM cells from GFP^+RFP^+ donor mice.

2.3.4 Thymus Transplant Chimera Construct

Thymic lobes were harvested from FFM or FCM newborn mice and transplanted under the kidney capsule of young (6-8 week old) OT-II/CD45.1/.2 (Rag sufficient) host mice [113]. Four to five days after surgery, the host mice received three doses of TM as above to induce atrophy of the transplanted thymuses. Approximately four weeks after surgery, the mice were sacrificed and the transplanted thymuses were analyzed. Surgery was performed with anesthesia/analgesia under aseptic conditions in accordance with our approved IACUC protocols.

2.3.5 Isolation of T cells from Pancreas

Freshly isolated mouse pancreas was digested by cutting into small pieces in 500µL of DMEM media and 50µL of digestion buffer (20µL collagenase, 5µL DNase, 25µL DMEM). After 30 mins of incubation at 37ºC, the tissues were gently mixed via pipetting, and then incubated for another 10 mins. After incubation, digested tissues were filtered through a 70µm filter with flow cytometry staining buffer into a 15mL tube and centrifuged at 1200rpm at 4ºC for 5mins. The cell pellets were resuspended and passed through a 40µm filter, then centrifuged again. Finally, the cell pellets were washed with staining buffer prior to staining for flow cytometry.

2.3.6 Flow Cytometric Analysis and Fluorescence-Assisted Cell Sorting (FACS)

Single cell suspensions of the homogenized and filtered thymus, spleen, and lymph nodes (LNs) were stained with various surface and intracellular markers for flow cytometry. Intracellular staining with FoxP3-FITC (eBiosciences #53-5773-82) utilized the FoxP3/Transcription Factor Staining Buffer Set (eBiosciences #00-5523-00) per the kit's protocol. All other antibodies were purchased from BioLegend and include CD4-APC/Cy7 (Cat#100414), CD25-PE/Cy7 (#102016), CD45.1-APC (#110714), Vα2-APC (#127809), Vβ5-APC (#139505), CD8-PE (#100708), FITC

isotype control (#400705), PE/Cy7 isotype control (#401908), CD16/32 blocking antibodies (#101302), CD4-BV711 (#100447), CD8-APC (#100712), B220-APC (#103211) and CD19-APC (#152409). An LSR-II cytometer (BD Biosciences) was used for sample acquisition and FlowJo software (v10) was used for data analysis.

For identification of Vα2Vβ5 TCR chains, the same flow cytometry staining channel was used based on results showing that WT mice have <3% of either Vα2, Vβ5, or double positive populations and that OT-II TCR-Tg mice are enriched for this population (see Fig. 8).

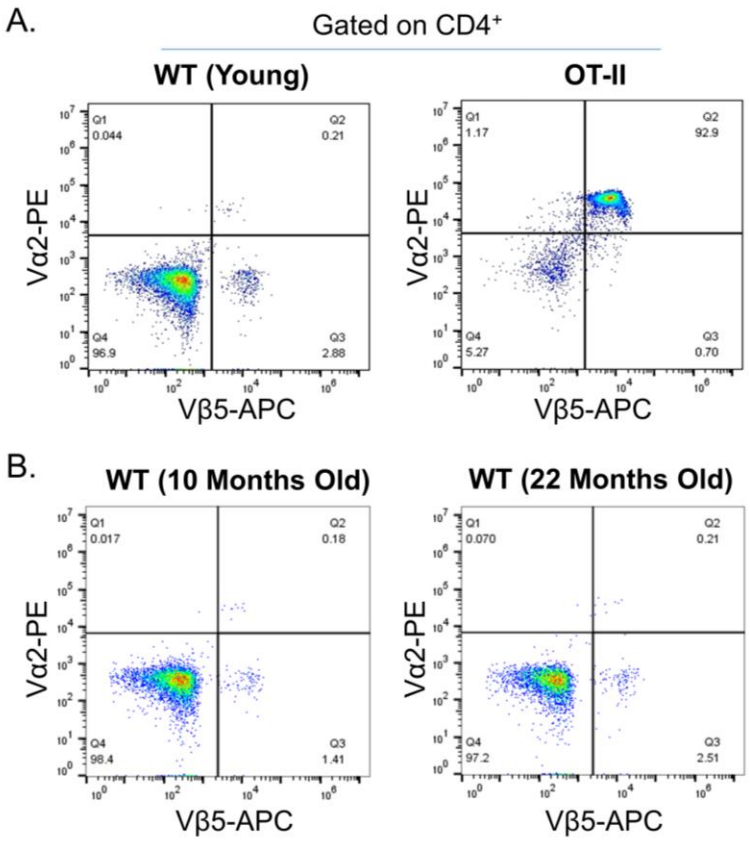

Figure 8: Flow Cytometric Staining of Vα2Vβ5 TCR chains in WT and OT-II TCR-Tg mice. Erythrocyte-depleted spleen cells were stained with CD4-FITC, Vα2-PE, and Vβ5-APC antibodies. Gating on $CD4^+$ cells shows an enriched $V\alpha2^+V\beta5^+$ clone (>90%) in OT-II TCR-Tg mice, but not in WT mice. Although there are few single Vα2 and/or Vβ5 positive CD4 T cells (a total of < 3%) in young WT mice (**A**), this percentage is decreased with natural thymic aging/atrophy as shown in an separate experiment with aged WT mice (**B**). Therefore, we used the same channel for both chains in order to conserve channels for more complex flow cytometry experiments, without compromising our ability to observe changes in the predominant OVA-specific T clone.

For FACS, single cell suspensions were either MACS bead-depleted of $CD8^+$ T cells and B cells ($CD19^+$ and $B220^+$) or these markers were labeled for a dump channel. A Sony Cell Sorter (SH800Z) was used for sample acquisition, and purity was verified using pre- and post-sorted fractions analyzed on the LSR-II cytometer and FlowJo software (v10).

2.3.7 In vitro Treg Suppression Assay

Approximately 6-8 weeks after immune reconstitution, spleen and LNs from the FFM and FCM chimeric mice were collected, stained, and sorted via FACS as described above, with a sorting purity of >95% (shown in Fig. 12). Next, the sorted OVA-Ag-specific ($CD45.1^+V\alpha2^+V\beta5^+$) Treg cells ($CD4^+CD25^+$, 1 x 10^4/well) were then co-cultured with irradiated splenic Ag-presenting cells (APCs, 4 x 10^4/well) from C57BL/6 mice, and MACS-bead negatively sorted (with CD8, CD19, B220, and CD25 antibodies) OVA-specific Teff cells ($CD4^+CD25^{--}$) cells (2 x 10^4/well) from the spleen of OT-II TCR-Tg mice. These co-cultured cells were treated with or without $OVA^{323-339}$ peptide (1µg/mL) (Bachem #4034255.1) stimulation. After co-culture for 72hrs, cell proliferation was quantified via CellTiter 96 Aqueous One Solution (Promega #PR-G3582) per the company's protocol, by adding 20µL of solution to each well for approximately 1.5hrs of culture. Then absorbance at 490nm was measured using an ELISA 96-well plate reader (BioTek ELx800).

2.3.8 Treg TCR Repertoire Sequencing & Analysis

Approximately 6-8 weeks after immune reconstitution, lymphocytes from the spleen and LNs of FF and FC Rag^{GFP}-$FoxP3^{RFP}$ dual-reporter chimera mice were collected and prepared for FACS. RTE (GFP^+) Treg ($CD4^+CD25^+RFP^+$) cells (Gate strategy in Fig. 15A) were collected and genomic DNA was isolated from each sorted sample with the Qiagen DNeasy Kit (#69504). TCRβ CDR3 survey-level sequencing was performed via Adaptive ImmunoSEQ customer service.

Information on the primers used by Adaptive can be found in their publication [300]. Data were analyzed via the Adaptive ImmunoSEQ data analysis software with the assistance of their technical support team. Per the Adaptive ImmunoSEQ Analyzer, Simpson's Clonality Index is a way of measuring how polyclonal or monoclonal a population is on a scale of 1 (monoclonal) to 0 (where no two clones are identical) and is calculated as "the square root of the sum over all observed rearrangements of the square fractional abundances of each rearrangement". Observed Richness is simply the number of clones sequenced, termed "productive rearrangements" and Relative Richness is the number of unique clones divided by the total number of clones in each sample. Adaptive ImmunoSEQ data analyzer for this project can be accessed via DOI: 10.21417/RT2021I or URL: https://clients.adaptivebiotech.com/pub/thomas-2021-immunology

2.3.9 Statistics

RQ-MFI was used to normalize MFI data to the control (FFM) group by dividing each raw MFI value by the average control group (FFM) MFI for each separate set of experiments. Prism GraphPad software (Prism-8) was used for data analysis. For evaluating differences between two groups, an unpaired two-tailed Student's t-test was used. For comparison between multiple groups, a one-way ANOVA with Bonferroni correction for multiple comparisons was employed. Differences were considered statistically significant for * $p<0.05$, ** $p<0.01$, and *** $p\leq0.001$.

2.4 RESULTS

2.4.1 Thymic atrophy affected agonist selection of a mock-self-Ag specific tTreg clone despite relatively normal tTreg polyclones

Previously, we have reported that the aged, atrophied thymus has reduced capacity for negative selection, exhibited by increased self-reactive T cells [113]; however, it does not impair

tTreg cell generation, as displayed by an increased ratio of RTE tTreg vs. tTcon cells either with naturally aged or with age-mimicking atrophied thymus [114]. This enhancement of tTreg generation may be compensatory, as it seems to result at the expense of negative selection [11,56]. However, we did not have sufficient evidence to determine whether a given self-antigen specific Treg clone was also normal or enhanced in the aged, atrophied thymus, although it was shown that a self-Ag (prostate)-specific Treg clone (TCAF3) was lost from the Treg repertoire in an *Aire*$^{-/-}$ mouse model [44,119].

Herein, we examined a mock-self-Ag (OVA) specific Treg clone in the murine thymus under conditions mimicking natural age-related thymic atrophy. In order to evaluate this, we utilized a T cell system reconstitution model via thymic transplantation, in which a chimera was generated by engrafting FCM (or FFM control) newborn mouse thymic lobes under the kidney capsule of OT-II TCR-Tg (*Rag* sufficient in order to provide an Ag-specific clone within a polyclonal background) recipient mice (Fig. 9A). Three to four weeks after the host mouse progenitors entered the engrafted thymic lobes, we then observed the developmental outcomes of the host progenitors (OT-II TCR-Tg) and donor thymus TECs, which express OVA and were induced with/without conditional *FoxN1* knockout. Although total cell numbers of tTreg and tTcon cells were reduced in atrophied thymuses, as expected (Figs. 9C and D), we found that the mock-self-Ag specific Treg clone was quantitatively impaired (in proportion and ratio of Treg/Tcon) in the FCM atrophied thymus, although the generation of pan-Treg cells remained relative unchanged (Figs. 9B and E, right panel), The results imply that although thymic atrophy does not reduce overall pan-tTreg generation, alterations in thymic function perturb the generation of this mock-self-Ag specific tTreg clone. In accordance with our previous findings that the atrophied thymus has impaired negative selection [113], we saw that specific tTcon cells were increased in proportion

in mice with thymic atrophy (Fig. 9F, right panel). The proportion of specific-tTreg cells (Fig. 9E, right panel) and ratio of specific-tTreg/specific-tTcon cells (Fig. 9G, right panel) in $CD4^{SP}$ were reduced. In sum, age-mimicking atrophied thymus impaired negative selection of antigen-specific tTcon (increased) and agonist selection of tTreg (decreased) cells, but not total, polyclonal tTreg cells.

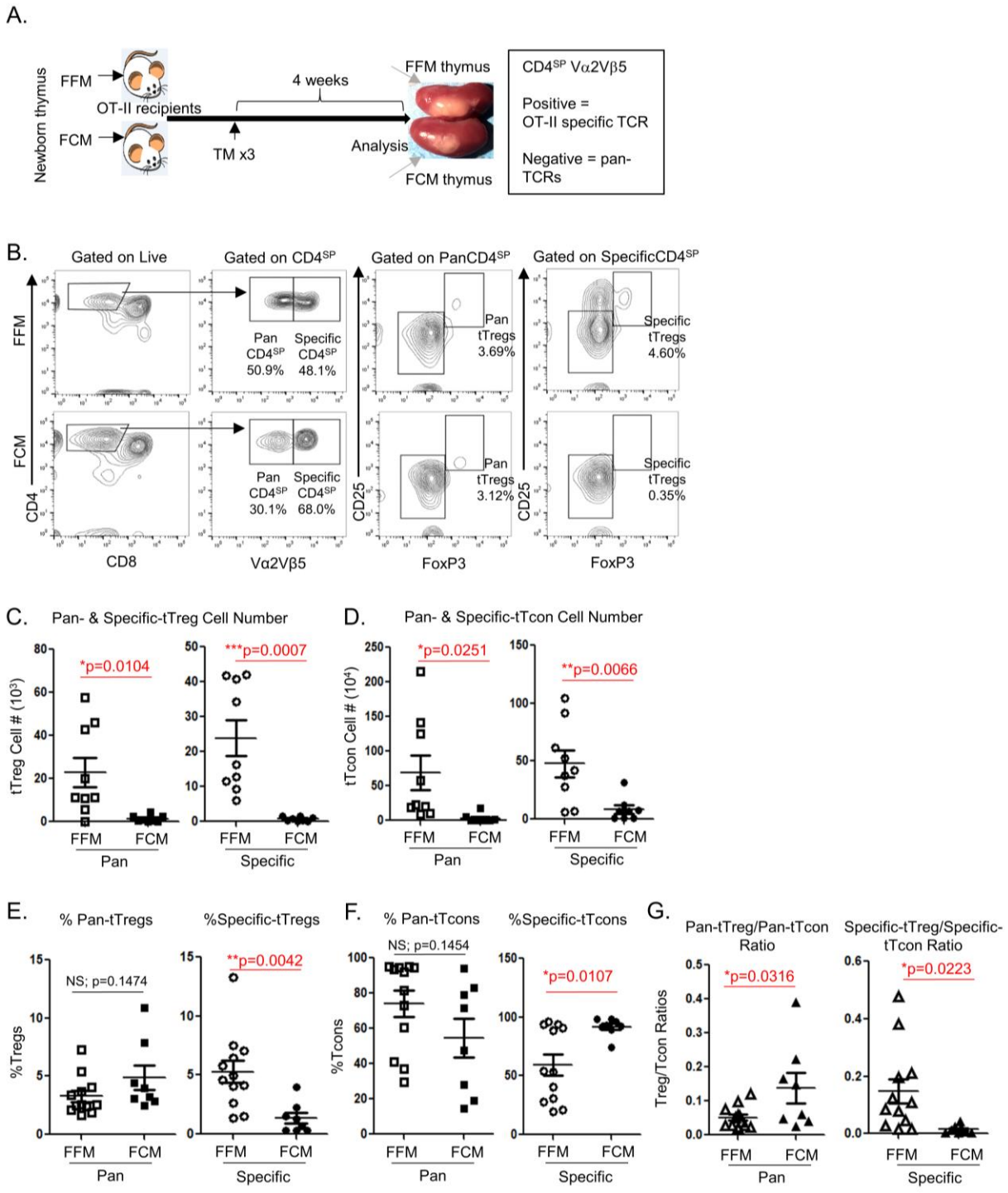

Figure 9: Selection of OT-II specific-tTreg cells was reduced, in mOVA atrophied thymus immune reconstitution model. (**A**) Workflow of immune reconstitution via OT-II TCR-Tg progenitor cells seeding into FFM or FCM newborn thymuses engrafted under the kidney capsule

of OT-II TCR-Tg mice. (**B**) Flow cytometric gating strategy, showing % pan-tTregs and % OT-II specific-Tregs in the engrafted thymuses. (**C**) pan- and specific-tTreg cell numbers in engrafted thymuses (**D**) pan- and specific-tTcon cell numbers in engrafted thymuses (**E**) % pan- and specific-tTreg cells in the engrafted thymuses (**F**) % pan- and specific-tTcon cells in the engrafted thymuses (**G**) ratios of pan-tTregs/pan-tTcons and specific-tTregs/specific-tTcons. A Student t-test was used to determine statistical significance between two groups. All p-values were calculated by mean ± SD, and each symbol represents an individual animal. "NS" = not significant. Experiment was repeated at least four times.

2.4.2 Thymic atrophy resulted in reduced proportion and number of a mock-self-Ag specific pTreg clone in the periphery, despite maintaining normal polyclonal (pan)-pTreg levels

Given that the generation of mock-self-Ag (OVA)-specific tTreg cells is impaired in the atrophied thymus (Fig. 9), we asked whether they can expand to comparable levels in the periphery. Thus, we examined the peripherally circulating (termed for our study, pTreg) cells in our immune reconstitution BM chimera model using OT-II TCR-Tg progenitors (on *Rag*$^{-/-}$ background) given with a fixed ratio with WT progenitors to provide a polyclonal set of progenitors to irradiated recipient mice (Fig. 10A, flow cytometric gating strategy in Fig. 10B). We observed that although total splenic lymphocytes and total pTreg cells were not decreased (Fig. 10C), the mock-self-Ag specific pTreg cells maintained the quantitative reduction found in both proportion (Fig. 10D, right panel) and absolute cell numbers (Fig. 10E, right panel) in the spleen of FCM mice with atrophied thymus. However, pan-pTreg cells in the FCM mice with the atrophied thymus still were increased in proportion (Fig. 10D, left panel) and showed no significance in absolute number (Fig. 10E, left panel) compared to their counterparts (FFM) with normal thymus.

Since the mOVA-Tg mice have mOVA expression in peripheral tissues, the OT-II TCR-Tg T cells cells encounter a low-level specific-Ag stimulation spontaneously. Therefore, CD4^{SP} pTcon cells could undergo expansion. We found that the Ag-specific pTcon cells were increased in proportion (Fig. 10F, right panel), though not in cell number (Fig. 10G, right panel). The pan-pTcon cells were decreased in both proportion and number (Fig. 10F, left panel) in FCM mice, suggesting that the source of the increased Ag-specific Tcon cells is likely the atrophied thymus rather than peripheral expansion. This resulted in an overall decreased ratio of specific-Treg/specific-Tcon cells (Fig. 10H, right panel), suggesting that the balance could be tipped against self-tolerance of this mock-self-Ag in the mice with thymic atrophy.

In addition, we analyzed $CD4^{SP}$ pTreg and pTcon cells in the pancreas since mOVA-Tg is driven by the rat insulin promoter, and OVA-specific Teff cells could induce pancreatitis if the OVA-specific Treg cells are impaired enough to break central tolerance in combination with specific $CD8^{SP}$ Teff cells and/or auto-antibodies [267,301-303]. The results showed that self-Ag-specific Treg cells were reduced and self-Ag-specific Teff cells were increased in the pancreas of mice with thymic atrophy (FCM) (Fig. 10I). However, we did not find manifestations of diabetes in these mice based on blood glucose measurement (Fig. 11), probably due to lack of intrinsic OVA-specific $CD8^{SP}$ Teff and/or antibodies [267,301-303]. In sum, these results suggest that this mock-self-Ag specific Treg clone in the peripheral lymphoid organs and specific tissues indeed has a quantitative defect.

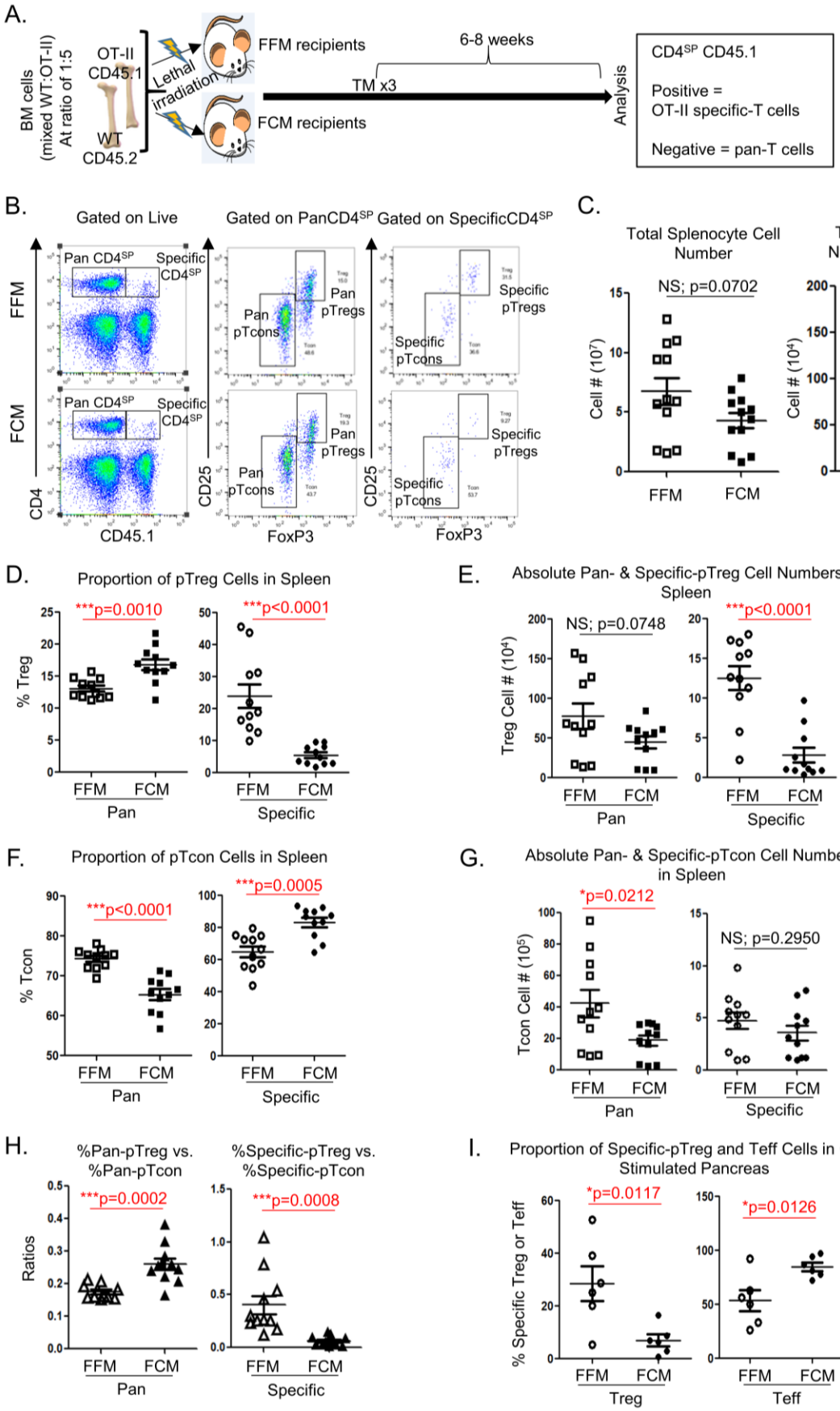
A.
BM cells
(mixed WT:OT-II)
At ratio of 1:5
OT-II
CD45.1
WT
CD45.2
Lethal irradiation
FFM recipients
FCM recipients
6-8 weeks
TM x3
Analysis
CD4SP CD45.1
Positive =
OT-II specific-T cells
Negative = pan-T cells
B.
Gated on Live
Gated on PanCD4SP
Gated on SpecificCD4SP
FFM
FCM
Pan CD4SP
Specific CD4SP
CD4
CD45.1
CD25
FoxP3
Pan pTregs
Pan pTcons
Specific pTregs
Specific pTcons
C.
Total Splenocyte Cell Number
NS; p=0.7002
Cell # (10^7)
Total pTreg Cell Number in Spleen
NS; p=0.0741
Cell # (10^4)
FFM
FCM
D.
Proportion of pTreg Cells in Spleen
***p=0.0010
***p<0.0001
% Treg
Pan
Specific
E.
Absolute Pan- & Specific-pTreg Cell Numbers in Spleen
NS; p=0.0748
***p<0.0001
Treg Cell # (10^4)
F.
Proportion of pTcon Cells in Spleen
***p<0.0001
***p=0.0005
% Tcon
G.
Absolute Pan- & Specific-pTcon Cell Numbers in Spleen
*p=0.0212
NS; p=0.2950
Tcon Cell # (10^5)
H.
%Pan-pTreg vs. %Pan-pTcon
***p=0.0002
%Specific-pTreg vs. %Specific-pTcon
***p=0.0008
Ratios
I.
Proportion of Specific-pTreg and Teff Cells in OVA-Stimulated Pancreas
*p=0.0117
*p=0.0126
% Specific Treg or Teff
Treg
Teff

Figure 10: OT-II specific-pTreg cells were reduced, in mOVA recipient mice with age-mimicking thymic atrophy. (A) Workflow of immune reconstitution via BM chimeras with mixed WT (CD45.2) and OT-II (CD45.1, *Rag*$^{-/-}$ background) progenitors into FFM or FCM recipient mice. (**B**) Flow cytometric gating strategy, showing % pan-pTregs and % OT-II specific-pTregs in the recipient spleen. (**C**) Absolute splenocytes and pTreg cell numbers from recipient spleens. (**D**) Proportions of pan- and specific-pTreg cells in the spleen. (**E**) pan- and specific-pTreg cell numbers in spleen. (**F**) % pan- and specific-pTcon cells in spleen. (**G**) pan- and specific-pTcon cell numbers in spleen (**H**) Ratios of pan-pTregs/pan-pTcons and specific-pTregs/specific-pTcons in spleen. (**I**) Proportions of specific-pTreg or -pTeff (OT-II TCR-Tg Tcon in the RIP-mOVA-Tg pancreas) cells in recipient pancreas. The statistical method is the same as in Fig. 9. Each symbol represents cells from an individual animal. Experiment was repeated at least four times.

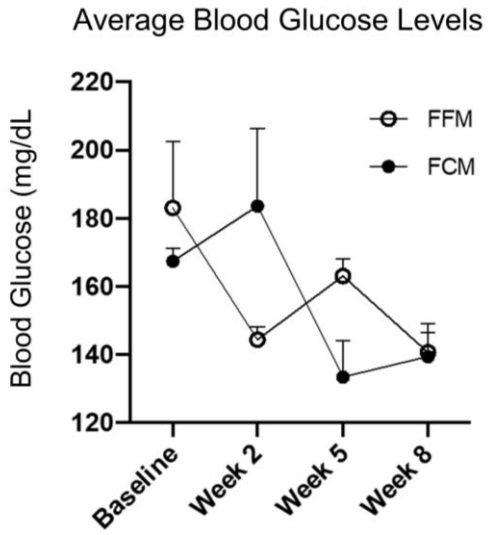

Figure 11: Blood glucose measurements in FFM and FCM BM chimera mice. Tail tip fed state blood glucose measurements were performed on chimera mice to assess potential onset of Type I diabetes since mOVA is expressed as a self-Ag on the pancreas of host mice. Baseline was the day of chimerization. A two-way repeated measures ANOVA was used. No difference was observed between groups.

2.4.3 The suppressive capacity of the mock-self-Ag specific pTreg clone from mice with thymic atrophy was functionally defective

Given the reduction in proportion and number of these specific-Treg cells in the periphery, we asked whether their function of suppressing specific-Teff cell proliferation was also defective when assessed at equal cell numbers. We therefore set up a Treg suppression assay using flow cytometrically sorted OVA-specific Treg-enriched ($CD25^{+}$) live cells from either FFM or FCM BM chimera mice (model in Fig. 10A). In order to exclude any possible alterations to the OVA-specific Teff cells from mice with thymic atrophy, OT-II specific Teff cells were taken from unmanipulated OT-II TCR-Tg (*Rag*$^{-/-}$ background) mice. These Teff cells were co-cultured with various groups of pTreg cells (WT, FFM, and FCM). The use of WT pTreg cells served as a control for non-specific pTreg bystander suppression, since WT mice do not express mOVA as a self-antigen and therefore should not generate mOVA-specific Treg cells.

The results showed that sorted OT-II TCR-Tg (based on Vα2Vβ5 staining) $CD4^{SP}CD25^{+}$ pTreg-enriched cells (Fig. 12A), derived from chimera mice with atrophied thymus (FCM), exhibited significantly reduced suppression of OT-II TCR-Tg $CD4^{SP}CD25^{-neg}$ Teff cells (Fig. 12B), compared to their counterparts derived from chimera mice with normal thymus (FFM) (Fig. 12B). Surprisingly, the specific-Treg cells from FCM chimeras suppressed similarly to the non-specific (pan) WT Treg control group, i.e. no Ag-specific suppressive function (Fig. 12B). Therefore, mice with atrophied thymus could contain a compromised OVA-specific Treg population that possesses not only a quantitative defect in proportion and number, but also exhibit a functional defect, since they cannot efficiently suppress OVA-specific Teff cells during OVA-peptide stimulation. In addition, this result was different from our previous report when we

observed that pan-tTreg cells derived from the atrophied thymus possessed normal suppressive function to pan-Teff cells under non-specific stimulation via CD3 and CD28 receptors [114].

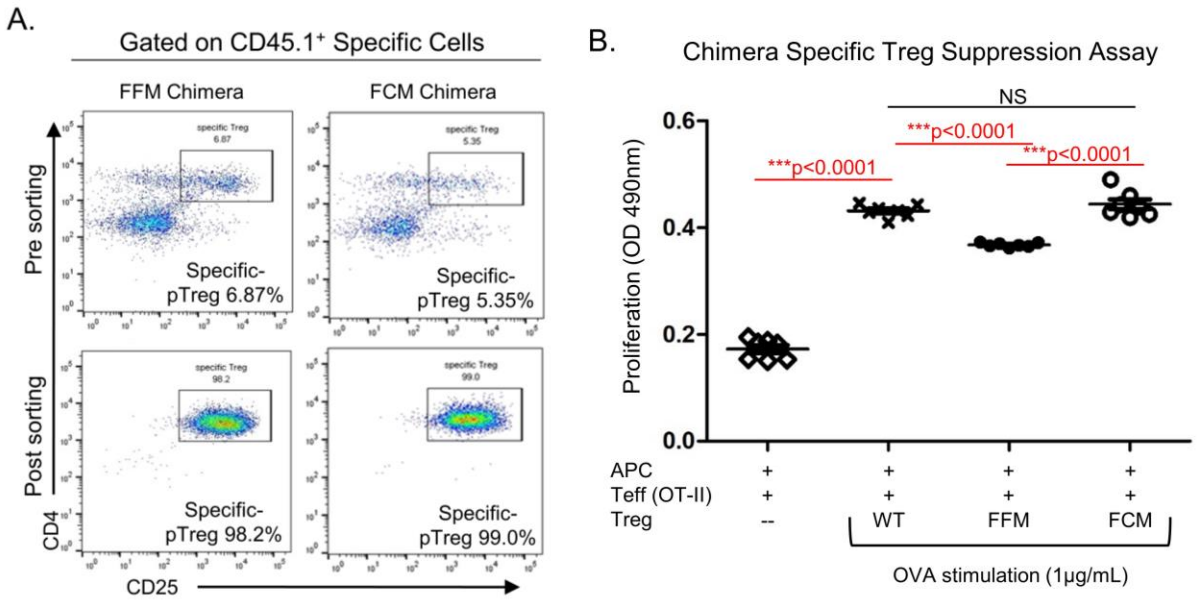

Figure 12: Suppressive capacity of OVA-specific pTregs was functionally defective in mice with age-mimicking thymic atrophy. (**A**) Flow cytometric gating sorting strategy showing purity of specific pTreg cells before (top panels) and after (bottom panels) FACS sorting from BM chimeric mice (Fig. 10A). (**B**) Summarized results of the suppressive capacity of OVA-specific pTreg cells on $CD4^{SP}$ effector T (Teff) cells isolated from the spleen of OT-II TCR-Tg mice. A one-way ANOVA with Bonferroni correction was used to determine statistical significance between multiple groups. Each symbol represents cells from an individual animal. Experiment was repeated three times.

2.4.4 The defective Ag-specific Treg clone in mice with thymic atrophy potentially possessed an intrinsic defect

Treg cell function is closely associated with the expression levels of FoxP3 [304]. Solid evidence also shows that once pTreg cells in individuals with autoimmune disease, such as multiple sclerosis patients or mice with experimental autoimmune encephalomyelitis (EAE), encounter self-Ag, FoxP3 expression is diminished or even undetectable. This phenotype is called Treg instability [269,277]. Therefore, the expression levels of FoxP3 could be indicative of intrinsic defects associated with autoimmune-prone Treg cells. To investigate potential intrinsic defects in our mock-self-Ag specific Treg population derived from mice with atrophied thymus, we investigated the expression intensity (relative quantitative – (geometric) mean fluorescence intensity, RQ-MFI) of FoxP3 protein levels in tTreg and pTreg populations using flow cytometry.

Upon investigation, the expression levels of FoxP3 in pTreg cells from the spleen and pancreas (Fig. 13) were significantly decreased in OVA-specific pTreg cells, but not in pan-pTreg cells in FCM chimeric mice (Figs. 13C and D) with low-level, endogenous OVA-specific stimulation. This might explain why the OVA-specific Treg cells from mice with thymic involution demonstrated reduced suppressive function, as shown by the Treg suppression assay (Fig. 12).

Treg development and function normally requires IL-2 and TGF-β signaling. We previously found that expression levels of IL-2 and TGF-β are normal in the atrophied thymus [114]. Herein, we examined expression levels of IL-2 receptor-α (CD25) in the atrophied thymus and peripheral lymphoid organs to determine whether IL-2 signaling capacity is affected in these Treg cells. CD25 levels were no different for pan- and specific-tTreg cells in the thymus of transplant chimera mice with and without thymic involution (Fig. 14).

These findings suggest that this mock-self-Ag specific Treg clone potentially possesses an intrinsic defect showing inability to elevate FoxP3 expression upon Ag stimulation, although likely not due to insufficient IL-2 signaling. This helps explain why these OVA-specific pTreg cells derived from the atrophied thymus are ill-equipped to suppress autoimmune Teff reactivity (Fig. 12).

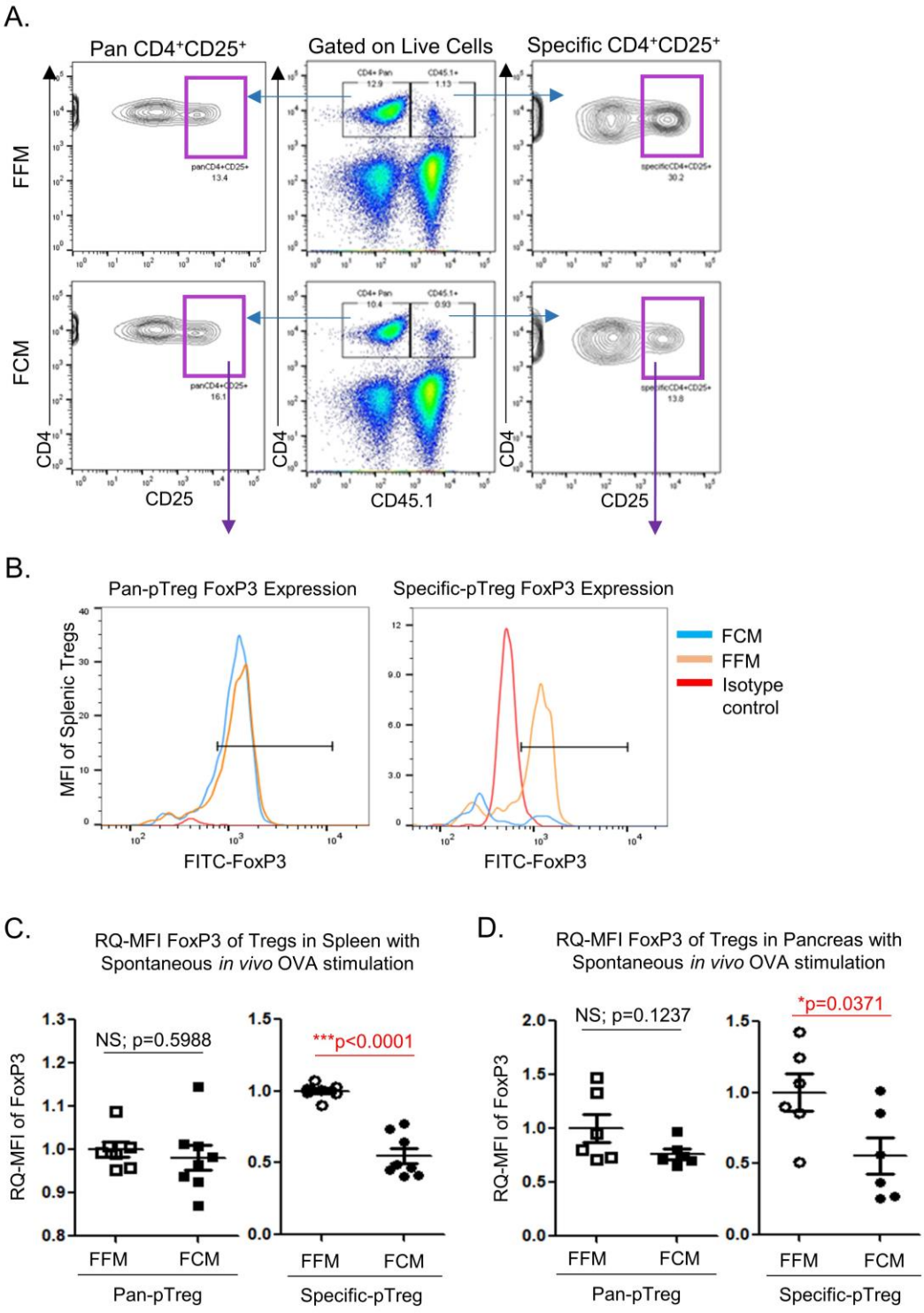

Figure 13: Reduced FoxP3 expression in OVA-specific-pTreg cells from mice with age-mimicking thymic atrophy post OVA-specific stimulation. Splenic or pancreatic cells from BM chimeric mice (workflow in Fig. 10A) were analyzed for FoxP3 expression (RQ-MFI) in

$CD4^+CD25^+$ population after spontaneous (*in vivo*) mOVA-Tg stimulation. (**A**) Flow cytometric gating strategy for MFI of FoxP3 quantification showing % pan-pTreg cells and % specific-pTreg cells (**B**) Representative histogram of FoxP3 MFI gating for pan-Treg and specific-Treg cells from FCM (blue line) and FFM (yellow line) mice compared to isotype control (red line). (**C**) Quantification for RQ-MFI of FoxP3 expression in pan- and specific-pTreg cells from FCM and FFM spleen. (**D**). Quantification for RQ-MFI of FoxP3 expression of pan- and specific-pTreg cells from FCM and FFM pancreas. The statistic method are the same as in Fig. 9. Each symbol represents cells from an individual animal. Experiment was repeated at least two times.

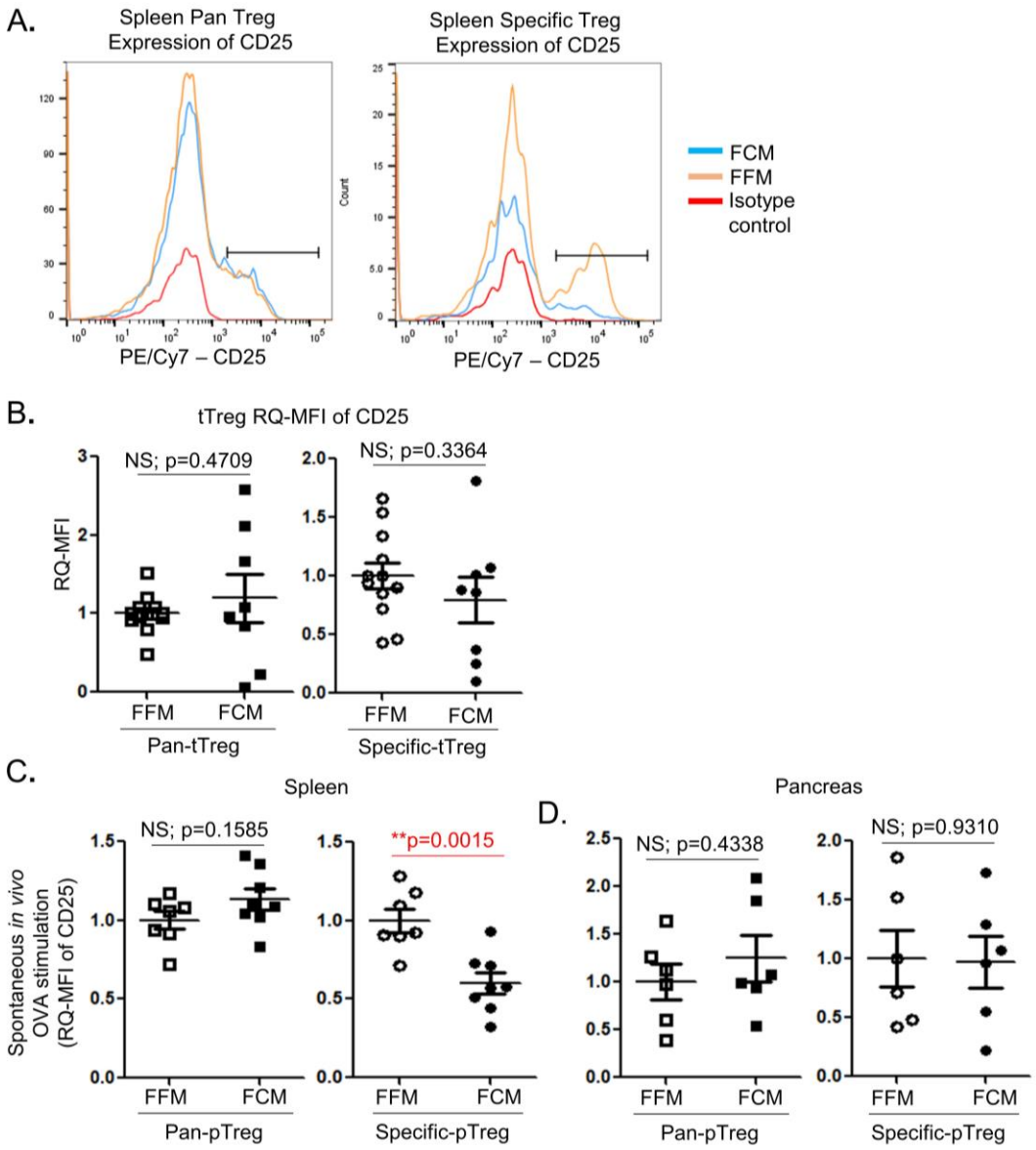

Figure 14: The specific-Treg population exhibited minimal change in IL-2Ra (CD25) expression in tTreg or pTreg cells in mice with age-mimicking thymic atrophy. (**A**) Representative histograms of MFI of $CD25^{+}$ gating in pan-pTreg (left panel) or specific-pTreg (right panel) cells from FCM (blue line) and FFM (yellow line) mice compared to isotype control (red line). (**B**) Quantification for RQ-MFI of CD25 expression of pan- and specific tTreg cells in the engrafted FFM and FCM thymuses (see Fig. 9A). (**C**) RQ-MFI of CD25 expression of pan- and specific pTreg cells from FCM and FFM BMT recipient spleens. (**D**) RQ-MFI of CD25

expression of pan- and specific pTreg cells from FCM and FFM BMT recipient pancreas. Workflow for panels C and D are the same as in Fig. 10A. A student's t-test was used to determine statistical significance between two groups. Each symbol represents cells from an individual animal. Experiments were repeated at least 3 times.

2.4.5 Thymic atrophy potentially affects Treg agonist selection resulting in decreased tTreg TCR repertoire diversity

Since the development of the TCR repertoire occurs in the thymus, age-associated restriction of total TCR repertoire diversity has been at least partly attributed to reduction of naïve T cell output from the aged, atrophied thymus [30,106,305]. We wanted to know whether the TCR repertoire diversity of Treg cells derived from age-mimicking atrophied thymus is also declined. We generated a radiation BM chimera via immune reconstitution of BM from *Rag*^{GFP} and *FoxP3*^{RFP} dual reporter mice into host mice carrying TECs with *floxed-FoxN1* and CreER^{T} for conditional KO (FC) or without Cre-Tg control (FF) with normal thymus [114]. Six to eight weeks after TM induction of thymic atrophy, the newly-generated (recent thymic emigrant, RTE) pan-pTreg cells, i.e. *Rag*^{GFP} and *FoxP3*^{RFP} double positive pTreg cells, from host mouse spleen and lymph nodes were flow cytometrically sorted (Fig. 5A). DNA sequencing was conducted for repertoire analysis of the TCR β-chain CDR3 region (Adaptive ImmunoSEQ).

The results showed that the top 10 clones in FC RTE pan-Treg cells (Fig. 15B, left 3 bars) comprised a greater proportion of their total repertoire, compared to FF RTE pan-Treg cells (Fig. 15B, right 3 bars). TCR repertoire diversity via clonality index was used to quantify the clonal dominance, with "1" indicating an entirely monoclonal sample (least possible diversity), and with "0" indicating that that no two TCRs are the same (highest possible diversity). The observed increased clonality in the FC group implies higher clonal expansion and thus less diversity (Fig. 15C). In addition, there was no statistical significant difference in observed richness (total number of productive rearrangements or clones sequenced), although the FC mice tended to have fewer total productive rearrangements (Fig. 15D). When accounting for number of unique rearrangements out of the total (relative richness), there was no difference between groups (Fig.

15E). Therefore, although preliminary, we observed a trend for increased RTE pan-Treg clonality, i.e. declined TCR repertoire diversity, in mice with age-mimicking thymic atrophy compared to normal thymus controls. Reduced TCR diversity suggests that certain tissue-specific Treg clones could be missing from the repertoire.

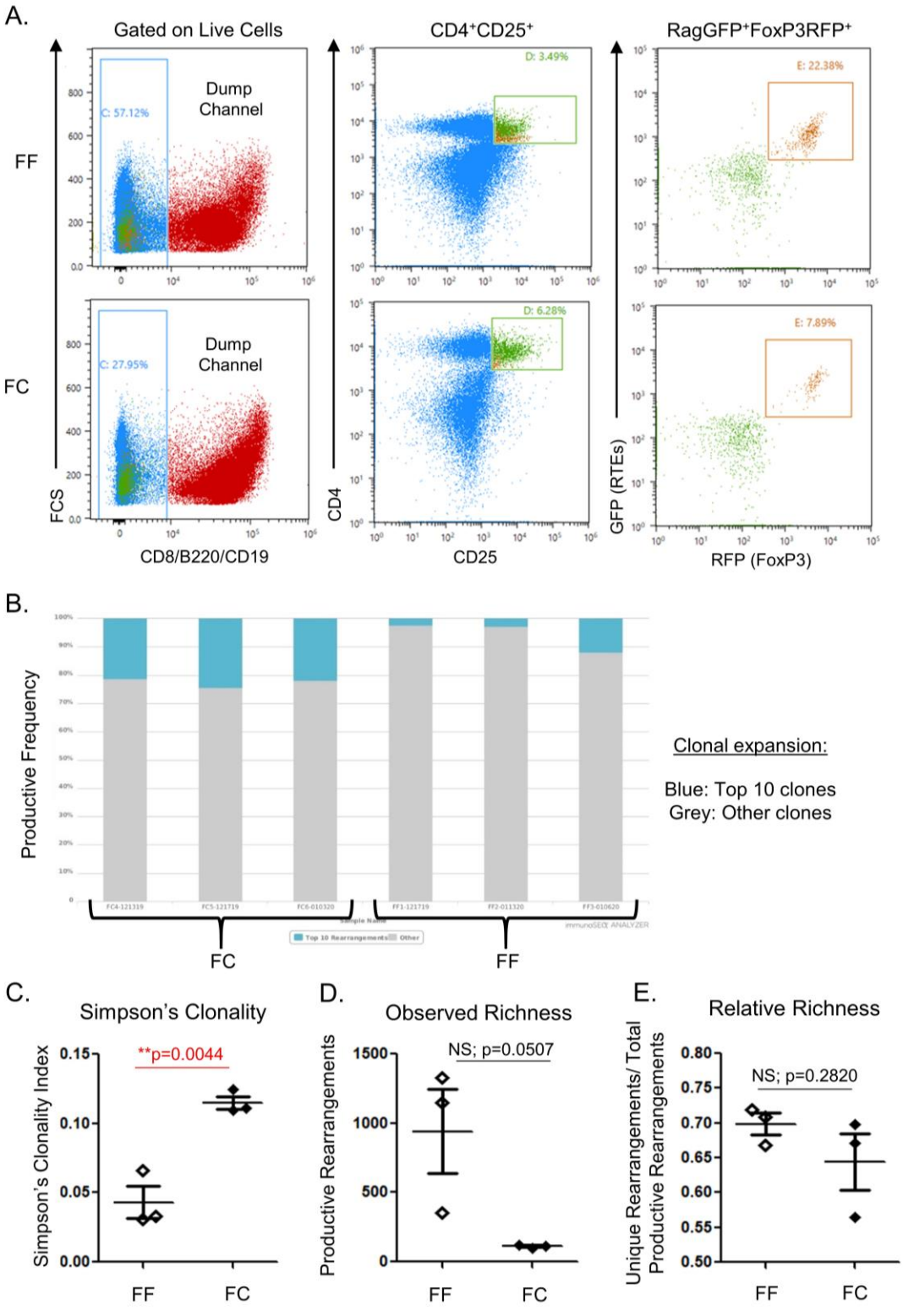
A.
Gated on Live Cells
CD4+CD25+
RagGFP+FoxP3RFP+
FF
FC
C: 57.12%
Dump Channel
C: 27.95%
Dump Channel
D: 3.49%
D: 6.28%
E: 22.38%
E: 7.89%
FCS
CD8/B220/CD19
CD4
CD25
GFP (RTEs)
RFP (FoxP3)
B.
Productive Frequency
Clonal expansion:
Blue: Top 10 clones
Grey: Other clones
FC
FF
C.
Simpson's Clonality
Simpson's Clonality Index
**p=0.0044
FF
FC
D.
Observed Richness
Productive Rearrangements
NS; p=0.0507
FF
FC
E.
Relative Richness
Unique Rearrangements/ Total Productive Rearrangements
NS; p=0.2820
FF
FC

Figure 15: **Declined TCR repertoire diversity in newly-released pTreg cells from mice with age-mimicking thymic atrophy.** RTE-pTreg cells were FACS sorted from peripheral T cells (lymph nodes and spleen) of dual reporter chimera mice, and the CDR3 regions of the TCRβ chain were analyzed by TCR repertoire sequencing. (**A**) Cell sorting strategy for obtaining recent thymic emigrants (RTEs), which express *Rag* promoter-driven GFP, and pTreg cells, which express *FoxP3*-driven RFP along with $CD4^+CD25^+$ markers. (**B**) Productive frequency of the top 10 clones (top blue portion of the bars) from three individual FC (with atrophied thymus) and three individual FF (with normal thymus) mice. (**C**) Results of Simpson's clonality index of RTE-pTreg cells from FF and FC mice. (**D**) Observed Richness based on total number of productive rearrangements. (**E**) Relative Richness based on unique rearrangements/total productive rearrangements. Simpson's clonality was calculated by ImmunoSEQ, while a Student's t-test was used to compare two groups. Each symbol represents cells from an individual animal.

2.5 CONCLUSIONS

Given that increased self-reactivity in the elderly is at least partially attributed to defects in thymocyte negative selection in the age-related, involuted thymus [292], we extended our investigation to Treg generation and function associated with the aged, atrophied thymus. Our intent was to decipher the contradiction of enhanced tTreg generation and pTreg accumulation with decreased ability to suppress self-reactivity in aged individuals. We focused on investigating the differences between pan-Treg and specific-Treg cells, and their TCR repertoires with various chimeric mouse models, carrying mock-self-Ag (mOVA) and OVA-recognizing TCR-Tg $CD4^{SP}$ T cells, coupled with age-mimicking (*FoxN1* conditional KO) atrophied thymus.

We identified a quantitative defect in this self-Ag-specific Treg clone, exhibited by a significant decline in percentage of tTreg cells, suggesting a shortcoming in tTreg agonist selection, as well as in the percentage and absolute numbers of pTreg cells, suggesting reduced expansion in the periphery. These OVA-specific Treg cells were significantly less able to suppress OVA-peptide induced proliferation of OVA-specific Teff cells *in vitro*. However, these defects could not be observed in polyclonal (pan)-Treg cells from the same mice with age-mimicking thymic involution, which is in agreement with our previous publication [114]. This may partially explain the seemingly contradictory phenomenon of immunosenscence and inflammaging. We do not rule out the role of pan-pTreg mediated bystander effects, which have been shown to suppress excessive immune responses to both self- and non-self-antigens to maintain immune homeostasis [306,307], although this effect is not as powerful as Ag-specific suppression. This could explain why auto-reactive Teff cells in the elderly usually induce a low-level, persistent inflammation (inflammaging) rather than induction of *bona fide* autoimmune diseases. We further found that these self-Ag recognizing Treg cells possessed a potential intrinsic defect exhibited as lower FoxP3 expression

after mOVA-Tg stimulation. We postulate that this could be rooted in potential defects during tTreg TCR agonist selection in the age-mimicking atrophied thymus, resulting in reduced repertoire diversity.

Our model of immune reconstitution with mock-self-Ag mOVA Tg and OVA-recognizing TCR-Tg OT-II cells follows the current paradigm for thymocyte central tolerance establishment, involving negative selection of clones with high avidity for self-antigens and the agonist selection of clones with intermediate to high avidity for self-antigens to develop tTreg cells [11,45]. Based on the current paradigm and the observations discussed herein, we provide an illustration of our hypothesized mechanism underlying these findings (Fig. 16).

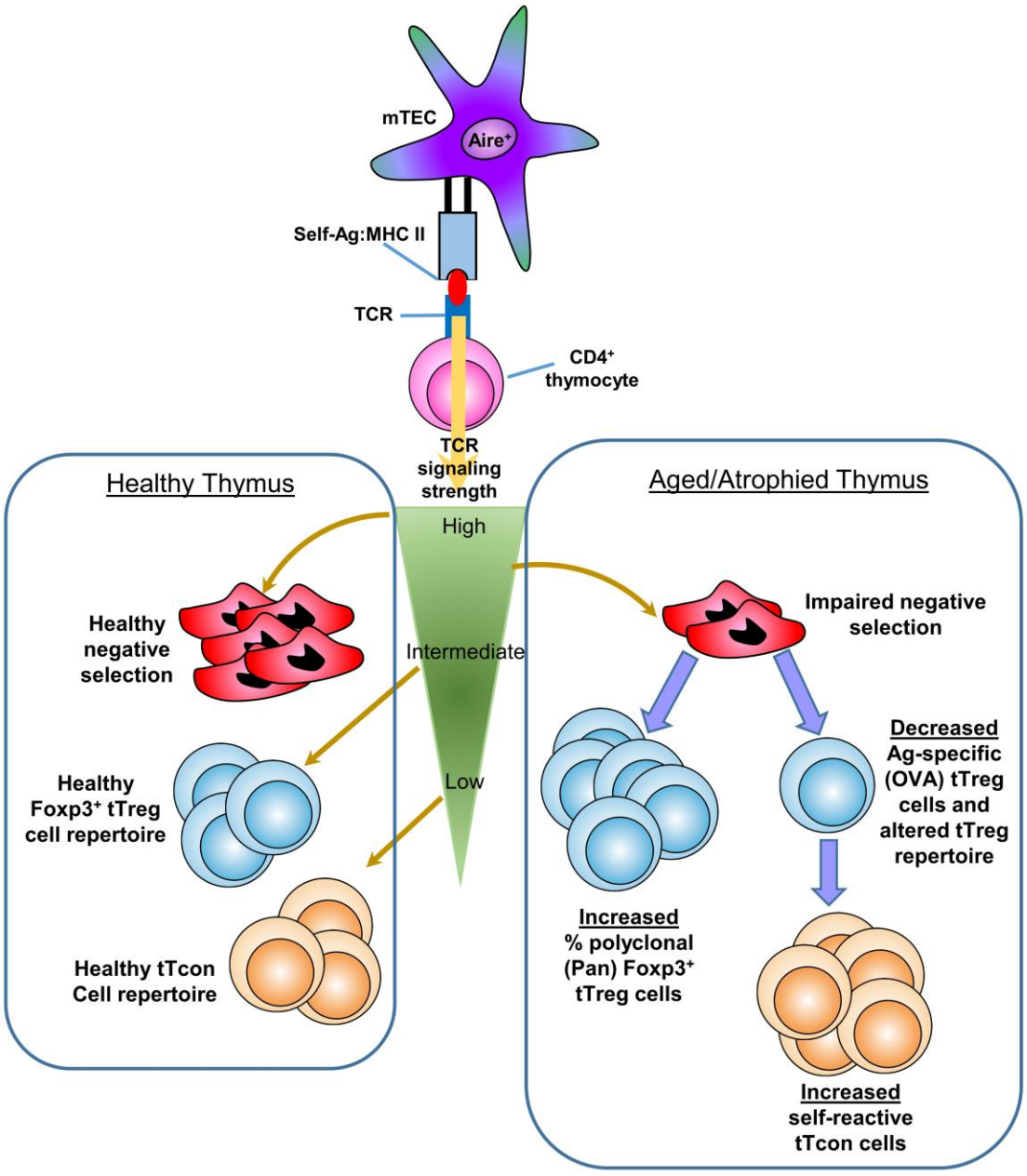

Figure 16: Hypothesized Mechanism. Alterations to the process of central tolerance induction in the aged, atrophied thymus have been described. Based on our observations that the proportion of pan-tTreg cells remain unchanged, but that an Ag-specific tTreg subset is impaired by age-mimicking thymic atrophy, we propose the paradigm by which overall signaling strength is

reduced in the atrophied thymus, leading to defects in central tolerance (right side) that potentially impact the tTreg cell repertoire by creating holes for certain Ag-specific tTreg clones.

tTreg cell development is dependent on multiple signals and cumulative signaling strength or avidity within the thymic microenvironment. In addition to interactions between self-peptide-MHC-II complex and TCR, IL-2 signaling via IL-2R on Treg cells is also critical. In our previous publication, we demonstrated that expression of IL-2 was not reduced in the FC nor in the naturally-aged atrophied thymus [114]. Similarly, herein, we found that expression of IL-2Rα, or CD25, was also no different between Treg cells in the normal versus the atrophied thymus. TGF-β is also an important factor involved with tTreg agonist selection [308], which was not reduced in our previous investigation [114]. Therefore, we believe these changes in certain tissue-specific Treg cells are mainly due to changes in TCR signaling strength [12,34,45,117,309]. The reduction in FoxP3 is likely responsible for the observed reduction in suppressive function, as FoxP3 is a master regulator of Treg function. Insufficient IL-2 signaling has been shown to exacerbate pTreg instability via decreased FoxP3 expression during specific antigen stimulation of certain specific pTreg cells [237,277], since signaling through CD25 is upstream from peripheral enhancement of FoxP3 expression [221,223]. However, we did not find reduced expression and IL-2Rα. Therefore, future study of the epigenetic modifications of FoxP3 expression [274] is needed to clarify this underlying mechanism.

As for other aspects of the thymic microenvironment, other stromal cells, such as thymic dendritic cells, have been shown to decrease in number in the aged thymus [310] and their function appears to be less efficient compared to young thymic-derived dendritic cells [311]. However, their overall ratio to the number of thymocytes developing in the aged thymus appears to remain intact [311]. It would be interesting to assess how age-related thymic atrophy impacts promiscuous self-antigen expression by thymic dendritic cells since they interaction with mTECs to facilitate this process [312]. It is also worth mentioning that other aspects of promiscuous self-antigen expression

for thymocyte selection and central tolerance induction, such as *FEZF2*, are beginning to be of interest, especially since the mTECs that express *AIRE* do not appear to overlap with those that express *FEZF2* [38]. Moreover, the effects of other age-related changes to the thymic microenvironment on Treg selection is an under-investigated field.

Finally, based on our findings in the TCR-Tg model, we wanted to assess a potential outcome of decreased Treg TCR diversity in a system in which only the thymus was manipulated to mimic thymic aging, namely our *FoxN1* conditional KO mice. Treg TCR repertoire normally encompasses a broad range of self- and some non-self-recognizing T cells [263,313]. Reduction of repertoire diversity of the Treg population results in unchecked autoimmunity [314,315]. The overall T cell TCR repertoire diversity declines with age due to thymic atrophy and increased oligoclonal expansion of peripheral memory T cells [30,106,305]. Herein, although our initial TCR diversity sequencing data is preliminary, due to limited animals and small sorted cell sample sizes, we observed a trend for declined diversity in the newly-generated (RTE) Treg pool from mice with age-mimicking thymic involution. This finding requires future investigation at the single-cell level. However, the prospect of a decreased tTreg repertoire diversity as a result of thymic atrophy is in line with current available evidence that certain tissue-specific Treg TCRs could not be agonist-selected from the dysfunctional thymus, such as the missing prostate TCAF3-specific Treg clone from *AIRE* gene deficient thymus [44,119], though total (pan-) Treg clones are not reduced.

Although there are some limitations to using a TCR-Tg system, such as the irregularities involved during early thymocyte development due to the early TCR-α and TCR-β expression, here we studied the impacts of thymic atrophy on the latter stages of Treg agonist selection, which occurs after $CD4^{SP}$ lineage is determined. We also maintained a polyclonal pool of T cell progenitors in these mice to ensure a more physiologically-relevant selection process. Given the

difficulties of identifying a single Ag-specific T cell clone within a naturally heterogeneous and unmanipulated pool, such a model was necessary and allowed us to avoid other artificial constructs required for such assessments in non-TCR-Tg models, such as artificial expansion of a single clone via immunization with cognate antigen [113], or the utilization of a fixed TCR-β chain [44] in order to ease identification of one clone via diminished overall diversity. These findings from our TCR-Tg model provided the foundation for our tTreg TCR diversity sequencing in a non-TCR-Tg system and are informative for future investigations of antigen-specific Treg selection in less manipulated models. Further investigation into the identification of various natural Treg clones that may be effected within the polyclonal repertoire of non-TCR-Tg mice will shed more light on the observations reported herein.

Taken together, our findings highlight the differential impacts of thymic involution on pan- and antigen-specific tTreg cell generation. In this scenario, the effects of age-related thymic involution did not affect pan-Treg cell generation, but resulted in intrinsic impairment of a mock-self-Ag specific Treg clone. Taken holistically, some self-Ag-specific tTreg clones may even fail to undergo agonist selection under these conditions, possibly creating TCR repertoire holes in the aged T cell regulatory system. Although, further study is needed, the implications of such Treg repertoire holes would further elucidate the underlying mechanisms of inflammaging and help explain why relatively increased pan-Treg cells are unable to attenuate inflammaging in the elderly.

CHAPTER III

Final Remarks and Future Directions

3.1 FINAL REMARKS

Herein we have discussed the hallmarks of age-related thymic atrophy and its underlying mechanisms. We have addressed the age-related changes to the aged immune system and expounded on the contributions of the aged thymus to these defects primarily through age-associated alterations to thymocyte development and central tolerance induction in the atrophied thymus. Next, we discussed the known impacts of thymic atrophy on Treg cell development and highlighted the need to explore antigen-specific Treg clones in the context of thymic atrophy, focusing on investigating "holes" in the Treg compartment. Finally, we presented our findings on the alterations to development and function of an antigen-specific Treg clone in mice with thymic atrophy using several comprehensive murine models. The overall conclusions of the investigations presented here are that thymic atrophy negatively alters certain self-antigen-specific Treg clones, while simultaneously generating an increased proportion of pan-tTreg/tTcon output. We propose that the changes to certain antigen-specific Treg clones might explain the various levels of self-reactivity associated with inflammaging via generation of "holes" in the tTreg repertoire during aging. Indeed, our preliminary tTreg TCR repertoire diversity results suggest decreased diversity in the tTreg repertoire of mice with thymic atrophy compared to normal thymus. Further areas of investigation are needed and some topics of interest for future work are explained below.

The findings discussed herein have contributed to the field of age-related thymic atrophy and aged immune dysfunction in several ways. First, we were able to demonstrate that despite the increased Treg/Tcon output from the atrophied thymus, one mock self-Ag-specific Treg clone was negatively impacted in both number/proportion as well as in functional capacity. To our knowledge, this concept has only been described in the $Aire^{-/-}$ thymus [44]. Second, we are among the first to perform TCR repertoire sequencing of Treg cells from the atrophied thymus, and to our

knowledge, we are the first to do so for *Rag*^{GFP}-based RTE Treg cells from mice with induced age-mimicking thymic atrophy. Although preliminary, due to limited animal numbers, the observed decreased in Treg TCR diversity in mice with thymic atrophy is novel and has clinical implications. Further investigation will enable scientists to determine how Treg repertoire "holes" may contribute to increased basal self-reactivity associated with inflammaging without inducing obvious autoimmune disease. It is our hope that these findings provide the framework for the development of therapeutic interventions that target thymic rejuvenation as a means of restoring T cell immune homeostasis in the elderly.

3.2 LIMITATIONS & FUTURE WORK

3.2.1 Aim 1 Limitations & Future Work

We were limited in continued experiments due to UNTHSC decommissioning the irradiation machinery at the end of 2019. Therefore, we put all of our effort and resources toward finalizing the BM chimera experiments that required irradiation. As an alternative strategy, we utilized the thymus transplant chimera model to confirm our results in the thymus without the need for irradiated host mice, however this model has limits in assessing peripheral Treg cells.

Since a prostate Ag-specific Treg clone was significantly declined in *Aire*$^{-/-}$ mice [44,119], it suggests that certain tissue-specific tTreg precursors cannot be selected in the dysfunctional thymus, and since the atrophied thymus has impaired promiscuous gene expression [125], this could potentially affect pancreatic Ag expression by mTECs as well [316]. As part of our lab's continued effort to understand the subtle and complex effects of thymic atrophy on T cell selection, we aim to explore natural self-Ag-specific populations (since here we use a mock-self-Ag). For example,

we are currently investigating MOG-specific T cells in the autoimmune model of EAE in young versus aged mice.

Further, since we did not observe pancreatitis or autoimmune diabetes in our RIP-mOVA chimeric mice with thymic atrophy (discussed in Chapter II), it would be interesting to study what conditions are required to induce autoimmune disease in these mice. This is an important area of investigation, since it is clinically relevant to determine at what level of imbalance and under what conditions could a sub-clinical condition develop into clinical disease in the elderly. One way to explore this question is to utilize a mixed BM chimera model, in which the host mice would receive both OT-II and OT-I ($CD4^+$ and $CD8^+$ cells that highly recognize mOVA), since other studies have demonstrated the importance of antigen-specific $CD8^+$ T cells in Type I diabetes pathogenesis [301]. Another experiment would be to use exogenous mOVA injection with adjuvant to simulate a pro-inflammatory insult and evaluate if this is sufficient to induce autoimmune disease. In our preliminary studies, we tested to effect of exogenous mOVA given without adjuvant and did not see any difference in pancreatic T cell infiltration or pathology. Therefore, additional immune components and/or inflammatory stimuli appear to be necessary to activate the existing imbalance of mock self-Ag-specific Tcon cells in the FCmOVA chimera mice with thymic atrophy.

3.2.2 Aim 2 Limitations & Future Work

We directly measured FoxP3 RQ-MFI as an indication of FoxP3 expression levels at the protein level, which can reflect relative Treg instability, since FoxP3 is closely associated with function, as confirmed by the Treg suppressive assay. Due to time constraints, we were unable to utilize cell-fate reporter mice [269], which label Treg cells that have lost expression of FoxP3 ("ex-FoxP3 cells"). Therefore, our model could not quantify Treg cells that had potentially lost FoxP3 expression. For future studies, it would be more informative to utilize Treg cell-fate reporter mice

so that we can more accurately assess Treg instability in our model. We could also quantify FoxP3 expression at the mRNA level to further elucidate the level at which FoxP3 down-regulation is occurring in the OVA-specific Treg cells from mice with thymic atrophy compared to normal thymus. In addition, it is known that Treg cell function, and specifically FoxP3 expression, increases with age, as a result of demethylation of FoxP3-regulating CpG sites [217]. Further, FoxP3 hypomethylation occurs after TCR engagement and has been used to identify the tTreg cell lineage, as opposed to induced Treg (iTreg) cells [274]. Therefore, it would be interesting to investigate how epigenetic changes to the FoxP3 promotor region or its enhancers might be influenced by thymic atrophy.

Finally, we would also like to perform single-cell RNA sequencing of the Treg cells in our mice with and without thymic atrophy. At this level of sequencing, we would be able to assess subtle changes in cytokine expression and evaluate Treg plasticity in a more comprehensive manner. For example, we originally included evaluation of Treg plasticity due to acquired IFN-γ expression, but we were unable to observe any changes between groups and this small sub-population was difficult to quantify via flow cytometry. Additionally, when we assessed activation marker levels on specific-Treg cells (such as $CD44^{+}Ki67^{+}$ or Nur77 MFI) we did not see any difference between groups. Therefore, subtle phenotypic alterations, such as this, could be more readily observed via transcriptomic analysis. We would also like to assess gene and protein expression levels of other Treg-associated markers of suppressive function, such as TGF-β, CTLA-4, and PD-1 in Treg cells from mice with and without thymic atrophy in order to further elucidate the mechanisms of decreased suppressive capacity we observed in OVA-specific pTreg cells from mice with thymic atrophy.

3.2.3 Aim 3 Limitations & Future Work

We acknowledge the technical challenge of attempting to quantify an individual TCR clone from an entire repertoire due to the large theoretical number of TCRs present (~10^{20}) [25,26]. Even in light of one study, which demonstrated a functional peripheral naïve T cell repertoire of 1-2 x 10^6, it is estimated that any one clone only comprises 1-10 cells of that peripheral pool[27]. Therefore, the results shown here can only provide trends to compare low and high levels of TCR diversity between the investigated Treg populations. Our future work would include adding more numbers to these sequencing groups; however, this would again require use of the irradiator, which has been decommissioned. An alternative to this would be to continue the cross-breeding of the double reporter mice and perform additional RTE tTreg TCR repertoire analysis on young versus naturally aged mice. This would circumvent the need for irradiation required for our dual reporter BM chimera model and remove any possible side effects of the irradiation on the thymus. Although this was initially part of my proposed work, we were unable to include these experiments here due to time constraints.

3.2.4 Additional Future Work

There are a few additional areas of future work, which do not directly fall under the previously outlined project aims. The first is the utilization of a mOVA:MHC-II tetramer in order to more accurately identify our mock self-Ag-specific ($CD4^+$) T cell populations. Although we attempted to utilize this tetramer in preliminary experiments using various staining protocols, we were unable to obtain acceptable staining, however, optimization of this tetramer system would ameliorate the limitation of our model in which there is a population of endogenous $V\alpha2^+V\beta5^+$ cells in the host mice (although very few, see Chapter II, Fig. 8).

Second, it is known that there are some differences in thymic atrophy and aging between males and females (also discussed in Chapter I section 1.6.2) [190]. We did not focus on sex differences in this study, however, given that estrogen has recently been implicated in acting as an epigenetic down-regulator of *Aire* [201,202], it would be interesting to analyze potential difference between male and female mice, as well as to evaluate changes that occur in overiectomized female mice, as a model of post-menopausal women.

Finally, in light of the goal of restoring immune homeostasis in the elderly, it would be interesting to see if restoration of *Aire* expression in our FCmOVA chimera mice is sufficient in our model to improve the imbalance in mock self-Ag-specific Treg/Tcon cells. Because we know that thymic atrophy causes decreased *Aire* expression and our mock self-Ag, mOVA is driven by RIP, which is under the regulatory control of Aire, it would follow that thymic rejuvenation, primarily via restored *Aire* expression, could be sufficient to rescue the observed imbalance. Further, it would also be intriguing if restoration of *Aire* expression also rescued the intrinsic functional defects observed in the Ag-specific Treg cells in these mice.

REFERENCES

1. Parkin, J. & Cohen, B. An overview of the immune system. *Lancet (London, England)* **357**, 1777-1789 (2001).
2. Riera Romo, M., Pérez-Martínez, D. & Castillo Ferrer, C. Innate immunity in vertebrates: an overview. *Immunology* **148**, 125-139 (2016).
3. Théry, C. & Amigorena, S. The cell biology of antigen presentation in dendritic cells. *Current opinion in immunology* **13**, 45-51 (2001).
4. Kotsias, F., Cebrian, I. & Alloatti, A. Antigen processing and presentation. *International review of cell and molecular biology* **348**, 69-121 (2019).
5. Bonilla, F.A. & Oettgen, H.C. Adaptive immunity. *The Journal of allergy and clinical immunology* **125**, S33-40 (2010).
6. Mueller, S.N. & Mackay, L.K. Tissue-resident memory T cells: local specialists in immune defence. *Nature reviews. Immunology* **16**, 79-89 (2016).
7. Inoue, T., Moran, I., Shinnakasu, R., Phan, T.G. & Kurosaki, T. Generation of memory B cells and their reactivation. *Immunological reviews* **283**, 138-149 (2018).
8. Netea, M.G.*, et al.* Trained immunity: A program of innate immune memory in health and disease. *Science (New York, N.Y.)* **352**, aaf1098 (2016).
9. Müller, L., Di Benedetto, S. & Pawelec, G. The Immune System and Its Dysregulation with Aging. *Sub-cellular biochemistry* **91**, 21-43 (2019).
10. Klein, L., Kyewski, B., Allen, P.M. & Hogquist, K.A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature reviews. Immunology* **14**, 377-391 (2014).
11. Klein, L., Robey, E.A. & Hsieh, C.S. Central CD4(+) T cell tolerance: deletion versus regulatory T cell differentiation. *Nature reviews. Immunology* **19**, 7-18 (2019).
12. Wirnsberger, G., Hinterberger, M. & Klein, L. Regulatory T-cell differentiation versus clonal deletion of autoreactive thymocytes. *Immunology and cell biology* **89**, 45-53 (2011).
13. Anderson, G. & Jenkinson, E.J. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* **1**, 31-40 (2001).
14. Krueger, A., Ziętara, N. & Łyszkiewicz, M. T Cell Development by the Numbers. *Trends in immunology* **38**, 128-139 (2017).
15. Mandl, J.N., Monteiro, J.P., Vrisekoop, N. & Germain, R.N. T cell-positive selection uses self-ligand binding strength to optimize repertoire recognition of foreign antigens. *Immunity* **38**, 263-274 (2013).
16. Stritesky, G.L., Jameson, S.C. & Hogquist, K.A. Selection of self-reactive T cells in the thymus. *Annual review of immunology* **30**, 95-114 (2012).
17. Palmer, E. Negative selection--clearing out the bad apples from the T-cell repertoire. *Nature reviews. Immunology* **3**, 383-391 (2003).
18. Hsieh, C.S., Lee, H.M. & Lio, C.W. Selection of regulatory T cells in the thymus. *Nature reviews. Immunology* **12**, 157-167 (2012).
19. Gellert, M. V(D)J recombination: RAG proteins, repair factors, and regulation. *Annual review of biochemistry* **71**, 101-132 (2002).
20. Fugmann, S.D., Lee, A.I., Shockett, P.E., Villey, I.J. & Schatz, D.G. The RAG proteins and V(D)J recombination: complexes, ends, and transposition. *Annual review of immunology* **18**, 495-527 (2000).

21. Zúñiga-Pflücker, J.C. T-cell development made simple. *Nature reviews. Immunology* **4**, 67-72 (2004).
22. Ceredig, R. & Rolink, T. A positive look at double-negative thymocytes. *Nature reviews. Immunology* **2**, 888-897 (2002).
23. Candéias, S., Muegge, K. & Durum, S.K. Junctional diversity in signal joints from T cell receptor beta and delta loci via terminal deoxynucleotidyl transferase and exonucleolytic activity. *The Journal of experimental medicine* **184**, 1919-1926 (1996).
24. Davis, M.M. & Bjorkman, P.J. T-cell antigen receptor genes and T-cell recognition. *Nature* **334**, 395-402 (1988).
25. Rosati, E.*, et al.* Overview of methodologies for T-cell receptor repertoire analysis. *BMC biotechnology* **17**, 61 (2017).
26. Goronzy, J.J., Qi, Q., Olshen, R.A. & Weyand, C.M. High-throughput sequencing insights into T-cell receptor repertoire diversity in aging. *Genome medicine* **7**, 117 (2015).
27. Casrouge, A.*, et al.* Size estimate of the alpha beta TCR repertoire of naive mouse splenocytes. *Journal of immunology (Baltimore, Md. : 1950)* **164**, 5782-5787 (2000).
28. Goronzy, J.J. & Weyand, C.M. Aging, autoimmunity and arthritis: T-cell senescence and contraction of T-cell repertoire diversity - catalysts of autoimmunity and chronic inflammation. *Arthritis research & therapy* **5**, 225-234 (2003).
29. Woodsworth, D.J., Castellarin, M. & Holt, R.A. Sequence analysis of T-cell repertoires in health and disease. *Genome medicine* **5**, 98 (2013).
30. Naylor, K.*, et al.* The influence of age on T cell generation and TCR diversity. *Journal of immunology (Baltimore, Md. : 1950)* **174**, 7446-7452 (2005).
31. Vallejo, A.N. Age-dependent alterations of the T cell repertoire and functional diversity of T cells of the aged. *Immunologic research* **36**, 221-228 (2006).
32. Yager, E.J.*, et al.* Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *The Journal of experimental medicine* **205**, 711-723 (2008).
33. Kyewski, B. & Klein, L. A central role for central tolerance. *Annual review of immunology* **24**, 571-606 (2006).
34. Klein, L., Robey, E.A. & Hsieh, C.S. Central CD4(+) T cell tolerance: deletion versus regulatory T cell differentiation. *Nat Rev Immunol* (2019).
35. Josefowicz, S.Z., Lu, L.F. & Rudensky, A.Y. Regulatory T cells: mechanisms of differentiation and function. *Annual review of immunology* **30**, 531-564 (2012).
36. Picca, C.C.*, et al.* Thymocyte deletion can bias Treg formation toward low-abundance self-peptide. *European journal of immunology* **39**, 3301-3306 (2009).
37. Perniola, R. Twenty Years of AIRE. *Frontiers in immunology* **9**, 98 (2018).
38. Takaba, H.*, et al.* Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance. *Cell* **163**, 975-987 (2015).
39. Chan, A.Y. & Anderson, M.S. Central tolerance to self revealed by the autoimmune regulator. *Annals of the New York Academy of Sciences* **1356**, 80-89 (2015).
40. Meredith, M., Zemmour, D., Mathis, D. & Benoist, C. Aire controls gene expression in the thymic epithelium with ordered stochasticity. *Nature immunology* **16**, 942-949 (2015).
41. Conteduca, G., Indiveri, F., Filaci, G. & Negrini, S. Beyond APECED: An update on the role of the autoimmune regulator gene (AIRE) in physiology and disease. *Autoimmunity reviews* **17**, 325-330 (2018).
42. Gallegos, A.M. & Bevan, M.J. Central tolerance: good but imperfect. *Immunological reviews* **209**, 290-296 (2006).

43. Bacchetta, R., Barzaghi, F. & Roncarolo, M.G. From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Annals of the New York Academy of Sciences* **1417**, 5-22 (2018).
44. Malchow, S.*, et al.* Aire Enforces Immune Tolerance by Directing Autoreactive T Cells into the Regulatory T Cell Lineage. *Immunity* **44**, 1102-1113 (2016).
45. Li, M.O. & Rudensky, A.Y. T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nature reviews. Immunology* **16**, 220-233 (2016).
46. Rezzani, R., Nardo, L., Favero, G., Peroni, M. & Rodella, L.F. Thymus and aging: morphological, radiological, and functional overview. *Age (Dordrecht, Netherlands)* **36**, 313-351 (2014).
47. Palmer, D.B. The effect of age on thymic function. *Frontiers in immunology* **4**, 316 (2013).
48. Hale, J.S., Boursalian, T.E., Turk, G.L. & Fink, P.J. Thymic output in aged mice. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 8447-8452 (2006).
49. Sudo, K., Ema, H., Morita, Y. & Nakauchi, H. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med* **192**, 1273-1280 (2000).
50. Min, H., Montecino-Rodriguez, E. & Dorshkind, K. Reduction in the developmental potential of intrathymic T cell progenitors with age. *Journal of immunology (Baltimore, Md. : 1950)* **173**, 245-250 (2004).
51. Kovtonyuk, L.V., Fritsch, K., Feng, X., Manz, M.G. & Takizawa, H. Inflamm-Aging of Hematopoiesis, Hematopoietic Stem Cells, and the Bone Marrow Microenvironment. *Front Immunol* **7**, 502 (2016).
52. Latchney, S.E. & Calvi, L.M. The aging hematopoietic stem cell niche: Phenotypic and functional changes and mechanisms that contribute to hematopoietic aging. *Semin Hematol* **54**, 25-32 (2017).
53. Sun, L., Brown, R., Chen, S., Zhuge, Q. & Su, D.M. Aging induced decline in T-lymphopoiesis is primarily dependent on status of progenitor niches in the bone marrow and thymus. *Aging* **4**, 606-619 (2012).
54. Su, D.M., Aw, D. & Palmer, D.B. Immunosenescence: a product of the environment? *Curr Opin Immunol* **25**, 498-503 (2013).
55. Vaidya, H.J., Briones Leon, A. & Blackburn, C.C. FOXN1 in thymus organogenesis and development. *European journal of immunology* **46**, 1826-1837 (2016).
56. Hinterberger, M.*, et al.* Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. *Nature immunology* **11**, 512-519 (2010).
57. Aw, D., Silva, A.B., Maddick, M., von Zglinicki, T. & Palmer, D.B. Architectural changes in the thymus of aging mice. *Aging cell* **7**, 158-167 (2008).
58. Chinn, I.K., Blackburn, C.C., Manley, N.R. & Sempowski, G.D. Changes in primary lymphoid organs with aging. *Seminars in immunology* **24**, 309-320 (2012).
59. Burnley, P.*, et al.* Role of the p63-FoxN1 regulatory axis in thymic epithelial cell homeostasis during aging. *Cell Death Dis* **4**, e932 (2013).
60. Masters, A.R., Haynes, L., Su, D.M. & Palmer, D.B. Immune senescence: significance of the stromal microenvironment. *Clin Exp Immunol* **187**, 6-15 (2017).
61. Sempowski, G.D.*, et al.* Leukemia inhibitory factor, oncostatin M, IL-6, and stem cell factor mRNA expression in human thymus increases with age and is associated with thymic atrophy. *J Immunol* **164**, 2180-2187 (2000).
62. Freund, A., Orjalo, A.V., Desprez, P.Y. & Campisi, J. Inflammatory networks during cellular senescence: causes and consequences. *Trends in molecular medicine* **16**, 238-246 (2010).
63. Pang, W.W.*, et al.* Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 20012-20017 (2011).

64. Linton, P.J. & Dorshkind, K. Age-related changes in lymphocyte development and function. *Nature immunology* **5**, 133-139 (2004).
65. Tyan, M.L. Age-related decrease in mouse T cell progenitors. *Journal of immunology (Baltimore, Md. : 1950)* **118**, 846-851 (1977).
66. Morrison, S.J., Wandycz, A.M., Akashi, K., Globerson, A. & Weissman, I.L. The aging of hematopoietic stem cells. *Nature medicine* **2**, 1011-1016 (1996).
67. Waterstrat, A. & Van Zant, G. Effects of aging on hematopoietic stem and progenitor cells. *Current opinion in immunology* **21**, 408-413 (2009).
68. Van Zant, G. & Liang, Y. Concise review: hematopoietic stem cell aging, life span, and transplantation. *Stem cells translational medicine* **1**, 651-657 (2012).
69. Min, H., Montecino-Rodriguez, E. & Dorshkind, K. Effects of aging on early B- and T-cell development. *Immunol Rev* **205**, 7-17 (2005).
70. Zhu, X.*, et al.* Lymphohematopoietic progenitors do not have a synchronized defect with age-related thymic involution. *Aging Cell* **6**, 663-672 (2007).
71. Peschon, J.J.*, et al.* Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *The Journal of experimental medicine* **180**, 1955-1960 (1994).
72. Porritt, H.E.*, et al.* Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* **20**, 735-745 (2004).
73. Gui, J.*, et al.* The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. *International immunology* **19**, 1201-1211 (2007).
74. Aspinall, R. & Andrew, D. Thymic atrophy in the mouse is a soluble problem of the thymic environment. *Vaccine* **18**, 1629-1637 (2000).
75. Henson, S.M., Snelgrove, R., Hussell, T., Wells, D.J. & Aspinall, R. An IL-7 fusion protein that shows increased thymopoietic ability. *Journal of immunology (Baltimore, Md. : 1950)* **175**, 4112-4118 (2005).
76. van Ewijk, W., Hollander, G., Terhorst, C. & Wang, B. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development* **127**, 1583-1591 (2000).
77. Shores, E.W., Van Ewijk, W. & Singer, A. Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *Eur J Immunol* **21**, 1657-1661 (1991).
78. Stanger, B.Z., Tanaka, A.J. & Melton, D.A. Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. *Nature* **445**, 886-891 (2007).
79. Jenkinson, W.E., Bacon, A., White, A.J., Anderson, G. & Jenkinson, E.J. An epithelial progenitor pool regulates thymus growth. *J Immunol* **181**, 6101-6108 (2008).
80. Foss, D.L., Donskoy, E. & Goldschneider, I. The importation of hematogenous precursors by the thymus is a gated phenomenon in normal adult mice. *J Exp Med* **193**, 365-374 (2001).
81. Donskoy, E., Foss, D. & Goldschneider, I. Gated importation of prothymocytes by adult mouse thymus is coordinated with their periodic mobilization from bone marrow. *J Immunol* **171**, 3568-3575 (2003).
82. Moore, K.A. & Lemischka, I.R. Stem cells and their niches. *Science* **311**, 1880-1885 (2006).
83. Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* **6**, 93-106 (2006).
84. Prockop, S.E. & Petrie, H.T. Regulation of thymus size by competition for stromal niches among early T cell progenitors. *J Immunol* **173**, 1604-1611 (2004).
85. Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H. & Boehm, T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* **372**, 103-107 (1994).

86. Nehls, M.*, et al.* Two genetically separable steps in the differentiation of thymic epithelium. *Science* **272**, 886-889 (1996).
87. Schlake, T. The nude gene and the skin. *Exp Dermatol* **10**, 293-304 (2001).
88. Brissette, J.L., Li, J., Kamimura, J., Lee, D. & Dotto, G.P. The product of the mouse nude locus, Whn, regulates the balance between epithelial cell growth and differentiation. *Genes Dev* **10**, 2212-2221 (1996).
89. Flanagan, S.P. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genetical research* **8**, 295-309 (1966).
90. Pantelouris, E.M. Absence of thymus in a mouse mutant. *Nature* **217**, 370-371 (1968).
91. Ortman, C.L., Dittmar, K.A., Witte, P.L. & Le, P.T. Molecular characterization of the mouse involuted thymus: aberrations in expression of transcription regulators in thymocyte and epithelial compartments. *International immunology* **14**, 813-822 (2002).
92. Rode, I.*, et al.* Foxn1 Protein Expression in the Developing, Aging, and Regenerating Thymus. *J Immunol* **195**, 5678-5687 (2015).
93. Cheng, L.*, et al.* Postnatal tissue-specific disruption of transcription factor FoxN1 triggers acute thymic atrophy. *The Journal of biological chemistry* **285**, 5836-5847 (2010).
94. Zhang, Z., Burnley, P., Coder, B. & Su, D.M. Insights on FoxN1 biological significance and usages of the "nude" mouse in studies of T-lymphopoiesis. *International journal of biological sciences* **8**, 1156-1167 (2012).
95. Matsuda, T. & Cepko, C.L. Controlled expression of transgenes introduced by in vivo electroporation. *Proc Natl Acad Sci U S A* **104**, 1027-1032 (2007).
96. Bleul, C.C.*, et al.* Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. *Nature* **441**, 992-996 (2006).
97. Sun, L.*, et al.* Declining expression of a single epithelial cell-autonomous gene accelerates age-related thymic involution. *Aging cell* **9**, 347-357 (2010).
98. Zook, E.C.*, et al.* Overexpression of Foxn1 attenuates age-associated thymic involution and prevents the expansion of peripheral CD4 memory T cells. *Blood* **118**, 5723-5731 (2011).
99. Bredenkamp, N., Nowell, C.S. & Blackburn, C.C. Regeneration of the aged thymus by a single transcription factor. *Development (Cambridge, England)* **141**, 1627-1637 (2014).
100. O'Neill, K.E.*, et al.* Foxn1 Is Dynamically Regulated in Thymic Epithelial Cells during Embryogenesis and at the Onset of Thymic Involution. *PloS one* **11**, e0151666 (2016).
101. Petrie, H.T. Role of thymic organ structure and stromal composition in steady-state postnatal T-cell production. *Immunological reviews* **189**, 8-19 (2002).
102. Aspinall, R. & Andrew, D. Thymic involution in aging. *J Clin Immunol* **20**, 250-256 (2000).
103. Hodes, R.J. Telomere length, aging, and somatic cell turnover. *The Journal of experimental medicine* **190**, 153-156 (1999).
104. Franceschi, C., Valensin, S., Fagnoni, F., Barbi, C. & Bonafe, M. Biomarkers of immunosenescence within an evolutionary perspective: the challenge of heterogeneity and the role of antigenic load. *Experimental gerontology* **34**, 911-921 (1999).
105. Swain, S., Clise-Dwyer, K. & Haynes, L. Homeostasis and the age-associated defect of CD4 T cells. *Seminars in immunology* **17**, 370-377 (2005).
106. Goronzy, J.J., Lee, W.W. & Weyand, C.M. Aging and T-cell diversity. *Experimental gerontology* **42**, 400-406 (2007).
107. Pawelec, G. Age and immunity: What is "immunosenescence"? *Experimental gerontology* **105**, 4-9 (2018).
108. McElhaney, J.E. & Effros, R.B. Immunosenescence: what does it mean to health outcomes in older adults? *Current opinion in immunology* **21**, 418-424 (2009).

109. Pawelec, G. Immunosenescence comes of age. Symposium on Aging Research in Immunology: The Impact of Genomics. *EMBO reports* **8**, 220-223 (2007).
110. Pawelec, G., Wagner, W., Adibzadeh, M. & Engel, A. T cell immunosenescence in vitro and in vivo. *Experimental gerontology* **34**, 419-429 (1999).
111. Palmer, S., Albergante, L., Blackburn, C.C. & Newman, T.J. Thymic involution and rising disease incidence with age. *Proc Natl Acad Sci U S A* (2018).
112. Guo, J.*, et al.* Deletion of FoxN1 in the thymic medullary epithelium reduces peripheral T cell responses to infection and mimics changes of aging. *PloS one* **7**, e34681 (2012).
113. Coder, B.D., Wang, H., Ruan, L. & Su, D.M. Thymic involution perturbs negative selection leading to autoreactive T cells that induce chronic inflammation. *Journal of immunology (Baltimore, Md. : 1950)* **194**, 5825-5837 (2015).
114. Oh, J., Wang, W., Thomas, R. & Su, D.M. Capacity of tTreg generation is not impaired in the atrophied thymus. *PLoS biology* **15**, e2003352 (2017).
115. Pohar, J., Simon, Q. & Fillatreau, S. Antigen-Specificity in the Thymic Development and Peripheral Activity of CD4(+)FOXP3(+) T Regulatory Cells. *Frontiers in immunology* **9**, 1701 (2018).
116. Lathrop, S.K., Santacruz, N.A., Pham, D., Luo, J. & Hsieh, C.S. Antigen-specific peripheral shaping of the natural regulatory T cell population. *The Journal of experimental medicine* **205**, 3105-3117 (2008).
117. Hwang, S.*, et al.* Reduced TCR signaling potential impairs negative selection but does not result in autoimmune disease. *J Exp Med* **209**, 1781-1795 (2012).
118. Leonard, J.D.*, et al.* Identification of Natural Regulatory T Cell Epitopes Reveals Convergence on a Dominant Autoantigen. *Immunity* **47**, 107-117.e108 (2017).
119. Malchow, S., Leventhal, D.S. & Savage, P.A. Organ-specific regulatory T cells of thymic origin are expanded in murine prostate tumors. *Oncoimmunology* **2**, e24898 (2013).
120. Kieback, E.*, et al.* Thymus-Derived Regulatory T Cells Are Positively Selected on Natural Self-Antigen through Cognate Interactions of High Functional Avidity. *Immunity* **44**, 1114-1126 (2016).
121. Lages, C.S.*, et al.* Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. *Journal of immunology (Baltimore, Md. : 1950)* **181**, 1835-1848 (2008).
122. Nikolich-Zugich, J. The twilight of immunity: emerging concepts in aging of the immune system. *Nature immunology* **19**, 10-19 (2018).
123. Zinger, A., Cho, W.C. & Ben-Yehuda, A. Cancer and Aging - the Inflammatory Connection. *Aging Dis* **8**, 611-627 (2017).
124. Machiela, M.J. Mosaicism, aging and cancer. *Curr Opin Oncol* **31**, 108-113 (2019).
125. Xia, J.*, et al.* Age-Related Disruption of Steady-State Thymic Medulla Provokes Autoimmune Phenotype via Perturbing Negative Selection. *Aging and disease* **3**, 248-259 (2012).
126. Coder, B. & Su, D.M. Thymic involution beyond T-cell insufficiency. *Oncotarget* **6**, 21777-21778 (2015).
127. De Martinis, M., Franceschi, C., Monti, D. & Ginaldi, L. Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. *FEBS letters* **579**, 2035-2039 (2005).
128. Franceschi, C.*, et al.* Inflamm-aging. An evolutionary perspective on immunosenescence. *Annals of the New York Academy of Sciences* **908**, 244-254 (2000).
129. Brunner, S., Herndler-Brandstetter, D., Weinberger, B. & Grubeck-Loebenstein, B. Persistent viral infections and immune aging. *Ageing research reviews* **10**, 362-369 (2011).
130. Franceschi, C.*, et al.* Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mech Ageing Dev* **128**, 92-105 (2007).

131. Callender, L.A*., et al.* Human CD8(+) EMRA T cells display a senescence-associated secretory phenotype regulated by p38 MAPK. *Aging Cell* **17**(2018).
132. Coppe, J.P*., et al.* Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* **6**, 2853-2868 (2008).
133. Coppé, J.P., Desprez, P.Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annual review of pathology* **5**, 99-118 (2010).
134. Fulop, T., Witkowski, J.M., Olivieri, F. & Larbi, A. The integration of inflammaging in age-related diseases. *Seminars in immunology* **40**, 17-35 (2018).
135. Fulop, T*., et al.* Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes? *Frontiers in immunology* **8**, 1960 (2017).
136. Gui, J., Mustachio, L.M., Su, D.M. & Craig, R.W. Thymus Size and Age-related Thymic Involution: Early Programming, Sexual Dimorphism, Progenitors and Stroma. *Aging and disease* **3**, 280-290 (2012).
137. Palmer, S., Albergante, L., Blackburn, C.C. & Newman, T.J. Thymic involution and rising disease incidence with age. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 1883-1888 (2018).
138. Fulop, T., Dupuis, G., Witkowski, J.M. & Larbi, A. The Role of Immunosenescence in the Development of Age-Related Diseases. *Revista de investigacion clinica; organo del Hospital de Enfermedades de la Nutricion* **68**, 84-91 (2016).
139. Dai, X., Zhang, D., Wang, C., Wu, Z. & Liang, C. The Pivotal Role of Thymus in Atherosclerosis Mediated by Immune and Inflammatory Response. *Int J Med Sci* **15**, 1555-1563 (2018).
140. Kennedy, B.K*., et al.* Geroscience: linking aging to chronic disease. *Cell* **159**, 709-713 (2014).
141. Accardi, G. & Caruso, C. Immune-inflammatory responses in the elderly: an update. *Immun Ageing* **15**, 11 (2018).
142. Hayflick, L. & Moorhead, P.S. The serial cultivation of human diploid cell strains. *Experimental cell research* **25**, 585-621 (1961).
143. He, S. & Sharpless, N.E. Senescence in Health and Disease. *Cell* **169**, 1000-1011 (2017).
144. Hernandez-Segura, A., Nehme, J. & Demaria, M. Hallmarks of Cellular Senescence. *Trends in cell biology* **28**, 436-453 (2018).
145. Campisi, J. Cellular senescence: putting the paradoxes in perspective. *Curr Opin Genet Dev* **21**, 107-112 (2011).
146. Marcotte, R., Lacelle, C. & Wang, E. Senescent fibroblasts resist apoptosis by downregulating caspase-3. *Mechanisms of ageing and development* **125**, 777-783 (2004).
147. Sanders, Y.Y*., et al.* Histone modifications in senescence-associated resistance to apoptosis by oxidative stress. *Redox biology* **1**, 8-16 (2013).
148. Chou, J.P. & Effros, R.B. T cell replicative senescence in human aging. *Current pharmaceutical design* **19**, 1680-1698 (2013).
149. Fukushima, Y., Minato, N. & Hattori, M. The impact of senescence-associated T cells on immunosenescence and age-related disorders. *Inflamm Regen* **38**, 24 (2018).
150. Crespo, J., Sun, H., Welling, T.H., Tian, Z. & Zou, W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Current opinion in immunology* **25**, 214-221 (2013).
151. Topalian, S.L*., et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine* **366**, 2443-2454 (2012).
152. Prata, L., Ovsyannikova, I.G., Tchkonia, T. & Kirkland, J.L. Senescent cell clearance by the immune system: Emerging therapeutic opportunities. *Semin Immunol* (2019).
153. Burton, D.G.A. & Stolzing, A. Cellular senescence: Immunosurveillance and future immunotherapy. *Ageing Res Rev* **43**, 17-25 (2018).

154. Sagiv, A*., et al.* Granule exocytosis mediates immune surveillance of senescent cells. *Oncogene* **32**, 1971-1977 (2013).
155. Plowden, J., Renshaw-Hoelscher, M., Engleman, C., Katz, J. & Sambhara, S. Innate immunity in aging: impact on macrophage function. *Aging cell* **3**, 161-167 (2004).
156. Solana, R*., et al.* Innate immunosenescence: effect of aging on cells and receptors of the innate immune system in humans. *Seminars in immunology* **24**, 331-341 (2012).
157. Tchkonia, T., Zhu, Y., van Deursen, J., Campisi, J. & Kirkland, J.L. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. *J Clin Invest* **123**, 966-972 (2013).
158. Franceschi, C*., et al.* Inflammaging 2018: An update and a model. *Seminars in immunology* **40**, 1-5 (2018).
159. Nikolich-Zugich, J. Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nature reviews. Immunology* **8**, 512-522 (2008).
160. High, K.P., Akbar, A.N. & Nikolich-Zugich, J. Translational research in immune senescence: Assessing the relevance of current models. *Semin Immunol* **24**, 373-382 (2012).
161. Lara, J*., et al.* A proposed panel of biomarkers of healthy ageing. *BMC medicine* **13**, 222 (2015).
162. Olsson, J*., et al.* Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. *Mechanisms of ageing and development* **121**, 187-201 (2000).
163. Wikby, A*., et al.* Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Experimental gerontology* **37**, 445-453 (2002).
164. Bektas, A., Schurman, S.H., Sen, R. & Ferrucci, L. Human T cell immunosenescence and inflammation in aging. *J Leukoc Biol* **102**, 977-988 (2017).
165. Tsukamoto, H*., et al.* Age-associated increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 18333-18338 (2009).
166. Tsukamoto, H., Huston, G.E., Dibble, J., Duso, D.K. & Swain, S.L. Bim dictates naive CD4 T cell lifespan and the development of age-associated functional defects. *Journal of immunology (Baltimore, Md. : 1950)* **185**, 4535-4544 (2010).
167. Chougnet, C.A*., et al.* A major role for Bim in regulatory T cell homeostasis. *Journal of immunology (Baltimore, Md. : 1950)* **186**, 156-163 (2011).
168. Parent, A.V*., et al.* Generation of functional thymic epithelium from human embryonic stem cells that supports host T cell development. *Cell Stem Cell* **13**, 219-229 (2013).
169. Sun, X*., et al.* Directed differentiation of human embryonic stem cells into thymic epithelial progenitor-like cells reconstitutes the thymic microenvironment in vivo. *Cell Stem Cell* **13**, 230-236 (2013).
170. Soh, C.L*., et al.* FOXN1 (GFP/w) reporter hESCs enable identification of integrin-beta4, HLA-DR, and EpCAM as markers of human PSC-derived FOXN1(+) thymic epithelial progenitors. *Stem cell reports* **2**, 925-937 (2014).
171. Barsanti, M*., et al.* A novel Foxn1(eGFP/+) mouse model identifies Bmp4-induced maintenance of Foxn1 expression and thymic epithelial progenitor populations. *Eur J Immunol* **47**, 291-304 (2017).
172. Kim, M.J., Miller, C.M., Shadrach, J.L., Wagers, A.J. & Serwold, T. Young, proliferative thymic epithelial cells engraft and function in aging thymuses. *Journal of immunology (Baltimore, Md. : 1950)* **194**, 4784-4795 (2015).
173. Bredenkamp, N*., et al.* An organized and functional thymus generated from FOXN1-reprogrammed fibroblasts. *Nature cell biology* **16**, 902-908 (2014).

174. Erickson, M.*, et al.* Regulation of thymic epithelium by keratinocyte growth factor. *Blood* **100**, 3269-3278 (2002).
175. Alpdogan, O.*, et al.* Keratinocyte growth factor (KGF) is required for postnatal thymic regeneration. *Blood* **107**, 2453-2460 (2006).
176. Bhandoola, A. & Artis, D. Immunology. Rebuilding the thymus. *Science* **336**, 40-41 (2012).
177. Dudakov, J.A.*, et al.* Interleukin-22 drives endogenous thymic regeneration in mice. *Science* **336**, 91-95 (2012).
178. Chaudhry, M.S., Velardi, E., Dudakov, J.A. & van den Brink, M.R. Thymus: the next (re)generation. *Immunol Rev* **271**, 56-71 (2016).
179. Pan, B.*, et al.* Acute ablation of DP thymocytes induces up-regulation of IL-22 and Foxn1 in TECs. *Clinical immunology (Orlando, Fla.)* **150**, 101-108 (2014).
180. Okoye, A.A.*, et al.* Effect of IL-7 Therapy on Naive and Memory T Cell Homeostasis in Aged Rhesus Macaques. *Journal of immunology (Baltimore, Md. : 1950)* **195**, 4292-4305 (2015).
181. Mackall, C.L., Fry, T.J. & Gress, R.E. Harnessing the biology of IL-7 for therapeutic application. *Nature reviews. Immunology* **11**, 330-342 (2011).
182. Perales, M.A.*, et al.* Recombinant human interleukin-7 (CYT107) promotes T-cell recovery after allogeneic stem cell transplantation. *Blood* **120**, 4882-4891 (2012).
183. Savino, W., Postel-Vinay, M.C., Smaniotto, S. & Dardenne, M. The thymus gland: a target organ for growth hormone. *Scandinavian journal of immunology* **55**, 442-452 (2002).
184. Hirokawa, K., Utsuyama, M. & Kikuchi, Y. Trade off situation between thymus and growth hormone: age-related decline of growth hormone is a cause of thymic involution but favorable for elongation of lifespan. *Biogerontology* **17**, 55-59 (2016).
185. Chen, B.J., Cui, X., Sempowski, G.D. & Chao, N.J. Growth hormone accelerates immune recovery following allogeneic T-cell-depleted bone marrow transplantation in mice. *Experimental hematology* **31**, 953-958 (2003).
186. De Mello-Coelho, V., Savino, W., Postel-Vinay, M.C. & Dardenne, M. Role of prolactin and growth hormone on thymus physiology. *Developmental immunology* **6**, 317-323 (1998).
187. Geenen, V.*, et al.* Evidence that insulin-like growth factor 2 (IGF2) is the dominant thymic peptide of the insulin superfamily. *Thymus* **21**, 115-127 (1993).
188. Ban, E.*, et al.* Specific binding sites for growth hormone in cultured mouse thymic epithelial cells. *Life sciences* **48**, 2141-2148 (1991).
189. Savino, W., Mendes-da-Cruz, D.A., Lepletier, A. & Dardenne, M. Hormonal control of T-cell development in health and disease. *Nature reviews. Endocrinology* **12**, 77-89 (2016).
190. Paton, D.N. The relationship of the thymus to the sexual organs: II. The influence of removal of the thymus on the growth of the sexual organs. *The Journal of physiology* **32**, 28-32 (1904).
191. Heng, T.S.*, et al.* Effects of castration on thymocyte development in two different models of thymic involution. *J Immunol* **175**, 2982-2993 (2005).
192. Sutherland, J.S.*, et al.* Activation of thymic regeneration in mice and humans following androgen blockade. *J Immunol* **175**, 2741-2753 (2005).
193. Goldberg, G.L.*, et al.* Sex steroid ablation enhances lymphoid recovery following autologous hematopoietic stem cell transplantation. *Transplantation* **80**, 1604-1613 (2005).
194. Gray, D.H.*, et al.* Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* **108**, 3777-3785 (2006).
195. Olsen, N.J., Olson, G., Viselli, S.M., Gu, X. & Kovacs, W.J. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology* **142**, 1278-1283 (2001).
196. Velardi, E.*, et al.* Sex steroid blockade enhances thymopoiesis by modulating Notch signaling. *J Exp Med* **211**, 2341-2349 (2014).

197. Min, H., Montecino-Rodriguez, E. & Dorshkind, K. Reassessing the role of growth hormone and sex steroids in thymic involution. *Clin Immunol* **118**, 117-123 (2006).
198. Griffith, A.V., Fallahi, M., Venables, T. & Petrie, H.T. Persistent degenerative changes in thymic organ function revealed by an inducible model of organ regrowth. *Aging Cell* **11**, 169-177 (2012).
199. Page, S.T.*, et al.* Effect of medical castration on CD4+ CD25+ T cells, CD8+ T cell IFN-gamma expression, and NK cells: a physiological role for testosterone and/or its metabolites. *Am J Physiol Endocrinol Metab* **290**, E856-863 (2006).
200. Brown, M.A. & Su, M.A. An Inconvenient Variable: Sex Hormones and Their Impact on T Cell Responses. *J Immunol* **202**, 1927-1933 (2019).
201. Bakhru, P. & Su, M.A. Estrogen turns down "the AIRE". *The Journal of clinical investigation* **126**, 1239-1241 (2016).
202. Dragin, N.*, et al.* Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. *The Journal of clinical investigation* **126**, 1525-1537 (2016).
203. Conboy, I.M.*, et al.* Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* **433**, 760-764 (2005).
204. Pishel, I.*, et al.* Accelerated aging versus rejuvenation of the immune system in heterochronic parabiosis. *Rejuvenation Res* **15**, 239-248 (2012).
205. Ruckh, J.M.*, et al.* Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell* **10**, 96-103 (2012).
206. Brack, A.S.*, et al.* Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* **317**, 807-810 (2007).
207. Loffredo, F.S.*, et al.* Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell* **153**, 828-839 (2013).
208. Katsimpardi, L.*, et al.* Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. *Science* **344**, 630-634 (2014).
209. Villeda, S.A.*, et al.* Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. *Nat Med* (2014).
210. Villeda, S.A.*, et al.* The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* **477**, 90-94 (2011).
211. Villeda, S.A. & Wyss-Coray, T. The circulatory systemic environment as a modulator of neurogenesis and brain aging. *Autoimmun Rev* **12**, 674-677 (2013).
212. Wang, W.*, et al.* Extracellular vesicles extracted from young donor serum attenuate inflammaging via partially rejuvenating aged T-cell immunotolerance. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, fj201800059R (2018).
213. Araki, T.*, et al.* Normal thymus in adults: appearance on CT and associations with age, sex, BMI and smoking. *European radiology* **26**, 15-24 (2016).
214. Duggal, N.A., Pollock, R.D., Lazarus, N.R., Harridge, S. & Lord, J.M. Major features of immunesenescence, including reduced thymic output, are ameliorated by high levels of physical activity in adulthood. *Aging cell* **17**(2018).
215. Duggal, N.A., Niemiro, G., Harridge, S.D.R., Simpson, R.J. & Lord, J.M. Can physical activity ameliorate immunosenescence and thereby reduce age-related multi-morbidity? *Nature reviews. Immunology* **19**, 563-572 (2019).
216. Liston, A. & Gray, D.H. Homeostatic control of regulatory T cell diversity. *Nature reviews. Immunology* **14**, 154-165 (2014).
217. Garg, S.K.*, et al.* Aging is associated with increased regulatory T-cell function. *Aging cell* **13**, 441-448 (2014).

218. Pacholczyk, R., Ignatowicz, H., Kraj, P. & Ignatowicz, L. Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity* **25**, 249-259 (2006).
219. Schmidt, A., Oberle, N. & Krammer, P.H. Molecular mechanisms of treg-mediated T cell suppression. *Frontiers in immunology* **3**, 51 (2012).
220. Zhao, H., Liao, X. & Kang, Y. Tregs: Where We Are and What Comes Next? *Frontiers in immunology* **8**, 1578 (2017).
221. Chinen, T.*, et al.* An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol* **17**, 1322-1333 (2016).
222. Barthlott, T.*, et al.* CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. *International immunology* **17**, 279-288 (2005).
223. Shevach, E.M. Foxp3(+) T Regulatory Cells: Still Many Unanswered Questions-A Perspective After 20 Years of Study. *Front Immunol* **9**, 1048 (2018).
224. Malek, T.R. & Bayer, A.L. Tolerance, not immunity, crucially depends on IL-2. *Nature reviews. Immunology* **4**, 665-674 (2004).
225. Hemmers, S.*, et al.* IL-2 production by self-reactive CD4 thymocytes scales regulatory T cell generation in the thymus. *The Journal of experimental medicine* **216**, 2466-2478 (2019).
226. Qureshi, O.S.*, et al.* Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science (New York, N.Y.)* **332**, 600-603 (2011).
227. Akkaya, B.*, et al.* Regulatory T cells mediate specific suppression by depleting peptide-MHC class II from dendritic cells. *Nature immunology* **20**, 218-231 (2019).
228. Colombo, M.P. & Piconese, S. Regulatory-T-cell inhibition versus depletion: the right choice in cancer immunotherapy. *Nature reviews. Cancer* **7**, 880-887 (2007).
229. Rosenzweig, N.*, et al.* PD-1/PD-L1 checkpoint blockade harnesses monocyte-derived macrophages to combat cognitive impairment in a tauopathy mouse model. *Nature communications* **10**, 465 (2019).
230. Tang, Q. & Bluestone, J.A. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nature immunology* **9**, 239-244 (2008).
231. Tarbell, K.V.*, et al.* Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *The Journal of experimental medicine* **204**, 191-201 (2007).
232. Krienke, C.*, et al.* A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. *Science (New York, N.Y.)* **371**, 145-153 (2021).
233. Bakhru, P.*, et al.* Combination central tolerance and peripheral checkpoint blockade unleashes antimelanoma immunity. *JCI insight* **2**(2017).
234. Overacre-Delgoffe, A.E. & Vignali, D.A.A. Treg Fragility: A Prerequisite for Effective Antitumor Immunity? *Cancer immunology research* **6**, 882-887 (2018).
235. Wolf, D., Sopper, S., Pircher, A., Gastl, G. & Wolf, A.M. Treg(s) in Cancer: Friends or Foe? *Journal of cellular physiology* **230**, 2598-2605 (2015).
236. Wang, W., Thomas, R., Sizova, O. & Su, D.M. Thymic Function Associated With Cancer Development, Relapse, and Antitumor Immunity - A Mini-Review. *Frontiers in immunology* **11**, 773 (2020).
237. Sawant, D.V. & Vignali, D.A. Once a Treg, always a Treg? *Immunological reviews* **259**, 173-191 (2014).
238. Dominguez-Villar, M. & Hafler, D.A. Regulatory T cells in autoimmune disease. *Nature immunology* **19**, 665-673 (2018).
239. Tan, T.G., Mathis, D. & Benoist, C. Singular role for T-BET+CXCR3+ regulatory T cells in protection from autoimmune diabetes. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 14103-14108 (2016).

240. McClymont, S.A*., et al.* Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *Journal of immunology (Baltimore, Md. : 1950)* **186**, 3918-3926 (2011).
241. Kleinewietfeld, M. & Hafler, D.A. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Seminars in immunology* **25**, 305-312 (2013).
242. Gillard, G.O., Dooley, J., Erickson, M., Peltonen, L. & Farr, A.G. Aire-dependent alterations in medullary thymic epithelium indicate a role for Aire in thymic epithelial differentiation. *Journal of immunology (Baltimore, Md. : 1950)* **178**, 3007-3015 (2007).
243. Naquet, P., Naspetti, M. & Boyd, R. Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. *Seminars in immunology* **11**, 47-55 (1999).
244. Sekai, M., Hamazaki, Y. & Minato, N. Medullary thymic epithelial stem cells maintain a functional thymus to ensure lifelong central T cell tolerance. *Immunity* **41**, 753-761 (2014).
245. Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nature immunology* **2**, 1032-1039 (2001).
246. Perry, J.S*., et al.* Distinct contributions of Aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus. *Immunity* **41**, 414-426 (2014).
247. Moon, J.J*., et al.* Quantitative impact of thymic selection on Foxp3+ and Foxp3- subsets of self-peptide/MHC class II-specific CD4+ T cells. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 14602-14607 (2011).
248. Raynor, J., Lages, C.S., Shehata, H., Hildeman, D.A. & Chougnet, C.A. Homeostasis and function of regulatory T cells in aging. *Current opinion in immunology* **24**, 482-487 (2012).
249. Haynes, L. & Swain, S.L. Why aging T cells fail: implications for vaccination. *Immunity* **24**, 663-666 (2006).
250. Boren, E. & Gershwin, M.E. Inflamm-aging: autoimmunity, and the immune-risk phenotype. *Autoimmun Rev* **3**, 401-406 (2004).
251. Goronzy, J.J. & Weyand, C.M. Immune aging and autoimmunity. *Cellular and molecular life sciences : CMLS* **69**, 1615-1623 (2012).
252. Prelog, M. Aging of the immune system: a risk factor for autoimmunity? *Autoimmunity reviews* **5**, 136-139 (2006).
253. Qi, Q*., et al.* Diversity and clonal selection in the human T-cell repertoire. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 13139-13144 (2014).
254. van der Geest, K.S*., et al.* Aging disturbs the balance between effector and regulatory CD4+ T cells. *Experimental gerontology* **60**, 190-196 (2014).
255. Sun, L*., et al.* Aged regulatory T cells protect from autoimmune inflammation despite reduced STAT3 activation and decreased constraint of IL-17 producing T cells. *Aging cell* **11**, 509-519 (2012).
256. Zhu, M.L., Nagavalli, A. & Su, M.A. Aire deficiency promotes TRP-1-specific immune rejection of melanoma. *Cancer research* **73**, 2104-2116 (2013).
257. Martinez-Jimenez, C.P*., et al.* Aging increases cell-to-cell transcriptional variability upon immune stimulation. *Science (New York, N.Y.)* **355**, 1433-1436 (2017).
258. Sato, K., Kato, A., Sekai, M., Hamazaki, Y. & Minato, N. Physiologic Thymic Involution Underlies Age-Dependent Accumulation of Senescence-Associated CD4(+) T Cells. *Journal of immunology (Baltimore, Md. : 1950)* **199**, 138-148 (2017).
259. Walker, L.S. CD4+ CD25+ Treg: divide and rule? *Immunology* **111**, 129-137 (2004).
260. Cohen, J.L., Trenado, A., Vasey, D., Klatzmann, D. & Salomon, B.L. CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. *The Journal of experimental medicine* **196**, 401-406 (2002).

261. Shafiani, S*., et al.* Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12. *Immunity* **38**, 1261-1270 (2013).
262. Kraj, P. & Ignatowicz, L. The mechanisms shaping the repertoire of CD4(+) Foxp3(+) regulatory T cells. *Immunology* (2017).
263. Lee, H.M., Bautista, J.L., Scott-Browne, J., Mohan, J.F. & Hsieh, C.S. A broad range of self-reactivity drives thymic regulatory T cell selection to limit responses to self. *Immunity* **37**, 475-486 (2012).
264. Wyss, L*., et al.* Affinity for self antigen selects Treg cells with distinct functional properties. *Nature immunology* **17**, 1093-1101 (2016).
265. Kurts, C*., et al.* Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med* **184**, 923-930 (1996).
266. Barnden, M.J., Allison, J., Heath, W.R. & Carbone, F.R. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunology and cell biology* **76**, 34-40 (1998).
267. Ryan, G.A*., et al.* B1 cells promote pancreas infiltration by autoreactive T cells. *Journal of immunology (Baltimore, Md. : 1950)* **185**, 2800-2807 (2010).
268. Konkel, J.E., Jin, W., Abbatiello, B., Grainger, J.R. & Chen, W. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E465-473 (2014).
269. Zhou, X*., et al.* Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nature immunology* **10**, 1000-1007 (2009).
270. Korn, T*., et al.* Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nature medicine* **13**, 423-431 (2007).
271. Kitz, A*., et al.* AKT isoforms modulate Th1-like Treg generation and function in human autoimmune disease. *EMBO reports* **17**, 1169-1183 (2016).
272. Kitz, A. & Dominguez-Villar, M. Molecular mechanisms underlying Th1-like Treg generation and function. *Cellular and molecular life sciences : CMLS* **74**, 4059-4075 (2017).
273. Kanamori, M., Nakatsukasa, H., Okada, M., Lu, Q. & Yoshimura, A. Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends in immunology* **37**, 803-811 (2016).
274. Ohkura, N*., et al.* T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* **37**, 785-799 (2012).
275. Ohkura, N. & Sakaguchi, S. Treg cells acquire new directions, cytokines navigate. *Immunity* **37**, 443-444 (2012).
276. Komatsu, N*., et al.* Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 1903-1908 (2009).
277. Bailey-Bucktrout, S.L*., et al.* Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity* **39**, 949-962 (2013).
278. Kuczma, M., Wang, C.Y., Ignatowicz, L., Gourdie, R. & Kraj, P. Altered connexin 43 expression underlies age-dependent decrease of regulatory T cell suppressor function in nonobese diabetic mice. *Journal of immunology (Baltimore, Md. : 1950)* **194**, 5261-5271 (2015).
279. Thiault, N*., et al.* Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. *Nature immunology* **16**, 628-634 (2015).
280. Rubtsov, Y.P*., et al.* Stability of the regulatory T cell lineage in vivo. *Science (New York, N.Y.)* **329**, 1667-1671 (2010).
281. Tang, Q*., et al.* Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* **28**, 687-697 (2008).

282. Wan, Y.Y. & Flavell, R.A. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* **445**, 766-770 (2007).
283. Anderson, M.S.*, et al.* Projection of an immunological self shadow within the thymus by the aire protein. *Science (New York, N.Y.)* **298**, 1395-1401 (2002).
284. Kraj, P. & Ignatowicz, L. The mechanisms shaping the repertoire of CD4(+) Foxp3(+) regulatory T cells. *Immunology* **153**, 290-296 (2018).
285. Perry, J.S. & Hsieh, C.S. Development of T-cell tolerance utilizes both cell-autonomous and cooperative presentation of self-antigen. *Immunological reviews* **271**, 141-155 (2016).
286. Ferreira, C., Palmer, D., Blake, K., Garden, O.A. & Dyson, J. Reduced regulatory T cell diversity in NOD mice is linked to early events in the thymus. *Journal of immunology (Baltimore, Md. : 1950)* **192**, 4145-4152 (2014).
287. Bolotin, D.A.*, et al.* MiXCR: software for comprehensive adaptive immunity profiling. *Nature methods* **12**, 380-381 (2015).
288. Yu, W.*, et al.* Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature* **400**, 682-687 (1999).
289. Boursalian, T.E., Golob, J., Soper, D.M., Cooper, C.J. & Fink, P.J. Continued maturation of thymic emigrants in the periphery. *Nature immunology* **5**, 418-425 (2004).
290. McCaughtry, T.M., Wilken, M.S. & Hogquist, K.A. Thymic emigration revisited. *The Journal of experimental medicine* **204**, 2513-2520 (2007).
291. Wan, Y.Y. & Flavell, R.A. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5126-5131 (2005).
292. Thomas, R., Wang, W. & Su, D.M. Contributions of Age-Related Thymic Involution to Immunosenescence and Inflammaging. *Immunity & ageing : I & A* **17**, 2 (2020).
293. Hogquist, K.A. & Jameson, S.C. The self-obsession of T cells: how TCR signaling thresholds affect fate 'decisions' and effector function. *Nat Immunol* **15**, 815-823 (2014).
294. Franceschi, C. & Campisi, J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci Med Sci* **69 Suppl 1**, S4-9 (2014).
295. Alexandropoulos, K. & Danzl, N.M. Thymic epithelial cells: antigen presenting cells that regulate T cell repertoire and tolerance development. *Immunol Res* **54**, 177-190 (2012).
296. Anderson, M.S.*, et al.* The cellular mechanism of Aire control of T cell tolerance. *Immunity* **23**, 227-239 (2005).
297. Hubert, F.X.*, et al.* Aire regulates transfer of antigen from mTEC to dendritic cells for induction of thymic tolerance. *Blood* (2011).
298. Bautista, J.L.*, et al.* Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nature immunology* **10**, 610-617 (2009).
299. Abbas, A.K.*, et al.* Regulatory T cells: recommendations to simplify the nomenclature. *Nature immunology* **14**, 307-308 (2013).
300. Carlson, C.S.*, et al.* Using synthetic templates to design an unbiased multiplex PCR assay. *Nature communications* **4**, 2680 (2013).
301. Graham, K.L.*, et al.* Pathogenic mechanisms in type 1 diabetes: the islet is both target and driver of disease. *Rev Diabet Stud* **9**, 148-168 (2012).
302. Espinosa-Carrasco, G.*, et al.* CD4(+) T Helper Cells Play a Key Role in Maintaining Diabetogenic CD8(+) T Cell Function in the Pancreas. *Front Immunol* **8**, 2001 (2017).
303. Harbers, S.O.*, et al.* Antibody-enhanced cross-presentation of self antigen breaks T cell tolerance. *J Clin Invest* **117**, 1361-1369 (2007).
304. Fontenot, J.D., Gavin, M.A. & Rudensky, A.Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature immunology* **4**, 330-336 (2003).

305. Zhang, X.*, et al.* Aging leads to disturbed homeostasis of memory phenotype CD8(+) cells. *J Exp Med* **195**, 283-293 (2002).
306. Oo, Y.H.*, et al.* Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. *J Immunol* **184**, 2886-2898 (2010).
307. Jun, S., Ochoa-Reparaz, J., Zlotkowska, D., Hoyt, T. & Pascual, D.W. Bystander-mediated stimulation of proteolipid protein-specific regulatory T (Treg) cells confers protection against experimental autoimmune encephalomyelitis (EAE) via TGF-beta. *J Neuroimmunol* **245**, 39-47 (2012).
308. Chen, W. & Konkel, J.E. Development of thymic Foxp3(+) regulatory T cells: TGF-β matters. *European journal of immunology* **45**, 958-965 (2015).
309. Vahl, J.C.*, et al.* Continuous T cell receptor signals maintain a functional regulatory T cell pool. *Immunity* **41**, 722-736 (2014).
310. Nakahama, M.*, et al.* Immunohistochemical and histometrical studies of the human thymus with special emphasis on age-related changes in medullary epithelial and dendritic cells. *Virchows Archiv. B, Cell pathology including molecular pathology* **58**, 245-251 (1990).
311. Varas, A.*, et al.* Age-dependent changes in thymic macrophages and dendritic cells. *Microscopy research and technique* **62**, 501-507 (2003).
312. Klein, L., Hinterberger, M., Wirnsberger, G. & Kyewski, B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nature reviews. Immunology* **9**, 833-844 (2009).
313. Hsieh, C.S., Zheng, Y., Liang, Y., Fontenot, J.D. & Rudensky, A.Y. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* **7**, 401-410 (2006).
314. Fohse, L.*, et al.* High TCR diversity ensures optimal function and homeostasis of Foxp3+ regulatory T cells. *Eur J Immunol* **41**, 3101-3113 (2011).
315. Yu, A.*, et al.* The Lower Limit of Regulatory CD4(+) Foxp3(+) TCRbeta Repertoire Diversity Required To Control Autoimmunity. *J Immunol* **198**, 3127-3135 (2017).
316. Smith, K.M., Olson, D.C., Hirose, R. & Hanahan, D. Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance. *International immunology* **9**, 1355-1365 (1997).